NON-ALCOHOLIC FATTY LIVER DISEASE.

FROM PATIENT TO POPULATION.

EDITH MARIANNE KOEHLER

Non-Alcoholic Fatty Liver Disease

From patient to population

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General introduction

Non-alcoholic fatty liver disease

Introduction

Non-alcoholic fatty liver disease (NAFLD) has become the most common chronic liver disease in Western countries, in parallel with epidemics in obesity and type 2 diabetes mellitus [1]. NAFLD comprises a wide range of histological findings, extending from simple steatosis to non-alcoholic steatohepatitis (NASH) with inflammation, ballooning degeneration and advanced fibrosis [2], which may eventually progress to end-stage cirrhosis (Figure 1). From an etiological perspective NAFLD can be divided by primary or secondary causality. Primary NAFLD is strongly associated with insulin resistance and its phenotypic manifestations, including visceral obesity and type 2 diabetes mellitus. Secondary NAFLD is less frequent and is due to a variety of medical or surgical conditions or use of pharmacological agents (e.g. methotrexate, amiodarone). Historically, primary NAFLD can only be diagnosed when other causes of liver disease and excessive ethanol consumption have been excluded.

Epidemiology

The estimated prevalence of NAFLD is dependent on the screening method used. In the general population the prevalence of NAFLD, diagnosed by ultrasonography, ranges from 15-35% [3-8]. When liver enzymes are adopted as a marker of NAFLD, the prevalence in the general population decreases to 3-9% [9-10]. However, considering up to 50% of NAFLD subjects have normal ALT levels, this marker appears to be insensitive for the diagnosis of NAFLD [3, 5, 11-12]. The current golden standard for determining NAFLD and NASH is liver biopsy, but this method is subject to sampling error and is unethical to perform in a population-based setting. As a result, data on prevalence of NASH is mainly extrapolated from post-mortem studies and studies of bariatric surgery patients, which constitute highly selected populations. Recently, Williams *et al.* studied 328 asymptomatic outpatient adults, recruited at Brooke Army Medical Center, whom completed a baseline questionnaire and ultrasonography of the liver, and demonstrated a prevalence of NAFLD of as high as 46%. Subsequently, they persuaded 134 of 156 participants with steatosis on ultrasound to undergo liver biopsies. The prevalence of NASH was as high as 12.2% in the total cohort and 29.9% in NAFLD participants.

Recently, efforts have been made to estimate the prevalence of advanced fibrosis in NAFLD using non-invasive methods, notably transient elastography. Transient elastography measures liver stiffness in a 1 cm wide by 5 cm long volume which is 100 times greater than the typical liver biopsy [13]. Since originally described in 2003, numerous studies have demonstrated a strong correlation between liver stiffness values and the stage of hepatic fibrosis in nearly all liver diseases [14-16]. In a study by Wong *et al.* the prevalence of liver stiffness measurement (LSM)

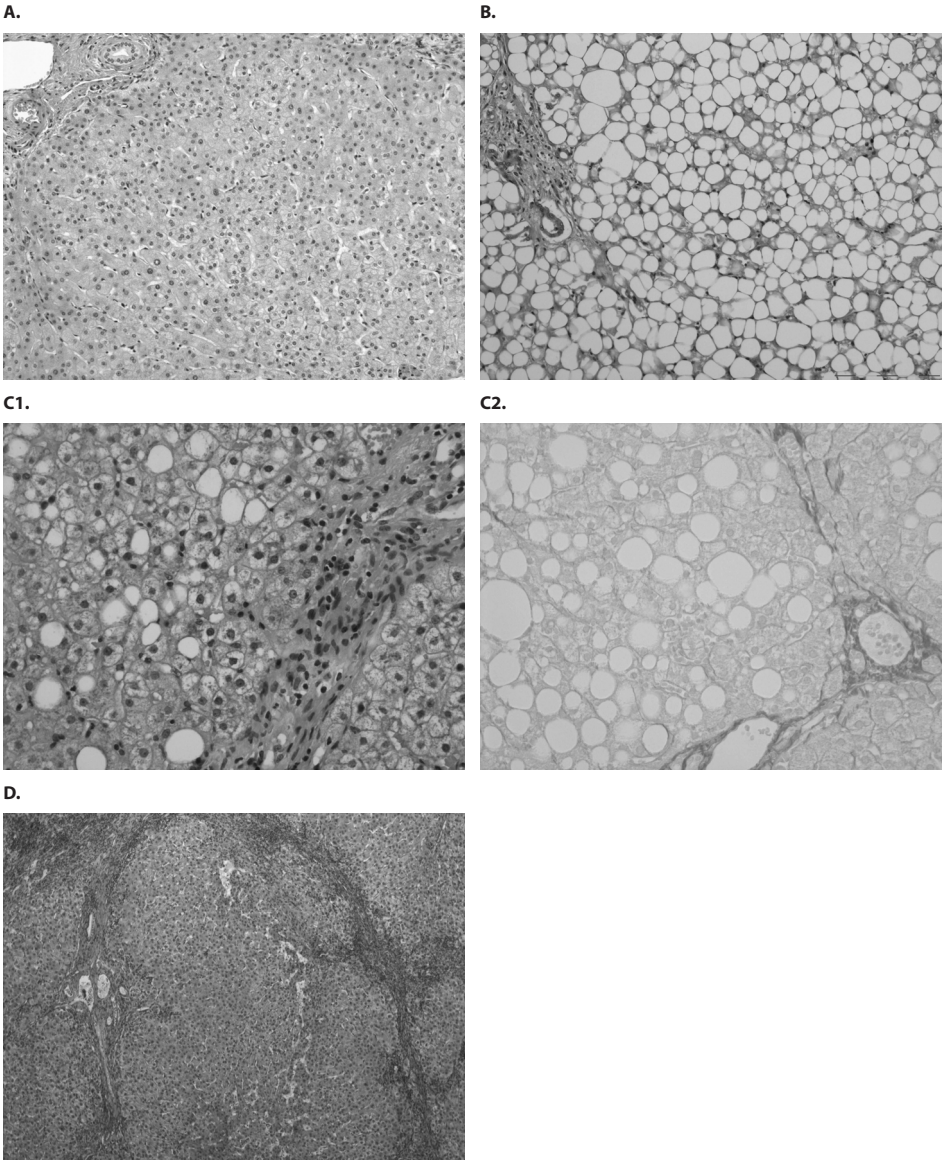

Fig 1. Histological sections illustrating normal liver (A) and the spectrum of NAFLD (B, simple steatosis; C1, NASH: ballooning degeneration and inflammation, hematoxylin-eosin staining; C2, NASH: fibrosis, Sirius red staining; D, NASH cirrhosis, steatosis has vanished).

>9.5 kPa, suggesting advanced liver fibrosis, in Hong Kong Chinese subjects with NAFLD, was 4.0% [17].

Etiology

Development of steatosis

Hepatic steatosis results from lipid accumulation in the liver. This occurs when the rate of fatty acid input is greater than the rate of fatty acid output, which depends on complex interaction among hepatic fatty acid uptake (through hydrolysis of triglycerides in plasma and adipose tissue), *de novo* lipogenesis, fatty acid oxidation and fatty acid export within very low-density lipoprotein (VLDL)- triglycerides [18]. Approximately 59% of hepatic fat is derived from circulating free fatty acids, with lesser contributions from *de novo* lipogenesis (26%) and diet (15%) [19]. *De novo* lipogenesis is governed by several nuclear transcription factors that are activated by insulin and glucose (sterol regulatory binding proteins, and carbohydrate-responsive sterol regulatory element binding proteins). These proteins are involved in the regulation of fatty acid and cholesterol biosynthesis and partially explain the link between insulin resistance and steatosis.

Recent insights indicate a key role for crosstalk between the liver and adipose tissue in the development of steatosis. C-Jun N-terminal kinase-1 (JNK1) has been shown to promote obesity-related insulin resistance [20]. JNK1 activation may impair insulin resistance directly through serine phosphorylation of insulin receptor substrate-1 (IRS-1) and indirectly through increased production of inflammatory cytokines [21]. When the liver is programmed for lipogenesis rather than fat disposal, insulin resistance may allow inappropriate sustained lipolysis with release of FFAs that are shunted to the liver.

Whether NAFLD is the cause or the consequence of insulin resistance remains an unresolved issue. Hepatic steatosis may generate or worsen insulin resistance, as prospective studies have shown that NAFLD, assessed by ultrasound or elevated liver enzymes, increases the risk of (pre-) diabetes and other metabolic comorbidities. In contrast, several studies in humans have shown that genetic defects associated with steatosis (e.g. mutations in or near PNPLA3, APOB, ATGL or CGI58) maintain normal insulin sensitivity over time [22-23]. Evidence that insulin resistance causes steatosis in humans comes from studies of metastatic insulinomas and of pancreatic islet cell transplants, that have shown that insulin directly promotes fat accumulation in liver cells, and from patients with mutations in AKT2 [23].

Development of NASH

One of the first hypotheses regarding progression of simple fatty liver to steatohepatitis was the 'two hit' model, proposed by Day and James in 1998 [24]. This model suggests that a first "hit", the development of steatosis, sensitizes the liver to a second "hit" – oxidative stress and cytokines – leading to apoptosis, necroinflammation and ultimately fibrosis and cirrhosis, through activation of hepatic stellate cells. This model has been reconsidered, since it does not explain the majority of cases of simple steatosis that do not progress to NASH and it does not recognize newer insights, including a role for 'lipotoxicity', vascular disturbances, and possibly gut

bacterial endotoxins [25-27]. Lipotoxicity is a term that signifies cellular injury caused by excessive free fatty acids and their metabolites, such as phosphatidic acid, ceramides, diacylglycerol, and other intermediaries [28]. Lipotoxicity may cause hepatocellular damage via several mechanisms. Firstly, FFA may injure the liver directly [29]. Secondly, FFA may indirectly cause injury through activation of nuclear factor-kappa β (NF-κβ) and proinflammatory cytokine production [30]. NF-κβ promotes the expression of inflammatory cytokines and death receptors, including Fas-1 receptor, tumor necrosis factor (TNF)-1 receptor and TNF-related apoptosis-inducing ligand (TRAIL) receptors 1 and 2, which in turn activate caspases that amplify the apoptotic signalling cascade [27]. Thirdly, FFAs are ligands for nuclear hormone receptors, such as peroxisome proliferator receptors (PPAR) alpha and gamma. PPAR-α, is expressed in various tissues including liver and muscle, and stimulates oxidation of FFAs in mitochondria, microsomes, and peroxisomes. PPAR-γ is expressed in adipose tissue and quiescent hepatic stellate cells. PPAR-γ activation inhibits activation of NF-kB, blocking production of TNF-a, which, in turn, increases adiponectin and decreases inflammation. Lastly, oxidation of FFAs generates reactive oxygen species (ROS) which may result in the generation of highly toxic lipid peroxides. These products may cause apoptosis, leading to inflammation and fibrosis via activation of hepatic stellate cells and kupffer cells with subsequent collagen deposition [31].

Furthermore, there is increasing attention for a role of the gut microbiota in the progression of NAFLD [32-33]. Although evidence is currently limited to studies in rodents and cross-sectional studies in humans, theories are very plausible. A different composition of gut microbiota may lead to an increased generation of fatty acids, insulin resistance and impaired VLDL secretion, all of which promote hepatic steatosis. Recently, Zhu *et al* found abundant differences in gut microbiota between healthy subjects and obese patients with or without NASH. Although fewer differences were observed between obese normal patients and obese NASH patients, they found significant differences in the microbioma with respect to phylum, family and genus of proteobacteria, enterobacteriaceae and escheria [34]. Given that patients with NASH also exhibited significantly elevated blood levels of ethanol, the authors concluded that their study suggested a role for alcohol producing microbiota in the pathogenesis of NASH.

Role of genetic factors

Finally, a mounting body of evidence suggests a role for genetic factors in the development and progression of NAFLD. In the past few years, there has been great activity in the identification of common susceptibility alleles for NAFLD using Genome-Wide Association Studies (GWAS). To date, two GWAS have reported genetic loci associated with non-alcoholic fatty liver assessed by means of computed tomography and magnetic resonance imaging [22, 35]. These studies demonstrated a strong association between NAFLD and rs738409, a single nucleotide polymorphism (SNP) positioned in the patatinlike phospholipase family 3 (PNPLA3) gene, that encodes an isoleucine-to-methionine substitution at amino acid position 148 (I148M) and presumably plays a role in acylation of lysophospholipids and hydrolysis of triglycerides [36]. This

genetic variant has also been associated with histological progression of NAFLD [37]. In addition, variants in GCKR have also been replicated in several studies and have been associated with NAFLD across ancestries. GCKR encodes glucokinase regulatory protein, an inhibitory protein, that regulates glucokinase (GK) in response to glucose and insulin [38]. GK is involved in the phosphorylation of glucose in the first step of glycolysis and enhances insulin secretion from pancreatic beta cells. It is hypothesized that variation of several SNPs in GCKR, e.g. rs780094, may increase glucokinase activity, leading to increased glycolysis and subsequent increases in malonyl co-enzyme A (CoA), a substrate for de novo lipogenesis, resulting in hepatic fat accumulation [39]. A recent GWAS performed by Anstee *et al*, identified new genetic variants associated with inflammation and fibrosis stage, suggesting that the genes in or near these variants may play a role in the progression of NAFLD.

Risk factors

Metabolic risk factors

NAFLD is strongly associated with insulin resistance and its phenotypic manifestations, including visceral adiposity, dyslipidemia, and diabetes [1]. Coherently, individuals with NAFLD are likely to have the metabolic syndrome. In a prospective observational study by Hamaguchi *et al*, NAFLD was less likely to regress in those participants with metabolic syndrome at baseline [40]. In addition, the likelihood of having NAFLD increases when more metabolic syndrome criteria are met [17]. One of the strongest correlates of NAFLD appears to be waist circumference, which reflects levels of visceral adipose tissue. Nevertheless, lean subjects may also develop NAFLD/NASH, which is often characterized by insulin resistance [41].

Lifestyle factors

In addition to metabolic factors, environmental factors, including exercise and diet, have been independently associated with NAFLD and NAFLD severity [42-44]. Diet may affect modulation of liver fat accumulation, regulation of antioxidant activity, insulin sensitivity and postprandial triglyceride metabolism. Physical activity may not only modulate liver fat content indirectly through weight loss, but may also directly reduce oxidative stress. By pooling current research Keating *et al.* demonstrated a clear benefit of exercise therapy on liver fat content, but not ALT levels, which was independent of weight loss [45]. Furthermore, intensity rather than duration of physical activity was associated with decreased odds of having NASH in a study by Kistler *et al.* [46]. Future studies will need to clarify the distinct role of physical activity with or without weight loss and its beneficial effects on histological features of NASH.

Age, gender and ethnicity

Studies have yielded controversial results with respect to gender predominance in NAFLD. Although the first studies of NAFLD described a higher prevalence of NAFLD in women, most community-based studies of adults up to age 50 to 60 years have found a male predominance of NAFLD and particularly NASH [47]. In turn, in the elderly, women again seem to take over [48-49] .

Regarding ethnicity, the prevalence of NAFLD seems to be higher in Hispanics compared to Black or Whites [5]. Interethnic differences in prevalence of NAFLD likely reflect the interethnic discrepancy in prevalence of metabolic features and differences in socio-demographic factors [50]. African Americans have lower prevalence of NAFLD than Caucasians, as they frequently have more subcutaneous, but less visceral fat than whites. Moreover, African Americans have a different lipoprotein metabolism [51]. In addition, genetic polymorphisms have been suggested to contribute to interethnic variation of NAFLD [52].

Prevalence and severity of NAFLD appears to increase with age, especially through the fourth to sixth decade of life. Age also appears to be a risk factor for NASH, NASH cirrhosis and complications from these conditions. In the elderly body fat distribution is shifted from subcutaneous to visceral adipose tissue, resulting in increased insulin resistance [53]. In addition, increased prevalence of NAFLD and NASH in the elderly may result from hepatic ageing. Livers of elderly subjects show a decline in blood flow, have less hepatic volume and have fewer, but larger hepatocytes and fewer mitochondria [54]. Furthermore, increased mitochondrial dysfunction in the elderly may cause insulin resistance and oxidative stress, phenomena that have both been associated with worse histology stages in NAFLD [55].

Natural history: fibrosis progression and clinical outcome

Fibrosis progression

Approximately 1 in 3 individuals with simple steatosis progress to NASH when metabolic factors persist [56-59]. Conversely, NASH may regress to simple steatosis in approximately 20%. Several factors have been associated with histological worsening of NAFLD. Argo *et al*. demonstrated that inflammation at initial liver biopsy was the strongest predictor of fibrosis progression over time [60]. Other factors that have been associated with fibrosis progression include higher age, higher body mass index, presence of diabetes mellitus, and male gender [61]. Individuals with NASH may develop cirrhosis, the end stage of fibrosis, over a course of 10 to 20 years. However, progression to cirrhosis may also occur in as little as 1-2 years [62].

Clinical outcome

Simple steatosis appears to be a relatively benign condition. However, some studies demonstrated that subjects with NAFLD may have an increased risk of cardiovascular and overall mortality [63-65]. Patients with NASH have increased liver-related mortality and overall

mortality (including liver failure, sepsis and variceal haemorrhage, or hepatocellular carcinoma (HCC)) [63, 66-68]. In addition, NASH has been associated with higher cardiovascular morbidity and mortality [69]. In a cohort of 247 patients with NASH and advanced stage fibrosis or cirrhosis, recruited from four international hospitals and followed up for a mean of 7 years, approximately 20% developed liver-related complications and 13% died or had a liver transplant [70]. When adjusting for baseline differences in age and gender, incidence of liver-related complications was lower in NAFLD patients than in patients with hepatitis C virus (HCV) infection (either non-responders to treatment or never treated), including incident HCC (6 vs. 18 respectively; p=0.03). Nevertheless, similar overall mortality was reported. Although the risk of developing HCC in NAFLD is much lower than in chronic HCV infection, given the high prevalence of NAFLD in the general population, population attributable risk of HCC is considerable [71]. The increasing rate of HCC in the United States has not only been attributed to increasing numbers of cirrhotic NASH, but it has been suggested that simple fatty liver itself directly promotes hepatic carcinogenesis independent of cirrhosis [72-74].

Approximately 5-10% of liver transplant recipients have NAFLD as the underlying cause of liver disease [75]. In contrast, the frequency of HCV as an indication for LT in the United States peaked at 28% in 2002 and has declined every year since. It has been projected that NAFLD will be the most common indication for LT in the next 10 to 20 years [76]. Unless a safe, effective, and widely prescribed therapy for NAFLD and NASH is identified, liver failure secondary to NASH is likely to become the most common indication for LT in Western countries. However, currently, the demand for organ grafts outweighs their availability. Moreover, as the prevalence of hepatic steatosis increases in parallel with the prevalence of obesity and type-2 diabetes, the proportion of donor organs that are unsuitable owing to steatosis may also be expected to increase, owing to the association of primary nonfunction with hepatic steatosis [77]. These data underline that NAFLD concerns an increasingly relevant public health issue.

Risk of cardiovascular disease

An expanding body of evidence suggests that patients with NAFLD may have a higher risk of developing cardiovascular disease independent of known metabolic risk factors [69]. Several cross-sectional studies have shown that NAFLD and NASH are associated with increased intima-media thickness of the carotid arteries [78]. In addition, there is a higher incidence of cardiovascular diseases (CVD) events in patients with NAFLD than in healthy controls [65]. In recent studies, cardiac structure and function of NAFLD patients was found to be profoundly different from that of non-NAFLD patients independent of metabolic factors [79-82]. From a pathophysiological perspective it is believed that lipotoxicity and a state of low-grade chronic inflammation in NAFLD play a role in the formation of atherosclerosis which subsequently leads

to higher cardiovascular risk. Therefore, cardiovascular risk management is advocated to be part of treatment regimens of NAFLD.

Diagnosis

Liver histology

Liver biopsy is considered the gold standard for the identification of NAFLD and NASH, for the semiquantitative assessment of the particular lesions (scoring), for diagnosis of concurrent disease processes, and for exclusion of other pathologic processes as the cause of clinical liver disease [83]. However, liver biopsy is not without controversies, for it is subject to sampling variability and inter-observer discordance [84]. NASH is histologically characterized by presence of steatosis (>5%), ballooning degeneration (apoptosis) and inflammation. Mostly biopsies of patient with NASH contain chronic inflammatory cells, including lymphocytes, plasma cells, monocytes and macrophages. Occasionally eosinophils and neutrophils may be present. In a majority of cases, one should be aware of non-NAFLD etiology (alcoholic hepatitis), when polymorphonuclear leucocytes encircle Mallory body-containing hepatocytes [85]. The NAS activity score, is often adopted in studies to evaluate pre- and post liver biopsies [2]. This score does not include fibrosis staging. Fibrosis stage is an important clinical outcome, for it is significantly associated with morbidity and mortality in NAFLD. It may be determined in the liver biopsy using the original proposed scoring system of Brunt *et al.*, as patterns of fibrosis in adult NASH differ from those of chronic viral hepatitis and biliary disease, both of which are initially portal-based [86]. In NASH the initial deposition of collagen is in the acinar zone 3, along the perisinusoidal spaces. Next stages include the presence of periportal fibrosis, then bridging fibrosis and finally, cirrhosis.

Non-invasive imaging modalities

Non-invasive imaging techniques to diagnose NAFLD include ultrasonography (US), magnetic resonance imaging (MRI), and computed tomography (CT). US has an acceptable sensitivity of 80-100% for detecting fatty liver, and its accuracy for diagnosis of fatty liver meets that of CT and MRI [87]. In addition, US is cheap and easily accessible. Nevertheless, all of these imaging modalities do not discriminate between simple fatty liver and steatohepatitis. Advanced liver fibrosis may be recognized at radiological examination by a granular or heterogeneous appearance of the liver parenchyma and indulations of the liver surface, nodularity and dysmorphia. In addition, cirrhosis may be accompanied by ascites, portal hypertension (splenomegaly) and vascular changes. However, advanced fibrosis and focal lesions may be less detectable at US when severe liver steatosis is present.

A recently developed, non-invasive US-based technique that is more frequently applied in clinical settings to assess presence of fibrosis in NAFLD is transient elastography using Fibroscan® (Echosens, Paris, France). Transient elastography can accurately diagnose severe

liver fibrosis or cirrhosis, also in NAFLD subjects [13, 88]. There are some pitfalls to this technique: it may not sufficiently discriminate when the liver is inflamed and does not perform well in elderly and obese subjects [89]. However, the recently developed XL-probe may perform better in obese subjects [90-91]. Recently, Fibroscan® also developed 'controlled attenuation parameter' (CAP), a method to evaluate and quantify liver steatosis at the same time one measures liver stiffness, which enlarges the spectrum of this non-invasive technique [92-93]. Other second-line non-invasive imaging techniques for the evaluation of fibrosis that are currently being investigated and validated include magnetic resonance elastography (MRE) and acoustic radiation force impulse imaging (ARFI) [94-95].

Non-invasive biomarkers and algorithms

Here we will briefly discuss some of the other non-invasive approaches for diagnosis of steatosis, steatohepatitis or fibrosis. It is clear from the literature that, although elevation of ALT or AST is most frequently caused by NAFLD, the single use of these traditional liver tests does not sufficiently discriminate between simple fatty liver, steatohepatitis activity or stage of liver fibrosis. Several algorithms have been developed to predict the presence or severity of NAFLD and/or the presence of fibrosis [96-101]. The Fatty Liver Index, an algorithm based on BMI, waist circumference, serum triglycerides and GGT, was developed to predict the presence of fatty liver, and was found to have good predictive abilities [102]. Non-invasive biomarkers, including cytokeratin 18, adiponectin, TNF-alpha, and IL-6 have been associated with NASH and fibrosis stage in NAFLD, but predictive ability is too low to discriminate between simple steatosis and NASH [103-107]. Widely validated fibrosis tests that are currently suggested to be of clinical usefulness, include the Enhanced Liver Fibrosis (ELF) panel and NAFLD fibrosis score [100, 108-109]. Nevertheless, it is important to keep in mind that longitudinal studies demonstrating the relevance of these markers for fibrosis progression or clinical outcome are currently not available.

Management

Lifestyle modification

Currently, increasing physical activity and introducing diet to reduce fat mass and correct insulin resistance is the mainstay for treatment of NAFLD and NASH. Patients who show histological improvement tend to exercise more [44, 46]. However, it has been difficult to differentiate the individual effects of physical activity and weight loss on the overall histological response in published studies [110]. It is often a challenge for patients to achieve sustainable weight loss through lifestyle modification. A majority of randomized clinical trials suggest that obtained weight loss is usually modest and returns to baseline within 1-3 years [111]. Patients with NASH, who do not respond to lifestyle changes, may benefit from pharmacological treatment.

Pharmacological therapy

One weight loss agent that has been studied in the context of NASH is orlistat, which reduces gastric and pancreatic lipases and prevents absorption of almost a third of dietary triglycerides. Studies have shown controversial results and drawing conclusions from these studies is complicated by methodological issues [112-113]. To reduce cardiovascular risk in patients with NAFLD, metabolic comorbidities including insulin resistance, hypertension, and dyslipidemia may be treated and monitored according to current protocols. In the Netherlands, the Dutch guidelines for general practitioners "cardiovascular risk management" may be consulted. Given the lack of evidence to show that patients with NAFLD and NASH are at increased risk for serious drug-induced liver injury from statins, statins can be used to treat dyslipidemia in patients with NAFLD and NASH, but until randomized controlled trials with histological end points prove their efficacy, statins are not recommended to be used to specifically treat NASH [109].

Given liver-related mortality is the third most common cause of mortality in NASH patients and prevalence of NASH is projected to increase substantially within the next decades, it is important to keep searching for pharmacological treatments. At present, there is no approved pharmacological therapy for NASH. Since lipotoxicity is considered a key player in mechanisms of hepatocellular injury in NAFLD, the focus of therapy for NAFLD and NASH in particular, should be to prevent or reverse hepatic injury induced by lipotoxicity. Therapies may be aimed at inhibition of lipid peroxidation and oxidative stress, or may have anti-inflammatory, anti-apoptotic or other hepatoprotective properties. Several hepatoprotective agents have been studied in recent years, including ursodeoxycholic acid, sartans, glitazones, vitamin E, betaine, omega 3 polyunsaturated fatty acids, pentoxyfilline and selective caspase inhibitors [114-116]. Most of these studies are limited by length of follow-up and small sample size as dual liver biopsies are difficult to obtain. To date, the majority of studies have shown controversial results. In addition, there is no knowledge on the long term effects and side effects of investigated drugs. Furthermore, several studies demonstrated that on discontinuation of drugs, like thiazolidiones, vitamin E, pentoxifylline and caspase inhibitors, liver biochemistry tests returned to baseline. Therefore, to date, almost none of the mentioned drugs have been implemented in current guidelines. A recent practice guideline from the American Association for the Study of Liver Diseases, American College of Gastroenterology, and American Gastroenterological Association, states that vitamin E (α -tocopherol) administered at daily dose of 800 IU / day improves liver histology in non-diabetic adults with biopsy-proven NASH and therefore it should be considered as a first-line pharmacotherapy for this patient population [109]. Furthermore, pioglitazone may be used to treat steatohepatitis in patients with biopsy-proven NASH. However, it is noted that the majority of the patients who participated in clinical trials that investigated pioglitazone for NASH were non-diabetic and that long-term safety and efficacy of pioglitazone in patients with NASH is not established.

Bariatric surgery

In selected patients with morbid obesity, long term weight reduction and improvement of NAFLD histology may be obtained by bariatric surgery [117-119]. In a meta-analysis of Mummadi et al, including 15 bariatric surgery studies (766 paired liver biopsies available), amelioration of steatosis was seen in 91.6% (95%CI 42.4%-90.8%) of patients, improvement of NASH in 81.3% (95%CI 61.9%-94.9%) of patients, and improvement of fibrosis in 65.5% (95%CI 38.2%-88.1%) of patients [118]. However, bariatric surgery is not without risks or complications [119].

Conclusion

NAFLD has become the most prevalent chronic liver disease is Western countries, in parallel with current epidemics in obesity and type II diabetes. Therefore, this condition concerns an increasingly relevant public health issue. NAFLD will most likely become the most common indication for LT in the next 10 to 20 years. Furthermore, another worrisome development concerns the increasing rate of HCC, which has been associated with NAFLD, independent of cirrhotic NASH. NAFLD is strongly associated with insulin resistance and metabolic risk factors. Although simple steatosis is a relatively benign condition, it has been associated with increased cardiovascular and all-cause mortality. NASH –fatty liver with inflammation and fibrosis- concerns a more advanced stage of NAFLD and has a worse prognosis. Therefore, it is important to discriminate simple fatty liver from NASH. This is preferably done by non-invasive techniques and not by liver biopsy. There are however, several pitfalls to the currently available non-invasive diagnostic techniques. Future studies are warranted to investigate the use of biomarker panels to discriminate between histological stages of NAFLD. To date, lifestyle modification, through exercise and weight loss efforts, is the cornerstone of management of NAFLD. This treatment regimen, however, is hard to sustain for many patients. There is no standard pharmacological therapy for NAFLD or NASH, although some treatments have shown to be of benefit on liver histology. Further research is necessary to determine long term efficacy and safety of these drugs. In addition, better understanding of pathophysiology and disease progression in NAFLD is needed to ensure that new therapies may be developed.

Aims and outline of the thesis

The aim of this thesis was to generate new insight into the prevalence of, risks of, and risk factors for NAFLD by means of clinical and epidemiological studies. **Chapter 1** describes the first results of a cross-sectional study on the prevalence of and risk factors for NAFLD in 2811 elderly participants of the population-based Rotterdam Study. Subsequently, in **Chapter 2**, we report on the association of liver enzyme levels with all-cause and cause specific mortality in

participants of the Rotterdam Study, with a maximum follow up of 19.5 years. In **Chapter 3** we validated the Fatty Liver Index, a non-invasive marker panel to predict NAFLD, enabling us to prospectively study risks of fatty liver in the nearby future. Furthermore, we studied the association of NAFLD with genetic factors in **Chapter 4**. In **Chapter 5,** we investigated the distribution of and factors associated with liver stiffness measurement in subjects with NAFLD. We studied biomarkers for the differentiation between simple steatosis and non-alcoholic steatohepatitis in **Chapter 6**. Finally, in **Chapter 7**, we studied the association between statin therapy and presence of NAFLD in a population-based study.

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CHAPTER 1

Prevalence of and risk factors for non-alcoholic fatty liver disease in the elderly: results from the Rotterdam Study

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Abstract

Background/Aims: The prevalence of non-alcoholic fatty liver disease (NAFLD) appears to increase with age. However, limited data are available concerning the prevalence of NAFLD in the elderly. Our objective was to determine the prevalence and risk factors of NAFLD in an elderly population. **Methods:** This study was based on participants of the population-based Rotterdam Study. Each participant was interviewed and had a clinical examination at the research center, including a fasting blood collection, liver ultrasonography and anthropometric assessment. Ordinal and logistic regression analysis were used to assess associations between covariables and (severity of) NAFLD. **Results:** Data from 2811 participants (mean age 76.4 ± 6.0 years) were analyzed. The prevalence of NAFLD was 35.1%. The prevalence of NAFLD decreased with advancing age (p<.001). In logistic regression analysis, age (OR 0.97; 95%CI 0.95-0.99; p<.001), total physical activity level (OR 0.98, 95% CI 0.96-0.99;p=.005), pack years of smoking (OR 1.01, 95%CI 1.00-1.01; p=.02), waist circumference > 88cm for women and >102 cm for men (OR 4.89; CI 4.00-5.96; p<.001), fasting glucose ≥100 mg/dL or drug treatment for elevated blood glucose (OR 2.11, 95%CI 1.72-2.59; p<.001), blood pressure ≥130/85 mmHg or drug treatment for elevated blood pressure (OR 1.80, 95%CI 1.08-3.01; p=.03), and triglycerides ≥150mg/dL or treatment with serum lipid reducing agents (OR 1.56, 95% CI 1.28-1.91; p<.001) were associated with NAFLD. **Conclusion:** NAFLD is common in the elderly, although the prevalence decreases with advancing age. Further studies are warranted exploring potential factors contributing to this apparent positive selection effect in the elderly.

