BMJ open

Fatty liver predicts the risk for cardiovascular events

Journal:	*BMJ Open*
Manuscript ID:	bmjopen-2013-003202
Article Type:	Research
Date Submitted by the Author:	10-May-2013
Complete List of Authors:	Pisto, Pauliina; Institute of Clinical Medicine, Department of Internal Medicine Santaniemi, Merja; Institute of Clinical Medicine, Department of Internal Medicine Bloigu, Risto; Medical Informatics and Statistics Research Group, Ukkola, Olavi; Institute of Clinical Medicine, Department of Internal Medicine Kesäniemi, Antero; Institute of Clinical Medicine, Department of Internal Medicine
Primary Subject Heading:	Cardiovascular medicine
Secondary Subject Heading:	Cardiovascular medicine, Diabetes and endocrinology
Keywords:	Coronary heart disease < CARDIOLOGY, Myocardial infarction < CARDIOLOGY, General diabetes < DIABETES & ENDOCRINOLOGY, Hepatology < INTERNAL MEDICINE

Fatty liver predicts the risk for cardiovascular events

Pauliina Pisto[1], Merja Santaniemi[1], Risto Bloigu[2], Olavi Ukkola[1], Y. Antero Kesäniemi[1]

[1]Institute of Clinical Medicine, Department of Internal Medicine and Biocenter Oulu, University of Oulu, and Clinical Research Center, Oulu University Hospital, Oulu, Finland, P.O. Box 20, 90029 OYS, Finland

[2] Medical Informatics and Statistics Research Group, University of Oulu, Aapistie 7, P.O. Box 5000, 90014 Oulu, Finland

Contact information: Pauliina Pisto, corresponding author

University of Oulu

Institute of Clinical Medicine

Department of Internal medicine

P.O. Box 5000, 90014 Oulu, Finland

Tel.: +35885376310

Fax: +35885376318

E-mail: pauliina.pisto@oulu.fi

Word count: 5093

Keywords: coronary disease, ectopic fat, insulin resistance, risk factors, stroke

Financial support: This study was supported by the Finnish Foundation for Cardiovascular Research, dated 16 Apr, 2012.

Disclosure summary: Authors report no conflict of interests.

ABSTRACT

Objective: We investigated if the differences in liver fat accumulation would predict the development of non-fatal and fatal atherosclerotic endpoints (coronary heart disease and stroke).

Design, setting and participants: Our study group is a population-based, randomly recruited cohort (OPERA), initiated in 1991. The cohort consisted of 988 middle-aged Finnish subjects.

Intervention: Total mortality and hospital events were followed up to 2009 based on the registry of the National Institute for Health and Welfare and the National death registry.

Main outcome measure: The severity of liver adiposity was measured by ultrasound and divided into three groups (0-2). Cox regression analysis was used in the statistical analysis.

Results: In the follow-up of years 1991-2009, 13.5% of the subjects with non-fatty liver, 24.2% of subjects having moderate liver fat accumulation and 29.2% of the subjects having severe fatty liver experienced a cardiovascular event during the follow-up time ($p < 0.001$). Severe liver fat accumulation predicted the risk for future risk of cardiovascular event even when adjusted for age, gender and study group (HR 1.92, CI 1.32-2.80, $p < 0.01$). When further adjustments for smoking, alcohol consumption, LDL-cholesterol, BMI and systolic blood pressure were conducted, the risk still remained statistically significant (HR 1.74, CI 1.16-2.63, $p < 0.01$). Statistical significance disappeared with further adjustment for QUICKI.

Conclusions: Liver fat accumulation increases the risk of future cardiovascular disease event in long-term follow-up but it is seems to be dependent on insulin sensitivity.

Article focus

1 To investigate if the differences in liver fat accumulation predict the risk for development of fatal or nonfatal atherosclerotic endpoints such as coronary heart disease and stroke.