Introduction

Non-alcoholic fatty liver disease (NAFLD) has become the most common chronic liver disease in Western countries in parallel with worsening epidemics of obesity and type II diabetes mellitus. NAFLD has been associated with an increased overall and cardiovascular mortality [1-3]. It has been projected that NAFLD will be the most common indication for liver transplantation in the next decades. In addition, global prevalence and impact of NAFLD are likely to increase as a result of population aging and increasing prevalence of obesity. Therefore, NAFLD constitutes a relevant public health issue.

The estimated prevalence of NAFLD in the adult general population ranges between 15 to 30 percent [4-8]. The prevalence of NAFLD appears to increase with age, especially through the fourth to sixth decade of life [9-10]. In two large studies, based on cohorts of the Dallas Heart Study and Framingham Heart Study, participants with fatty liver were significantly older than participants without fatty liver [5-6]. However, these studies only included a small number of participants older than 65 years. Prevalence and risk factors of NAFLD may vary in the elderly, as a result of metabolic changes at old age, including fat redistribution, and mitochondrial dysfunction [11-12].

The association of obesity, diabetes, dyslipidemia, and insulin resistance with NAFLD has been extensively investigated in adult subjects [5-6, 13-17]. These metabolic traits are now well-recognized risk factors for NAFLD. However, fewer data are available concerning the association of NAFLD with some environmental traits, including physical activity and smoking. Regarding the latter, studies have yielded contradictory results [18-19].

The aim of this study was to determine the prevalence of NAFLD in an elderly population and to generate insight into the association of NAFLD with metabolic risk factors, smoking, physical activity and markers of liver injury.

Subjects and methods

Study population

The Rotterdam Study is a large prospective population-based cohort study conducted among elderly inhabitants of Ommoord, a district of Rotterdam, The Netherlands. The rationale and study design have been described previously [20]. The medical ethics committee at Erasmus University of Rotterdam approved the study, and written informed consent was obtained from all participants.

Abdominal ultrasonography was added to the core protocol at the fifth survey of the Rotterdam Study (February 2009- February 2012), which constitutes the baseline survey for the present study. Each participant completed an extensive interview and clinical examination that included a fasting blood sample, liver ultrasonography, and anthropometric assessment.

Interview

The interview preceded the clinical examination and was designed to obtain data concerning demographics, medical history, comorbid conditions, smoking behaviour, physical activity, excessive alcohol intake (>14 drinks/week), and drug use. Detailed information on drug prescriptions was dispensed from automated pharmacies, where nearly all participants (98%) are registered. Physical activity was assessed using a validated questionnaire [21], which contained questions about walking, housekeeping activities, diverse sports, and hobbies. Durations of all activities were recalculated to hours/week (h/wk) and multiplied by activity expenditure costs, expressed in the ratio of work metabolic rate to resting metabolic rate (MET), estimated by a review of published and unpublished data by Ainsworth et al [22-23]. Vigorous activities included all activities with a MET-value ≥ 4 (e.g. cycling, swimming, gardening, and fitness), moderate activities included all activities with a MET-value between 2 and 4 (e.g. walking, light housekeeping activities, bowling, and volleyball). Highest attained educational level was assessed during the first survey that took place between 1990 and 1993 and was split into three categories: lower, intermediate or higher education. Data concerning current and past smoking behaviour was obtained by questionnaire. Pack years was calculated as years of smoking (excluding years of non-smoking) multiplied by the average number of packs smoked per day. A pack contains 20 cigarettes.

Biochemistry

Fasting blood samples were collected on the morning of ultrasound examination. Blood lipids, 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, HBsAg and anti-HCV antibodies were measured by automatic immunoassay (Roche Diagnostics GmbH, Mannheim, DE).

Diagnosis and grading of NAFLD

Abdominal ultrasonography was performed by certified and experienced technicians on Hitachi HI VISION 900 in all study participants. Images were stored digitally and re-evaluated by a hepatologist with more than five years experience in ultrasonography. The diagnosis and grading of fatty liver was determined by the ultrasound technician according to the protocol by Hamaguchi et al [24]. 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). Individuals with any of the following possible secondary causes of fatty liver were excluded from the analyses: 1) excessive alcohol consumption 2) positive serum HBsAg or anti-HCV, and 3) use of pharmacological agents historically associated with fatty liver (i.e. amiodarone, corticosteroids, methotrexate, and tamoxifen).

Metabolic covariables

Anthropometric measurements were performed by well trained nurses. Body Mass Index (BMI) was calculated as weight (kg)/ length (m^2). Waist and hip circumference were measured in centimeters. The average of two blood pressure measurements, obtained at a single visit in sitting position after a minimum of 5 minutes rest, was used for analysis. The metabolic syndrome was defined, according to Adult Treatment Panel III criteria [25], 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 <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. Diabetes was defined as fasting plasma glucose (FPG) ≥126 mg/dL (7.0 mmol/L) or drug treatment for elevated blood glucose. 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 [26]. Fatty Liver Index (FLI), a validated algorithm to detect fatty liver, based on BMI, waist circumference, triglycerides, and GGT was calculated by the formula previously described by Bedogni *et al* [27]. We used a cut-off of ≥60 to rule in hepatic steatosis.

Statistical analysis

Baseline analyses 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 NAFLD or severity of NAFLD we performed logistic or ordinal logistic regression analyses respectively. Tests for parallel lines in ordinal regression analysis were performed. Models were also tested for interaction. A p-value of <.05 was considered as statistically significant. Statistical analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

Results

Study population

A total of 3205 participants underwent abdominal ultrasonography. Three hundred ninety-four participants were excluded (excessive alcohol consumption (n= 255), positive HBsAg (n=3) or anti-HCV (n=24), use of pharmacological agents historically associated with fatty liver (n=121)). The total number of eligible study participants was 2811.

Table 1. General characteristics of participants with and without non-alcoholic fatty liver disease

	total (n=2811)	no NAFLD (n=1825)	NAFLD (n=986)	p-value*
	100	*64.9*	*35.1*	
Covariables				
Age (years)	76.4 (±6.0)	76.7 (±6.3)	75.8 (±5.4)	< .001
Female (%)	60.2	59.3	61.8	.2
Caucasian (%)	95.5	95.7	95.0	.4
BMI (kg/m2)	27.4 (±4.2)	26.0 (±3.4)	30.0 (±4.2)	<.001
Normal; BMI < 25 (%)	29.4	40.2	11.5	
Overweight; 25 ≤ BMI < 30 (%)	47.2	48.4	44.8	
Obese; BMI ≥ 30 (%)	23.4	11.4	45.6	
Waist/Hip-ratio	0.90 (±0.09)	0.88 (±0.09)	0.93 (±0.09)	<.001
Alcohol intake (drinks/week)	3.7 (±3.7)	3.7 (±3.6)	3.7 (±3.8)	.6
Physical activity (MET-h/wk)[1]	47.3 (25.6-78.5)	51.3 (28.0-82.5)	42.1 (21.6-68.1)	<.001
Moderate activities (MET-h/wk)	27.0 (13.6-44.6)	25.6 (11.8-42.0)	26.5 (12.8-42.3)	.001
Vigorous activities (MET-h/wk)	12.0 (1.5-29.0)	13.5 (2.1-32.0)	9.5 (0.0-24.0)	<.001
Smoking				.003
Never (%)	36.6	38.5	33.1	
Former (%)	54.9	52.5	59.3	
Pack years	23.0 (11.7-37.9)	21.0 (10.4-35.0)	26.0 (13.8-40.0)	<.001
Current (%)	8.5	9.0	7.6	
Pack years	26.1 (15.8-42.9)	26.5 (15.7-41.6)	25.4 (15.7-45.2)	.9
Educational level				<.001
Low (%)	46.1	42.9	52.0	
Intermediate (%)	37.9	39.3	35.1	
High (%)	16.0	17.8	12.8	
Hypertension (%)	86.4	83.5	91.4	<.001
Diabetes Mellitus (%)	14.7	9.4	24.5	<.001
Metabolic syndrome (%)	54.5	43.6	74.5	<.001
Fasting glucose >100 mg/dL or drug treatment for elevated blood glucose	49.2	40.8	64.7	<.001
Waist circumference >88cm (♀) or >102 cm (♂)	42.3	27.5	70.1	<.001
Triglycerides >150 mg/dL or drug treatment for elevated triglycerides	44.5	38.0	56.4	<.001
HDL-C <40 mg/dL(♂) or <50 mg/dL(♀) or drug treatment for low HDL-C	42.7	37.2	53.1	<.001
BP ≥130/85 mmHg or drug treatment for elevated BP	93.7	92.1	96.7	<.001
Fatty Liver Index	42 (23-67)	31 (17-50)	67 (49-83)	<.001
<30/ ≥ 60	34.9/32.6	49.0/17.7	8.5/60.4	

Table 1. (continued)

	total (n=2811)	no NAFLD (n=1825)	NAFLD (n=986)	p-value*
Laboratory data				
ALT (U/L)	18 (14-23)	17 (14-21)	21 (16-27)	<.001
AST (U/L)	25 (22-28)	25 (22-28)	25 (22-29)	.06
GGT (U/L)	22 (17-32)	21(16-29)	26 (20-37)	<.001
HOMA-IR	2.6 (1.8-4.0)	2.2 (1.5-3.2)	3.9 (2.7-5.9)	<.001

Data are represented as mean (± standard deviation), median (25^{th}-75^{th} percentile) or percentages.
*Based on T-test, Wilcoxon rank sum test or Chi-square test.
[1]complete data on physical activity were available for 2388 participants.
Abbreviations: BMI, Body Mass Index; MET-h/wk, metabolic task hours per week; HDL-C, high-density lipoprotein cholesterol; BP, blood pressure; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma glutamyl transferase; HOMA-IR, Homeostasis Model Assessment of Insulin Resistance.

General characteristics are shown in Table 1. Sixty percent of the study population were women. Mean age of participants was 76.4 ± 6.0 years (range 65.3-98.7 years) and mean BMI was 27.4 ± 4.2 kg/m^2. Participants were predominantly of Caucasian ethnicity (95.5%).

Prevalence of NAFLD

The overall prevalence of nonalcoholic fatty liver, assessed by ultrasound, was 35.1%. The vast majority (84.4%) had moderate to severe fatty liver. The prevalence of NAFLD was lower with advancing age (Figure 1; p<.001). NAFLD was found in 35.8% of participants <70 years (n=455), in 36.6% of participants aged 70-74 (n=809), in 39.6% of participants aged 75-79 (n=787), in 32.1% of participants aged 80-84 (n=495) and in 21.1% of participants of age ≥85 (n=265). FLI was also significantly lower with advancing age (p<.001). FLI was ≥60 in 35.5% of participants <70 years, in 33.6% of participants aged 70-74, in 33.5% of participants aged 75-79, in 31.0% of participants aged 80-84 and in 24.3% of participants of age ≥85. Age remained significantly associated with NAFLD after adjustment for gender, alcohol consumption, total physical activity level, educational level, smoking status, and all metabolic syndrome criteria in logistic regression analysis (OR 0.97, 95% CI 0.95-0.99; p<.001). Thirty-six percent of women had NAFLD, versus 33.7% of men (p=.2). The prevalence of NAFLD in non-obese participants was 24.9%.

Association between NAFLD and metabolic features

The metabolic syndrome was present in 54.5% of the total population. Of metabolic covariables, impaired fasting glucose, hypertension and decreased HDL levels were more prevalent in participants ≥ 75 years (p-values <.007). Increased waist circumference was more frequent in women than in men (49.3% vs. 31.8%; p<.001), whereas impaired fasting glucose was more frequent in men than in women (54.5% vs. 45.7%; p<.001).

In univariable analysis all metabolic/ anthropometric traits were significantly associated with NAFLD and severity of NAFLD. In logistic regression analysis -after adjustment for age, gender, educational level, pack years of smoking, total physical activity level and alcohol consumption-,

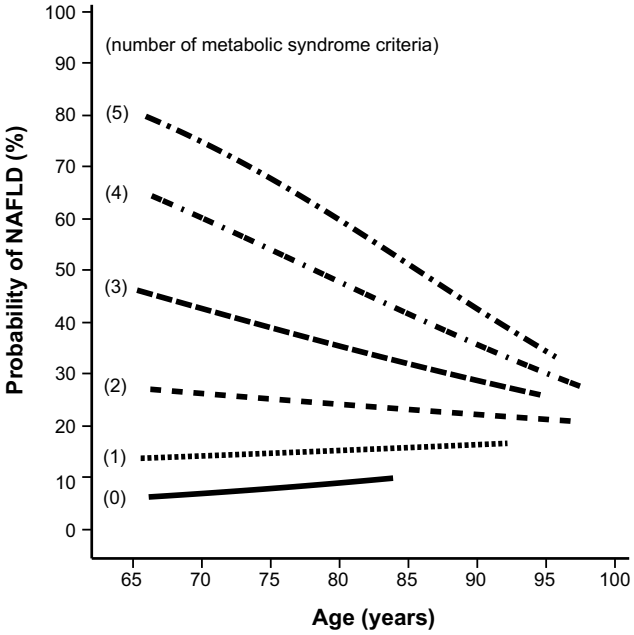

Fig. 1. Association of the sum of metabolic syndrome criteria with probability of nonalcoholic fatty liver disease for age. At high age metabolic features are significantly associated with NAFLD (*P*<.001). However, the association between the number of metabolic syndrome criteria and probability of NAFLD weakens with advancing age (*P*=.002).

waist circumference > 88cm for women and >102 cm for men (OR 4.89; CI 4.00-5.97; p<.001), fasting glucose ≥100 mg/dL or drug treatment for elevated blood glucose (OR 2.11, 95%CI 1.72-2.59; p<.001), blood pressure ≥130/85 mmHg or drug treatment for elevated blood pressure (OR 1.80, 95%CI 1.08-3.01; p=.03), and triglycerides ≥150mg/dL or treatment with serum lipid reducing agents (OR 1.56, 95% CI 1.28-1.91; p<.001) were independent predictors of NAFLD (Table 2). Lowered HDL cholesterol was no longer associated with NAFLD. There was no interaction between age and alcohol consumption in multivariable analysis (p=.6). Substitution of metabolic syndrome criteria for continuous data in logistic regression did not alter results. When each metabolic syndrome criterion and the aggregate metabolic syndrome diagnosis was analyzed separately, adjusting only for age, gender, educational level, pack years of smoking and alcohol consumption, OR's decreased for all variables with increasing age. However, there was only significant interaction between age and fasting glucose ≥100 mg/dL (p=.044), lowered HDL cholesterol (p=.003), waist circumference (p<.001), and the aggregate metabolic syndrome (p=.02).

Participants meeting more metabolic syndrome criteria were more likely to have NAFLD (no criteria OR (= reference) 1.00 (95% CI); one criterion OR 3.22 (0.98-10.60); two criteria OR 8.70 (2.68-28.23); three criteria OR 13.80 (4.25-44.73); four criteria OR 20.13 (6.20-65.42); five criteria OR 49.65 (15.12-163.04)). This association weakened with increasing age (*P*=.002; Figure 1).

Table 2. Multivariable adjusted model for non-alcoholic fatty liver disease

	Normal Liver vs. NAFLD	
	OR (95% CI)	p-value*
Age (years)	0.97 (0.95-0.99)	<.001
Gender (female)	0.83 (0.67-1.03)	.3
Educational level (low)	1.14 (0.94-1.40)	.2
Physical activity (MET-h/day)	0.97 (0.96-0.99)	.005
Smoking, pack years	1.01 (1.00-1.01)	.02
Alcohol intake (drinks/week)	0.99 (0.96-1.02)	.4
Fasting glucose >100 mg/dL or drug treatment for elevated blood glucose	2.11 (1.72-2.59)	<.001
Waist circumference >88 cm (♀) or >102 cm (♂)	4.89 (4.00-5.96)	<.001
Triglycerides >150 mg/dL or drug treatment for elevated triglycerides	1.56 (1.28-1.91)	<.001
HDL-C <40 mg/dL(♂) or <50 mg/dL(♀) or drug treatment for low HDL-C	1.07 (0.82-1.40)	.6
BP ≥130/85 mmHg or drug treatment for elevated BP	1.80 (1.08-3.01)	.03

*Based on value of *P* associated with the likelihood ratio test.
Abbreviations: MET-h/day, metabolic task hours per day; HDL-C, high-density lipoprotein cholesterol; BP, blood pressure.

Insulin resistance –calculated by HOMA-IR- also showed a strong association with presence and severity of NAFLD in elderly (p-values <.001). HOMA-IR decreased with increasing age, after age 80.

Association between NAFLD and physical activity

Total time spent on physical activity in men was 12.0 h/wk versus 16.0 h/wk in women (p<.001). Higher total physical activity level (in MET-h/wk) was associated with a lower prevalence of NAFLD (p<.001; Figure 2). Total physical activity level remained inversely associated with prevalence and severity of NAFLD after adjustment for age, BMI and gender in logistic and ordinal regression analysis (p=.002 and p=.005 respectively). There was no effect modification of total physical activity level and these covariables. MET-h/wk spent on vigorous activities were also inversely correlated with NAFLD (p=.01), as were MET-h/wk spent on moderate activities (p=.02). Predicted probability of NAFLD decreased by 11% for every 3 MET-h/day (equal to half an hour of cycling per day) spent on vigorous physical activity and by 9% for every 3 MET-h/day spent on moderate activities (equal to an hour of walking per day).

Association between NAFLD and smoking

The overall prevalence of current smoking was 8.5%. Fifty-five percent of participants were former smokers. Nine percent of women and 7.8% of men were current smokers (p=.3). More men (70.9%) than women (44.3%) were former smokers (p<.001). No significant association between

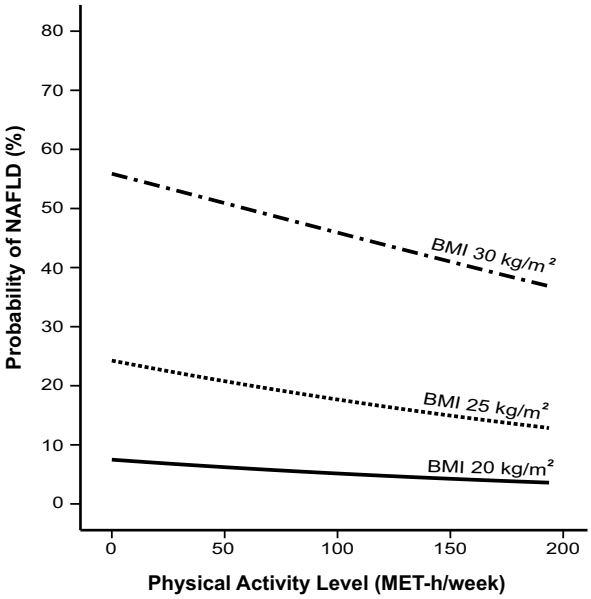

Fig. 2. Association of total physical activity level with probability of nonalcoholic fatty liver disease in the elderly. Results are illustrated for mean age (76.4 years).

current smoking and NAFLD was observed. Median pack years in current smokers with NAFLD was 25.4 (IQR 15.7-45.2) versus 26.5 (IQR 15.7-41.6) in smokers without NAFLD (p=.9). In univariable analysis there was no association between NAFLD with pack years of smoking in current smokers (p=.6), whereas there was a statistically significant association observed for pack years of smoking in former smokers and former and current smokers combined (p-values <.001). In multivariable analysis adjusting for metabolic features, education, physical activity level, age and gender, smoking status was not associated with NAFLD, whereas pack years of smoking in former and current smokers was associated with higher probability of NAFLD (OR 1.01, 95%CI, 1.00-1.01;p=.02). Similar results were attained when using ordinal regression analysis to assess associations between smoking and severity of NAFLD. There was no interaction between BMI and smoking status (p=.8).

Association between NAFLD and liver enzymes

Participants with NAFLD had higher serum ALT and GGT levels than participants without NAFLD (p-values <.001). ALT and GGT were also significantly associated with severity of NAFLD independent of age and gender. Median ALT in participants without NAFLD was 16 U/L (IQR 13-20) in women versus 18 U/L (IQR 14-23) in men, whereas median ALT in participants with NAFLD was 19 U/L (IQR 16-25) in women versus 24 U/L (IQR 18-30) in men. The 95% percentile of ALT in participants with NAFLD was higher than that in participants without NAFLD, notably 45 U/L versus 30 U/L respectively. Eighty-eight percent of participants with NAFLD had a normal serum ALT level, defined according to local guidelines (ALT <31 U/L for women and <41 U/L for

men). Sixty-two percent of participants with NAFLD had ALT <19 U/L for women and <30 U/L for men.

Discussion

The present study demonstrated that NAFLD is prevalent in more than one-third of the elderly and shows strong association with metabolic traits, including abdominal obesity, dyslipidemia, and impaired fasting glucose. Furthermore, we observed a decreasing prevalence of NAFLD with advancing age, suggesting positive selection of elderly without NAFLD.

This is the first large population-based study to describe the prevalence of, and risk factors for NAFLD in the elderly. Although several studies reported an increasing prevalence of NAFLD with advancing age, only few elderly participants were included [9-10]. To date, only one small study has investigated the prevalence of NAFLD in an aged population [28]. The authors of this study demonstrated that 46.2% of 91 octogenarian patients had NAFLD, as diagnosed by ultrasonography. However, this study concerned a selected study population, as subjects were recruited from rehabilitation departments of a geriatric hospital.

The prevalence of NAFLD we observed was remarkably higher than reported in previous population-based studies. In unselected adult populations, the estimated prevalence of NAFLD ranges between 15-30% [4-8]. NAFLD, detected by computed tomography, was found in 17% of subjects drawn from The Framingham Heart Study (mean age 51 years). In that study only 31.5% of subjects met the definition of metabolic syndrome, whereas 54.5% of participants in our study had the metabolic syndrome. Therefore, the discrepancy in prevalence of NAFLD may simply be attributed to an increased prevalence of metabolic traits in elderly compared to adults. At old age, dysfunction of preadipocytes impairs the capacity of fat tissue to store lipids [11, 29]. The consequent increase in systemic free fatty acids is postulated to aggravate metabolic disease and cause fat redistribution from subcutaneous to intra-abdominal visceral depots as well as other ectopic sites, including the liver [30].

A compelling observation in our study concerned the lower prevalence of NAFLD with advancing age. Firstly, the observed lower prevalence of NAFLD at old age may be the result of selective mortality. NAFLD has been independently associated with an increased overall and cardiovascular-related mortality [1, 31-32]. Therefore, the present study may reflect this association. Genetic, inflammatory and anti-oxidant profiles may have favoured selection of participants, in spite of presence of metabolic risk factors [33]. Secondly, lower prevalence of fatty liver in octogenerians may result from a lower prevalence of insulin resistance and metabolic syndrome in these participants. A lower prevalence of insulin resistance and metabolic syndrome with increasing age in the elderly has been demonstrated in previous studies [34]. However, a causal relationship may not be clarified by this study, given its cross-sectional design. Thirdly, increased fibrosis correlates with loss of fatty infiltration [35]. Accordingly, fibrosis may be worse

in the 'older old' than in the 'younger old', resulting in a lower prevalence of NAFLD in octo- and nonagenarians. In a retrospective cohort study including 351 patients with biopsy proven NAFLD, older patients had significantly more fibrosis on biopsy with less steatosis [36]. However, participants with more fibrosis also had more metabolic risk factors, which was not true for the older participants in our study. We do not have information on liver biopsy, the golden standard for assessment of necroinflammation and liver fibrosis, as this is unethical to perform in a population-based setting. Nevertheless, ultrasound characteristics of severe fibrosis and/or cirrhosis, including irregular liver surface, signs of portal hypertension and altered hepatic configuration, were not more prevalent with advancing age in our cohort (data not shown). Finally, dietary composition or intake may vary with age, which in turn may influence prevalence of NAFLD in the highest age categories. Unfortunately, we do not have information on diet of participants.

The association of NAFLD with metabolic traits weakened with advancing age. This effect was most pronounced after surpassing the age of 80 years. These findings suggest that metabolic risk factors may play a less important role in the pathophysiology and susceptibility of NAFLD in the very old. Undefined age-related mechanisms possibly have a larger contribution in fat accumulation. At high age, muted responses to oxidative stress, and altered endocrine functioning may affect mechanisms of fatty infiltration [37-38].

Although there is a strong theoretical basis supporting physical activity as a lifestyle change in patients with NAFLD, epidemiological evidence of an independent association is limited. We demonstrated that physical activity level was independently associated with a decreased prevalence of NAFLD in elderly participants. Moreover, total expenditure on activities with vigorous intensity showed stronger association with lower probability of NAFLD than activities with moderate intensity. Our results are in agreement with previous small cross-sectional and retrospective studies in adults [39-43].

Recently, experimental studies established a link between NAFLD and smoking [44-45]. Only few studies in humans have investigated this relationship, with contrasting results [18-19, 46-47]. Although there was no association between smoking status with NAFLD, pack years of smoking for former and current smokers combined was independently associated with NAFLD in the present study. However, this study does not provide definite evidence that smoking exacerbates NAFLD or results in a higher risk of NAFLD, since the study has a cross-sectional design.

We found that serum ALT levels were significantly higher in participants with NAFLD than in participants without NAFLD. Therefore, this study provides further support, that subjects with hepatic steatosis on ultrasound should be excluded from studies to establish upper reference levels of serum transaminases [48]. Moreover, we demonstrated that serum ALT was an insensitive marker of NAFLD in elderly, since the vast majority of participants (88%) with NAFLD had normal ALT levels, defined by local guidelines. Even when applying more strict upper limits of normal for ALT [49], sensitivity of ALT for detecting NAFLD was merely 62%.

The strength of the present study concerns the large number of elderly participants that were included. Extensive data were available for characterization of metabolic and environmental traits. Moreover, the district where the Rotterdam Study is conducted has a social-economic structure adequately corresponding with that of the overall Dutch population. Our study was also subject to potential limitations. Firstly, NAFLD was diagnosed by means of ultrasonography, which may underestimate the prevalence of NAFLD, for it is unable to identify a degree of steatosis of less than 30%. Nevertheless, abdominal ultrasonography has an acceptable sensitivity of 80-100% for detecting fatty liver, and its accuracy for diagnosis of fatty liver meets other imaging modalities [50-52]. Secondly, our study mainly concerned elderly Caucasian participants. Therefore, our results may not be generalized to other age groups and ethnicities. Finally, we cannot exclude a selection bias as non-responders may have had higher morbidity. Volunteers tend to be better educated, healthier, and lead better lifestyles[53]. Consequently, we may have underestimated the prevalence of NAFLD in elderly.

In conclusion, NAFLD is common in the elderly. In the current setting of greater longevity and increasing prevalence of obesity, we found that the prevalence of NAFLD in the elderly appears to decrease with advancing age. Further studies are warranted exploring potential factors contributing to this apparent positive selection effect in the elderly.

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CHAPTER 2

Serum liver enzymes are associated with all-cause mortality in an elderly population

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Submitted

Abstract

Background: Little is known about the association of serum liver enzymes with long-term outcome in the elderly. **Aim:** We sought to clarify the association of serum gamma-glutamyltransferase (GGT), alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) with all-cause and cause-specific mortality in an elderly population. **Methods:** This study was embedded in the Rotterdam Study, a large population-based cohort of persons aged 55 years or older. Cox-regression analyses were performed to examine the association of baseline serum GGT, ALP, and aminotransferase levels with mortality, adjusted for age, sex, education, smoking status, alcohol intake, hypertension, diabetes mellitus, body mass index, and total cholesterol levels. Liver enzyme levels were categorized according to sample percentiles, levels <25th percentile were taken as a reference. **Results:** During a follow-up of up to 19.5 years, 2997 of 5186 (57.8%) participants died: 672 participants died of causes related to cardiovascular diseases (CVD) and 703 participants died of cancer. All liver enzymes were associated with increased all-cause mortality (all p-values <.001). Moreover, GGT was associated with increased CVD mortality (p<.001), and ALP and AST with increased cancer-related mortality (p=.03 and p=.005 respectively). Participants with GGT and ALP in the top 5% had the highest risk for all-cause mortality (HR 1.55; 95%CI 1.30-1.85 and HR 1.49; 95%CI 1.25-1.78, respectively). AST and ALT <25th percentile were also associated with a higher risk of all-cause mortality. **Conclusions:** All serum liver enzymes were positively associated with long-term mortality in this elderly population. Why participants with low ALT and AST levels have higher risk of mortality remains to be elucidated.

Introduction

Serum gamma-glutamyltransferase (GGT), alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels are commonly used markers of liver injury. ALT is predominantly found in the liver, whereas GGT and AST are expressed in multiple other tissues, including heart, skeletal muscle, kidneys, and brain [1]. ALP is also present at many locations, but is primarily expressed in liver and bone [2]. GGT is responsible for the extracellular catabolism of glutathione, a thiol antioxidant that protects specific components of mammalian cells from damage by reactive oxygen species [3-4]. ALP catalyzes the hydrolysis of organic phosphate esters and AST and ALT catalyze the transfer of amino groups to form the hepatic metabolites pyruvate and oxaloacetate, respectively.

Elevations of serum liver chemistry tests are reported in up to 24.5% of individuals in Western community- or population-based studies [5-7]. The majority of liver test abnormalities may be attributed to the presence of alcoholic and nonalcoholic fatty liver disease [8-10]. Additionally, elevations of ALP are associated with cholestatic liver diseases and bone diseases. However, a considerable proportion of liver enzyme elevations remains unexplained [11].

Currently, there is increasing interest for the role of liver enzymes as independent predictors of non liver-related morbidity and mortality. Elevations of GGT, ALT, and AST have been associated with a higher incidence of cancer and CVD, and higher risk of overall and CVD-related mortality [6, 12-13]. To date, two studies examined the association between elevation of ALP and mortality [14-15]. However, only few studies investigated associations of the whole range of liver biochemistries with mortality [16-19]. Moreover, the aging population is the greatest consumer of health care, and given the majority of studies included mainly younger adults, the role of these inexpensive blood tests as predictors of mortality in the elderly remains to be determined.

The aim of this study is to investigate the association of serum GGT, ALP, ALT and AST levels with all-cause and cause-specific mortality in an elderly population.

Materials and methods

Study population

The current study was embedded in the Rotterdam Study, a large prospective population-based cohort study, with the objective to examine the occurrence and risk factors of chronic diseases in the elderly. The study design and objectives have been described in detail previously [20]. From 1990 to 1993, all inhabitants of the Ommoord district of Rotterdam, the Netherlands, aged 55 years or older, were invited for participation. Of 10,275 invitees 7,983 (78%) agreed to participate. During baseline examinations participants had an extensive at home interview and subsequently visited the research center for a clinical examination and blood collection. The

medical ethics committee at Erasmus University of Rotterdam approved the study, and written informed consent was obtained from all participants.

Serum liver chemistry tests

Non-fasting and fasting blood samples were collected by venapuncture, and immediately frozen (−20ºC). Serum GGT, ALP, ALT and AST levels were determined within two weeks using a Merck Diagnostica kit on an Elan Autoanalyzer (Merck). All liver biochemistry measurements were obtained in the laboratory of the Department of Epidemiology, Erasmus University Medical Center. Non-fasting samples were considered acceptable, for normal food-intake does not greatly affect serum liver enzyme levels [21-22]. Liver enzyme tests were only performed until December 31, 1992, when they were stopped because of financial constraints. According to local cut-offs elevation of GGT was defined as >34U/L for women and >49U/L for men, and elevation of ALP was defined as >97U/L for women and >114U/L for men. Elevation of ALT was defined as >30U/L for women and >40U/L for men and elevation of AST was defined as >30 U/L for women and >36 U/L for men.