Key messages

1 Subjects with ultrasound-diagnosed fatty liver have cardiovascular disease more often compared to the subjects without fat in the liver

2 Severe liver fat accumulation increases the risk of a future cardiovascular event and mortality to cardiovascular disease over the long-term follow-up but it does seem to be dependent on insulin sensitivity

3 Severe fatty liver predicts the risk for overall mortality but the association is dependent on traditional metabolic risk factors

Strengths and limitations of the study

1 Study seems to be the first follow-up study with a large population-based study group and a very long follow-up time

2 Official registers used in event diagnoses - data is accurate and the classification is systematic

3 Grade of liver brightness was measured by ultrasound, which has a high specificity but low sensitivity

Introduction

Non-alcoholic fatty liver disease (NAFLD) refers to liver disorders such as abnormal fat accumulation, which exists in a spectrum ranging from steatosis with no inflammation to non-alcoholic steatohepatitis (NASH), which can ultimately lead to liver cirrhosis [1]. The prevalence of NAFLD is estimated to range from 20 to 30% of population in Western countries, being the leading cause of liver disorders [2]. It is associated with obesity, type 2 diabetes mellitus (T2DM) and hyperlipidemia [1]. NAFLD is commonly regarded as a hepatic manifestation of the metabolic syndrome and both conditions share several risk factors for cardiovascular disease (CVD) [2, 3].

In 2008, the prevalence of CVD in adults (≥ 20 years) in United States was 36.2% [4]. Every year, 4.3 million subjects die for CVD in Europe causing nearly half of the all deaths (48%) [5]. So-called traditional risk factors for cardiovascular disease are age, gender, smoking, high low-density lipoprotein (LDL) cholesterol concentration, hypertension and diabetes [6]. In addition, total body fatness as well as abdominal fat accumulation increase independently the risk of CVD and insulin resistance is regarded to be an important factor linking visceral adiposity to cardiovascular risk [7]. Adipose tissue is now recognized as a significant endocrine organ as adipocytes and macrophages infiltrating adipocytes secrete a number of bioactive mediators, such as adipokines, proinflammatory cytokines and hypofibrinolytic markers [6] that may lead to oxidative stress and endothelial dysfunction, finally leading to atherosclerosis [8].

NAFLD and CVD share several molecular mechanisms [9, 10]. Fatty liver might play a part in the pathogenesis of CVD through the overexpression and systemic release of several inflammatory, hemostatic [11] and oxidative-stress mediators or via contributing to whole-body insulin resistance and atherogenic dyslipidemia [2]. NAFLD has also been reported to be linked with circulatory endothelial dysfunction [3, 12]. Several investigators have reported that NAFLD is associated with coronary artery disease [3, 12] and increased carotid intima-media thickness [13, 14].

It is known that subjects with fatty liver disease have an increased risk of suffering CVD [3], but whether NAFLD is an independent indicator of cardiovascular disease is still far from clear. Long-term follow-up studies are needed to clarify the correlation between fatty liver and CVD. The aim of our study was to investigate if fatty liver could predict independently the

risk for total mortality as well as non-fatal and fatal cardiovascular endpoints with a 19-year follow-up after adjusting for all known conventional risk factors.

Materials and methods

Human subjects

OPERA (Oulu Project Elucidating Risk of Atherosclerosis) is a population-based, epidemiological prospective cohort study designed to address the risk factors and disease end points of atherosclerotic cardiovascular diseases. Selection criteria of the study subjects have been described earlier [15]. In short, a total of 520 men and 525 women participated: 259 control men, 261 hypertensive men, 267 control women and 258 hypertensive women aged 40-59. Hypertensive participants were randomly selected from the national register for reimbursement of the costs of antihypertensive medication. For each hypertensive subject, an age- and sex-matched control subject was randomly selected from the same register. Informed consent in writing was obtained from each patient. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and this study was approved by the Ethical Committee of the Faculty of Medicine, University of Oulu.