Assessment of mortality

Information on vital status was obtained on a regular basis from the central registry of the Municipality of Rotterdam, from collaborating general practitioners and by obtaining information during follow-up rounds. The Central Registry of Genealogy of the Netherlands was consulted for data on participants with missing information on vital status. Two research physicians independently classified events according to the International Classification of Diseases, 10th revision (ICD-10) [23]. We used the underlying cause of death, which is the disease or injury, which initiated the train of events leading directly to death. In case of disagreement, consensus was reached in a separate session. A medical expert in the field reviewed all coded events for a final classification. The following ICD codes were considered for cause-specific mortality: 1) *CVD*: I20-25, I42, I46, I50, I 63, I66, I67.2, I67.8, I69.3, I70, I70.9, I74; 2) *cancers*: all C-codes. Liver diseases had ICD codes K70, K72, or K73.

Participants were followed up until death or January 1st, 2009, whichever came first.

Assessment of covariables

In the interview preceding the clinical examination data was obtained concerning demographics, medical history, comorbid conditions, smoking behaviour, and drug use. Additionally, detailed information on drug prescriptions was dispensed from local pharmacies. Weekly alcohol consumption was obtained by means of a validated food-frequency questionnaire and recalculated into grams per day. Anthropometric measurements were performed at the research center. BMI was calculated as weight (kg)/ height (m^2). Waist and hip circumference were measured in centimeters. The average of two blood pressure measurements, obtained at a single visit in sitting position after 5 minutes rest, was used for analysis. Hypertension was

defined as blood pressure ≥140/90 mmHg or drug treatment for elevated blood pressure. Diabetes mellitus was defined as fasting plasma glucose ≥126 mg/dL (7.0 mmol/L) or non-fasting plasma glucose ≥200 mg/dL (11.1 mmol/L) or drug treatment for elevated blood glucose. Cholesterol levels were defined as desirable (<200 mg/dL or <5.1 mmol/L), borderline high (200-240 mg/dL or 5.1-6.1 mmol/L), or high (≥240 mg/dL or ≥6.2 mmol/L), according to Adult Treatment Panel (ATP) III criteria. Highest attained educational level was grouped according to the Dutch Standard Classification of Education and split into three categories: lower (primary education only), intermediate (lower vocational or general education) or higher education (intermediate or higher vocational or general education, university) [24]. Presence of CVD at baseline was defined as prior myocardial infarction, stroke, coronary artery bypass graft, or percutaneous transluminal coronary angioplasty. This information was initially self-reported and later confirmed using physician or hospital records or an electrocardiogram showing characteristics of prior myocardial infarction.

Statistical analyses

Baseline analyses 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. Serum GGT, ALP, ALT and AST levels were categorized into percentiles for men and women separately (<25^{th}, 25^{th}-<50^{th}, 50-<75^{th}, 75^{th}-<95^{th} and ≥95^{th} percentile). To reduce bias, missing values on covariables were imputed by multiple imputation applying the Markov chain Monte Carlo method. All variables, covariables and outcome variables were used to generate imputations of missing values and 10 datasets were imputed (MI procedure and MI analyze procedure)[25-26]. The Cox proportional hazard model was used to evaluate associations between serum GGT, ALP, ALT and AST levels and all-cause or cause-specific mortality. After adjustment for age and sex, further models were adjusted for education, smoking status, alcohol intake, hypertension, diabetes mellitus, BMI, and total cholesterol levels. Age was modelled as a continuous variable. Covariables like smoking status and hypertension were replaced by continuous variables (packyears and both systolic and diastolic blood pressure, respectively) in additional analyses to check for consistency of the model. In addition, BMI was replaced by waist circumference, for there is collinearity between these two covariables. Liver enzyme levels <25th percentile were taken as a reference. Models were tested for interaction with age, sex, BMI and alcohol intake. To adjust for multiple testing, level for interaction was set to 0.01. Additional sensitivity analyses were performed excluding 1) participants with cardiovascular disease at baseline, 2) former drinkers, 3) participants with >8 times the upper limit of normal of serum liver biochemistries (for these participants may have had acute hepatitis), and 4) participants that died during the first three years of follow-up (because these participants may have had underlying terminal illness). Furthermore, covariables that might be associated with ALP levels, including haemoglobin levels and serum phosphate levels, were added to models of ALP. A cubic spline regression was performed to

illustrate the association of the continuous liver test measurements with all-cause mortality. Knots were placed at the 5th, 27.5th, 50th, 72.5th and 95th percentile; graphs were plotted using the SAS LGTPHCURV9 Macro [27-28]. SAS version 9.2 (SAS Institute Inc, Cary, NC, USA) was used for all statistical analyses. A p-value of <.05 was considered statistically significant.

Results

Baseline characteristics

Serum GGT, ALP, ALT, and AST levels were tested for 5186 participants. Baseline characteristics are illustrated in Table 1. Mean age of participants was 70.3 ± 9.1 years (range: 55-99 years) and 61.6% of the population was female. The vast majority of the population was of Caucasian ethnicity. According to local cut-offs of liver enzyme levels 15.1% of the population had elevated GGT, 13.8% had elevated levels of ALP, 5.2% had elevated levels of ALT, and 4.2% had elevated levels of AST. Values for percentiles of each liver enzyme in the study population are shown in Table 2.

Association of liver enzymes with established risk factors

In univariable analysis higher serum GGT levels were associated with lower age, male sex, presence of diabetes mellitus, hypertension, and current smoking, and higher BMI, cholesterol levels and alcohol intake (Table 3). Higher serum ALP levels were associated with higher age, female gender, presence of diabetes mellitus, current smoking and hypertension, lower cholesterol levels, and lower alcohol intake. Serum ALT levels were inversely associated with age and positively associated with female sex, current smoking, presence of diabetes mellitus, hypertension and alcohol intake. Finally, AST levels were inversely associated with age, and positively associated with male gender, hypertension, and alcohol intake.

Mortality in the study population

Median follow-up was 14.0 years (interquartile range (IQR) 6.8-16.3 years, with a maximum of 19.5 years). During follow-up 2997 (57.8%) participants died; 672 participants died of cardiovascular related causes (22.4% of total deaths) and 703 participants died of cancer (23.4% of total deaths). Stroke was the cause of death in 9.9% of total deaths and ischemic heart disease in 5.3% of total deaths. Eight percent of total deaths were due to neurodegenerative diseases and 6.5% due to diseases of the respiratory system. Five participants died from liver diseases (4 deaths were alcohol-related) and 7 participants died from primary liver cancer (hepatocellular carcinoma: 3; cholangiocarcinoma: 4).

Association of cholestatic liver enzymes with all-cause and cause-specific mortality

GGT was positively associated with all-cause mortality (p<.001) and CVD-related mortality (p<.001) after adjustment for age, sex, and all other potential confounders, if studied

Table 1. Baseline characteristics of the study population (n=5186)

Characteristic	
Age (years)	70.3 ± 9.1
Female	61.6
Caucasian	91.7
Education, low	56.3
Smoking	
Never	34.1
Former	39.5
Current	23.0
BMI (kg/m^2)	26.3 ± 3.8
Diabetes Mellitus	8.1
Hypertension	56.8
Total Cholesterol	
< 200 mg/dL	10.7
200-239 mg/dL	25.5
≥ 240 mg/dL	63.8
Alcohol intake	
Non-drinker	36.1
≤ 10 grams/day	29.4
>10 ≤ 30 grams/day	24.8
>30 grams/day	9.7
Cardiovascular disease	33.6
Inorganic phosphate (mg/dL)	3.7 (3.3-4.1)
Haemoglobin (g/dL)	14.1 (13.2-15.0)
GGT (U/L)	23 (17-32)
ALP (U/L)	76 (63-91)
ALT (U/L)	16 (12-20)
AST (U/L)	19 (16-22)

Values are mean ± standard deviation, percentage, or median (interquartile range).
Abbreviations: BMI, Body Mass Index; eGFR, estimated glomerular filtration rate; GGT, gamma-glutamyltransferase; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

Table 2. Percentile values of serum GGT, ALP, ALT and AST levels for men and women in the study population.

		25th percentile	50th percentile	75th percentile	95th percentile
GGT (U/L)	male	20	26	37	79
	female	16	21	28	58
ALP (U/L)	male	61	73	89	119
	female	64	77	92	124
ALT (U/L)	male	13	17	22	35
	female	12	15	19	33
AST (U/L)	male	17	20	33	31
	female	16	19	22	30

Table 3. Association between liver enzymes and covariables in univariate analyses. Illustrated are correlation coefficients and standard errors.

Variable	GGT (U/L)	ALP (U/L)	ALT (U/L)	AST (U/L)
Age, years	–0.24 (0.05)**	0.31 (0.04)**	–0.21 (0.02)**	–0.04 (0.02)*
Sex, female	–8.10 (0.88)**	3.50 (0.78)**	1.78 (0.36)**	–0.95 (0.37)*
Smoking, current	4.40 (1.03)**	3.03 (0.89)**	–0.13 (0.40)	–0.37 (0.41)
BMI, kg/m2	0.49 (0.11)**	–0.10 (0.10)	0.40 (0.05)**	0.04 (0.05)
Waist circumference, cm	0.37 (0.04)**	0.09 (0.04)*	0.17 (0.02)**	0.04 (0.02)*
Diabetes Mellitus	9.03 (1.68)**	9.28 (1.43)**	3.24 (0.68)**	–0.22 (0.71)
Hypertension	2.57 (0.87)**	2.47 (0.75)**	0.92 (0.33)**	0.90 (0.36)*
Total cholesterol mg/dL	0.86 (0.35)*	–0.93 (0.31)**	0.05 (0.14)	–0.15 (0.15)
Alcohol intake(grams/day)	0.38 (0.03)**	–0.13 (0.03)**	0.11 (0.01)**	0.11 (0.01)**

**Correlation is significant < 0.01 level
*Correlation is significant < 0.05 level

continuously (Table 4). Figure 1a illustrates adjusted hazard ratios (HR) and 95% confidence intervals (CI) for GGT percentiles. The HR for participants with GGT ≥95th percentile (58U/L for women and 79U/L for men) was 1.62 (95% CI 1.36-1.92) after adjustment for age and sex, and 1.55 (95% CI 1.30-1.85) after adjustment for all other potential confounders. When GGT was fitted as a spline with median GGT as a reference, an almost linear relationship was observed (Figure 1b).

After adjustment for all potential confounders, ALP was positively associated with all-cause mortality (p<.001) and cancer-related mortality (p=.03) and showed a trend for association with CVD mortality (p=.08) (Figure 1c, Supplementary Table 2). Adjusted HR for all-cause mortality for participants with ALP ≥95th percentile (124U/L for women and 119U/L for men) was 1.50 (95%CI 1.25-1.79). Findings remained consistent when haemoglobin levels and serum phosphate levels were added in the model and when analyses were restricted to participants with normal haemoglobin levels. Cubic spline regression for ALP is illustrated in Figure 1d.

Table 4. Association of cholestatic liver enzymes with all-cause and cause-specific mortality. Percentiles are shown in categories: (1) <25th percentile; (2) 25th-<50th percentile; (3) 50th-<75th percentile; (4) 75th-<95th percentile; (5) ≥95th percentile.

Category	HR	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value
	Crude univariate model			Adjusted for age and sex			Adjusted for all covariables		
Category	**HR**	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**
GGT (1)	*1(ref)*		0.099	*1(ref)*		<0.001	*1(ref)*		<0.001
GGT (2)	0.95	0.85-1.05		1.08	0.97-1.20		1.09	0.98-1.21	
GGT (3)	0.94	0.85-1.05		1.16	1.05-1.29		1.18	1.06-1.31	
GGT (4)	0.95	0.86-1.06		1.25	1.12-1.39		1.22	1.09-1.37	
GGT (5)	1.17	0.99-1.40		1.62	1.36-1.92		1.55	1.30-1.85	

Table 4. (continued)

Association of GGT CVD related mortality

	Crude univariate model			Adjusted for age and sex			Adjusted for all covariables		
Category	**HR**	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**
GGT (1)	*1(ref)*		0.016	*1(ref)*		<0.001	*1(ref)*		<0.001
GGT (2)	1.29	1.02-1.64		1.49	1.17-1.88		1.40	1.10-1.77	
GGT (3)	1.41	1.11-1.78		1.73	1.37-2.19		1.58	1.24-2.01	
GGT (4)	1.32	1.03-1.68		1.74	1.36-2.22		1.47	1.14-1.91	
GGT (5)	1.73	1.21-2.47		2.38	1.66-3.40		2.07	1.43-2.99	

Association of GGT with cancer related mortality

	Crude univariate model			Adjusted for age and sex			Adjusted for all covariables		
Category	**HR**	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**
GGT (1)	*1(ref)*		0.6	*1(ref)*		0.3	*1(ref)*		0.4
GGT (2)	0.90	0.73-1.12		0.95	0.76-1.17		0.96	0.77-1.20	
GGT (3)	0.91	0.74-1.14		0.96	0.77-1.19		0.98	0.78-1.22	
GGT (4)	1.03	0.83-1.29		1.14	0.92-1.42		1.15	0.92-1.45	
GGT (5)	1.06	0.74-1.53		1.18	0.82-1.70		1.17	0.80-1.70	

Association of ALP with all-cause mortality

	Crude univariate model			Adjusted for age and sex			Adjusted for all covariables		
Category	**HR**	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**
ALP (1)	*1(ref)*		<0.001	*1(ref)*		<0.001	*1(ref)*		<0.001
ALP (2)	1.08	0.97-1.20		1.07	0.96-1.19		1.07	0.96-1.19	
ALP (3)	1.19	1.07-1.32		1.20	1.08-1.33		1.19	1.07-1.32	
ALP (4)	1.39	1.25-1.55		1.28	1.15-1.42		1.20	1.07-1.34	
ALP (5)	2.09	1.78-2.45		1.69	1.44-1.98		1.51	1.28-1.77	

Association of ALP CVD related mortality

	Crude univariate model			Adjusted for age and sex			Adjusted for all covariables		
Category	**HR**	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**
ALP (1)	*1(ref)*		<0.001	*1(ref)*		0.004	*1(ref)*		0.08
ALP (2)	1.34	1.07-1.68		1.34	1.07-1.67		1.30	1.04-1.63	
ALP (3)	1.17	0.93-1.48		1.19	0.94-1.50		1.15	0.91-1.45	
ALP (4)	1.57	1.25-1.98		1.46	1.16-1.84		1.32	1.04-1.67	
ALP (5)	2.12	1.50-3.00		1.73	1.22-2.45		1.43	1.01-2.04	

Association of ALP with cancer related mortality

	Crude univariate model			Adjusted for age and sex			Adjusted for all covariables		
Category	**HR**	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**
ALP (1)	*1(ref)*		0.001	*1(ref)*		0.005	*1(ref)*		0.03
ALP (2)	0.94	0.76-1.18		0.96	0.77-1.19		0.96	0.77-1.19	
ALP (3)	1.18	0.96-1.46		1.20	0.97-1.48		1.19	0.96-1.47	
ALP (4)	1.21	0.97-1.52		1.19	0.95-1.48		1.13	0.90-1.41	
ALP (5)	1.86	1.34-2.59		1.70	1.22-2.37		1.57	1.12-2.20	

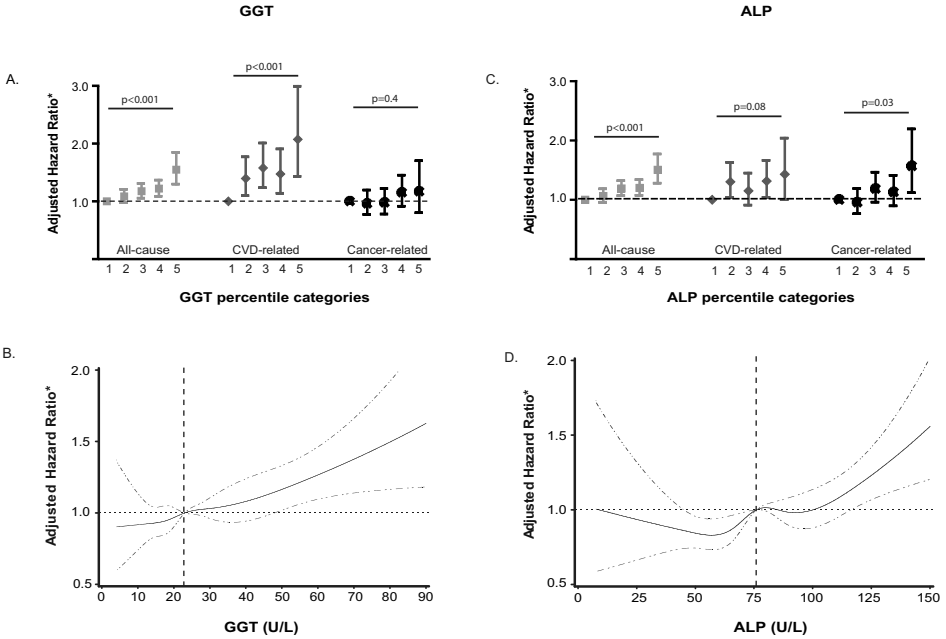

Fig. 1. Association of cholestatic liver enzymes with all-cause and cause-specific mortality.
(A) Adjusted Hazard Ratios (HRs) for mortality by percentiles of serum GGT. Reference: <25th percentile. HRs are adjusted for age, sex, education, smoking status, alcohol intake, hypertension, diabetes mellitus, BMI, and total cholesterol levels. Percentiles are illustrated as following: category 1: <25th percentile; category 2: 25th-<50th percentile; category 3: 50th-<75th percentile; category 4: 75th-<95th percentile; category 5: ≥95th percentile.
(B) Association of all-cause mortality with serum GGT levels, illustrated as a spline with the median as a reference.
(C) Adjusted HRs for mortality by percentiles of serum ALP.
(D) Association of all-cause mortality with serum ALP levels, illustrated as a spline with the median as a reference.

Elevation of both cholestatic liver enzymes was associated with higher all-cause mortality (HR 1.44, 95%CI 1.16-1.79; p=.001), CVD mortality (HR 1.81, 95%CI 1.22-2.69; p=.003) and cancer-related mortality (HR 1.68, 95% CI 1.10-2.55, p=.016).

Association of aminotransferases with all-cause and cause-specific mortality

ALT was positively associated with all-cause mortality (p<.001; Figure 2a, Table 5). Highest risks were observed for participants with ALT <25th percentile (<12U/L for women and <13U/L for men; HR 1 (*ref*)) and ALT ≥ 95th percentile (33U/L for women and 35U/L for men; HR 0.92, 95% CI 0.76-1.11). This J-shaped relationship is also illustrated in Figure 2b, where ALT is fitted as a spline with the median as a reference.

AST was positively associated with all-cause mortality (p<.001) and cancer-related mortality (p=.005; Figure 2c, Supplementary Table 3). Participants with AST ≥ 95th percentile (30 U/L for women and 31U/L for men) had a HR of 1.12 (95% CI 0.95-1.32). Similar to ALT, a J-shaped curve was observed (Figure 2d). AST showed an interaction with age. AST was not associated with all-cause mortality for age >76.8 years.

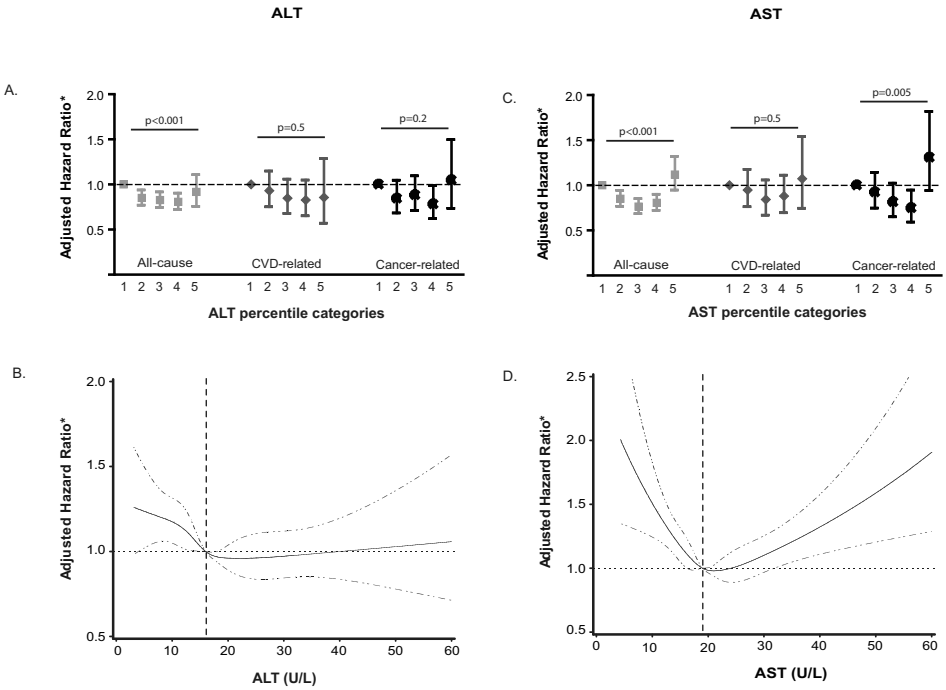

Fig. 2. Association of aminotransferases with all-cause and cause-specific mortality.
(A) Adjusted HRs for mortality by percentiles of serum ALT. Reference: <25th percentile. HRs are adjusted for age, sex, education, smoking status, alcohol intake, hypertension, diabetes mellitus, BMI, and total cholesterol levels. Percentiles are illustrated as following: category 1: <25th percentile; category 2: 25th-<50th percentile; category 3: 50th-<75th percentile; category 4: 75th-<95th percentile; category 5: ≥95th percentile.
(B) Association of all-cause mortality with serum ALT levels, illustrated as a spline with the median as a reference.
(C) Adjusted HRs for mortality by percentiles of serum AST.
(D) Association of all-cause mortality with serum AST levels, illustrated as a spline with the median as a reference.

Elevation of both ALT and AST was associated with higher all-cause mortality (HR 1.43, 95%CI 1.13-1.80; p=.002) and cancer-related mortality (HR 1.99, 95%CI 1.34-2.96; p=.001), but not with CVD related mortality (HR 1.21, 95%CI 0.74-2.01, p=.4).

The model including AST and all potential confounders had lower −2 log likelihood than models including GGT, ALP and ALT (37131 vs. 37138, 37136 and 37145 respectively), suggesting a stronger fit of the model.

In the Cox-regression analyses, no interaction was found between any of the liver biochemistries and sex, BMI or alcohol consumption. Age was not an effect modifier for the association of GGT, ALP and ALT with mortality.

Sensitivity analyses were performed regarding all liver tests and results remained consistent. Only when analyses were restricted to participants without a baseline history of CVD, no association was observed between GGT and CVD mortality. However, the pattern did almost not diverge from the original pattern. Adjustment for waist circumference instead of BMI did not

Table 5. Association of aminotransferases with all-cause and cause-specific mortality. Percentiles are shown in categories: (1) <25th percentile; (2) 25th-<50th percentile; (3) 50th-<75th percentile; (4) 75th-<95th percentile; (5) ≥95th percentile.

Association of ALT with all-cause mortality

	Crude univariate model			Adjusted for age and sex			Adjusted for all covariables		
Category	**HR**	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**
ALT (1)	*1(ref)*		<0.001	*1(ref)*		<0.001	*1(ref)*		<0.001
ALT (2)	0.77	0.70-0.85		0.84	0.76-0.93		0.85	0.77-0.94	
ALT (3)	0.66	0.60-0.73		0.82	0.74-0.91		0.83	0.75-0.92	
ALT (4)	0.54	0.48-0.60		0.80	0.72-0.90		0.81	0.72-0.90	
ALT (5)	0.56	0.47-0.67		0.95	0.79-1.15		0.92	0.76-1.11	

Association of ALT with CVD related mortality

	Crude univariate model			Adjusted for age and sex			Adjusted for all covariables		
Category	**HR**	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**
ALT (1)	*1(ref)*		<0.001	*1(ref)*		0.9	*1(ref)*		0.5
ALT (2)	0.86	0.70-1.06		0.94	0.76-1.16		0.93	0.75-1.15	
ALT (3)	0.74	0.59-0.91		0.91	0.73-1.13		0.85	0.68-1.06	
ALT (4)	0.62	0.49-0.78		0.92	0.73-1.16		0.83	0.65-1.05	
ALT (5)	0.60	0.40-0.88		1.07	0.68-1.50		0.87	0.57-1.29	

Association of ALT with cancer related mortality

	Crude univariate model			Adjusted for age and sex			Adjusted for all covariables		
Category	**HR**	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**
ALT (1)	*1(ref)*		0.008	*1(ref)*		0.01	*1(ref)*		0.2
ALT (2)	0.82	0.66-1.01		0.84	0.68-1.03		0.85	0.68-1.05	
ALT (3)	0.82	0.67-1.01		0.86	0.70-1.06		0.88	0.71-1.10	
ALT (4)	0.66	0.53-0.82		0.75	0.60-0.93		0.78	0.62-0.99	
ALT (5)	0.84	0.60-1.18		1.02	0.73-1.44		1.05	0.74-1.50	

Association of AST with all-cause mortality

	Crude univariate model			Adjusted for age and sex			Adjusted for all covariables		
Category	**HR**	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**
AST (1)	*1(ref)*		<0.001	*1(ref)*		<0.001	*1(ref)*		<0.001
AST (2)	0.76	0.69-0.85		0.78	0.71-0.87		0.85	0.77-0.95	
AST (3)	0.67	0.60-0.74		0.72	0.64-0.79		0.76	0.69-0.85	
AST (4)	0.69	0.62-0.77		0.75	0.67-0.84		0.81	0.72-0.90	
AST (5)	1.02	0.86-1.19		1.11	0.94-1.31		1.12	0.95-1.32	

Association of AST CVD related mortality

	Crude univariate model			Adjusted for age and sex			Adjusted for all covariables		
Category	**HR**	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**
AST (1)	*1(ref)*		0.008	*1(ref)*		0.06	*1(ref)*		0.5
AST (2)	0.80	0.64-0.99		0.82	0.66-1.02		0.95	0.76-1.18	
AST (3)	0.70	0.56-0.88		0.76	0.61-0.95		0.84	0.67-1.06	

Table 5. (continued)

AST (4)	0.70	0.55-0.87		0.76	0.61-0.96		0.88	0.70-1.11	
AST (5)	0.94	0.66-1.34		1.04	0.73-1.48		1.07	0.75-1.54	
	Association of AST with cancer related mortality								
	Crude univariate model			**Adjusted for age and sex**			**Adjusted for all covariables**		
Category	**HR**	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**
AST (1)	*1(ref)*		<0.001	*1(ref)*		0.001	*1(ref)*		0.005
AST (2)	0.85	0.69-1.05		0.88	0.72-1.09		0.92	0.75-1.14	
AST (3)	0.72	0.57-0.90		0.77	0.62-0.97		0.82	0.65-1.02	
AST (4)	0.68	0.54-0.85		0.72	0.57-0.90		0.75	0.59-0.95	
AST (5)	1.21	0.88-1.67		1.28	0.93-1.77		1.31	0.94-1.81	

change results, and adjustment for continuous variables for smoking status (pack years) and hypertension (diastolic and systolic blood pressure) did not diverge the model.

Discussion

In this large population-based cohort study of participants older than 55 years we demonstrated that serum GGT, ALP, ALT, and AST levels are associated with all-cause mortality. Secondly, GGT was associated with CVD-related mortality, and ALP and AST with cancer-related mortality.

A unique aspect of this study is its well characterized design, long-term follow up, and large number of events. Moreover, this study was population-based, has an excellent participation rate of 78%, and mortality was continuously and accurately monitored through linkage with records of GPs, hospitals and nursing homes.

We analyzed our data using percentiles of serum liver tests, for we were interested in studying the association of the whole range of serum GGT, ALP and aminotransferase levels with mortality. Moreover, no upper limits of normal (ULN) have been defined for elderly participants, since blood donor studies mainly include younger adults and ULN of ALT decreases with age [29-31]. When we analyzed the association between serum liver biochemistries by spline regression, similar outcomes were obtained.

For GGT, our results are consistent with previous studies demonstrating increased overall and CVD mortality with increasing GGT levels in various, younger populations [6, 12, 17-18, 32-35]. Few studies observed an interaction with age. Lee *et al.* showed that the relationship between elevated GGT and increased CVD mortality was only present in subjects younger than 55 years and not in elderly subjects [18]. Our study mainly consisted of elderly participants and we did not find interaction between age and GGT. Although GGT levels were not significantly associated with CVD mortality after excluding participants with CVD at baseline, these results should be interpreted carefully, since almost one third of participants were excluded for these sensitivity analyses and results showed an almost similar pattern. Given our results and evidence from

previous studies, GGT appears to be an important indicator of overall health and CVD health, even in the elderly. It has been suggested that GGT levels may capture processes relevant to atherogenesis or may play a role in the development of oxidative stress [36-37].

Only two studies investigated the relation between elevation of ALP and mortality in population samples [14-15]. Tonelli *et al.* demonstrated an independent relation of ALP with all-cause and CVD mortality in adult subjects from the general US population [14]. In the present study serum ALP levels are associated with all-cause and cancer-related mortality and showed a trend for association with CVD mortality. To date, little is known about the physiological functioning of ALP. ALP may be involved in vascular calcification processes outside the context of renal failure, which may explain the observed trend for association between ALP and CVD mortality [14, 38].

In contrast to the strong body of evidence for GGT, recent studies of community or population-based samples investigating the relation of ALT and AST with mortality are less congruent. A large Korean study confirmed the association of the whole range of ALT with increased mortality in men, but not in women [16]. However, Asian populations have a different distribution of mortality causes than that of Western populations [39-40]. Secondly, using data from NHANES III, Ong *et al.* found that elevated serum aminotransferases in the absence of significant alcohol intake and other liver diseases, was also significantly associated with higher mortality risk [41]. An analysis with the same dataset using slightly different selection criteria and a different statistical calculation of variance did not demonstrate a statistically significant association [6]. We demonstrated that aminotransferases are associated with all-cause mortality in an elderly population. However, for AST the association with all-cause mortality was not significant above age 76.

In the present study AST showed stronger association with all-cause mortality than ALT. In part, this may be due to the association of AST with cancer-related mortality. Moreover, from a biological perspective, ALT is abundantly expressed in liver tissue, whereas AST is expressed in multiple other tissues. Given the small number of events of specific causes of CVD or cancer, leading to large confidence intervals, we were unable to determine the association with cause-specific mortality in more detail.

Remarkably, GGT and ALP levels showed an almost linear relationship with mortality risk, whereas ALT and AST showed a J-shaped relationship. Similar observations have been published previously and it has been suggested that overall or hepatic frailty, or hepatic aging may underlie these observations [42]. Hepatic aging has been associated with greater oxidative stress and hepatic cell apoptosis in rat livers [43]. Furthermore, in early studies on aminotransferases it was demonstrated that low transaminase levels may be caused by pyridoxine deficiency [44]. In turn, pyridoxine deficiency is generally the result of decreased intake of vitamin B6. In our analyses we were not able to adjust for pyridoxine levels or intake of vitamin B6, but did correct for BMI. Furthermore, there was no interaction of BMI with any of the other covariables. Nevertheless, low BMI may also reflect low nutritional intake and is associated with higher mortality [45]. Additional research is required to further elucidate the mechanism of these observations.

To date, individuals over 55 years of age constitute the fastest growing segment in Western populations and greatest consumers of health care [46]. Liver chemistry tests may represent valuable markers of long term outcome in this segment of the population. Although screening for these tests may aid clinicians to identify conditions that may lead to significant morbidity and mortality in elderly persons, the enormous burden for health care practice in terms of cost effectiveness and provision of services and health care providers should also be considered.

A limitation of this study is the inability of excluding subjects with viral hepatitis at baseline. However, the prevalence of hepatitis B and C in the Dutch population is estimated to be very low [47-48]. We performed additional sensitivity analyses excluding participants with possible acute hepatitis, and did not obtain significant differences on results. Moreover, in the present study we performed analyses using only a single measurement of GGT, ALP, ALT or AST. Liver enzyme examinations were stopped before all participants had visited the research center. Because participants were invited in a semi-random order, by postal code, it is unlikely that this affected our results. Finally, our results may not be generalized to other ethnic populations as the vast majority of this study population were of Caucasian ethnicity.

In summary, the current study sought to clarify the association between serum GGT, ALP, ALT and AST levels with long-term mortality in a large prospective population-based study of mainly Caucasian elderly. All liver enzymes were positively associated with all-cause mortality. Therefore, these tests may represent useful indicators of longevity in the elderly. Moreover, in this elderly population we found an association between low levels of ALT and AST and increased mortality of all causes. Although clinicians are generally concerned about significant increases in transaminases, moderate increases or very low levels may also be clinically relevant.

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CHAPTER 3

External validation of fatty liver index for identifying non-alcoholic fatty liver disease in a population-based study

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Abstract

Background & Aim: We aimed to validate the fatty liver index (FLI), an algorithm based on waist circumference, body mass index (BMI), and levels of triglyceride and γ-glutamyltransferase (GGT). We calculated its ability to identify fatty liver disease from any cause or non-alcoholic fatty liver disease (NAFLD) in large population of Caucasian elderly persons. **Methods:** Participants of the Rotterdam Study completed an interview and clinical examination that included abdominal ultrasonography, a fasting blood sample and anthropometric assessment. The ability of FLI to detect (non-alcoholic) fatty liver was assessed using Area Under the Receiver Operator Characteristic (AUROC) curve analysis. **Results:** Data on FLI and ultrasonography were available in 3034 elderly participants. In total, 282 participants were excluded for possible secondary causes of fatty liver, leaving 2652 participants (mean age 76.3±6.0 years) for analyses regarding NAFLD. FLI was strongly associated with NAFLD in multivariable analysis (OR 1.05, 95%CI 1.04-1.05; p<.001). AUROC of FLI was 0.813 (0.797-0.830) for predicting NAFLD, and 0.807 (0.792-0.823) for predicting fatty liver due to any cause. **Conclusion:** The FLI (an algorithm based on waist circumference, BMI, and levels of triglyceride and GGT) has good ability for identifying NAFLD, confirmed via ultrasonography, in a large, Caucasian, elderly population.

Introduction

Nonalcoholic fatty liver disease (NAFLD) constitutes an emerging public health problem, as its prevalence and incidence are rapidly increasing due to epidemics in obesity and type II diabetes mellitus. It has been projected that NASH will be the most common indication for liver transplantation in the next decades [1]. A great body of evidence has demonstrated that NASH increases the risk of liver-related, cardiovascular and overall morbidity and mortality [2-4]. Whether simple fatty liver increases this risk independent of metabolic risk factors remains a controversial issue, mainly because of inadequate diagnostic approaches of studies, e.g. defining NAFLD by elevation of serum aminotransferases, whereas only 50% of subjects with NAFLD have elevated liver enzymes [2, 5-7]. Using data from the Dionysos Nutrition and Liver Study, Bedogni *et al* developed a simple algorithm for the prediction of fatty liver, the 'Fatty Liver Index (FLI)' [8]. This score appears more accurate for prediction of fatty liver than elevation of liver enzymes but, to date, has not been externally validated in a Caucasian general population. The aim of this study was to validate the FLI in a large population of Caucasian elderly persons for prediction of both fatty liver due to any cause, and NAFLD in particular.

Methods

Study population

We analyzed data from the Rotterdam Study, a large prospective population-based cohort study conducted among elderly inhabitants of a district of Rotterdam, The Netherlands. The rationale and study design have been described previously [9]. The medical ethics committee at the Erasmus University of Rotterdam approved the study, and written informed consent was obtained from all participants.

Abdominal ultrasonography was performed in the first two cohorts of the Rotterdam Study between February 2009 and February 2012. In addition, each participant completed an extensive interview and clinical examination, including a fasting blood sample and anthropometric assessment.

Serum chemistry tests

Fasting blood samples were collected on the morning of ultrasound examination. Blood lipids, glucose, and gamma-glutamyltransferase (GGT) were measured using automatic enzymatic procedures (Roche Diagnostics GmbH, Mannheim, DE). Insulin, HBsAg and anti-HCV antibodies were measured by automatic immunoassay (Roche Diagnostics GmbH, Mannheim, DE).

Diagnosis of non-alcoholic fatty liver

Abdominal ultrasonography was performed by certified and experienced technicians on Hitachi HI VISION 900. Images were stored digitally and re-evaluated by a hepatologist with more than ten years experience in ultrasonography. The diagnosis and grading of fatty liver was determined by the ultrasound technician according to the protocol by Hamaguchi et al [10]. 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 presence of fatty liver on ultrasound, in the absence of excessive alcohol consumption (more than 14 alcoholic beverages weekly for men and women), positive HBsAg or anti-HCV, and use of pharmacological agents historically associated with fatty liver (e.g. amiodarone, tamoxifen, corticosteroids and methotrexate).

Covariables

At the research center, anthropometric measurements were performed by well-trained nurses. Body Mass Index (BMI) was calculated as weight (kg)/ length (m^2). Waist and hip circumference were measured in centimeters. The average of two blood pressure measurements obtained at a single visit was used for analysis. Insulin resistance index was calculated using the Homeostasis Model Assessment of Insulin Resistance (HOMA-IR): fasting glucose (mmol/L)xfasting insulin (mU/L)/22.5 [11]. Lipid Accumulation Product (LAP) was calculated for men and women separately as previously described by Kahn et al [12-14]. Fatty Liver Index (FLI) was calculated by the formula previously described by Bedogni *et al* [8].

Statistical analyses

Baseline analyses were performed using descriptive statistics. To study the association of FLI with NAFLD and severity of NAFLD, we performed logistic and ordinal logistic regression analyses, respectively, adjusting for age, gender, alcohol consumption, HDL-cholesterol levels, systolic and diastolic blood pressure and HOMA-IR. Performance of FLI as marker of (nonalcoholic) fatty liver was examined using Area Under the Receiver Operator Characteristic (AUROC) curves. Comparison between AUROCs of FLI versus separate components of FLI, HOMA-IR and LAP was done using the method of DeLong [15]. A p-value of <0.05 was considered statistically significant. Statistical analyses were performed using SAS version 9.2 (SAS Institute Inc., Cary, NC, USA).

Results

FLI could be calculated in 3034 of 3205 elderly participants in whom liver ultrasonography was performed. In total, 382 participants were excluded for possible secondary causes of fatty liver (excessive alcohol consumption (n= 251), positive HBsAg or anti-HCV (n=21), use

of pharmacological agents associated with fatty liver (n=119)), leaving 2652 participants for analyses. General characteristics of the study population are shown in Table 1. Sixty percent of the study population were women and mean age of participants was 76.3±6.0 years. Participants were predominantly of Caucasian ethnicity (95.3%).

Table 1. General characteristics of participants with and without NAFLD

	Total	No NAFLD	NAFLD	p-value*	AUROC (95%CI)
	n=2652	n=1727	n=925		
	(100%)	*(65.1%)*	*(34.9%)*		
Age (years)	76.3 (±6.0)	76.7 (±6.3)	75.7 (±5.4)	< .001	
Female (%)	60.1	59.6	60.9	.5	
Caucasian (%)	95.3	95.7	94.7	.3	
BMI (kg/m2)	27.2 (±4.2)	26.0 (±3.5)	30.0 (±4.1)	<.001	0.776 (0.758-0.794)
Waist circumference	92 (84-101)	88 (81-96)	100 (92-107)	<.001	0.759 (0.740-0.777)
Systolic blood pressure	151 (137-166)	150 (136-165)	153 (140-167)	<.001	
Diastolic blood pressure	85 (78-92)	84 (77-91)	86 (79-93)	<.001	
Alcohol intake (drinks/ week)	3.7 (±3.7)	3.7 (±3.6)	3.7 (±3.8)	.6	
Fatty Liver Index	42 (23-67)	31 (17-50)	67 (49-83)	<.001	0.813 (0.797-0.830)
<30/ ≥ 60	34.9/32.6	49.0/17.7	8.5/60.4		
GGT (U/L)	22 (17-32)	21(16-29)	26 (20-37)	<.001	0.640 (0.618-0.661)
HOMA-IR	2.6 (1.8-4.0)	2.2 (1.5-3.2)	3.9 (2.7-5.9)	<.001	0.771 (0.752-0.789)
Triglycerides (mmol/L)	1.27 (0.98-1.70)	1.17 (0.91-1.52)	1.53 (1.16-2.01)	<.001	0.682 (0.661-0.703)
HDL-C (mmol/L)	1.43 (1.18-1.71)	1.50 (1.24-1.81)	1.31 (1.11-1.55)	<.001	
Lipid Accumulation Product	39 (25-60)	31 (21-46)	58 (41-82)	<.001	0.786 (0.768-0.804)

Data are represented as mean (± standard deviation), median (25^{th}-75^{th} percentile) or percentages.
*Based on T-test, Wilcoxon rank sum test or Chi-square test.
Abbreviations: BMI, Body Mass Index; GGT, gamma glutamyl transferase; HOMA-IR, Homeostasis Model Assessment of Insulin Resistance; HDL-C, high-density lipoprotein cholesterol; AUROC, Area Under the Receiver Operator Characteristic,

NAFLD was present in 925 of 2652 (34.9%) participants: 139 (5.2%) participants had mild fatty liver, and 786 (29.6%) participants had moderate to severe fatty liver according to the criteria reported by Hamaguchi *et al*. Median FLI was 42.2 (25^{th}-75^{th} percentile: 22.6-67.3). Applying cut-offs proposed by Bedogni *et al*. 926 (34.9%) participants had FLI<30, and 864 (32.6%) participants had FLI≥60. In univariable analyses, continuous FLI and both cut-offs for FLI were strongly associated with NAFLD (p-values<.001). In logistic regression analysis, adjusting for age, gender, alcohol consumption, systolic and diastolic blood pressure, HDL-cholesterol levels, and HOMA-IR, FLI remained strongly associated with presence of NAFLD (OR1.05, 95%CI 1.04-1.05; p<.001). FLI was also strongly associated with severity of NAFLD in ordinal regression analysis (correlation coefficient 0.048, 95%CI 0.043-0.052; p<0.001).

Area under the receiving operator characteristic (AUROC) of FLI for predicting NAFLD was 0.813 (95%CI 0.797-0.830). AUROC for FLI was significantly higher than AUROC for HOMA-IR (0.771; 95%CI 0.752-0.789), waist circumference (0.759; 95%CI 0.740-0.777), BMI (0.776; 95%CI 0.758-0.794), GGT (0.640; 95%CI 0.618-0.661) or LAP (0.786; 95%CI 0.769-0.804; all p-values<.0001; Figure 1). Sensitivity and specificity of FLI<30 for predicting absence of NAFLD was 91.5% and 49.0%, respectively. Sensitivity and specificity of FLI≥60 for predicting presence of NAFLD was 60.4% and 82.3%, respectively. Performance of FLI was lower for higher age, although the association was not significant (AUROC of 0.833 for age category 65-69 years versus AUROC of 0.790 for age category ≥80; p=.3).

Results were comparable when we did not exclude participants with possible secondary causes of fatty liver. AUROC of FLI for predicting fatty liver due to any cause was 0.807 (95%CI 0.792-0.823), with sensitivity of 62% and specificity of 81% at a cut-off of 60 to rule in fatty liver.

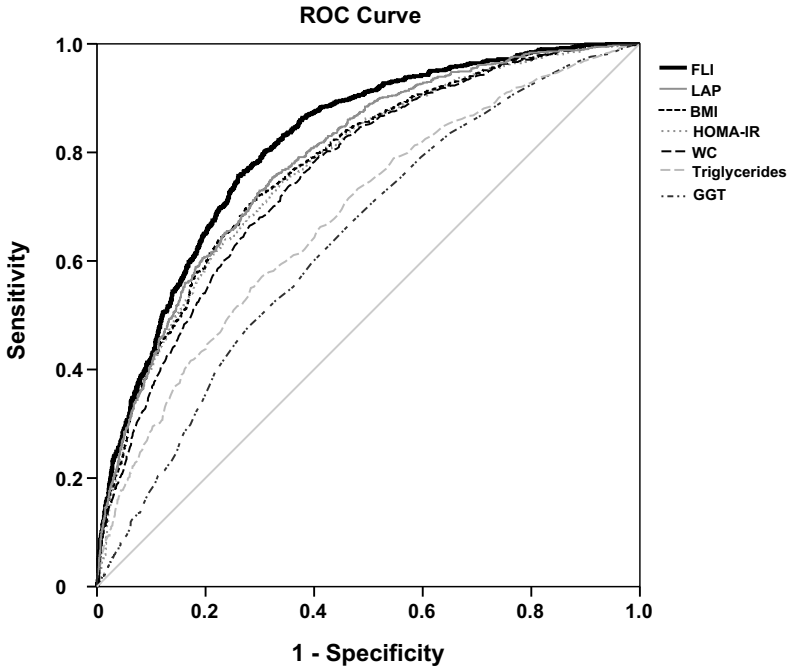

Fig. 1. ROC curves for Fatty Liver Index, Lipid Accumulation Product (LAP), Body Mass Index (BMI; kg/m^2), Homeostasis Model Assessment of Insulin Resistance (HOMA-IR), waist circumference (WC; cm), serum triglyceride levels (mmol/L) and serum GGT levels (U/L). FLI performed significantly better than its separate components, LAP or HOMA-IR (all p-values <0.0001).

Discussion

In the present study we validated the Fatty Liver Index in 2652 elderly participants of the population-based Rotterdam Study. We demonstrated that FLI has good predictive values in the elderly for both ultrasonographically diagnosed fatty liver due to any cause as well as non-alcoholic fatty liver in this population, independent of age.

Sensitivity and specificity for fatty liver at the recommended cut-off of 60 (to rule in fatty liver) in the original study was 61% and 86% respectively and 62% and 81% respectively, in the present study [8]. For the prediction of NAFLD performance was similar, with a good AUROC of 0.813. Although application of FLI for screening individuals at risk for NAFLD may be less adequate, given insufficient sensitivity of FLI at recommended cut-offs, FLI as continuous measurement strongly correlates with presence and severity of NAFLD, assessed by ultrasonography, making the score an adequate marker of NAFLD for research purposes. Interestingly, FLI has been shown to be associated with cardiovascular morbidity and mortality and incident diabetes [16-18]. These diseases have the highest incidence and prevalence rates in the elderly population. To date, elderly persons constitute the fastest growing segment in Western populations and greatest consumers of health care. To perform epidemiological studies in the elderly applying FLI, it is important that the FLI is replicated in such a population.

At higher age, metabolic disease aggregates and fat redistribution from subcutaneous to intra-abdominal visceral depots as well as other ectopic sites, including the liver, occurs. In addition, the prevalence of NAFLD may decrease in subjects older than 80 years [19]. Therefore, we investigated interaction between FLI and age, and found that the association of FLI and NAFLD was independent of age or age categories.

Given the high prevalence of NAFLD in the general population and uncertainties about ultrasonography screening, a validated non-invasive score may help physicians select individuals for ultrasonography screening. The parameters included in the FLI are easily accessible for physicians. Noteworthy, FLI performed better than any of its single components, HOMA-IR or LAP. LAP was recently also evaluated by Bedogni *et al*, who demonstrated adequate performance of this formula for detection of fatty liver in the Dionysos Study [13].

The strengths of the present study include the large number of elderly participants that were included and extensive characterization of subjects. Unfortunately, we do not have information on severity of liver disease in terms of inflammation and fibrosis, as a liver biopsy is unethical to perform in a population-based study.

In conclusion, we validated FLI for the prediction of ultrasonographically detected non-alcoholic fatty liver disease in a large Western population-based study, enabling its use in epidemiological studies including elderly subjects.

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CHAPTER 4

Interaction between insulin resistance and common variants for non-alcoholic fatty liver disease

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Submitted

Abstract

Background/Aim: Recently, several common susceptibility alleles for non-alcoholic fatty liver disease (NAFLD) were identified in genome-wide association studies (GWAS). The objective of this study was to investigate the association of several of these genetic variants with NAFLD in a large population and to study interaction with metabolic traits. **Methods:** The present study was part of the Rotterdam Study, a large prospective population-based cohort study conducted among older individuals. Five single nucleotide polymorphisms (SNPs), including rs2228603 near neurocan (NCAN), rs12137855 near lysophospholipase-like protein 1 (LYPLAL1), rs780094 in glucokinase regulator (GCKR), rs738409 in patatinlike phospholipase family 3 (PNPLA3), and rs4240624 near protein phosphatise 1, regulatory subunit 3B (PPP1R3B), were extracted from a GWAS-genotype dataset and analyzed for their association with presence and severity of NAFLD, which was assessed by ultrasonography (US). In addition, association and interaction with metabolic covariables was tested for all SNPs. **Results:** Data of 2285 participants (mean age 76.6 ± 6.1 years; 60% women) were analyzed. The prevalence of NAFLD determined by ultrasonography (US-NAFLD) was 35.1%. In logistic regression analysis, rs738409C>G in PNPLA3 (p<.001), rs2228603C>T near NCAN (p=.001) and rs780094C>T in GCKR (p=.02) were associated with presence of NAFLD. In ordinal regression analysis, rs738409C>G ($p=5x10^{-8}$), rs2228603C>T (p=.001) and rs780094C>T (p=.007) were associated with severity of US-NAFLD. Furthermore, significant interaction was demonstrated between rs738409, rs4240624, and rs780094 with Homeostasis Model Assessment of Insulin Resistance (HOMA-IR; p-values for interaction term <.001), such that lower HOMA-IR amplified the effects of the variant in PNPLA3 and higher HOMA-IR amplified the effects of variants in or near PPP1R3B and GCKR. In multiple linear regression analysis, none of the SNPs were associated with HOMA-IR, or levels of fasting insulin; only rs780094C>T in GCKR was associated with lower fasting glucose levels (β −1.48, SE 0.65; p=.02) and higher triglyceride levels (β 7.34, SE 1.5; $p= 9x10^{-7}$), while rs738409C>G was associated with lower triglyceride levels (β −6.43, SE 1.72; $p=2x10^{-4}$). **Conclusion:** The present study demonstrated the association between genetic variants in or near PNPLA3, PPP1R3B, NCAN and GCKR with presence of US-NAFLD in a large population-based study. There was significant interaction between rs738409 in PNPLA3, rs4240624 near PPP1R3B, and rs780094 in GCKR with insulin resistance, such that the genetic effect of rs738409C>T was stronger in subjects with lower HOMA-IR, and the genetic effects of rs780094C>T and rs4240624G>A were stronger in subjects with higher HOMA-IR.

Introduction

The term NAFLD describes a spectrum ranging from simple fatty liver through nonalcoholic steatohepatitis (NASH) and NASH cirrhosis, and is currently the most common chronic liver disease in Western countries [1]. Primary NAFLD is strongly associated with insulin resistance and its phenotypic manifestations, including obesity and type 2 diabetes mellitus [2]. The first clues that host genetic variation influences NAFLD came from family studies and studies demonstrating differences in susceptibility to NAFLD between ethnicities [3]. In the past few years, there have been great advances in our understanding of genetic susceptibility through the identification of common susceptibility alleles for NAFLD using GWAS. To date, two large GWAS have reported genetic loci associated with NAFLD, which was assessed by means of computed tomography and magnetic resonance imaging [4-5]. These studies demonstrated a strong association between NAFLD and rs738409, a single nucleotide polymorphism (SNP) positioned in the PNPLA3 gene that presumably plays a role in acylation of lysophospholipids and hydrolysis of triglycerides [6]. However, exact mechanisms on how the variant allele exhibits its effects remain unknown. In their genome-wide association analysis, Speliotes *et al.* identified four additional loci associated with NAFLD, of which two (i.e. rs12137855 near LYPLAL1 and rs780094 in GCKR) were not genome wide significant ($p \geq 5*10^{-8}$) in the GOLD sample, however, were replicated in participants from the NASH Clinical Research Network (CRN) [5]. Whether these findings may be generalized to elderly individuals, whom have been exposed to lifestyle factors for a longer time period and in whom metabolic factors are highly prevalent, remains to be unknown. Furthermore, although association of these SNPs with metabolic factors has been investigated in few replication studies, effect modification of SNPs by metabolic factors was -to our knowledge- not always reported in these studies, and may extend our knowledge on gene-environmental interaction. Therefore, the aim of this study was to investigate whether identified genetic variants were associated with a higher prevalence of NAFLD, assessed by ultrasonography, in a large population-based study of Caucasian elderly participants. Furthermore, we sought to study the association and interaction of these genetic variants with metabolic traits and elevation of alanine aminotransferase (ALT), used as a marker of liver inflammation.

Subjects and methods

Study population

The present study was part of the Rotterdam Study, a large prospective population-based cohort study conducted among elderly inhabitants of Ommoord, a district of Rotterdam, The Netherlands. The rationale and study design have been described previously [7]. The medical

ethics committee of the Erasmus Medical Center of Rotterdam approved the study, and written informed consent was obtained from all participants.

Abdominal ultrasonography was added to the core protocol at the fifth survey of the Rotterdam Study (February 2009-February 2012), which constitutes the baseline survey for the present study. Each participant completed an extensive interview and clinical examination that included a fasting blood sample, liver ultrasonography, and anthropometric assessment.

Diagnosis of nonalcoholic fatty liver disease

Abdominal ultrasonography was performed at the research center by certified and experienced technicians on Hitachi HI VISION 900 in all study participants. Images were stored digitally and re-evaluated by a hepatologist with more than ten years experience in ultrasonography (JNLS). The diagnosis and grading of fatty liver was determined by the ultrasound technician according to the protocol by Hamaguchi et al [8]. 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). Individuals with any of the following possible secondary causes of fatty liver were excluded from the analyses: 1) excessive alcohol consumption (>14 drinks/week) 2) serum HBsAg or anti-HCV positivity, and 3) use of pharmacological agents associated with fatty liver (i.e. amiodarone, corticosteroids, methotrexate, and tamoxifen).

Serum chemistry tests

Fasting blood samples were collected on the morning of ultrasound examination. Blood lipids, glucose, aspartate aminotransferase (AST), and alanine aminotransferase (ALT), were measured using automatic enzymatic procedures (Roche Diagnostics GmbH, Mannheim, DE). Insulin, HBsAg and anti-HCV antibodies were measured by automatic immunoassay (Roche Diagnostics GmbH, Mannheim, DE).

Genotyping, imputation and extraction of single nucleotide polymorphisms

Genomic DNA was extracted from whole blood samples using the salting out method [9]. Micro-array genotyping was performed in the original Rotterdam Study cohort with proper quality DNA samples by using the Infinium II HumanHap550K Genotyping Bead-Chip® version 3 (Illumina Inc., San Diego, CA, USA) [10]. Genotyping procedures were followed according to the manufacturer's protocols.

Single nucleotide polymorphisms (SNPs), previously identified in GWAS of NAFLD (initially determined by radiology), were extracted from the dataset (Table 1)[4-5]. Three of five SNPs, i.e., rs2228603 near NCAN, rs12137855 near LYPLAL1, and rs780094 in GCKR were measured with the bead-chip. SNPs that were not measured by the bead-chip, i.e., rs738409 in PNPLA3, and rs4240624 near PPP1R3B, were imputed using the MACH software (versions 1.0.15 and 1.0.16). The reference phased datasets for imputation were downloaded from HapMap (http://hapmap.org, release 22). Imputation quality for imputed SNPs was very high (rsq>0.98). The

SNP effect allele frequencies (EAF) in the present study were similar to the EAF described in previous publications regarding populations of Caucasian origin. A power calculation by SAS Proc Power (SAS Inc, Cary, NC, USA) was performed to assess the ability to detect odds ratios previously published using the present population sample of the Rotterdam Study. Using previously published odds ratios (ORs) of NAFLD, EAF of SNPs, a prevalence of US-NAFLD of 35% and population sample size of 2285, power calculation revealed that power to detect effects was >90% for all five SNPs.

Table 1. Single nucleotide polymorphisms associated with NAFLD in recent genome wide association studies.

SNP id	Chr	In/ near Gene	Function	Imputed or genotyped	Imputation quality (Rsq)	Allele	EA/F RS	EA/F Hapmap
rs738409	22q13.31	PNPLA3	Missense	Imputed	0.9843	C/G	G/ 0.2311	G/ 0.233
rs2228603	19p13.11	NCAN	Missense	Genotyped	-	C/T	T/ 0.0710	T/ 0.075
rs4240624	8p23.1	PPP1R3B	Unknown	Imputed	0.9863	A/G	A/ 0.9133	A/ 0.925
rs12137855	1q41	LYPLAL1	Unknown	Genotyped	-	C/T	C/ 0.7954	C/ 0.783
rs780094	2p23.3	GCKR	Intron	Genotyped	-	C/T	T/ 0.3572	T/ 0.383

Abbreviations: PNPLA3, Patatinlike phospholipase family 3; NCAN, Neurocan; PPP1R3B, Protein phosphatase 1, regulatory subunit 3b; LYPLAL1, Lysophospholipase-like 1; GCKR, Glucokinase regulatory protein; EA/F, effect allele and its frequency in either the Rotterdam Study (RS) or in the Caucasian population in HapMap phase 2, release 22 (www.hapmap.ncbi.nlm.nih.gov).

Assessment of covariables

In the interview preceding the clinical examination, detailed data was obtained concerning demographics, medical history, comorbid conditions, alcohol consumption, and drug use. Excessive alcohol consumption was defined as consumption of more than 14 alcoholic beverages weekly for both men and women. Detailed information on drug prescriptions was dispensed from automated pharmacies. Anthropometric measurements were performed at the research center by well trained nurses. Body Mass Index (BMI) was calculated as weight (kg)/ length (m^2). The metabolic syndrome was defined, according to Adult Treatment Panel III criteria [11]. Diabetes was defined as fasting plasma glucose ≥126 mg/dL (7.0 mmol/L) or drug treatment for elevated blood glucose. Insulin resistance index was calculated using the Homeostasis Model Assessment of Insulin Resistance (HOMA-IR): fasting glucose (mmol/L) x fasting insulin (μU/mL) / 22.5 [12]. Elevation of ALT was defined as ALT>40U/L for men and >30U/L for women, according to local cut-off values.

Statistical analysis

In univariate analysis, 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. Host genotype was coded as 0, 1, or 2 based on the number of effect alleles. Uncertainty of imputation was considered using the fractional allele count

(imputed dosage of the effect allele). To examine associations between SNPs and US-NAFLD or severity of US-NAFLD we performed logistic or ordinal logistic regression analyses respectively, adjusting for age, sex, BMI, HOMA-IR, serum triglyceride levels and serum HDL-C levels. In additional models effect modification was tested for age, sex and metabolic traits by adding interaction terms ((SNP)*(trait: age, sex, BMI, serum triglyceride levels or serum HDL-C levels)). Furthermore, the association between SNPs and ALT levels or elevation of ALT was examined using a linear or logistic multivariable model respectively. A *P*-value of .05 was considered statistically significant. Statistical analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

Results

Study population

A total of 3205 participants underwent abdominal ultrasonography. Three hundred ninety-four participants were excluded (excessive alcohol consumption (n= 255), positive HBsAg (n=3) or anti-HCV (n=24), use of pharmacological agents historically associated with fatty liver (n=121)). In total, 2285 of 2811 eligible participants were successfully genotyped and of Caucasian origin. General characteristics are shown in Table 2. Sixty percent of the study population were women. Mean age of participants was 76.6 ± 6.1 years and mean BMI was 27.5 ± 4.2 kg/m^2. The prevalence of US-NAFLD was 35.1%; 5.6% had mild fatty liver and 29.5% had moderate to severe fatty liver.

Association of single nucleotide polymorphisms with NAFLD

Univariate analyses

Univariate analyses regarding the association of SNPs with US-NAFLD are illustrated in Table 3. Rs738409C>G showed the strongest association with a higher prevalence of NAFLD (p=.002). In addition, rs738409C>G in PNPLA3 (p=4x10^{-4}) and rs780094C>T (p=.004) in GCKR were associated with severity of US-NAFLD. None of the five SNPs were correlated with either age, sex, BMI, HOMA-IR, serum ALT or AST levels, or HDL-cholesterol levels. Serum triglyceride levels were associated with copies of G allele in rs738409 in PNPLA3 (correlation coefficient (r)= −0.06; p=.008) and copies of T allele in rs780094 in GCKR (r=0.11;2x10^{-7}). There was no significant correlation between SNPs (all r<0.03, p-values>.2).

Multivariate analyses

In logistic regression analysis, rs738409 in PNPLA3 (p<.001), rs2228603 near NCAN (p=.001) and rs780094 in GCKR (p=.02) were associated with presence of US- NAFLD (Table 3). No interaction was present between SNPs. In ordinal regression analysis, rs738409 (p=5x10^{-8}), rs2228603

Table 2. General characteristics of the study population according to presence of US-NAFLD

	Total (n=2285)	Normal (n=1484)	US-NAFLD (n=801)	p-value*
	100	*64.9*	*35.1*	
Age (years)	76.6 (±6.1)	76.9 (±6.4)	76.0 (±5.4)	.001
Female (%)	59.3	58.3	61.0	.2
BMI (kg/m2)	27.5 (±4.2)	26.1 (±3.4)	30.0 (±4.2)	<.001
Normal; BMI < 25 (%)	29.4	40.2	9.4	
Overweight; 25 ≤ BMI < 30 (%)	47.2	48.2	45.2	
Obese; BMI ≥ 30 (%)	23.5	11.6	45.4	
Alcohol intake (drinks/week)	3.7 (±3.7)	3.8 (±3.6)	3.7 (±3.9)	.6
Metabolic syndrome (%)	54.7	44.1	73.9	<.001
Diabetes Mellitus (%)	14.5	9.3	24.1	<.001
ALT (U/L)	18 (14-23)	17 (14-21)	21 (16-27)	<.001
AST (U/L)	25 (22-29)	25 (22-28)	25 (22-30)	.02
GGT (U/L)	23 (17-32)	21(16-29)	27 (20-38)	<.001
HOMA-IR	2.6 (1.8-4.0)	2.2 (1.5-3.2)	3.8 (2.6-5.9)	<.001
Triglycerides (mg/dL)	112 (87-149)	103 (81-134)	134 (102-177)	<.001
HDL-C (mg/dL)	55 (46-66)	58 (48-69)	51 (42-60)	<.001

Data are represented as mean (± standard deviation), median (25^{th}-75^{th} percentile) or percentages.
*Based on T-test, Wilcoxon rank sum test or Chi-square test.
Abbreviations: US-NAFLD, non-alcoholic fatty liver disease determined by ultrasonography; BMI, Body Mass Index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma glutamyl transferase; HOMA-IR, Homeostasis Model Assessment of Insulin Resistance; HDL-C, high-density lipoprotein cholesterol.

(p=.001) and rs780094 (p=.007) were associated with severity of US-NAFLD. In multiple linear regression analysis, none of the SNPs were associated with HOMA-IR, or levels of fasting insulin; only rs780094C>T in GCKR was associated with lower fasting glucose levels (β −1.48, SE 0.65; p=.02) and higher triglyceride levels (β 7.34, SE 1.5; p= $9x10^{-7}$) and rs738409C>G was associated with lower triglyceride levels (β −6.43, SE 1.72; p=$2x10^{-4}$).

Table 3. Association of genetic variants with US-NAFLD in univariable and multivariable analysis. Odds ratios for NAFLD per copy of the effect allele.