Determination of liver adiposity

The determination of liver adiposity was based on liver-kidney contrast measured with ultrasonography [16] by one trained radiologist with extensive experience in abdominal ultrasound examinations. The severity of liver adiposity was based on the brightness of the liver and it was classified into three groups ranging from 0 to 2 (0 = normal bright, indicating

a non-fatty liver, 1 = medium bright, a moderate lipid accumulation and 2 = clearly bright, a severe lipid accumulation and fatty liver) [17].

Follow-up

Both the hypertensive and the control men were recruited during December 1990 to May 1992 and the women approximately one year later (n=1045). In total, 1023 subjects had a liver ultrasound result available at baseline. Mortality data were obtained from the National Death Registry and the diagnoses of cardiovascular events were based on the registry of the National Institute for Health and Welfare. The follow-up time ended December 31, 2009 or whenever the first event occurred. Cardiovascular events included fatal and non-fatal endpoints. Subjects with a previous hospital-diagnosed myocardial infarction or stroke (n=41) at baseline were excluded. In total, 988 subjects participated in this part of the study.

CVD included a major coronary heart disease event (CHD) and stroke (excluding subarachnoid hemorrhage, SAH) - whichever of these happened first. The evidence of CHD was based on the following diagnosis: I20.0, I21, I22 [ICD-10, International Statistical Classification of Diseases and Related Health Problems] / 410, 4110 [ICD-8/9] as the main diagnosis (symptom or cause) and I21, I22 [ICD-10] / 410 [ICD-8/9] as a first side diagnosis (symptom or cause) or second side diagnosis (symptom or cause) and third side diagnosis (ICD-8/9 only) or if a subject had undergone coronary artery bypass graft (CABG) surgery or angioplasty. CHD as a cause of death included I20–I25, I46, R96, R98 [ICD-10] / 410-414, 798 (not 7980A) [ICD-8/9] as the underlying cause of death or immediate cause of death and I21 or I22 [ICD-10] / 410 [ICD-8/9] as first to third contributing cause of death. Stroke

(excluding SAH) included I61, I63 (not I636), I64 [ICD -10] / 431, 4330A, 4331A, 4339A, 4340A, 4341A, 4349A, 436 [ICD-9] / 431 (except 43101, 43191) 433, 434, 436 [ICD-8] as main diagnosis (symptom or cause) or as a first or second side diagnosis (symptom or cause) or as a third side diagnosis (ICD-8/9 only) or as an underlying cause of death or immediate cause of death or as a first to third contributing cause of death [18].

Laboratory analyses

Waist circumference, body mass index (BMI) and blood pressure were measured as described in previous study [15].

Blood insulin and glucose concentrations were analyzed at 0, 60, and 120 min after administration of 75 g glucose [17]. Insulin sensitivity was assessed using fasting plasma insulin concentrations and a quantitative insulin sensitivity check index (QUICKI) {QUICKI=1/[log (fasting insulin)+log (fasting glucose)]}[19].

Very-low-density lipoprotein (VLDL), high-density lipoprotein (HDL), low-density lipoprotein (LDL) and hs-CRP concentrations [17] as well as alanine aminotransferase (ALT) and gamma-glutamyltransferase (GGT) levels were measured as described previously [16]. Alcohol consumption and smoking history were determined by validated questionnaires [20]. Alcohol consumption was divided into three groups: 0 (n=161) mean alcohol consumption less than 1g/week in men and women, 1 (n=767) mean consumption less than 210g/week in men and less than 140 g/week in women, 2 (n=76) mean alcohol consumption more than

210g/week in men and more than 140g/week in women. Group 2 designates large-scale alcohol consumers according to the guidelines [21].