	Crude univariate model		Multivariate adjusted model*	
SNP	**OR (95%CI)**	**p**	**OR (95% CI)**	**p**
(PNPLA3) rs738409; dosage, G allele	1.26 (1.09-1.45)	.002	1.51(1.27-1.78)	$2x10^{-6}$
(NCAN) rs2228603; dosage, T allele	1.27 (1.00-1.61)	.047	1.59 (1.20-2.10)	.001
(PPP1R3B) rs4240624; dosage, A allele	1.01 (0.82-1.25)	.920	1.18 (0.91-1.52)	.216
(LYPLAL1) rs12137855; dosage, C allele	0.96 (0.83-1.12)	.620	0.99 (0.83-1.18)	.891
(GCKR) rs780094; dosage, T allele	1.18 (1.04-1.34)	.009	1.20 (1.03-1.39)	.018

* adjusted for age, sex, homeostasis model assessment of insulin resistance, body mass index, serum triglyceride levels, serum HDL levels, and the other 4 single nucleotide polymorphisms.

Interaction

No interaction was observed between all five genetic variants with age, sex, BMI, serum triglyceride levels and serum HDL-C levels. Only HOMA-IR, analyzed a continuous variable, significantly modified the effect of rs738409, rs4240624, and rs780094 in logistic as well as ordinal regression analyses (p-values for interaction term <.001). Association of the effect allele G in rs738409 was weaker with higher HOMA-IR. We further analyzed interaction of SNPs with HOMA-IR by calculating ORs for US-NAFLD and level of significance per 0.5 increase in HOMA-IR. In participants with HOMA-IR >4.5, rs738409 was not associated with US-NAFLD (Figure 1). On the contrary, association with Us-NAFLD was stronger in participants with higher HOMA-IR per copy of the A allele in rs4240624 (p<.01 when HOMA-IR>6) and T allele in rs780094 (p<0.01 when HOMA-IR>4.0; Figure 1). When the effects of insulin, glucose and presence of diabetes mellitus were separately investigated, all of these variables modified the effect for rs738409 in PNPLA3; whereas only insulin modified the effect for variants in or near PPP1R3B and GCKR. There was no significant interaction between SNPs and the metabolic syndrome or sum of metabolic syndrome criteria when metabolic factors were replaced by the metabolic syndrome or sum of metabolic syndrome criteria in both the logistic as well as the ordinal regression model.

The association of rs738409 with BMI was modified by presence of US-NAFLD: rs738409C>G was significantly associated with a lower BMI in individuals without NAFLD (β-0.75, SE 0.19; $p=1x10^{-5}$), but not in individuals with US-NAFLD (β –0.17, SE 0.16; p=.2).

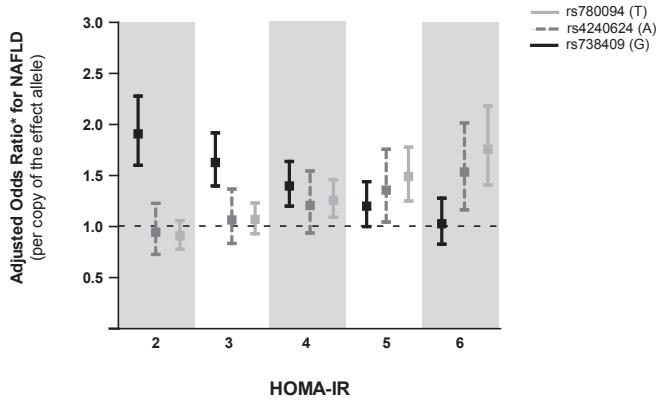

Fig. 1. Multivariable adjusted odds ratios (ORs) for US-NAFLD per copy of the effect allele for rs738409 in PNPLA3, rs4240624 near PPP1R3B and rs780094 in GCKR according to HOMA-IR, analyzed as a continuous variable (p-values for interaction term <0.001). *ORs were adjusted for age, sex, body mass index, serum triglyceride levels, serum HDL levels, and every other single nucleotide polymorphism.

Association of single nucleotide polymorphisms with elevation of ALT

Overall no associations were observed between the investigated SNPs and higher ALT or elevation of ALT, suggesting liver inflammation, either in linear or logistic regression adjusting for age, sex, presence of fatty liver, BMI, HOMA-IR, triglyceride level, and HDL-cholesterol level. Rs738409 in PNPLA3 showed significant association with elevated ALT (OR –per copy of the G-allele- 1.63, 95%CI 1.15-2.32; p=.006), but only in participants with US-NAFLD.

Discussion

The present study confirmed the association between genetic variants in or near PNPLA3, PPP1R3B, NCAN and GCKR with presence of US-NAFLD in a large population-based study of older subjects. The association of rs738409C>G in PNPLA3, rs4240624A>G near PPP1R3B, and rs780094C>T in GCKR was modified by insulin resistance as determined by HOMA-IR, such that lower HOMA-IR amplified the effects of the variant in PNPLA3 and higher HOMA-IR amplified the effects of variants in or near PPP1R3B and GCKR.

In elderly individuals the association between NAFLD and genetic variants may be expected to differ, as a results of longer maintenance of exposure to lifestyle factors, and because metabolic features or hepatic steatosis may have a distinct pathophysiology. Moreover, survivorship may cause the genetic background of studied individuals to vary. Consistent with previous studies in children, adolescents and adult subjects, rs738409C>G in PNPLA3 was strongly associated with higher prevalence of US-NAFLD in elderly individuals, and no interaction with age was demonstrated [4-5, 13-14]. Current knowledge is that PNPLA3, or adiponutrin, is involved in lipid metabolism. Several studies demonstrated that PNPLA3 plays a role in hydrolysis of glycerolipids; the isoleucine-to-methionine substitution at amino acid position 148 (I148M), resulting from the rs738409 polymorphism, may cause a loss of function [15-16]. More recently, Kumari *et al* demonstrated that human and murine adiponutrin acts as nutritionally regulated acyl-CoA-dependent lysophosphatidic acid acyltransferase (LPAAT) and that the I148M variant of adiponutrin exhibits elevated LPAAT activity in comparison to the wild-type protein, arguing in favour for a gain of fuction resulting from the I148M variant [17]. Therefore, it was suggested that PNPLA3 may exhibit distinct activities, dependent on nutritional environment, by mediating acylation of lysophospholipids in an acyl-CoA-dependent manner and to a lesser extend, by hydrolyzing TG in liver [18]. However, exact pathophysiological mechanisms remain unknown. Interestingly, rs738409C>G was also associated with elevation of serum ALT in elderly participants with US-NAFLD, suggesting an increased risk of liver inflammation in subjects carrying a G allele. These findings are in line with previous biopsy studies assessing the association of the rs738409 variant in PNPLA3 with histological NASH, and inflammation in hereditary hemochromatosis and hepatitis C [19-22]. We did not demonstrate an association between rs738409 and ALT or elevation of ALT outside the context of US-NAFLD. However, this variant was associated

with ALT in a large meta-analysis of GWAS that included a larger sample of Rotterdam Study participants, with earlier data on ALT [23].

In addition, this is the first study to replicate findings of an association of rs2228603C>T near NCAN and rs780094C>T in GCKR with NAFLD in adult subjects. NCAN is a chondroitin sulfate proteoglycan thought to be involved in the modulation of cell adhesion and migration [24]. Although this variant is in high linkage disequilibrium with variants that affect gene functioning, the pathophysiological mechanism by which this variant may increase susceptibility to NAFLD is yet unknown [5]. The role of GCKR is defined at greater length; it encodes glucokinase regulatory protein, an inhibitory protein, that regulates glucokinase (GK) in response to glucose and insulin [25]. GK is involved in the phosphorylation of glucose in the first step of glycolysis and enhances insulin secretion from pancreatic beta cells. GCKR deficient mice display reduced GK expression and show impaired glycemic control, whereas hepatic overexpression of GCKR in mice is associated with improvement of fasting plasma glucose and insulin sensitivity, but higher triglyceride levels [26-27]. Consistent with previous studies in humans we found that the T allele of rs780094 in GCKR is associated with lower fasting plasma glucose levels, but oppositely higher triglyceride levels [28-30]. It is hypothesized that variation of rs780094 in GCKR, or a SNP in strong linkage disequilibrium with rs780094, may increase glucokinase activity, leading to increased glycolysis and subsequent increases in malonyl co-enzyme A (CoA), a substrate for de novo lipogenesis, resulting in hepatic fat accumulation [31-32].

Although various studies in humans have investigated associations between rs738409C>G and measures of insulin resistance or sensitivity, BMI and fasting levels of triglycerides, cholesterol, glucose and insulin, these studies did –to our knowledge- not specifically examine effect modification. Noteworthy, although none of the SNPs were associated with HOMA-IR or fasting insulin levels, we demonstrated significant interaction with insulin resistance for variants in or near PNPLA3, PPP1R3B and GCKR. Effect modification was not observed for the variants in or near NCAN and LYPLAL1. Insulin resistance is an important correlate of NAFLD in epidemiological as well as clinical studies, and may be both a cause as well as a consequence of fatty liver [33]. Our findings suggest that expression of the deleterious phenotype may be dependent on certain internal environmental conditions in the elderly, such as insulemic or glycemic status. *In vitro* and *in vivo* studies have demonstrated that adiponutrin expression is upregulated by insulin and glucose, possibly indirectly by altering expression and cleavage of transcription factors carbohydrate-responsive sterol regulatory element binding protein (ChREBP) and sterol regulatory binding protein (SREBP)1C [34-36]. Consequently, expression of adiponutrin in insulin sensitive subjects may be lower than in insulin resistant subjects, and thus an impairment of the functional protein, caused by an I148M change, may yield a more pronounced effect on NAFLD in insulin sensitive subjects. However, rs738409 may also influence NAFLD by modifying the effect of insulin resistance. Currently, the knowledge regarding the role of adiponutrin in the adipocyte or hepatocyte is still limited, making it difficult to functionally understand a link between adiponutrin expression and metabolic status.

There was also a significant interaction of the variants in or near GCKR and PPP1R3B with insulin resistance. Both PPP1R3B as well as GCKR were considered susceptibility genes in type II diabetes mellitus and maturity onset diabetes of the young. PPP1R3B acts as a glycogen-targeting subunit for a phosphatase, PP1, which is involved in the modulation of glycogen synthesis in liver and muscle. Expression of the hepatic glycogen-targeting subunit of PP1 is regulated by insulin [37]. Given the involvement of PPP1R3B and GCKR in glucose metabolism and replication of the variants in our near these genes in elderly participants with insulin resistance, it is reasonable to initiate further studies to identify causative mechanisms regarding these genes for susceptibility to NAFLD.

Finally, we also found paradoxical lower plasma triglyceride levels in participants with rs738409C>G. Although most studies on NAFLD in humans have demonstrated no association of variants in PNPLA3 with serum lipid levels, an association between rs738409C>G and lower triglyceride levels was previously demonstrated in obese individuals with the I148M variant in a study by Palmer *et al* [38]. Possibly, because of the high prevalence of obesity in this elderly population, power to detect such an association was greater than in previous population-based studies, in which the prevalence of obesity was not as pronounced. Furthermore, Speliotes *et al* also demonstrated a favourable effect of the G allele on metabolic profile in samples from the NASH CRN, when glucose intolerant PIVENS (Proglitazone versus vitamin E versus placebo for treatment of non-diabetic patients with non-alcoholic steatohepatitis trial) samples were excluded [39]. The decrease in fasting serum triglycerides has been suggested to be the result of impaired hepatic triglyceride efflux due to decreases in very low-density lipoprotein (VLDL) lipidation caused by the variant I148M [38].

The strength of the present study concerns the large number of elderly participants that were included. Power calculation showed that the power to detect previously reported odds ratios was more than ninety percent. Furthermore, extensive data were available for characterization of metabolic and environmental traits, which made it possible to investigate effect modification by these traits.

Our study was also subject to potential limitations. Firstly, NAFLD was diagnosed by means of ultrasonography, which is unable to identify a degree of steatosis of less than 30%. Nevertheless, abdominal ultrasonography has an acceptable sensitivity of 80-100% for detecting fatty liver, and its accuracy for diagnosis of fatty liver meets other imaging modalities [40-42]. Secondly, this study has a cross-sectional design, and thus it is difficult to differentiate cause and consequence concerning observed associations. Finally, we are unable to support our epidemiological study with functional or experimental data. Future experimental studies are warranted addressing these putative gene-trait interactions.

In conclusion, in this cross-sectional population-based study, we demonstrated an association between rs738409 in PNPLA3 and the presence of US-NAFLD, especially in insulin sensitive subjects. Furthermore, we replicated the association of rs2228603C>T near NCAN with US-NAFLD for the first time, and demonstrated an association between rs780094C>T,

rs4240624G>A and US-NAFLD in insulin resistant subjects. Further studies are warranted investigating the role of these genes and variants in the genetic susceptibility of NAFLD. Our findings concerning interaction may have substantial implications for public health care, given current epidemics in diabetes mellitus type 2 and obesity. Elucidation of genetic factors that predispose to NAFLD would allow prevention strategies to be targeted at those most at risk, and may identify those that would respond best to certain treatments.

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CHAPTER 5

Distribution of and factors associated with liver stiffness in older adults: transient elastography in the Rotterdam Study

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Submitted

Abstract

Background and Aim: Transient elastography (TE) measures liver stiffness, which correlates well with histological stages of liver fibrosis. By means of TE, we aimed to investigate the distribution and factors associated with liver stiffness measurement in older individuals. **Methods:** This study was part of the Rotterdam Study, a large population-based cohort study among older individuals. All participants underwent abdominal ultrasound and TE. TE was considered reliable if ≥60% success rate (≥10 valid measurements) and IQR/median liver stiffness measurement (LSM) ≤0.3. LSM>9.5kPa and >13kPa were taken as cut-offs suggesting advanced liver fibrosis and cirrhosis, respectively. Associations between traits and continuous log-transformed LSM as or LSM>9.5kPa were assessed using linear or logistic regression analysis, respectively. **Results:** Of 1927 participants who underwent TE, 1328 participants (mean age 68.5±7.5 years) had reliable LSM (TE failure: 4.0%, unreliable LSM: 27.1%). In multivariable analysis, unsuccessful LSM was associated with higher age, higher BMI and female gender. Thirty-seven participants had LSM >9.5kPa (2.8%), 9 had LSM >13kPa (0.7%). In multivariable linear regression analysis, higher age, male gender, greater HOMA-IR, greater spleen size, higher ALT, lower BMI and presence of fatty liver were associated with higher log-transformed LSM. Fourteen (3.8%) of 365 participants with non-alcoholic fatty liver disease (NAFLD) had LSM>9.5kPa. In linear regression analyses higher age, higher ALT and greater HOMA-IR were associated with higher log-transformed LSM in subjects with NAFLD. **Conclusion:** In the present population-based study, we demonstrated that higher age, male gender, greater HOMA-IR, greater spleen size, higher ALT, lower BMI and presence of fatty liver were associated with higher log-transformed LSM in older adults.

Introduction

Advanced liver fibrosis and cirrhosis affect hundreds of millions worldwide and comprise a major cause of morbidity and mortality [1-2]. In the United States and Europe liver cirrhosis alone is the twelfth leading cause of mortality [3]. Considering population ageing and current epidemics in obesity and type II diabetes, the burden of chronic liver diseases is projected to increase substantially in the next decades [4-6]. Currently, there is a lack of knowledge on prevalence and risk factors of advanced liver disease in the general population, as data are mainly derived from autopsy studies or biopsy studies in selected populations. So far, no studies have examined the prevalence of advanced liver fibrosis in the elderly.

In theory, the best 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. Newer non-invasive techniques have begun to circumvent the need for liver biopsy. One of these techniques is transient elastography (TE). TE measures liver stiffness, which is strongly associated with histological stages of liver fibrosis [7-12]. This technique performs particularly well in discriminating between stages of advanced liver fibrosis (or cirrhosis) from absence of liver fibrosis. Therefore, our aim was to investigate the distribution of and factors associated with liver stiffness measurement (LSM) in a population-based study of mainly older Caucasian individuals by means of TE.

Subjects and methods

Study population

The Rotterdam Study is a large prospective population-based cohort study conducted among older adults and elderly persons living in Ommoord, a district of Rotterdam, The Netherlands. The rationale and study design have been described previously [13]. The medical ethics committee at Erasmus University of Rotterdam approved the study, and written informed consent was obtained from all participants.

All participants that visited the research center between January 2011 and September 2012 (age range 51-98 years) were included in the study. Each participant completed an extensive interview and clinical examination that included abdominal ultrasonography, transient elastography, 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 drug prescriptions was dispensed from automated pharmacies, where nearly all participants (98%) are registered.

Biochemistry

Fasting blood samples were collected on the morning of ultrasound examination. Blood lipids, 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, HBsAg and anti-HCV were measured by automatic immunoassay (Roche Diagnostics GmbH, Mannheim, DE). According to local cut-off criteria, the upper limit of normal (ULN) of ALT was defined as 40U/L for men and 30U/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 experience in ultrasonography (JNLS). The diagnosis of fatty liver was determined by the ultrasound technician according to the protocol by Hamaguchi *et al* [14]. Non-alcoholic fatty liver disease (NAFLD) was defined as fatty liver in absence of any of the following possible secondary causes of fatty liver: 1) excessive alcohol consumption (>14 drinks/week), 2) positive HBsAg or anti-HCV tests, and 3) use of pharmacological agents associated with fatty liver (i.e. amiodarone, corticosteroids, methotrexate, and tamoxifen). In addition, spleen size, presence of collaterals, and Doppler examination of hepatic veins, artery and portal vein were evaluated during US examination. Splenomegaly was defined as a spleen size >12.0 cm.

Transient elastography

Liver stiffness measurements (Fibroscan, EchoSens, Paris, France) were performed by a single operator, who carried out more than 200 examinations before start of the study. Liver stiffness measurement (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. Failure was recorded when no LSM was obtained after at least 10 shots. Reliable LSM was defined as ≥ 60% success rate (≥ 10 valid measurements) and IQR/M >0.3. LSM >9.5kPa and >13 kPa were taken as cut-offs suggesting advanced liver fibrosis and cirrhosis, respectively. These cut-off levels were deliberately chosen, for they yield high positive predictive value for presence of advanced fibrosis in various liver diseases, including (N)AFLD and viral hepatitis [9, 15].

Covariables

Anthropometric measurements were performed by well trained research assistents. Body Mass Index (BMI) was calculated as weight (kg)/ length (m^2). Waist and hip circumference were measured in centimeters. The average of two blood pressure measurements, obtained at a single visit in sitting position after a minimum of 5 minutes rest, was used for analysis. The metabolic syndrome was defined, according to Adult Treatment Panel III criteria [16], 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 <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. Diabetes mellitus (DM) was defined as fasting plasma glucose (FPG) ≥126 mg/dL (7.0 mmol/L) or drug treatment for elevated blood glucose. 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 [17]. Echocardiographic assessment of the pulmonary artery systolic pressure (PASP) was performed with a commercially available ultrasonography system (Vivid i, GE Healthcare, with a 2.5 MHz transducer) using a standardized protocol. The PASP was calculated as the sum of the estimated right atrial pressure (based on inferior vena cava diameter and respiratory collapse) and the pressure gradient over the tricuspid valve [18]. The pressure gradient was computed from the highest Doppler tricuspid regurgitation velocity gathered from several windows using the simplified Bernoulli equation [18]. In those with sufficient tricuspid regurgitation to estimate PASP, a 40 mmHg cut-off was set to define echocardiographic pulmonary hypertension.

Statistical analysis

Baseline analyses 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. We used logistic regression analysis, adjusted for age, gender, BMI, presence of fatty liver, and ALT to study factors associated with unsuccessful LSM (TE failure and unreliable LSM). Choice of probe and presence of LSM greater than 9.5kPa was additionally adjusted for in a second model, including all participants in whom LSM was obtained. To examine associations between traits and LSM as continuous (log-transformed) dependent variable or LSM >9.5kPa, we performed linear or logistic regression analysis, respectively. For there is some discordancy regarding the cut-off level of LSM for presence of advanced liver fibrosis using XL-probe, we tested our model applying a one point lower cut-off for XL-probe (corresponding to 8.5kPa) [8, 19]. All models were tested

for interaction with age and gender. A *P*-value of <.05 was considered statistically significant. Statistical analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

Results

Study population

Of 1950 participants aged 51 or above, a total of 1927 participants underwent transient elastography at our research center. LSM was successful in 1328 participants (Figure 1). Baseline characteristics of the study population according to TE feasibility are shown in Table 1. Fifty-five percent of 1927 participants were women, mean age of participants was 69.2 ± 7.6 years and mean BMI was 27.6 ± 4.3 kg/m^2. Participants were predominantly of Caucasian ethnicity (95.2%). In multivariable analysis, failure and unreliable LSM were associated with higher age, female gender, and higher BMI (all p-values <.003; Table 2).

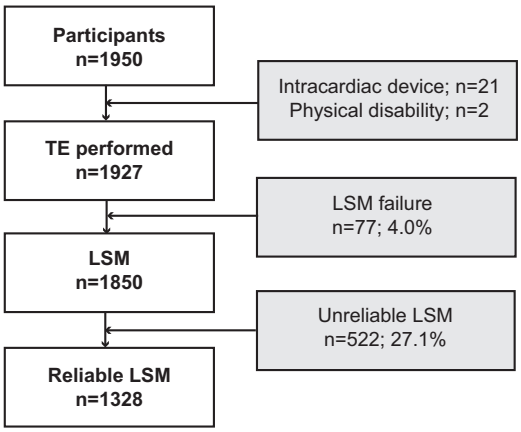

Fig. 1. Flow chart of the study. In total, 1328 participants had reliable liver stiffness measurement.

Factors associated with liver stiffness

Univariable analysis

Data on LSM in 1328 elderly participants were analysed. Distribution of LSM is illustrated in Figure 2. Median LSM was 4.9kPa (interquartile range (IQR) 4.0-6.1).

In univariable analysis, log-transformed (log) LSM was correlated with higher age (correlation coefficient (ρ)=0.16; p<.001), higher waist circumference (ρ=0.16; p<.001), higher BMI (ρ=0.06; p=0.02), greater spleensize (ρ=0.23; p<.001) and was higher in men than in women (0.74 log kPa (0.72-0.75) vs. 0.66 log kPa (0.65-0.67) respectively, p=<.001). In addition, log LSM

Table 1. Baseline characteristics according to feasibility of TE.

	Total	TE Failure	Unreliable LSM	Reliable LSM	p*
Characteristic	*n=1927*	*n=77*	*n=522*	*n=1328*	
Age (years)	69.2 (7.6)	69.5 (7.4)	70.7 (7.4)	68.5 (7.5)	<.001
Female (%)	54.6	54.5	60.3	52.3	.008
BMI (kg/m2)	27.6 (4.3)	31.2 (7.0)	28.1 (4.2)	27.3 (4.0)	<.001
Normal; BMI < 25 (%)	27.4	16.9	23.3	29.6	
Overweight; 25 ≤ BMI < 30 (%)	48.9	31.2	49.1	49.8	
Obese; BMI ≥ 30 (%)	23.7	51.9	27.6	20.6	
Waist/Hip-ratio	0.91 (0.09)	0.95 (0.09)	0.92 (0.09)	0.90 (0.09)	.002
Alcohol consumption (drinks/week)	6.1 (7.6)	5.6 (7.1)	5.3 (6.7)	6.5 (7.9)	.008
Diabetes Mellitus (%)	13.4	19.7	15.2	12.3	.1
Fatty liver (%)	37.8	44.2	37.2	37.7	.5
ALT (U/L)	18 (14-23)	17 (15-22)	17 (13-22)	18 (14-24)	.3
HOMA-IR	2.6 (1.7-4.0)	3.3 (2.2-5.6)	2.6 (1.8-4.2)	2.6 (1.7-3.9)	<.001
LSM (kPa)	4.9 (4.0-6.2)†	.	4.9 (3.8-6.6)	4.9 (4.0-6.1)	.3‡
LSM >9.5 kPa (%)	3.2	.	4.2	2.8	.1‡

Data are represented as mean (± standard deviation), median (25^{th}-75^{th} percentile) or percentages.
*Based on T-test, Wilcoxon rank sum test or Chi-square test.
†Results for n=1850.
‡ P-value for unreliable versus reliable LSM.
Abbreviations: BMI, Body Mass Index; ALT, alanine aminotransferase; HOMA-IR, Homeostasis Model Assessment of Insulin Resistance; LSM, liver stiffness measurement; TE, Transient Elastography.

Table 2. Factors associated with TE failure and unreliable LSM in multivariable analysis for participants in whom TE was performed (n=1927; A) and for participants in whom LSM was obtained (n=1850; B), respectively.

(A) Participants in whom TE was performed (n=1927)			**(B)** Participants in whom LSM was obtained (n=1850)		
	OR (95% CI)	**p**		**OR (95% CI)**	**p**
Age, years	1.04 (1.03-1.06)	<.001	Age, years	1.05 (1.03-1.06)	<.001
Gender, female	1.29 (1.05-1.59)	.01	Gender, female	1.40 (1.13-1.72)	.002
BMI, kg/m^2	1.08 (1.06-1.11)	<.001	BMI, kg/m^2	1.06 (1.03-1.09)	<.001
Fatty liver	0.85 (0.67-1.08)	.2	Fatty liver	0.77 (0.58-1.01)	.06
M-probe	1.04 (0.81-1.33)	.8	M-probe	1.15 (0.94-1.41)	.08
			LSM >9.5kPa	1.37 (0.78-2.38)	.3

was correlated with higher serum levels of ALT (ρ=0.19; p<.001), AST (ρ=0.22; p=<.001), GGT (ρ=0.31; p<.001), ALP (ρ=0.06; p=.04), and HOMA-IR (ρ=0.15; p<.001), and lower platelet count (ρ=−0.12; p<.001). Thirteen participants had positive viral serology (3 HBsAg positive, 10 anti-HCV positive). Log LSM was correlated with positive viral serology (0.69 log kPa (0.69-0.70) vs. 0.79 log kPa (0.71-0.87); p=.02). Data on estimated PASP were available for 819 participants;

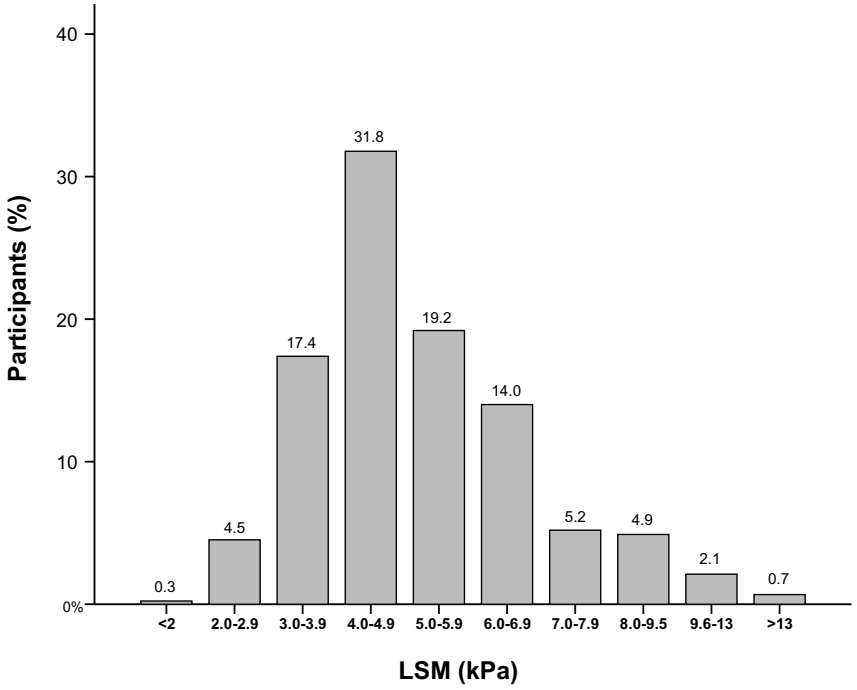

Fig. 2. Distribution of reliable liver stiffness measurements in 1328 participants.

PASP >40 mmHg was significantly associated with higher log LSM (0.69 log kPa (0.68-0.70) vs. 0.82 log kPa (0.75-0.89); p<.001).

Multivariable analysis

In linear regression analysis, log LSM was associated with higher age, male gender, greater HOMA-IR, greater spleen size, higher ALT, use of M-probe, lower BMI, and presence of fatty liver (Table 3). There was a trend for association between higher LSM and presence of positive viral serology. Waist circumference was not associated with higher LSM, when we replaced BMI with waist circumference in the model. Presence of diabetes mellitus was associated with higher LSM when we replaced HOMA-IR with presence of diabetes mellitus in the model (correlation coefficient (β) 0.054, SE 0.012, p=<.001). Since data on PASP were available for only 819 participants, we adjusted for PASP >40mmHg in a separate model. Adjustment for PASP did not alter the association of other covariables and estimated PASP >40mmHg remained associated with higher log LSM (β 0.094, SE 0.032; p=.003).

When we performed linear regression analysis in 781 participants without apparent liver disease, excluding participants with positive HBsAg, anti-HCV, fatty liver, and ALT above the upper limit of normal, only higher age (β 0.003, standard error (SE) 0.001; p<.001), male gender (β 0.080, SE 0.012; p<.001), and lower BMI (β −0.005, SE 0.002; p=.008) remained associated

with log LSM. There was a trend for association between higher log LSM and greater spleen size (β 0.007, SE 0.004; p=.08). In these participants, 5th and 95th percentile values of LSM were 3.0kPa and 7.9kPa for M-probe and 2.8kPa and 7.8kPa for XL-probe, respectively. Age (β 0.002, SE 0.001; p=.003), male gender (β 0.081, SE 0.011; p<.001) and lower BMI (β −0.005, SE 0.002; p=.005) remained associated with higher log LSM, after excluding an additional 12 cases with LSM >9.5 kPa.

Table 3. Factors associated with (log-transformed) LSM in multivariable analysis.

Variable	B	SE	p
Age, years	0.003	0.001	<.001
Gender, male	0.052	0.010	<.001
HOMA-IR	0.004	0.001	.001
Platelet count, *10^9/L	−3.1e−5	6.8e−5	.7
Spleen size, cm	0.015	0.003	<.001
ALT, U/L	0.002	3.7e−4	<.001
XL probe	−0.022	0.010	.03
Hypertension	0.002	0.010	.9
BMI, kg/m^2	−0.003	0.001	.02
Fatty liver	0.029	0.011	.004
Alcohol consumption, drinks/week	−3.0e−4	0.001	.9
HBsAg or anti-HCV positive	0.082	0.046	.08

Factors associated with elevated liver stiffness

Of 1328 participants, 727 (54.7%) participants had LSM ≤5kPa, suggesting absence of liver fibrosis, 37 (2.8%) participants had LSM >9.5kPa, suggesting presence of advanced liver fibrosis and 9 (0.7%) participants had LSM >13kPa, suggesting presence of liver cirrhosis. General characteristics of participants according to a LSM cut-off of 9.5kPa are shown in Table 4. One participant with LSM >9.5kPa had positive serum anti-HCV and none had positive serum HBsAg. In multivariable logistic regression analysis higher age, presence of diabetes mellitus, and higher ALT were associated with LSM >9.5kPa (Table 5). Although presence of fatty liver was more prevalent in subjects with LSM >9.5kPa (59.5%) than in subjects with LSM ≤9.5kPa (37.1%), the association was not significant in multivariable analysis. Eight of 9 (89%) participants with LSM>13kPa were female, mean age was 72.5 (±8.4) years, and mean BMI 28.5 (±5.1) kg/m^2. One participant drank more than 14 alcoholic beverages weekly and 5 participants had fatty liver on ultrasonography. Two of 7 participants with LSM >13kPa had PASP >40mmHg and splenomegaly was present in 1 of 8 participants with LSM >13kPa.

Table 4. Baseline characteristics of elderly participants according to LSM (≤/>9.5kPa).

	Total	≤9.5kPa	>9.5kPa	p*
Characteristic	*n=1328*	*n=1291*	*n=37 (2.8%)*	
Age (years)	68.5 (7.5)	68.3 (7.5)	72.5 (8.4)	.001
Female (%)	52.3	52.3	54.1	.8
Caucasian (%)	94.8	94.7	100.0	.2
BMI (kg/m2)	27.5 (4.0)	27.3 (3.9)	28.5 (5.1)	.1
Normal; BMI < 25 (%)	29.6	29.6	30.6	
Overweight; 25 ≤ BMI < 30 (%)	49.8	50.2	36.1	
Obese; BMI ≥ 30 (%)	20.6	20.2	33.3	
Waist/Hip-ratio	0.91 (0.09)	0.91 (0.09)	0.94 (0.10)	.05
Alcohol consumption (drinks/week)	6.5 (7.9)	6.5 (7.9)	7.2 (7.4)	.6
Smoking				.3
Never (%)	34.7	34.9	27.0	
Former (%)	53.0	53.0	54.1	
Current (%)	12.3	12.1	18.9	
Hypertension (%)	69.7	69.2	83.8	.06
Diabetes Mellitus (%)	12.3	11.7	34.3	<.001
Metabolic syndrome (%)	47.7	47.5	57.1	.3
FPG >100 mg/dL or drug treatment for elevated blood glucose	44.7	44.2	62.9	.03
Waist circumference >88cm (♀) or >102 cm (♂)	44.0	43.7	54.1	.2
Triglycerides >150 mg/dL or drug treatment for elevated triglycerides	38.9	44.4	38.8	.5
HDL-C <40 mg/dL(♂) or <50 mg/dL(♀) or drug treatment for low HDL-C	35.6	35.4	44.4	.3
BP ≥130/85 mmHg or drug treatment for elevated BP	83.1	80.6	89.2	.3
Ultrasound characteristics				
Fatty liver (%)	37.7	37.1	59.5	.006
Splenomegaly; spleen size >12 cm (%)†	2.0	1.7	11.8	.004
Portal vein flow velocity (cm/s)‡	23.0 (5.0)	23.1 (5.3)	22.4 (5.1)	.3
Estimated PASP >40 mmHg§	2.3	1.9	19.0	<.001
Laboratory data				
ALT (U/L)	18 (14-24)	18 (14-23)	21 (17-37)	.003
AST (U/L)	25 (21-29)	25 (21-28)	33 (24-41)	<.001
Bilirubin (umol/L)	8 (6-11)	8 (6-11)	9 (6-15)	.1
ALP (U/L)	67 (56-78)	67 (56-78)	75 (64-98)	.01
GGT (U/L)	24 (17-36)	23 (17-33)	42 (27-100)	<.001
HOMA-IR	2.6 (1.7-3.9)	2.6 (1.7-3.8)	4.0 (2.1-6.2)	.001
Platelet count (*10^9/L)	256 (220-303)	257 (220-303)	227 (186-311)	.04

Table 4. (continued)

Characteristic	Total n=1328	≤9.5kPa n=1291	>9.5kPa n=37 (2.8%)	p*
Transient elastography				
IQR/M	0.17 (0.12-0.22)	0.17 (0.12-0.22)	0.19 (0.13-0.25)	.1
LSM (kPa)	4.9 (4.0-6.1)	4.8 (4.0-5.9)	11.5 (10.2-13.5)	<.001

Data are represented as mean (± standard deviation), median (25^{th}-75^{th} percentile) or percentages.
*Based on T-test, Wilcoxon rank sum test or Chi-square test.
† Spleen size measurement was available for 1153 participants
‡ Portal blood flow velocity was available for 1286 participants.
§ Echocardiographic parameters for estimation of PASP were available for 819 participants.
Abbreviations: BMI, Body Mass Index; FPG, fasting plasma glucose; HDL-C, high-density lipoprotein cholesterol; BP, blood pressure; PASP, pulmonary artery systolic 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.

Table 5. Factors associated with LSM >9.5kPa (n=37) in multivariable analysis.

Variable	OR (95%CI)	p
Age, years	1.09 (1.03-1.14)	.001
Gender, male	0.78 (0.38-1.61)	.5
Diabetes Mellitus	2.84 (1.31-6.13)	.008
ALT, U/L	1.03 (1.02-1.05)	<.001
Hypertension	1.16 (0.44-3.03)	.8
BMI, kg/m^2	1.03 (0.94-1.13)	.5
Fatty liver	1.59 (0.71-3.55)	.3
Alcohol consumption, drinks/week	1.01 (0.97-1.06)	.6

We performed several sensitivity analyses to determine the robustness of our findings. Consistency was shown, when one point lower cut-off for elevated LSM was applied for use of XL-probe (LSM >8.5kPa). Furthermore, when participants with ALT >2x the ULN were excluded from the analyses, for severe inflammation of the liver may have influenced positive predictive value of presence of advanced liver fibrosis, the prevalence of LSM >9.5kPa was 2.5% and similar factors remained associated with elevated LSM. No interaction between age, gender and any of the other covariables was observed in all models.

Liver stiffness in participants with NAFLD

A sub analysis was performed to investigate factors associated with LSM in participants with NAFLD. Of 1328 participants, 244 participants were excluded due to presence of secondary causes associated with fatty liver (excessive alcohol consumption (n=204), positive anti-HCV (n=10), positive HBsAg (n=3), pharmacological agents associated with fatty liver (n=36)). Three hundred sixty-five (34.4%) participants had NAFLD. Mean age of participants with NAFLD was 68.1±6.9 years, mean BMI was 29.8±3.9 kg/m^2, and median LSM was 5.1kPa (4.3-6.3). Fourteen

(3.8%) participants had LSM>9.5kPa. In univariable analysis, only higher ALT (ρ=0.27; p<.001), and greater HOMA-IR (ρ=0.15; p=.004) were correlated with log LSM in participants with NAFLD. In linear regression analysis, adjusting for age, gender, BMI, ALT, HOMA-IR, alcohol consumption and presence of hypertension, higher age (β 0.003, SE 0.001; p=0.02), greater HOMA-IR (β 0.004, SE 0.002; p=.03), and higher ALT (β 0.003, SE 0.001; p=<.001) were associated with higher log LSM.

Discussion

This is the first study to investigate the distribution of and factors associated with liver stiffness in a Caucasian general population of mainly older individuals. In this cross-sectional study the prevalence of liver stiffness measurement >9.5kPa, suggestive of advanced liver fibrosis, was around 3.0%. Higher age, male gender, greater HOMA-IR, greater spleen size, higher ALT, use of M probe, lower BMI, and presence of fatty liver were independently associated with higher log-transformed LSM.

To date, only few community-based studies have used TE to assess liver stiffness in adults of the general population. A cross-sectional community-based study by Wong *et al*. assessed the prevalence of LSM >9.5kPa in 759 Hong Kong Chinese subjects in whom MRI was performed to assess intrahepatic triglyceride content [20]. Four percent of subjects with NAFLD and 1.9% of total subjects (without hepatitis B or C infection) had LSM >9.5kPa. In the present study prevalence of LSM >9.5kPa was 2.8%. The high prevalence of fatty liver and metabolic syndrome, and high age of this population may explain the slight discrepancy in results. Only few participants had viral hepatitis or excessive alcohol consumption, and thus these risk factors for liver disease are less likely to explain the higher prevalence of advanced liver fibrosis. In a community-based study by Roulot *et al*, in which TE was performed in 1358 subjects older than 45 years attending for a free medical check-up, prevalence and risk factors of LSM >8kPa were rather consistent with our findings (data not shown) [19].

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 [19, 21-23]. Indeed, the incidence of liver diseases increases with advancing age, which may in part explain a higher prevalence of LSM >9.5kPa at higher age [24]. However, we demonstrated that higher age remained associated with higher LSM after exclusion of subjects with HBsAg, anti-HCV, fatty liver and LSM >9.5kPa. Elastic properties of the normal liver may change as a result of ageing for several reasons. Firstly, age-related changes in histological structure 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 [25-26]. Hepatocytes in aged livers show increased polyploidy [27]. Secondly, livers of older individuals may be stiffer as a result of minimal accumulation of collagen.

It is hypothesized that older individuals have reduced collagenolytic activity [28]. 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 [24].

We observed an association of greater insulin resistance with higher LSM and LSM >9.5kPa. Indeed, HOMA-IR is a well known predictor of advanced liver fibrosis in (non)alcoholic steatohepatitis (NASH) and hepatitis C virus infection [29-30]. In a sub analysis of participants with NAFLD, HOMA-IR was independently associated with log LSM. Unfortunately, we were unable to determine if this is a cause or a consequence of greater liver stiffness or advanced liver fibrosis, due to cross-sectional design of this study. Nevertheless, these findings underline the relevance of targeting insulin resistance in the management of NASH in older individuals.

Interestingly, there was a strong independent association between greater spleen size and higher liver stiffness, suggesting that even slight increases in liver stiffness or liver fibrosis may be associated with increases in portal hypertension. Furthermore, in a separate analysis, presence of echocardiographic pulmonary hypertension, was associated with higher liver stiffness. However, it remains uncertain whether higher liver stiffness in participants with elevated PASP truly reflects presence of liver fibrosis. Other underlying conditions or epiphenomena of liver disease, including lung and heart disease, may cause pulmonary hypertension and increase liver stiffness as a result of congestion of the liver or increased vascular resistance [31-33]. Unfortunately, we were unable to specify these causes in the present study. This finding therefore warrants further research in this area.

In contrast to earlier community-based studies applying TE, we found a high rate of unreliable LSM (27.1%). The high rate of unsuccessful measurements can probably be explained by the fact that we investigated a population of individuals with a mean age of 69 years, in whom the metabolic syndrome, including obesity, is highly prevalent. Indeed, obesity, and in particular increased waist circumference, is considered to be a pivotal predictor of TE failure or unreliable LSM [19, 22, 34-35]. Furthermore, in a small study from France, in which TE was performed in elderly inpatients without liver disease, TE failure was observed in 12 of 66 subjects (18%) and subsequent success rates were significantly lower in elderly subjects than in adult subjects [21]. Given the high rate of unsuccessful TE, feasibility of TE in the elderly is debatable.

The strengths of this study encompass the large number of elderly participants that were included and extensive data that were available for characterization of metabolic and environmental traits. Our study also has several limitations. Firstly, we used either M- or XL- probe to assess liver stiffness. Feasibility of XL-probe has only been reported in few studies and to date, results are controversial [8, 36-37]. XL-probe may have a lower cut-off value for advanced liver fibrosis. However, choice of probe was adjusted for in our analyses if applicable, and when one point lower cut-off for elevated LSM was applied for XL-probe in multivariable analyses, results did not change. Secondly, the rather high rate of unreliable LSM may have led to a selection bias, presumably an underestimation of the prevalence of advanced liver fibrosis.

Lastly, we were unable to report on liver biopsy findings since the majority of participants with LSM >9.5kPa did not undergo liver biopsy. This decision was at the discretion of the physician and the additional value of liver biopsy in aged individuals may be questionable, especially in individuals with (non-alcoholic) fatty liver disease.

In summary, prevalence of LSM higher than 9.5kPa, suggestive of advanced liver fibrosis, was 3.0% in this older adult population. Higher age, male gender, greater HOMA-IR, greater spleen size, higher ALT, use of M-probe, lower BMI, and presence of fatty liver were independently associated with higher log-transformed LSM.

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CHAPTER 6

Growth hormone, sulfated dehydroepiandrosterone and adiponectin levels in non-alcoholic steatohepatitis: an endocrine signature for advanced fibrosis in obese patients

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Abstract

Background and aims: Liver related clinical consequences of non-alcoholic fatty liver disease (NAFLD) are seen only in the minority of patients with advanced fibrosis. The aim of our study was to generate insight into a potential endocrine basis of steatohepatitis and advanced fibrosis in NAFLD. **Methods:** Liver biopsy and blood samples were prospectively collected from patients with medically complicated Class III obesity. Patients were categorized, according to liver histology, into: i) normal, ii) simple steatosis (SS), iii) NASH with fibrosis stage (FS) 0-1 and iv) NASH with FS≥2. A broad panel of potential biomarkers, included sulfated dehydroepiandrosterone (DHEA-S), growth hormone (GH), homeostasis model assessment of insulin resistance (HOMA-IR), leptin, resistin, adiponectin, and cytokeratin 18 (CK-18) fragments. **Results:** We studied 160 patients (mean BMI 46.8± 8.2 kg/m^2). Liver biopsies demonstrated normal histology in 10%, SS in 45%, NASH with FS 0-1 in 37.5% and NASH with FS≥2 in 7.5%. C-reactive protein (CRP), interleukin 6 ((IL-6), GH, CK-18, adiponectin, HOMA-IR and quantitive insulin sensitivity check index (QUICKI) were significantly associated with NASH in univariate analysis, but overall predictivity of these parameters was low (AUROC=0.62-0.68). In contrast, all patients with NASH with FS≥2 had insulin resistance, as measured by QUICKI, and GH levels <0.45 ng/mL and all but one patient with NASH FS≥2 had low DHEA levels (<123μg/dL). **Conclusions:** Low serum levels of GH and DHEA are very common in patients with NASH with more advanced fibrosis. Other biomarkers, including CK-18 fragments, have predictivity characteristics that would be of low clinical utility for distinguishing patients with normal histology or SS from those with NASH. These findings demonstrate an endocrine profile associated with advanced fibrosis.

Introduction

Non Alcoholic Fatty Liver Disease (NAFLD) comprises a range of histological findings, from simple steatosis (SS) to non alcoholic steatohepatitis (NASH) with advanced fibrosis. Based on current prevalences of obesity and type 2 diabetes, non-alcoholic fatty liver disease (NAFLD) may conservatively be estimated to affect over 30 million people in the United States [1], and has been projected to become the leading indication for liver transplantation in the next decade [2]. Although the prevalence of NAFLD is high, the great majority of patients with NAFLD do not develop NASH with advanced fibrosis. The basis of susceptibility of a minority of patients with NAFLD to the development of NASH with advanced hepatic fibrosis is not known.

Growth hormone (GH) deficiency states, including Alstrom's syndrome [3] and hypopituitarism [4] are associated with severe NASH and advanced fibrosis. NASH can be reversed by exogenous growth hormone supplementation [3]. Obesity is associated with low growth hormone levels [5-7], at least in part due to low circulating levels of ghrelin [8] (in response to caloric excess), which, in addition to affecting appetite, is a somatotrope [9]. In addition, hyperinsulinemia, a consequence of insulin resistance and overnutrition, is known to inhibit GH release[10].The potential physiological implications of growth hormone deficiency are substantial. In this study, we sought to generate insight into the biological basis of advanced fibrosis in NAFLD through the prospective analysis of serum biomarkers, including a detailed endocrine and enzymatic profile, to determine their correlation with liver histology in patients with NAFLD.

Patients and methods

The study was approved by the Institutional Review Board (IRB) of the Mayo Clinic, Rochester, MN. All participants gave informed written consent.

From November 2006 to April 2009, liver biopsy and fasting blood samples were collected prospectively from patients with medically complicated Class III obesity (defined by BMI $\geq$ 40kg/m^2) who were scheduled to undergo bariatric surgery. Data with respect to age, gender, race, BMI, medications, alcohol consumption history and routine chemical blood values (including aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lipid profile) were collected from the medical records of patients. Comorbidities that were registered included hypertension, diabetes mellitus (DM) or impaired fasting glucose (IFG), dyslipidemia, metabolic syndrome, obstructive sleep apnea (OSA), and hypothyroidism. Dyslipidemia was defined according to the National Cholesterol Education Program III [11]. Metabolic syndrome was defined according to the Adult Treatment Panel III (ATP III) [12], in which waist circumference was replaced for a BMI $\geq$ 30. Patients were excluded if there was any clinical, laboratory, or histological suspicion of steatosis and chronic liver disease other than NAFLD (by means of history of alcohol consumption, medication history, viral serologies for hepatitis C and B,

iron studies, alpha-1 antitrypsin phenotype, ceruloplasmin concentration, antimitochondrial, antinuclear and anti smooth-muscle antibodies; all data available within six months of a subject entering the study). Alcohol consumption was assessed by history. Participants drank <20g/day of ethanol.

Fasting morning blood samples, drawn within 24hrs of liver biopsy, were analyzed for a broad panel of endocrine, metabolic and immune biomarkers including: sulfated dehydroepiandrosterone (DHEA-S, Diagnostic Systems Laboratories; GH, Diagnostic Systems Laboratories; free insulin like growth factor (IGF-1), Human Free IGF-1 ELISA, Diagnostic Systems Laboratories; insulin, Diagnostic Systems Laboratories; adiponectin, Diagnostic Systems Laboratories; interleukin-6 (IL-6), Diagnostic Systems Laboratories; C-peptide, Diagnostic Systems Laboratories; glucose, leptin, Diagnostic Systems Laboratories; tumor necrosis factor alpha (TNF-α), Diagnostic Systems Laboratories; C-reactive protein (CRP), Diagnostic Systems Laboratories; cytokeratin 18 (CK-18) fragments, M30-Apoptosense® ELISA, Peviva AB, Sweden; complement C4 (cC4), AssayMax Human Complement C4 ELISA, Assaypro, MO; human resistin, Human Resistin ELISA, Millipore, MA). All analyses were carried out in blinded fashion (histology unknown during analysis of serum biomarkers). Plasma glucose concentrations were measured enzymatically with an auto analyzer (Beckman Instruments, Fullerton, CA, USA).

Insulin resistance was determined using the homeostasis model assessment (HOMA-IR) [13], and insulin sensitivity was determined using the quantitative insulin sensitivity check index (QUICKI) [14].

Histology

Liver biopsies were performed at the time of bariatric surgery. All histological results are based on the interpretation of these single timepoint biopsies. A liver specimen with at least 6 portal tracts was considered adequate for histological evaluation. Liver biopsy specimens were reviewed in a blinded fashion by experienced hepatopathologists and judged on grade/stage of steatosis, necroinflammation, and fibrosis according to the Brunt criteria.[15] NASH was defined as a necroinflammatory grade ≥ 1 (mild: steatosis involving up to 66% of biopsy; occasional ballooned zone 3 hepatocytes may be present; scattered rate intra-acinar polymorphonuclear ± intraacinar lymphocytes; no or mild chronic inflammation). All patients with NASH in our study had ballooning.

Patients who met these criteria for NASH were then divided into those with mild (stage 0-1) or advanced (stage ≥ 2) fibrosis, also according to the staging system suggested by Brunt *et al* [15].

Patients classified as having SS in our study had steatosis in the absence of necroinflammation and fibrosis.

Patients were thus categorized prospectively into four groups according to liver histology:

i) normal,
ii) simple steatosis (SS),

iii) NASH with fibrosis stage (FS) 0-1 (NASH mild) and
iv) NASH with FS ≥2 (NASH advanced).

Groups 1 and 2 were considered as 'no NASH'; the latter two were considered as 'NASH'.

Data Analysis

Statistical analysis was performed using descriptive statistics using JMP® 7.0.1 Software (SAS Institute Inc., NC, USA). For comparison between continuous clinical and biochemical parameters and the 4 different histology groups we applied the non-parametric Kruskal-Wallis test. Comparisons between NASH and non NASH subjects were done using the Wilcoxon test. Pearson's correlation test was used for comparison between categorical data. In multivariate analysis, we adjusted for confounders that were significantly different between groups in univariate analysis, using logistic regression. A *P*-value of <.05 was considered as statistically significant. For each parameter we calculated the Area Under Receiver Operating Characteristic (AUROC) curve.

Results

Patient characteristics

A total of 160 participants were included (85% women, mean age of 47.7 ± 11.2 years and mean BMI of 46.8 ± 8.2 kg/m^2). Liver biopsies demonstrated normal histology in 10%, SS in 45%, NASH with FS 0-1 in 37.5% and NASH with FS ≥2 in 7.5% of patients. Clinical and demographic characteristics are shown in Table 1.

The prevalence of NAFLD was 90%. Forty-three percent had SS, and 36% had NASH. NASH advanced was seen in 6.8%.

Mean necroinflammation grade was higher in patients with NASH advanced than in patients with NASH mild (1.33±0.60 vs. 1.09±0.30, respectively, p>.05).

Comorbidities

Common comorbidities in patients with NASH were dyslipidemia (97.3%), OSA (75.0%) and metabolic syndrome (85.4%). Only T2DM was significantly more prevalent in NASH patients than in non-NASH patients (45.8% versus 29.9%). Both hypertension and OSA were significantly more prevalent in patients with NASH with FS 2-3 compared to all of the other histology groups.

Biomarkers of non-alcoholic steatohepatitis

There was an association between the four groups for CRP, IL-6, GH, CK-18, adiponectin, HOMA-IR and QUICKI (Table 1). Lower growth hormone was associated with higher steatosis grade,

Table 1. Clinical and demographical characteristics per histological category.

	Normal	Simple Steatosis	NASH and FS 0-1	NASH and FS≥2	P1	P2	AUROC 1	AUROC 2
	n=16	n=72	n=60	n=12				
Female (%)	75.0	88.9	86.7	66.7	.1	.6		
Caucasian (%)	93.8	94.4	93.3	100	.6	.4		
Age (years)	50.0±13.2	47.8±11.1	46.4±10.9	50.7±10.7	.5	.5		
BMI (kg/m^2)	47.3±7.0	45.8±7.3	47.1±9.1	50.4±8.7	.3	.2		
Comorbidities								
Hypertension	68.8	54.2	58.3	91.7	.09	.4		
T2DM	25.0	31.0	43.3	58.3	.1	.04		
IFG	25.0	38.0	35.0	25.0	.7	.8		
Dyslipidemia	91.7	98.2	95.5	100	.6	.8		
OSA	62.5	61.1	70.0	100	.06	.07		
Metabolic Syndrome	63.6	72.6	81.6	100	.1	.07		
Laboratory data								
AST/ALT ratio	1.06 (0.83-1.28)	1.00 (0.79-1.19)	0.79 (0.70-1.06)	0.75 (0.55-1.00)	.007	.001	0.658	0.666
Triglycerides (mg/dL)	133 (93-171)	154 (126-201)	155 (116-203)	132 (99-179)	.02	.9		
Platelets (*10^9/L)	249 (219-342)	258 (236-308)	262 (215-287)	234 (197-356)	.8	.4		
IL-6 (pg/mL)	4.0 (1.9-7.3)	3.5 (2.6-5.3)	4.7 (2.8-8.4)	7.5 (4.1-25.3)	.002	<.001	0.661	0.756
CK-18 (U/L)	235 (164-288)	192.3 (140-267)	219 (165-337)	330 (226-8960)	.005	.004	0.632	0.735
CRP (mg/dL)	0.69 (0.29-1.73)	0.48 (0.32-0.94)	0.87 (0.45-1.65)	1.41 (0.69-1.89)	.004	.002	0.651	0.673
cC4 (µg/mL)	374 (329-447)	393 (335-478)	380 (345-400)	350 (284-358)	.1	.4		
Free IGF-1 (ng/ml)	0.98 (0.84-1.06)	0.93 (0.83-1.13)	0.99 (0.72-1.17)	0.93 (0.73-1.08)	.9	.8		
GH (ng/mL)	0.45 (0.16-1.16)	0.21 (0.09-0.49)	0.10 (0.03-0.37)	0.14 (0.04-0.22)	<.001	<.001	0.666	0.616
DHEA-S (µg/mL)	74 (14-202)	100 (14-461)	95 (14-347)	72 (14-367)	.1	.6		
C-peptide (nM)	0.9 (0.6-1.2)	1.2 (0.8-1.5)	1.2 (0.9-1.7)	1.6 (1.2-2.2)	.007	.02	0.617	0.693

Table 1. (continued)

	Normal	Simple Steatosis	NASH and FS 0-1	NASH and FS≥2	P1	P2	AUROC 1	AUROC 2
	n=16	n=72	n=60	n=12				
QUICKI	0.32 (0.30-0.37)	0.32 (0.30-0.34)	0.30 (0.29-0.33)	0.30 (0.27-0.30)	<.001	<.001	0.671	0.693
HOMA-IR	2.7 (1.3-4.7)	3.5 (2.4-5.3)	4.9 (2.9-7.8)	6.1 (4.8-11.4)	<.001	<.001	0.676	0.735
Leptin (ng/mL)	40.6 (32.0-60.5)	44.6 (28.4-60.4)	41.0 (32.3-56.0)	48.3 (38.8-70.1)	.6	.9		
Adiponectin (ng/mL)	12909 (8050-19292)	8563(6261-12063)	6833 (5035-11513)	3930 (3235-6453)	<.001	<.001	0.682	0.801
Resistin (ng/ml)	15.1 (13.7-23.4)	15.3 (12.7-19.0)	14.9 (12.6-17.8)	17.5 (14.2-19.7)	.4	.9		
TNF-alpha (pg/mL)	1.5 (1.2-2.0)	1.4 (1.2-1.9)	1.5 (1.2-1.9)	1.5 (1.3-2.2)	.8	.9		

Laboratory data are expressed as median (25th-75th percentile).
P1 is calculated by One-way ANOVA, Kruskall-Wallis test or Chi-squared test (univariable analysis between groups)
P2 is calculated by Students T-test, Wilcoxon test or Chi-squared test (univariable analysis between NASH (FS 0-3) and no NASH (Normal and SS)
AUROC1 is the Area Under Receiver Operating Characteristic for NASH (FS 0-3) vs. no NASH (Normal and SS).
AUROC2 is the Area Under Receiver Operating Characteristic for NASH (FS0-1) vs. NASH (FS≥2).
Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; cC4, complement C4; CK-18, cytokeratin 18; DHEA-S, sulphated dehydroepiandrosterone; HOMA-IR, homeostasis model assessment of insulin resistence; GH, growth hormone; NASH, non-alcoholic steatohepatitis; OSA, obstructive sleep apnea; QUICKI, quantitive insulin sensitivity index.

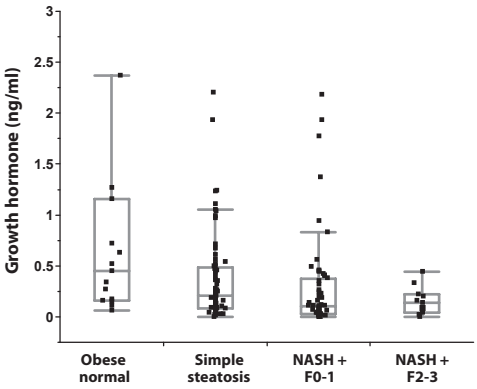

Fig. 1. Variation in growth hormone (GH) levels for the different histology groups are shown. All patients with NASH with fibrosis stage 2-3 had circulating levels of GH within criteria for adult growth hormone deficiency (<0.45 ng/mL; p<.001).

necroinflammatory grade and fibrosis stage. All patients with FS≥2 had low levels of GH (Table 1, Figure 1).

All biochemical parameters had overall predictivity in differentiating NASH (any FS) and no NASH (normal and SS) that was too low to have any clinical utility (AUROC between 0.63 and 0.68). Using AUROC curves we identified the best cut-off point for HOMA-IR (to differentiate between 'NASH' or 'no NASH') to be 4.8, with a sensitivity of 60% and a specificity of 71%, with negative and positive predictive values (PPV) of 67% and 61% respectively. For QUICKI the best cut-off point would be 0.30 to differentiate NASH, with similar sensitivity and specificity. The best cut-off point for adiponectin was 7149 (AUROC was 0.682). The specificity, sensitivity, PPV, and negative predictive value for this cut-off would be 71.4%, 39.4%, 64.2% and 68.2% respectively.

In a multivariate model, including the five strongest predictors in univariate analysis (GH, adiponectin, QUICKI, CK-18 and IL-6), only adiponectin and insulin resistance, measured by QUICKI, remained independently predictive of NASH (p=.03 for both).

Biomarkers for identification of non-alcoholic steatohepatitis with fibrosis

All patients with NASH mild (NASH + FS 0-1) and NASH advanced (NASH + FS ≥2) had insulin resistance, as measured by QUICKI. The maximum level and best cut-off point for QUICKI in identifying patients with NASH advanced was 0.31 (100% sensitivity, 50% specificity). The AUROC was 0.74 (same AUROC for HOMA-IR). The minimum HOMA-IR and best cut-off point was 3.9 (sensitivity 100%, specificity 50%).

All patients with NASH advanced also had low GH levels <0.45 ng/mL, and all but one had low DHEA-S levels (Figure 2) and adiponectin levels (Figure 3). Variation in GH levels with histology is shown in Figure 1. The P-value for DHEA-S for NASH FS2-3 vs. NASH FS0-1 was .03. For all other comparisons, the DHEA-S levels between all study groups are shown in Table 2.

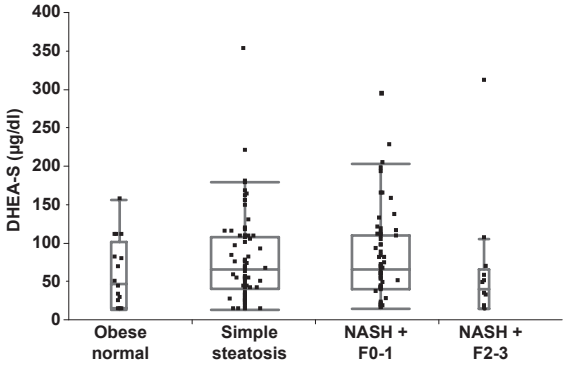

Fig. 2. Variation in dehydroepiandroastenedione sulfate (DHEA-S) levels for the different histology groups are shown. All but one patient with NASH with fibrosis stage >/=2 had low circulating levels of DHEA-S (p=.04 for DHEA –S levels in 'NASH advanced' (FS ≥2) versus all other groups combined)

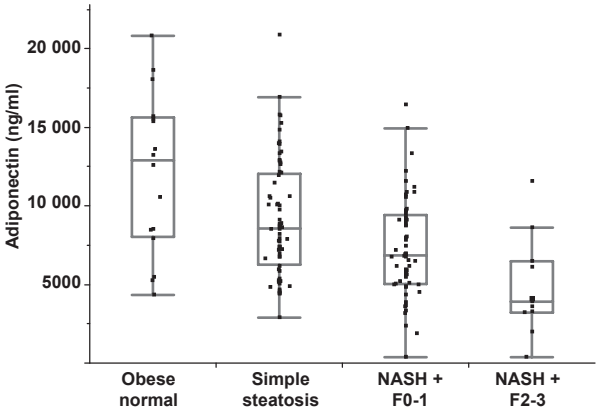

Fig. 3. Variation in adiponectin levels for the different histology groups are shown. In multivariate analysis, only adiponectin was an independent predictor of NASH with FS>/=2 (p-value=.01). The AUROC curve for adiponectin in this group was .801. The best cut-off point for adiponectin to distinguish NASH-mild from NASH-advanced was 4080ng/ml, with a sensitivity of 63% and specificity of 94%.

In multivariate analysis, after adjusting for other variables that were predictive in univariate analysis, only adiponectin was an independent predictor of NASH with FS ≥2 (p=.01). The AUROC curve for adiponectin in this group was 0.80. The best cut-off point for adiponectin to distinguish NASH mild from NASH advanced was 4080ng/ml, with a sensitivity of 63% and specificity of 94%.

Discussion

The primary purpose of this study was to generate new insights into the biological basis of steatohepatitis and advanced fibrosis (steatofibrosis) in NAFLD. We found significant associations of several serum biomarkers, including CRP, IL-6, CK-18 fragments, adiponectin, and indices

Table 2. Significance of differences in DHEA-S levels between study groups. Levels of DHEA-S per histology category are shown in Table 1.

DHEA-S	
Univariate analysis	**p-value**
Obese normal vs. NASH advanced	.60
Simple steatosis vs. NASH advanced	.03
NASH mild vs. NASH advanced	.03
NASH vs. no NASH	.76
Obese normal +Simple steatosis + NASH mild vs. NASH advanced	.04

Abbreviations: DHEA-S, sulphated dehydroepiandrosterone; NASH, non-alcoholic steatohepatitis

of insulin resistance with NASH when compared to non-NASH NAFLD. We also confirmed the previously reported strong associations of NASH with low circulating levels of adiponectin and DHEA. Importantly, this study produced the novel observation that obese patients with NASH and advanced fibrosis have low serum levels of GH. While many patients with normal liver histology, simple steatosis and NASH without advanced fibrosis also had low growth hormone level in our study, a normal growth hormone level essentially excluded the presence of NASH with advanced fibrosis. Low growth hormone levels were thus a highly sensitive but less specific in identifying patients with NASH and advanced fibrosis. Together these observations suggest a potential endocrine basis of susceptibility to advanced fibrosis in NASH and provide an endocrine signature of NASH with advanced fibrosis.

All of the patients in this study had medically complicated class III obesity and had been obese for decades at the time of liver biopsy. While NAFLD was present in 90% of our patients, only a small percentage (7.5%) had NASH with FS ≥2. Patients with simple steatosis in our study had, by definition, a slowly or non-advanced histological course. It is notable that none of the 72 patients with steatosis in the absence of inflammation had abnormal fibrosis, suggesting that simple steatosis is highly unlikely to be associated with important hepatic fibrosis. Whether patients in our study who had normal liver histology or simple steatosis would ever develop NASH and/or any degree of hepatic fibrosis is unknown. The lack of steatohepatitis and fibrosis despite decades of obesity suggests that liver disease in these patients is either nonprogressive or very slowly progressive. We cannot, of course, exclude a contribution from undeclared alcohol consumption to the histological changes that we observed.

Distinguishing chronically overnourished, obese patients with normal liver histology and/or simple steatosis from patients with and NASH with advanced fibrosis is important from several perspectives. The identification of patients who are at risk of clinically important liver injury from NAFLD for consideration of initiation of treatments might reduce the histological impact of NAFLD. In the largest community-based study reported to date NAFLD had an attributable standardized mortality ratio of 1.34 (95% CI, 1.003-1.76; P=0.03)[16], with three percent of patients with NAFLD developing liver-related complications (e.g. hepatocellular carcinoma) within 10 years. It is highly probable that patients with NAFLD who develop cirrhosis will be

similar to the patients in our study who had NASH with advanced fibrosis. We evaluated the utility of published biomarkers for identifying patients with histologically severe NAFLD, including several adipocytokines. As for GH, while many patients with normal histology, simple steatosis and NASH without advanced fibrosis also had low adiponectin levels in our study, a normal adiponectin level essentially excluded the presence of NASH with advanced fibrosis. Adiponectin, a key regulator of energetic and glycol-lipidic homeostasis, was the only adipocytkine that was independently predictive of NASH (vs. simple steatosis and normal histology) and NASH with advanced fibrosis (vs. NASH with mild fibrosis). We did not find other adipocytokines to be strongly predictive of NASH regardless of fibrosis stage. Our results are consistent with earlier findings of hypoadiponectinemia in association with steatosis, inflammation, fibrosis, obesity and insulin resistance [17-18]. The physiological basis and impact of hypoadiponectinemia on hepatic histology is unclear. In our study adiponectin levels were significantly associated with insulin resistance and sensitivity measured by HOMA-IR and QUICKI, respectively.

Cytokeratin 18 levels have been reported to be predictive of NASH vs. normal histology and simple steatosis [19-20]. In our cohort, CK18 fragments were also significantly associated with NASH overall and NASH with advanced fibrosis. The degree of predictivity observed in our study suggests that the predictivity of CK-18 fragments may be too low to be of probable clinical utility (AUROC 0.63-0.74).

The most unique aspect of our study was the identification of an endocrine signature of NASH with advanced fibrosis. All of our subjects with NASH with advanced fibrosis had insulin resistance (defined as QUICKI<0.33), low serum levels of GH and, except for one patient, low DHEA levels. Insulin resistance is central to the physiological abnormalities of the metabolic syndrome and NAFLD [21]. Our results are consistent with a long term study of NAFLD demonstrating that progression of fibrosis is associated with more pronounced insulin resistance[22]. Others have also demonstrated insulin resistance, expressed by HOMA-IR, as an independent predictor of NASH[18, 23]. We have previously demonstrated, in a multicenter prospective study, that low circulating dehydroepiandrosterone (DHEA) levels are associated with NAFLD with advanced fibrosis [24]. DHEA mediates reactive oxygen species scavenger synthesis [25], insulin sensitivity [26-27], and activation of peroxisome proliferators [28-29]. Moreover, there is a high frequency of NASH in patients with panhypopituitarism, who have low DHEA and GH levels [4]. Although the basis of low DHEA and GH levels in patients with NASH with advanced fibrosis cannot be determined from our current analysis, some degree of relative GH deficiency in NAFLD is not unexpected. Obesity is associated with low GH levels [5-7], at least in part due to low circulating levels of ghrelin [8] (in response to caloric excess), which, in addition to affecting appetite, is a somatotrope [9]. In addition, hyperinsulinemia, a consequence of insulin resistance and overnutrition, inhibits GH release [10]. Although the basis of marked GH deficiency in patients with steatofibrosis is not known, the potential physiological implications are substantial. Phenotypically, patients with hypopituitarism have features of metabolic syndrome, including visceral obesity, insulin resistance, hypertension and dyslipidemia [30-31]. GH inhibits

11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), which catalyses the conversion of inactive cortisone to active cortisol [32]. In addition, as DHEA is known to have a dose response effect on inhibiting 11β-HSD1 activity [33], low circulating levels of DHEA may have a synergistic effect with GH deficiency on increasing 11β-HSD1 activity. Moreover, many patients with NASH are phenotypically similar to patients with adult growth hormone deficiency: increased BMI, visceral adiposity, dyslipidemia and insulin resistance. In addition, patients with growth hormone resistance have severe NASH, responsive to GH supplementation [3, 34]. GH therapy has also been reported to reverse histological features of NASH in panhypopituitarism [35], and to stimulate lipolysis [36-37]. There are few studies that have assessed GH levels in patients with NAFLD. Low GH levels were reported to be an independent predictor of NAFLD in adult males in a study by Lonardo et al. [38], and to be associated with NASH as a whole [39-40]. Low GH levels have not previously been associated with fibrosis in NAFLD/NASH.

GH levels can be regulated by adiponectin via the adiponectin receptor [41]. A regulatory association between GH and adipose tissue is suggested by the observation of normalization of GH levels with reduced body weight [42]. GH may regulate the expression of AdipoR1 and AdipoR2 in human adipose tissue and reduce adiponectin secretion [43]. Adiponectin directly regulates GH secretion from somatotrophs by binding to an adiponectin receptor [44-45]. Moreover, GH may decrease 11β-HSD, which positively relates to increased adiponectin expression and decreased adiponectin levels [46-47]. Thus higher glucocorticoid production in adipose tissue may also favour the development of metabolic disorders through a decrease in adiponectin release.

An important consideration in interpreting these results is that we did not look for predictors or associations with necroinflammation grade and it is possible such associations exist. Mean necroinflammation grade was higher in patients with NASH advanced (fibrosis stage ≥) than in patients with NASH mild (fibrosis stage 0-1) (1.33±0.60 vs. 1.09±0.30 respectively, p=ns). We cannot exclude the possibility that some of the observed differences in biomarkers of fibrosis were related to differences in necroinflammation. Regardless of cause, endocrine parameters, including adiponectin, insulin resistance and GH, were near ubiquitous among patients with NASH who had advanced fibrosis in our study. Although morbidity and mortality may occur as a result of any of the myriad complications of obesity and insulin resistance, clinical consequences of liver disease are seen almost exclusively in patients who develop progressive fibrosis.

AST/ALT ratios have been variably reported to predict advanced fibrosis. Specifically a ratio of >1 has been associated with cirrhosis [48-50]. We did not have patients with cirrhosis in our study cohort. AST/ALT ratios were very similar, 0.79 in patients with fibrosis stage 0-1, versus 0.75 for those with fibrosis stage ≥2. The AST/ALT ratios are both below the 1.0 cut-off that has been suggested as a marker for cirrhosis. The predictivity of AST/ALT ratios in distinguishing between non-cirrhotic stages of fibrosis is much lower than for cirrhosis. It is also possible that serum transaminases were affected by medications, such oral hypoglycemic agents or statins.

In conclusion, although cause and effect may not be extrapolated from these studies, endocrine parameters, including adiponectin, insulin resistance and GH, are near ubiquitous among patients with NASH who have advanced fibrosis. CRP, IL-6, GH, CK-18, adiponectin, HOMA-IR and QUICKI are also statistically significantly associated with NASH, but overall predictivity is probably too low to have clinical utility. An endocrine basis of susceptibility to NASH with advanced fibrosis merits further investigation.

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CHAPTER 7

Association between statin therapy and non-alcoholic fatty liver disease in a large population-based study

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Abstract

Background & Aims: Non-alcoholic fatty liver disease (NAFLD) is considered as the hepatic manifestation of the metabolic syndrome. Statin therapy is effective in the treatment of metabolic syndrome, but the effect and safety of statins in NAFLD is not well established. The objective was to study the association between statin therapy and the presence and severity of NAFLD, and elevated alanine aminotransferase (ALT) levels, in a large cross-sectional population-based study. **Methods:** In the Rotterdam Study, a prospective population-based cohort study, we identified 2578 subjects who underwent liver ultrasonography, and for whom statin prescription data were available. We used logistic regression models, and investigated the effect of both current and past use, and duration of use. The analyses were adjusted for age, sex, statin dose, total cholesterol level, alcohol consumption, metabolic syndrome, history of cardiovascular disease (CVD), and use of fibrates or other cholesterol-lowering drugs. **Results:** The prevalence of NAFLD was 35.3%. In total, 990 participants had ever used statin therapy (631 current users and 359 past users). In multivariable analyses, ever use of statin therapy was neither associated with NAFLD, nor with elevated serum ALT concentrations (OR 0.94, 95%CI 0.66 – 1.33 and OR 1.23, 95%CI 0.68 – 2.23, respectively). However, current statin use for >2 years was associated with a significantly lower prevalence of NAFLD (OR 0.44, 95%CI 0.21 – 0.96).

Conclusions: Within the Rotterdam study, more than 2 years current use of statin therapy was associated with a lower prevalence of NAFLD. No association was found with elevated serum ALT.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common cause of serum alanine aminotransferase (ALT) elevation in Western countries, and the prevalence is increasing. The term NAFLD encompasses a spectrum of disease activity, ranging from simple hepatic steatosis, to non-alcoholic steatohepatitis (NASH) and NASH cirrhosis, which may lead to a decreased liver function, hepatocellular carcinoma, and liver failure[1-3]. NAFLD is considered as the hepatic manifestation of the metabolic syndrome. It is frequently associated with dyslipidemia, with elevated serum triglycerides and low-density lipoprotein (LDL) cholesterol, and a decrease in serum high-density lipoprotein (HDL) cholesterol[1-5]. Furthermore, NAFLD has been independently associated with the risk of incident cardiovascular disease (CVD), independent of the components of the metabolic syndrome, and the major cause of death in NAFLD is CVD[2, 5-10].

Statins interfere with cholesterol metabolism in the liver by inhibiting HMG-CoA reductase, the rate-limiting enzyme of the cholesterol synthesis pathway. This leads to up-regulation of LDL receptors in the liver, increased uptake of circulating LDL cholesterol, and subsequently a decrease in LDL cholesterol concentration[11-16]. Inhibition of HMG-CoA reductase may also limit the availability of cholesterol for very low density lipoprotein (VLDL) production and decrease hepatic VLDL secretion, and accordingly lead to lower plasma triglyceride levels. In general, statins are well-tolerated and safe drugs[17]. The most common effect of statins therapy on the liver is an elevation of the serum ALT concentration[4].

Although statins are frequently prescribed drugs, there is some discussion as to whether statins are safe and effective in NAFLD, and whether they should be prescribed in the presence of liver biochemistry abnormalities. Some studies suggest that statins increase hepatotoxicity, worsen hepatic steatosis, and significantly increase serum liver enzymes, despite improvement of serum lipid concentration[18-23]. The safety of statins in NAFLD is not well established, and the effect of statins on the lipid concentration in the liver and the effect of these drugs on NAFLD progression is unknown. Further clarification of this topic is important, since statins are increasingly prescribed, and higher doses and more potent statins are used to reach target serum LDL cholesterol levels[4]. Moreover, due to the co-existence of dyslipidemia and NAFLD, and a higher risk of CVD mortality in NAFLD patients, they will often be treated with statins for the primary and secondary prevention of CVD. Therefore, a better understanding of the association between statin therapy and NAFLD prevalence, and statin therapy and serum ALT concentration, is clinically relevant.

In the present study, the objective was to investigate whether statin therapy is associated with the presence and severity of NAFLD, considering both current and past use of statin therapy, and duration of use. Secondly, we investigated whether statin therapy was associated with elevated serum ALT concentrations in a large prospective cohort study in community-dwelling elderly.

Materials and methods

Setting

The Rotterdam Study is a prospective population-based cohort study of chronic diseases in the elderly population. From 1990 to 1993, 7,983 inhabitants of the suburb Ommoord in Rotterdam, The Netherlands, aged 55 years or older, participated in the Rotterdam Study (RS-I) and gave written informed consent. Ethical approval was obtained from the Medical Ethical Committee of the Erasmus Medical Center, Rotterdam, The Netherlands. Baseline examinations took place from March 1990 through July 1993. Follow-up examinations were conducted periodically, every 4-5 years. In 2000, an extended cohort was enrolled, the Rotterdam Study II (RS-II). Three-thousand-eleven inhabitants entered the study and have been continuously followed since then. Furthermore, in 2006, a younger cohort was enrolled, the Rotterdam Study III (RS-III), containing 3,932 inhabitants aged 45 years or older.

Abdominal ultrasonography was added to the core protocol at the fifth survey of the Rotterdam Study (February 2009 – February 2012), which constitutes the baseline survey for the present study.

Medication prescription data were obtained from the fully computerized pharmacies in the Ommoord suburb. Information on all filled prescriptions from January 1st 1991 until December 1st 2011 was available and included information on the product name of the drug, the Anatomical Therapeutical Chemical code, the amount dispensed, the prescribed dosage regimen and the date of dispensing[24].

Detailed information on design, objectives and methods of the Rotterdam Study have been described before[25-26].

Study population

The study population consisted of all participants with complete data on the extensive interview and clinical examination at the fifth survey of the Rotterdam Study (February 2009 – February 2012). The clinical examination included a fasting blood sample, abdominal ultrasonography, and anthropometric assessment. Medication prescription data on the use of statin therapy was available until December 1st, 2011. Therefore, all participants with an interview and clinical examination date after December 1st, 2011 were excluded.

Exposure to statins

For every prescription of a statin, the duration was calculated by dividing the number of dispensed tablets by the prescribed daily number. Repeated prescriptions which were filled within seven days after ending a previous one, were considered as one single episode of continuous use. At the date of ultrasonography, every cohort participant was classified into mutually exclusive categories: 'current use' if the ultrasonography was performed within a prescription episode; 'past use' if the patient had been treated with statins in the past but did not use statins

on the day of ultrasonography; 'non-use' meant that the participant had not used statins at all during the study period. An 'ever-use' category was defined and included current statin users and past statin users combined in one category.

Outcome

The primary outcome of interest was the presence of non-alcoholic fatty liver disease, assessed by abdominal ultrasonography in all study participants. Abdominal ultrasonography was performed by certified and experienced technicians on a Hitachi HI VISION 900. Images were stored digitally and re-evaluated by a hepatologist with more than ten years experience in ultrasonography. The diagnosis and grading of fatty liver was determined according to the protocol by Hamaguchi et al[27]. 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). Individuals with any of the following possible secondary causes of fatty liver were excluded from the analyses: 1) current excessive alcohol consumption or a history of excessive alcohol consumption, 2) positive HBsAg or anti-HCV, and 3) use of pharmacological agents historically associated with fatty liver (i.e. amiodarone, corticosteroids, methotrexate, and tamoxifen).

The secondary outcome of interest was elevated fasting serum ALT, which was defined as serum ALT ≥31 U/L for women and ≥41 U/L for men, according to local cut-offs. Blood samples were collected on the morning of ultrasound examination and were measured using automatic enzymatic procedures (Roche Diagnostics GmbH, Mannheim, DE).

Covariables

To control for confounding, we adjusted the analyses for age, sex, prescribed dose of statin therapy, serum total cholesterol level, number of ethanol consumptions weekly, presence of the metabolic syndrome, presence of CVD in history, and use of fibrates or other cholesterol-lowering medication. CVD in history was defined as a myocardial infarction (MI), percutaneous transluminal coronary angioplasty (PTCA), or a coronary artery bypass grafting (CABG) in the history. Information on co-variables was obtained by an interview at home, laboratory measurements, and anthropometric assessments at the research center.

The interview was designed to obtain data concerning demographics, medical history, co-morbid conditions, smoking behaviour, physical activity, alcohol consumption, and drug use. The detailed information on drug data was derived from the computerized pharmacies, as described above. To facilitate direct dose comparisons between drugs from the same therapeutic group, the prescribed daily dose of statin therapy was expressed in standardized defined daily doses (DDD), according to the World Health Organization[24].

Fasting blood samples were collected on the morning of ultrasound examination. Blood lipids, serum glucose, ALT, aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT), alkaline phosphatase (ALP), and total bilirubin were measured using automatic enzymatic

procedures (Roche Diagnostics GmbH, Mannheim, DE). HbsAg and anti-HCV antibodies were measured by automatic immunoassay (Roche Diagnostics GmbH, Mannheim, DE).

Anthropometric measurements were performed by well trained nurses. Waist and hip circumference were measured in centimeters. The average of two blood pressure measurements, obtained at a single visit in sitting position after a minimum of 5 minutes rest, was used for analysis. The metabolic syndrome was defined, according to Adult Treatment Panel III criteria[28]. However, in two criteria, the presence of cholesterol lowering drug treatment was excluded, since in the analysis we consider the effect of statin therapy on the risk of NAFLD. The metabolic syndrome was defined 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), 3) serum HDL cholesterol <40 mg/dL (1.0 mmol/L) in men and <50 mg/dL (1.3 mmol/L) in women, 4) blood pressure ≥130/85 mmHg or drug treatment for elevated blood pressure, 5) Elevated fasting glucose ≥100 mg/dL (5.6 mmol/L) or drug treatment for hyperglycaemia.

Statistical analysis

Differences in the distributions of characteristics between statin users and non statin users were tested for significance with a t-test (means) for continuous variables and a Wilcoxon rank sum test (medians) for categorical variables.

We used logistic regression analysis to investigate the association between statin therapy and the presence of NAFLD. We considered both current, past, and never use of statin therapy, as well as the duration of current and past use. Duration of current use was distinguished a priori, based on the median duration of past and current use in the population: a cut off point of 730 days for past use and a cut off point of 183 days for current use. For comparison with past use, also current use for >730 days was investigated. In an extended analysis, we investigated the association between the use of statin therapy and the severity of NAFLD, using logistic regression models. Furthermore, we investigated the association between use of statin therapy and elevated serum ALT levels.

Statistical analyses were performed using SPSS software (SPSS Inc., version 20.0, Chicago, Illinois, USA).

Results

Baseline characteristics

In total, 3,205 participants underwent abdominal ultrasonography. Three hundred ninety-four participants were excluded because of the presence of potential secondary causes of fatty liver (excessive alcohol consumption (n=255), positive HBsAg (n=3) or anti-HCV (n=24), use of pharmacological agents historically associated with fatty liver (n=121)). Of these 2,811 remaining

participants, 2,578 had information on statin prescription data available and were eligible for the analysis.

Differences in characteristics between statin users and non statin users are shown in Table 1. Of the 2,578 study participants, 1,588 (61.6%) had never used any statin, 631 (24.5%) were current users of statin therapy and 359 (13.9%) were past users of statin therapy.

The prevalence of NAFLD was 35.3%; 134 participants (5.2%) had 'mild fatty liver' and 776 participants (30.1%) had 'moderate to severe fatty liver'.

Table 1. Characteristics of 2578 study participants

	Statin users (n = 990)	Non statin users (n= 1588)	p-value
Age (mean, years)	76.8 ± 5.5	76.2 ± 6.2	.014
Gender, male (n, %)	423 (42.7%)	604 (38.0%)	.018
Ethanol use (drinks/week)	3.6 ± 3.7	3.8 ± 3.8	.3
Serum total cholesterol (mean ± SD, mmol/L)	4.9 ± 1.1	5.7 ± 0.9	<.001
Serum HDL cholesterol (mean ± SD, mmol/L)	1.3 ± 0.3	1.5 ± 0.4	<.001
Serum triglycerides (mean ± SD, mmol/L)	1.6 ± 0.8	1.3 ± 0.6	<.001
Serum ALT (mean ± SD, mmol/L)	21.6 ± 11.3	19.5 ± 10.2	<.001
Serum AST (mean ± SD, mmol/L)	26.8 ± 10.4	25.7 ± 7.0	.004
Serum GGT (mean ± SD, mmol/L)	33.7 ± 29.8	28.5 ± 34.0	.001
Serum bilirubin (mean ± SD, mmol/L)	8.8 ± 4.2	9.1 ± 4.1	.08
Serum ALP (mean ± SD, mmol/L)	71.8 ± 23.5	71.3 ± 20.8	.6
Hypertension (n, %)	960 (97.0%)	1440 (90.7%)	<.001
Waist circumference (mean ± SD, cm)	94.5 ± 12.3	91.2 ± 11.8	<.001
Insulin resistance (HOMA-IR > 3) (n, %)	520 (52.5%)	537 (33.8%)	<.001
Diabetes Mellitus (n, %)	251 (26.0%)	125 (8.1%)	<.001
Metabolic syndrome (n,%)	519 (52.4%)	515 (32.4%)	<.001
Cardiovascular disease in history (n, %)	203 (20.5%)	37 (2.3%)	<.001

Abbreviations: HDL: high-density lipoprotein; ALT: alanine aminotransferase; AST: aspartate aminotransferase; GGT: gamma-glutamyltransferase; ALP: alkaline phosphatase; HOMA-IR: Homeostasis Model of Assessment – Insulin Resistance

Statin therapy and the prevalence of NAFLD

The results of the logistic regression analysis on the association between the use of statin therapy and NAFLD are shown in Table 2. In this analysis, current and past use of statin therapy were compared with never use as the reference category, and duration of use was considered by dividing current and past use into categories with different durations of use, based on the median duration of use.

In the multivariable analysis, adjusted for age and sex, ever use of statin therapy (including current and past use) was associated with a higher prevalence of NAFLD (OR 1.51, 95%CI

Table 2. Logistic regression: the association between the use of statin therapy and the presence and severity of non-alcoholic fatty liver disease

Ever use: (current + past) vs. never use[1]	**Crude OR**[3]	**95% CI**	**P-value**	**Adjusted OR**[4]	**95% CI**	**p-value**
Additive model	1.51	1.28 – 1.78	<.001	0.94	0.66- 1.33	.709
Categorical model						
never use (n = 1588)	*1.00*	*(ref)*	*(ref)*	*1.00*	*(ref)*	*(ref)*
>730 days past use (n = 189)	1.69	1.24 – 2.30	.001	0.99	0.63 - 1.55	.958
1-730 days past use (n = 170)	1.75	1.27 – 2.41	.001	0.99	0.62 - 1.57	.954
1-183 days current use (n = 326)	1.41	1.10 – 1.80	.007	0.91	0.60 - 1.37	.648
184-730 days current use (n = 153)	1.53	1.17 – 2.01	.002	0.94	0.62 - 1.44	.783
>730 days current use (n = 152)	0.81	0.43 – 1.55	.526	0.44	0.21 - 0.96	.038
Mild fatty liver[2]						
Additive model	1.26	0.88 – 1.81	.215	1.15	0.54 – 2.43	.714
Categorical model						
never use (n = 80)	*1.00*	*(ref)*	*(ref)*	*1.00*	*(ref)*	*(ref)*
>730 days past use (n = 13)	1.67	0.90 – 3.10	.107	1.53	0.61 – 3.87	.368
1-730 days past use (n = 8)	1.16	0.55 – 2.48	.697	1.09	0.39 – 3.05	.868
1-183 days current use (n = 20)	1.37	0.82 – 2.30	.228	1.30	0.55 – 3.07	.556
184-730 days current use (n = 12)	1.08	0.58 – 2.03	.808	0.87	0.34 – 2.23	.778
>730 days current use (n = 1)	0.38	0.051 – 2.81	.344	0.32	0.038 – 2.74	.300
Moderate to severe fatty liver[2]						
Additive model	1.56	1.31 – 1.85	<.001	0.87	0.60 – 1.25	.865
Categorical model						
never use (n = 426)	*1.00*	*(ref)*	*(ref)*	*1.00*	*(ref)*	*(ref)*
>730 days past use (n = 69)	1.69	1.22 – 2.34	.002	0.87	0.54 – 1.40	.567
1-730 days past use (n = 68)	1.87	1.34 – 2.60	<.001	0.92	0.57 – 1.50	.739
1-183 days current use (n = 108)	1.41	1.09 – 1.83	.010	0.83	0.54 – 1.29	.416
184-730 days current use (n = 93)	1.62	1.22 – 2.15	.001	0.91	0.58 – 1.42	.674
>730 days current use (N = 12)	0.90	0.46 – 1.75	.749	0.44	0.20 – 0.97	.043

[1]In all analyses, never use is the reference category.
[2]mild fatty liver/moderate to severe fatty liver against no fatty liver
[3]Adjusted for age and sex.
[4]Adjusted for age, sex, statin dose, total cholesterol level, number of ethanol containing drinks per week, presence of metabolic syndrome, cardiovascular disease in history, and use of fibrates or other cholesterol-lowering drugs.

1.28 – 1.78, P <.001) compared with never use, whereas this association disappeared when the analysis was adjusted for all co-variables (full model: OR 0.94, 95%CI 0.66 – 1.33, p=.709).

When we analysed ever use as a categorical variable, dividing past use into two categories (>2 year past use and ≤2year past use), and current use into three categories (≤ half year current use, >half year – ≤2 year current use, >2 year current use), and adjusted for all co-variables, past use of statin therapy was not significantly associated with NAFLD (OR 0.99, 95%CI 0.63 – 1.55, p=.958 for >2 year past use; OR 0.99, 95%CI 0.62 – 1.57, p=.954 for ≤2 year past use). Current use for more than 2 years was significantly associated with a lower prevalence of NAFLD (OR 0.91, 95%CI 0.60 – 1.37, p=.648 for ≤half year current use; OR 0.94, 95%CI 0.62 – 1.44, P .783 for >half year – ≤2 year current use; OR 0.44, 95%CI 0.21 – 0.96, p=.038 for >2 year current use).

Table 2 shows the results of the logistic regression analysis using the severity of NAFLD as dependent variable. In this analysis, the outcome measures 'mild fatty liver' and 'moderate to severe fatty liver' were compared to 'no fatty liver'. Results of these analyses were comparable with the logistic regression analysis regarding presence of NAFLD: there was no association between ever use of statin therapy and severity of NAFLD in the full model. Regarding the association with mild NAFLD, there was a non-significant trend for an association between current statin use and a decreased prevalence of NAFLD (OR 1.30, 95%CI 0.55 – 3.07, p=.556 for ≤half year current use; OR 0.87, 95%CI 0.34 – 2.23, p=.778 for >half year – ≤2 year current use; OR 0.32, 95%CI 0.038 – 2.74, p=.300 for >2 year current use). Regarding the association with moderate to severe NAFLD, current use >2 years was associated with a significant lower prevalence of NAFLD (OR 0.83, 95% CI 0.54 – 1.29, p=.416 for ≤half year current use; OR 0.91, 95%CI 0.58 – 1.42, p=.674 for >half year – ≤2 year current use; OR 0.44, 95%CI 0.20 – 0.97, p=.043 for >2 year current use).

Statin therapy and elevated serum ALT concentrations

We performed logistic regression analyses to investigate the association between the use of statin therapy and elevated serum ALT concentrations.

In multivariable analysis, adjusting for age and sex, ever use of statin therapy (including current and past use) was associated with an elevated serum ALT (OR 1.69, 95% CI 1.22 – 2.34, p=.001) compared with never use, whereas this association disappeared when the analysis was adjusted for all co-variables (full model: OR 1.23, 95%CI 0.68 – 2.23, p=.494). Use of statin therapy was not associated with elevated serum ALT in participants with NAFLD (p-value for interaction term:.302).

When we analysed ever use as a categorical variable, dividing past use into two categories (>2 year past use and ≤2year past use), and current use in three categories (≤ half year current use, >half year – ≤2 year current use, >2 year current use), and adjusted for all co-variables, both past and current use of statin therapy was not significantly associated with NAFLD. Current use showed a non-significant trend for association with a lower prevalence of elevated serum ALT for longer duration of current statin use (OR 1.31, 95%CI 0.65 – 2.63, p=.047 for ≤ half year current use; OR 0.92, 95%CI 0.43 – 1.97, p=.829 for > half year – ≤2 year current use; OR 0.87, 95%CI 0.23 – 3.33, p=.843 for >2 year current use).

Discussion

In this cross-sectional analysis in a large population-based prospective cohort study, ever use of statin therapy was not associated with a higher prevalence of NAFLD, after adjustment for age, sex, dose of statin therapy, total cholesterol level, number of ethanol containing drinks weekly, presence of metabolic syndrome, and a history of cardiovascular disease. Furthermore, ever use of statin therapy was not associated with the severity of NAFLD. However, current use of statin therapy for more than 2 years was significantly associated with an approximately two times lower prevalence of NAFLD. Moreover, both current and past use of statin therapy were not associated with elevated serum ALT concentrations, after full adjustment. Current use showed a non-significant trend for association with a lower prevalence of elevated serum ALT for longer duration of current statin use (longer than 1 year).

It has been hypothesized that statins may exacerbate or worsen NAFLD by increasing hepatic de novo cholesterol and fatty acid synthesis through induction of the expression of transcription factor sterol response regulatory element-binding protein-2 that activates genes involved in the synthesis of cholesterol and the LDL receptor. Furthermore, by inhibition of HMG-CoA reductase, the number of hepatic LDL receptors increases, leading to an enhanced uptake of LDL cholesterol. Both effects might increase hepatic fatty infiltration and thereby exacerbate or worsen NAFLD[5-6]. Conversely, the effects of statins might be beneficial as well, both by their most widely known lipid lowering function, as well as through their pleiotropic effects acting on other mechanisms than the HMG-CoA reductase pathway, independent of their cholesterol-lowering effect. Anti-inflammatory and immunomodulatory effects, anti-oxidant effects, and an improvement of the endothelial function are examples of other beneficial effects of statins[5]. Although the exact pathogenesis of NAFLD is currently not clarified and probably many factors contribute, lipid abnormalities and chronic inflammation are considered to be the central pathway for the development of diseases related to obesity such as NAFLD and CVD [4-6, 29].

In the present study, we did not demonstrate an overall association between ever use of statin therapy and the prevalence of NAFLD. These findings are consistent with findings of the Dallas Heart Study.[7] We did demonstrate a protective effect of >2 years current statin use and the prevalence of NAFLD, and this has not been demonstrated before. Compared to other studies, in the Rotterdam Study medication prescription data is continuously collected through linkage with fully computerized pharmacies. Therefore, we had the ability to consider the duration of use in the analysis in current and past users. Also, we could control for the prescribed dosage in the analysis, in contrast with other studies which mostly obtained medication data by questionnaire such as the Dallas Heart Study[7].

For current clinical practice, the crucial question remains whether statins are hepatotoxic. In this study, we did not demonstrate an association between the use of statin therapy and elevated serum ALT levels. Other studies also did not show a difference between statin users and non users, or even demonstrated an improvement in patients using statin therapy while

liver enzymes worsened in those who did not receive statin therapy[2, 7, 30-31]. Although studies have suggested that statins might induce hepatic injury, statin therapy was infrequently associated with acute or chronic liver failure, and significant liver injury from statins was rare[18-23]. Elevation of aminotransferases appears to be a class effect of statins and the mechanism of this effect is unknown, but it is probably related to their pharmacological effect of cholesterol reduction within the hepatocytes. However, serum aminotransferases elevation in these patients can also be related to comorbid conditions that are often present in NAFLD patients, such as diabetes mellitus or obesity, concurrent alcohol consumption, or comedication with potential hepatotoxic effects. Studies indicate that statins are safe from a hepatic standpoint, the primary cause of death in NAFLD is CVD, currently the marketed statins have a favourable benefit-to-risk relation with respect to their hepatic effects and to date liver biochemistry testing is not recommended by previous studies and by the FDA [2, 7, 12, 30-34]. The relationship between NALFD and cardiovascular events and especially the increased cardiovascular mortality, which is much higher than the hepatic-related mortality, underlines the importance of statin therapy in NAFLD patients[2, 9, 35]. In the GREACE study, use of statin therapy demonstrated a 68% relative risk reduction for cardiovascular events in patients with abnormal liver tests[2].

This study is the first observational study on this topic with continuous information on medication data, whereby past, current and duration of use was investigated. Another strength of the study was that we were able to adjust our analyses for a history of CVD, whereas no other studies did control for this. Adjustment for CVD is important to minimize confounding by indication. Since statin therapy is mainly prescribed for the primary and secondary prevention of CVD, and previous studies suggest a strong link between NAFLD and CVD, independently from the metabolic syndrome, the prevalence of NAFLD may also be higher in patients that use statins for CVD[8-9]. When no adjustment is made for a history of CVD in the analyses, one may not detect or underestimate a potential favourable effect of statin therapy on NAFLD.

Potential limitations and biases in our study should be considered. The risk of information or selection bias is unlikely since the Rotterdam Study is a population-based cohort study, in which data are collected prospectively without prior knowledge of the aim of this study. A 'healthy user' effect seems unlikely because in that case ever use would also have been associated with a lower prevalence of NAFLD. In the analysis we controlled for potential confounding factors such as dose of the prescribed statin therapy and a history of cardiovascular disease. In the analysis on the severity of NAFLD, we had insufficient power to demonstrate a significant association between >2 years current statin use and mild fatty liver, because of a small sample size (n = 134), while this association was significant for moderate to severe fatty liver (n = 776). Furthermore, in the present study, the diagnosis and severity of hepatic steatosis was assessed by ultrasonography. Ultrasonography may be less sensitive than more advanced imaging techniques such as CT/MRI, since ultrasonography is not appropriate for the detection of less than 30 percent steatosis. However, Hernaez et al.[36] showed that ultrasonography is comparable with other imaging modalities in the detection of NAFLD with an acceptable sensitivity of

80-100%. Moreover, in clinical practice, ultrasonography is the most frequently used imaging modality. Unfortunately, no histology was available in this population-based study. Therefore, we could not investigate the effect of statin therapy on hepatic histology by liver biopsy, since other studies demonstrated that the use of statin therapy was associated with improvement in liver steatosis[5, 37-38]. Finally, we had too low number of users of cholesterol-lowering medication other than statins, such as ezetimibe, to investigate the effect of these drugs in NAFLD patients.

In conclusion, we did not demonstrate an overall association between current and past use of statin therapy and the presence of NAFLD in this large population-based cohort study. However, current use of statin therapy for more than two years was significantly associated with a lower prevalence of NAFLD. We think that this protective association warrants further investigation through replication in an independent cohort. The present study also provides further evidence for the safety of statins in patients at high risk of NAFLD, as we did not find an association between statin therapy and elevated serum ALT concentrations in participants with and without NAFLD. Therefore, it seems that clinicians may prescribe and continue statin therapy in patients at risk of NAFLD with reasonable safety. Given the association between NAFLD and CVD, lipid lowering treatment with statins should be an important aspect of the treatment of NAFLD patients.

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Summary and discussion

Nederlandse samenvatting

Abbreviations

Summary and discussion

As a result of the obesity epidemic, non-alcoholic fatty liver disease (NAFLD) has become the most common chronic liver disease in Western countries [1]. In the general population the prevalence of NAFLD, diagnosed by ultrasonography, ranges from 15- 35% [2]. The prevalence of NAFLD increases through the fourth to sixth decade of life [3-4]. Moreover, prevalence rates are higher in populations that have a higher prevalence of metabolic risk factors, including obesity and type II diabetes. In **Chapter I**, we investigated the prevalence of NAFLD in Dutch elderly individuals, by means of ultrasonography of the liver. In addition, we sought to clarify the association of NAFLD with metabolic and life style factors in the elderly. This study, as well as several other studies described in this thesis, was part of the Rotterdam Study, a large prospective population-based cohort study, with the objective to examine the occurrence and risk factors of chronic diseases in the elderly [5]. From 1990 to 1993, all inhabitants of the Ommoord district of Rotterdam, the Netherlands, aged 55 years or older, were invited for participation. Of 10,275 invitees 7,983 (78%) agreed to participate. During baseline and follow-up examinations (every three to five years) participants had an extensive at home interview and subsequently visited the research center for a clinical examination and blood collection. A second and third cohort was added to the Rotterdam Study in 1999 and 2006, respectively. Ultrasonography of the abdomen and transient elastography of the liver, a non-invasive technique that measures liver stiffness, were added to the core protocol of the Rotterdam Study in 2009, at the start of the fifth follow-up examination of the first cohort. By studying ultrasonographies of the participants of the first and second cohort that visited the research center from 2009-2012, we found that approximately one in three elderly individuals have NAFLD, which is in line with the high prevalence of metabolic factors at old age. Indeed, the prevalence of fatty liver increased when more metabolic syndrome criteria were present. In addition, the prevalence of NAFLD was significantly lower in individuals over eighty years of age, than in younger elderly individuals. Furthermore, physical activity was associated with a lower prevalence of NAFLD, which underlines the importance of increasing physical activity as part of first line treatment modalities for NAFLD.

Nonalcoholic fatty liver disease comprises a spectrum of histological findings, ranging from simple steatosis to non-alcoholic steatohepatitis (NASH) with inflammation, ballooning degeneration and advanced fibrosis, and NASH cirrhosis [6]. Patients with NASH have increased overall and liver-related mortality (including liver failure, sepsis and variceal haemorrhage, and hepatocellular carcinoma (HCC)) [7-10]. In addition, NASH has been associated with higher cardiovascular morbidity and mortality [11]. Simple steatosis is considered to be a relatively benign condition. However, some studies demonstrated that subjects with NAFLD may have an increased risk of cardiovascular and overall mortality [9, 12-13], although discordant data have

been reported [14]. In **Chapter II** we report the results of our study on the association of liver enzymes and elevation of liver enzymes (of which the most frequent underlying condition is NAFLD) with all-cause and cause-specific mortality. We demonstrated an association between liver enzymes and all-cause mortality. Although elevation of both aminotransferases and both cholestatic liver enzymes were associated with an increased risk of all-cause mortality in elderly individuals, we –unexpectedly- found that low levels of serum aminotransferases were also associated with higher all-cause mortality. These findings have recently been replicated by the American 'National Health and Nutrition Examination Survey' (NHANES).

Considering the finding that up to 50% of NAFLD subjects have normal ALT levels, this marker appears to be insensitive and therefore is not the best method to prospectively investigate associations between NAFLD and mortality. In order to prospectively study the association of NAFLD with morbidity and mortality more accurately, we tried to validate a noninvasive score that identifies NAFLD more accurately than using single liver enzymes [15]. The Fatty Liver Index, an algorithm based on waist circumference, body mass index, triglyceride levels and gamma glutamyltransferase, which predicts presence of fatty liver on ultrasonography, is validated in **Chapter III**.

The role of genetic factors in NAFLD remains largely unknown. In the past few years, there has been great activity in the identification of common susceptibility alleles for NAFLD using Genome-Wide Association Studies (GWAS). To date, two GWAS have reported genetic loci associated with non-alcoholic fatty liver, as assessed by means of computed tomography and magnetic resonance imaging [16-17]. In **Chapter IV** we validated and investigated these single nucleotide polymorphisms in relation to NAFLD and other metabolic traits in more detail. We studied rs2228603 near neurocan (*NCAN*), rs12137855 near lysophospholipase-like protein 1 (*LYPLAL1*), rs780094 in glucokinase regulator (*GCKR*), rs738409 in patatinlike phospholipase family 3 (*PNPLA3*), and rs4240624 near protein phosphatise 1, regulatory subunit 3B (*PPP1R3B*) in our Rotterdam Study cohort of elderly individuals and demonstrated an association between rs738409C>G in PNPLA3 and the presence of NAFLD, especially in insulin sensitive subjects. Furthermore, we replicated the association of rs2228603C>T near *NCAN* with NAFLD for the first time, and demonstrated an association of rs780094C>T in *GCKR* and rs4240624G>A near *PPP1R3B* with NAFLD in insulin resistant subjects.

Data regarding the prevalence of NASH is mainly extrapolated from post-mortem studies and studies of bariatric surgery patients, which constitute highly selected populations. Only few studies have been able to estimate the prevalence of advanced fibrosis in NAFLD in community-based studies, using non-invasive methods [18-19]. In **Chapter V** we report the first results of liver stiffness measurements that were performed in a Western population-based study. In several common chronic liver diseases a liver stiffness greater than ~ 9.5kPa has been correlated

to presence of advanced liver fibrosis. We demonstrated that approximately 4% of individuals with NAFLD have a liver stiffness measurement of greater than 9.5 kPa, which suggests presence of advanced fibrosis. The factors that were associated with greater (log-transformed) liver stiffness measurement in individuals with NAFLD were higher age, greater insulin resistance and higher serum alanine aminotransferase levels.

Currently, liver biopsy is the golden standard for diagnosing the severity of NAFLD. As this is an invasive procedure, it is of great importance to improve the accuracy to develop non-invasive methods to diagnose NASH with improved accuracy and determine the stage of fibrosis noninvasively, e.g. by using biomarkers or biomarker panels. Moreover, development of non-invasive methods that detect liver inflammation and fibrosis need further investigation to capture the natural history of this disease more fully and to test the efficacy of therapeutic approaches that reverse or stop disease from progress to worse stages. In **Chapter VI**, we studied the association and predictive abilities of several biomarkers that have been identified to play a role in pathophysiological pathways suggested to be involved in the progression of NAFLD. Parameters that were measured included insulin, tumor necrosis factor alpha, interleukin 6, cytokeratin 18, adiponectin, C-reactive protein, growth hormone (GH) and sulfated dehydroepiandrosterone (DHEA-S). Liver biopsies were obtained from class III obese patients that underwent bariatric surgery. We found that endocrine parameters, including adiponectin, insulin resistance and GH, are near ubiquitous among the obese patients with NASH who had advanced fibrosis. CRP, IL-6, GH, CK-18, adiponectin, HOMA-IR and QUICKI were also statistically significantly associated with NASH, but overall predictivity was too low to be of clinical use.

Increasing physical activity and introducing diet to reduce fat mass and correct insulin resistance is the mainstay for treatment of NAFLD and NASH. In addition, metabolic comorbidities including insulin resistance, hypertension, and dyslipidemia may be treated. Dyslipidemia may be present in up to 80% of subjects with NAFLD and may be effectively treated with statins [20]. However, there remains to be uncertainty as to whether statins are safe and effective in subjects with NAFLD, and whether they should be prescribed in the presence of liver biochemistry abnormalities. Some studies suggest that statins increase hepatotoxicity, worsen hepatic steatosis, and significantly increase serum liver enzymes, despite improvement of serum lipid concentration [21-24]. Therefore, in **Chapter VII**, we sought to clarify the association between the use of statins and NAFLD and/or elevation of serum alanine aminotransferase levels in the population-based Rotterdam Study. Although we overall did not demonstrate an overall association between current and past use of statins and the presence of NAFLD, current use of more than two years was associated with a lower prevalence of NAFLD. Furthermore, our study provides additional evidence that statins may be safe, as no association was demonstrated between statins and elevation of ALT in participants with or without NAFLD.

Directions and future research

NAFLD has become the most prevalent chronic liver disease in Western countries, in parallel with current epidemics in obesity and type II diabetes mellitus. Therefore, this condition concerns an increasingly relevant public health issue. NAFLD will most likely become the most common indication for liver transplantation in the next 10 to 20 years. Age is an important risk factor for progression of NAFLD and for the development of liver-related morbidity and mortality from advanced liver disease. Thus, in the setting of an ageing population, and current lack of knowledge regarding NAFLD in the elderly, it is important that we gain more insight into etiology, diagnosis and treatment of this condition in this segment of the population, especially since these individuals are the most frequent users of healthcare.

With respect to etiology, the Rotterdam Study provides an excellent opportunity to study genetic risk factors for NAFLD and liver stiffness (which is strongly associated with liver fibrosis). In addition, future studies may investigate the role of the gut microbiota in development and progression of disease, as stool samples are currently being collected in the Rotterdam Study. Furthermore, NAFLD is believed to be associated with a state of low-grade chronic and systemic inflammation. Hence, it would be interesting to study the association with inflammatory markers and other inflammatory (& vascular) diseases into more detail using data from this large well-characterized study.

There is a great body of evidence that patients with NASH and fibrosis have a greater risk of overall and liver-related morbidity and mortality, and liver biopsy is, to date, necessary to ascertain the diagnosis of these stages of disease. It is of great importance to develop and improve the accuracy of tools for non-invasive diagnosis of inflammation and fibrosis of the liver. In addition, the development of non-invasive methods that detect liver inflammation and fibrosis need further investigation to capture the natural history of this disease more fully and to test the efficacy of therapeutic approaches that reverse or stop disease from progress to worse stages.

Lifestyle modification, through exercise and weight loss efforts, is currently the cornerstone of management of NAFLD. This treatment regimen, however, is hard to sustain for many patients. On societal level, this means that governments will need to take more leadership and responsibility and invest in sustainable *prevention* programs addressing obesity and obesity-related diseases. In addition, at present, there is no standard pharmacological therapy for NAFLD or NASH, although some treatments have shown to be of benefit on liver histology. Further research is necessary to determine long term efficacy and safety of currently available drugs. Furthermore, better understanding of the pathophysiology and disease progression in NAFLD is needed to ensure that new therapies may be developed.

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Nederlandse samenvatting

Door de obesitas epidemie is niet-alcoholische vetleverziekte ('non-alcoholic fatty liver disease', NAFLD) nu de meest voorkomende chronische leverziekte in Westerse landen[1]. In Westerse bevolkingsgroepen heeft ongeveer 15 tot 35% van de volwassenen individuen een zgn. niet-alcoholische vette lever[2-3]. De prevalentie oftewel het voorkomen van deze aandoening neemt toe met de leeftijd. Bovendien is NAFLD sterk geassocieerd met metabole risicofactoren en dus komt deze aandoening meer voor in bevolkingsgroepen waar suikerziekte en obesitas meer voorkomen. In **Hoofdstuk I** hebben we de prevalentie van NAFLD, gebaseerd op echografisch onderzoek, bij Nederlandse ouderen in kaart gebracht. Daarnaast hebben wij gekeken welke metabole en leefstijlfactoren geassocieerd waren met NAFLD in deze groep ouderen. Net als diverse andere studies in dit proefschrift, is deze studie onderdeel geweest van het Erasmus Rotterdam Gezondheid Onderzoek (ERGO), ook wel de "Rotterdam Study" genoemd. Dit is een groot longitudinaal bevolkingsonderzoek naar de oorzaken van chronische ziekten en beperkingen bij ouderen[4]. Voor deze studie, die gestart is in 1990, zijn alle mensen van 55 jaar of ouder, die in Ommoord (een wijk van Rotterdam) woonden uitgenodigd voor deelname. Van 10.275 genodigden hebben 7983 (78%) mensen deelgenomen. Gedurende het uitgangs-onderzoek en meerdere vervolgonderzoeken (welke elke 3 tot 5 jaar plaatsvonden) kreeg elke deelnemer een uitgebreid thuis-interview en bezocht vervolgens het onderzoekscentrum voor meerdere klinische onderzoeken en een bloedafname. Een tweede en derde onderzoeksgroep werd aan de studie toegevoegd in respectievelijk 1999 en 2006. Vanaf 2009 worden er bij alle drie de onderzoeksgroepen ook echografieën van de buik gemaakt en wordt er een Fibro-scan® gedaan, een niet-invasief onderzoek waarmee de leverstijfheid wordt gemeten. Door het bestuderen van de echografieën van deelnemers die tussen 2009 en 2012 het centrum bezochten, vonden we dat ongeveer een op de drie ouderen een niet-alcoholische vette lever heeft, hetgeen goed correspondeert met de hoge prevalentie van metabole risicofactoren op hogere leeftijd. We zagen ook dat de prevalentie toenam naarmate ouderen meer metabole risicofactoren hadden, zoals bijvoorbeeld een buikomtrek van meer dan 88cm voor vrouwen en meer dan 102 cm voor mannen, een te hoge bloeddruk en glucose boven de normaalwaarde. Bovendien vonden wij dat de prevalentie van NAFLD afnam na het bereiken van een leeftijd van ongeveer 80 jaar en dat naarmate men meer fysieke activiteit verrichtte, NAFLD minder vaak voorkwam. Dit laatste benadrukt hoe belangrijk fysieke activiteit is voor de behandeling van NAFLD.

De term 'NAFLD' omvat verschillende stadia van deze aandoening, van 'simpele' vette lever en niet-alcoholische steatohepatitis (NASH) met ontsteking en littekenvorming ('fibrose') tot het eindstadium levercirrose[5]. NASH is een belangrijke oorzaak van algehele en levergerelateerde morbiditeit en mortaliteit [6-9]. Cirrose van de lever, het eindstadium van leverfibrose, kan leiden

tot leverfalen en hepatocellulair carcinoom (HCC). Een 'simpele' vette lever, waarbij dus geen ontsteking of fibrose wordt gezien en wat het overgrote deel van NAFLD omvat, is een relatief goedaardige aandoening. Sommige studies hebben echter laten zien dat de sterfte als gevolg van hart- en vaatziekten hoger is bij mensen met een vette lever dan bij mensen zonder een vette lever[8, 10-11]. In **Hoofdstuk II** hebben wij onderzocht of leverenzymen dan wel een verhoging van leverenzymen boven de normaalwaarde (meestal veroorzaakt door NAFLD), geassocieerd is met een hogere sterfte. Wij vonden dat alle onderzochte leverenzymen geassocieerd waren met algehele sterfte gedurende een vervolgperiode van bijna 20 jaar. Opmerkelijk was dat niet alleen een stijging van alanine aminotransferase (ALAT) en aspartaat aminotransferase (ASAT) gepaard gaan met een hogere sterfte, maar ook hele lage waarden van deze enzymen lijken gepaard te gaan met een hogere sterfte. Deze bevindingen zijn recent bevestigd door de Amerikaanse equivalent van het ERGO, de NHANES (National Health and Nutrition Examination Survey).

Aangezien ongeveer de helft van mensen met een vette lever een stijging van het ALAT heeft boven de normaalwaarde, is dit niet de meest betrouwbare methode om longitudinaal onderzoek te doen naar de risico's van een vette lever voor de gezondheid. Daarom hebben wij een bestaande niet-invasieve score, de zogenaamde "Fatty Liver Index", gevalideerd in **Hoofdstuk III** [12].

De rol van genetische factoren in NAFLD is vooralsnog grotendeels onbekend. In de afgelopen jaren is er veel activiteit geweest op het gebied van het identificeren van veel voorkomende varianten van nucleotidecodes (van 1 nucleotide lang: 'single nucleotide polymorphism', SNP) in of nabij bepaalde genen die een rol zouden kunnen spelen bij het ontstaan van een vette lever, door middel van 'Genome-Wide Association Studies' (GWAS). Tot en met 2012 zijn er twee van deze studies gepubliceerd die meerdere SNPs identificeerden die geassocieerd waren met NAFLD, wat gediagnosticeerd was middels computed tomography (CT) of magnetic resonance imaging (MRI)[13-14]. In **Hoofdstuk IV** hebben wij gekeken of deze vijf SNPs ook in het ERGO geassocieerd waren met een hogere prevalentie van NAFLD, en hebben wij in meer detail de associatie en interactie met metabole risicofactoren bestudeerd. Wij vonden dat de SNP genaamd 'rs738409' in het gen 'patatinlike phospholipase family 3' (PNPLA3) geassocieerd was met NAFLD, in het bijzonder bij insuline gevoelige personen. Daarnaast was ook rs2228603 gelegen nabij het gen 'neurocan' (*NCAN*) geassocieerd met NAFLD en was rs780094 in het gen 'glucokinase regulator' (*GCKR*) en rs4240624 nabij het gen 'protein phosphatise 1, regulatory subunit 3B' (*PPP1R3B*) geassocieerd met NAFLD in insuline resistente ouderen.

Er is weinig bekend over de prevalentie van NASH in de algemene bevolking. De meeste data komen van geselecteerde populaties, bijvoorbeeld post-mortem studies en studies waarin obese patiënten bariatrische chirurgie ondergingen en zodoende per-operatief een leverbiopt kon worden afgenomen. Er zijn enkele studies die hebben getracht de prevalentie van ernstige

fibrose in NAFLD te schatten, gebruik makende van non-invasieve methoden[15-16]. In **Hoofdstuk V** rapporteren wij de eerste resultaten van leverstijfheidmetingen die werden uitgevoerd in het ERGO. Bij verschillende leverziekten is het aangetoond dat een leverstijfheidmeting boven de 9.5kPa is geassocieerd met de aanwezigheid van ernstige fibrose, dat wil zeggen fibrose stadium 3 of 4 uit 4. Wij demonstreren in dit hoofdstuk dat ongeveer 4% van de personen met NAFLD een leverstijfheid hebben van >9.5kPa, hetgeen zou kunnen duiden op de aanwezigheid van een ernstige leverfibrose. De factoren die geassocieerd waren met hogere leverstijfheid waren hogere leeftijd, meer insuline resistentie en hogere ALAT waarden.

De gouden standaard voor het vaststellen van de ernst van NAFLD is het leverbiopt. Aangezien leverbiopsie een invasieve procedure is, is het belangrijk de betrouwbaarheid van niet-invasieve technieken om leverfibrose of ontsteking op te sporen te verbeteren. Daarnaast is het belangrijk dat er non-invasieve methoden ontwikkeld worden die ontsteking en fibrose kunnen opsporen. Hiermee kan dan onderzoek worden gedaan het natuurlijk beloop van de ziekte en om de effectiviteit van eventuele behandelingen. In **Hoofdstuk VI** hebben wij gekeken hoe bepaalde markers in het bloed geassocieerd zijn met de ernst van NAFLD en of deze markers betrouwbaar genoeg zijn om het stadium of de activiteit van de aandoening vast te stellen. Wij hebben onder andere gekeken naar tumor necrosis factor alpha, interleukin 6, cytokeratin 18, adiponectine, C-reactief proteïne, groeihormoon en sulfated dehydroepiandrosteron. Leverbiopsieën werden verkregen tijdens bariatrische chirurgie van zeer obese patiënten. Wij vonden dat endocriene parameters, zoals bijvoorbeeld adiponectine, insuline en groeihormoon goed geassocieerd waren met de ernst van leverziekte, echter dat de voorspellende waarden van deze testen niet goed genoeg waren om in de kliniek te gebruiken voor het onderscheid tussen patiënten met of zonder ontsteking en/of fibrose.

Tot op heden is het verhogen van fysieke activiteit en het introduceren van een dieet om de vetmassa te verlagen en insuline resistentie te corrigeren de beste therapie voor NAFLD en NASH. Ook het behandelen van metabole risicofactoren, indien aanwezig, is belangrijk. Veel patiënten met NAFLD hebben dyslipidemie, dat wil zeggen een hoge triglyceride waarde in het bloed en/of een te laag HDL-cholesterol en/of een te hoog totaal cholesterol of LDL-cholesterol. Hiervoor kan men bijvoorbeeld een statine, een cholesterolverlagend middel voorschrijven. Er blijft echter onzekerheid bestaan over het feit of statines wel effectief zijn bij de behandeling van NAFLD en of zij, bij de aanwezigheid van een vette lever, niet juist verdere leverschade kunnen veroorzaken[17-20]. In **Hoofdstuk VII** hebben wij gekeken naar de associatie tussen het gebruik van statines en het voorkomen van NAFLD. Hoewel we geen associatie vonden tussen het gebruik van statines en de aanwezigheid van NAFLD, vonden wij dat meer dan twee jaar actueel gebruik van statines geassocieerd was met een lagere prevalentie van NAFLD. Bovendien ondersteunt de studie de veiligheid van het gebruik van statines in de aanwezigheid van NAFLD.

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Abbreviations

11β-HSD1	11β-hydroxysteroid dehydrogenase type 1
ALP	alkaline phosphatise
ALT	alanine aminotransferase
AST	aspartate aminotransferase
ATP III	adult treatment panel III
AUROC	area under receiver operating characteristic
BMI	body mass index
BP	blood pressure
CABG	coronary artery bypass grafting
cC4	complement C4
CI	confidence interval
CK-18	cytokeratin 18
CT	computed tomography
CVD	cardiovascular disease
DDD	defined daily doses
DHEA-S	sulphated dehydroepiandrosterone
EAF	effect allele frequency
ERGO	Erasmus Rotterdam Gezondheid Onderzoek
FFA	free fatty acid
FLI	fatty liver index
FPG	fasting plasma glucose
FS	fibrosis stage
GCKR	glucokinase regulator
GGT	gamma glutamyl transferase
GH	growth hormone
GK	glucokinase
GWAS	genome wide association studies
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HDL-C	high-density lipoprotein cholesterol
HOMA-IR	homeostasis Model Assessment of Insulin Resistance
IFG	impaired fasting glucose
IL-6	interleukin 6
IRS-1	insulin receptor substrate-1
JNK1	C-Jun N-terminal kinase-1
IQR/M	interquartile range devided by the median of LSM

LSM	liver stiffness measurement
LAP	lipid Accumulation Product
LDL	low-density lipoprotein
LYPLAL1	lysophospholipase-like protein 1
MET-h	metabolic task hours
MRI	magnetic resonance imaging
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
NCAN	neurocan
NF-κβ	nuclear factor kappa beta
ON	obese normal
OR	odds ratio
OSA	obstructive sleep apnea
PASP	pulmonary artery systolic pressure
PNPLA3	patatinlike phospholipase family 3
PPAR	peroxisome proliferator receptor
PPP1R3B	protein phosphatise 1, regulatory subunit 3B
PPV	positive predictive value
PTCA	percutaneous coronary angioplasty
QUICKI	quantitive insulin sensitivity check index
RS	Rotterdam Study
SNP	single nucleotide polymorphism
SS	simple steatosis
TE	transient elastography
T2DM	type 2 diabetes mellitus
TNF-α	tumor necrosis factor alpha
US-NAFLD	NAFLD as determined by ultrasonography
VLDL	very low-density lipoprotein

List of publications

PhD Portfolio

Curriculum Vitae

List of publications

1. Koehler EM, Swain J, Sanderson SO, Viker K, Krishnan A, Kendrick M, Thompson G, Que FG, Sarr M, Charlton MR. *Growth Hormone, DHEA and adiponectin levels in NASH: Endocrine Signature for advanced fibrosis in obese patients.* Liver Int. 2012 Feb; 32(2):279-86.
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PhD Portfolio

Name PhD student: Edith M. Koehler
PhD period: 2009-2013
Promotor: Prof. Dr. H.L.A. Janssen
Co-promotor: Dr. J.N.L. Schouten
Affiliation: Erasmus MC Rotterdam
Dept.: Gastroenterology and Hepatology

International conferences

	YEAR	WORKLOAD
63rd Annual Meeting of the American Association for the Study of Liver Diseases Boston, USA	2012	28 hours
47th Annual Meeting of the European Association for the Study of the Liver Barcelona, Spain	2012	28 hours
62nd Annual Meeting of the American Association for the Study of Liver Diseases San Francisco, USA	2011	28 hours
46th Annual Meeting of the European Association for the Study of the Liver Berlin, Germany	2011	28 hours
61st Annual Meeting of the American Association for the Study of Liver Diseases Boston, USA	2010	28 hours
45th Annual Meeting of the European Association for the Study of the Liver Vienna, Austria	2010	28 hours

Oral presentations

	YEAR	WORKLOAD
Prevalence of and risk factors for advanced fibrosis in the elderly: results from the Rotterdam Study NVH, Zeist, the Netherlands	2012	12 hours
Gamma glutamyltransferase and aminotransferase levels are independently associated with all-cause mortality in the elderly: results of a population-based study AASLD, San Francisco, USA	2011	12 hours
Higher physical activity level is associated with decreased prevalence and severity of non-alcoholic fatty liver in the elderly NVGE, Veldhoven, the Netherlands	2011	12 hours
Decreased prevalence of hepatic steatosis in the elderly: a population-based study. NVGE, Veldhoven, the Netherlands	2010	12 hours

Poster presentations

	YEAR	WORKLOAD
Prevalence of and risk factors for advanced fibrosis in the elderly: results from the Rotterdam Study AASLD, Boston, USA	2012	12 hours
Factors associated with non-alcoholic fatty liver disease in non-diabetic lean elderly individuals AASLD, Boston, USA	2012	12 hours
Association between statin use and non-alcoholic fatty liver disease in a population-based study AASLD, Boston, USA	2012	12 hours
Higher physical activity level is associated with decreased prevalence and severity of non-alcoholic fatty liver in the elderly AASLD, San Francisco, USA	2011	12 hours
Non-alcoholic fatty liver disease is not associated with cognitive impairment in the elderly: results of a population-based study AASLD, San Francisco, USA	2011	12 hours
Decreased prevalence of hepatic steatosis in the elderly: a population-based study. AASLD, Boston, USA	2010	12 hours
Prospective evaluation of serum biomarkers in NASH in patients with class III obesity: evidence of an endocrine basis for progressive fibrosis. AASLD, Boston, USA.	2009	12 hours

Courses and workshops

	YEAR	WORKLOAD
Adobe Illustrator, Photoshop and Indesign	2012	16 hours
Introductory course on statistics & survival analysis	2011	16 hours
Good Clinical Practice (BROK) course	2011	20 hours
Methodology of patient-based studies and preparation of subsidy applications (CPO)	2010	8 hours
Ultrasonography course (Dutch Liver Week)	2010	8 hours
SNP course	2010	36 hours

Teaching activities

	YEAR	WORKLOAD
Vascular liver disorders. Lecture for 2nd year medical students	2012	6 hours
Liver research in the Rotterdam Study. Lecture for participating general practitioners	2011	6 hours

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Curriculum Vitae

Edith Marianne Koehler werd op 20 juni 1984 geboren te 's Gravenhage. In 2002 behaalde zij haar gymnasiumdiploma aan het Bernardinus College te Heerlen. In september 2002 startte zij haar studie University College aan de Universiteit van Maastricht. Na het behalen van haar propedeuse in 2003, begon zij vervolgens datzelfde jaar aan de opleiding Geneeskunde aan de Universiteit van Maastricht. Na het verrichten van haar afstudeeronderzoek naar niet-alcoholische vetleverziekte in Rochester (Verenigde Staten), behaalde zij in januari 2010 haar basisartsexamen. Diezelfde maand begon zij aan haar promotieonderzoek bij de afdeling Maag-Darm- en Leverziekten van het Erasmus MC te Rotterdam, onder supervisie van prof. dr. Harry Janssen en dr. Jeoffrey Schouten. Sinds april 2013 is zij in opleiding tot Maag- Darm- Leverarts (opleider dr. R.A. de Man). Momenteel is zij werkzaam in Deventer Ziekenhuis (opleider interne geneeskunde: dr. C.J. Vermeij, opleider Maag- Darm- en Leverziekten: dr. F. ter Borg).