Statistical analysis

Statistical analysis was performed by using IBM SPSS Statistics for Windows, Version 20.0 (Armonk, NY: IBM Corp.). Analysis of variance was used to compare the means of the variables measured. Post hoc tests were performed using the Tukey method. Statistical significances between percentages were measured by using χ^2 test. Cumulative survival rates were estimated using Kaplan-Meier method. Cox regression analysis was performed to investigate if liver brightness (fat) could predict the future risk for total mortality, cardiovascular death or hospital events. A p value < 0.05 was regarded as significant.

Skewed variables (smoking, alcohol consumption, fasting insulin, fasting glucose, triglyceride, ALT, GGT concentration, hs-CRP level) were logarithmically transformed to improve normality before analysis of variance. We used three models with progressive degrees of adjustments. Model 1 included study group (subjects with medicine-treated hypertension and their age- and sex-matched controls), age and gender. Model 2 included further adjustments for smoking, alcohol consumption, systolic blood pressure, LDL-cholesterol level and body mass index. Model 3 included further adjustment for QUICKI. We carried out sensitivity analyses: in the analyses of cardiovascular events, we added all covariates one by one and investigated if the hazard ratios (HR) changed or remained stable when further adjustment with one covariate was performed. Model 4 included variables which

were stable and were statistically significant in intermediate phases. Model 5 included stable and significant covariates without QUICKI (Table 2).

C-index was calculated for the model 1, model 3, model 4 and model 5 to assess the discrimination of the risk markers. The analyses were performed in 250 bootstrap resamplings to obtain 95% CI for c-index of each model.

Results

The main baseline characteristics of the study group are shown in Table 1.

Table 1 about here

Incidence of cardiovascular disease

The median follow-up time was 212 (maximum 228) months. During the follow-up time, 13.5% of the subjects with no fat in the liver (97/720), 24.2% (30/124) of subjects having moderate liver fat accumulation and 29.2% (42/144) of the subjects having severe fatty liver experienced a CVD event ($p < 0.001$). CVD was the cause of death in 3.6% of the subjects with non-fatty liver and 8.1% of the subjects with moderate liver fat accumulation, while 12.5% of the subjects with severe fatty liver ($p < 0.001$).

Severe liver fat accumulation predicted the risk for future risk of cardiovascular event when adjusted for age, gender and study group (Model 1: HR 1.92, CI 1.32-2.80, $p < 0.01$) (Table 2). When further adjustments were made for smoking, alcohol consumption, LDL-cholesterol, BMI and systolic blood pressure (Model 2: HR 1.74, CI 1.16-2.63), the risk still remained statistically significant ($p < 0.01$). Statistical significance disappeared when further adjustment for QUICKI was performed (Model 3: HR 1.49, CI 0.97-2.30, p=0.071). In the CVD event sensitivity analyses, all covariates were added one by one and it was examined whether the hazard ratios would change or remain stable. After adjusting for the statistically significant variables (including quick index) in the sensitivity analyses, the association between severe fatty liver was no longer significant (Model 4: HR 1.43, CI 0.93-2.18, NS). When QUICKI was not added into Model 5, severe fatty liver did predict the risk for future risk for CVD event (HR 1.76, CI 1.21- 2.56, $p < 0.001$) (Table 2). The c-index decreased when the risk factors were removed from the model (Table 3).

Tables 2and 3 about here

The future risk of death from CVD in participants with severe fat accumulation was significant when age, gender and study group were added as covariates (Model 1: HR 2.95, CI 1.58-5.51, $p < 0.01$). Even after further adjustments with other conventional risk factors (Model 2: HR 2.04, CI 1.03-4.05), statistical significance remained ($p < 0.05$). When QUICKI was added as the covariate, then significance disappeared (Model 3: HR 1.64, CI 0.79-3.43, NS) (Fig 1.).

Figure 1 about here

Fatty liver and total mortality

In total, 11.9% of the participants not having fatty liver, 18.5% of the subjects having moderate fatty liver and 22.2% of the subjects with severe fatty liver died from all causes ($p < 0.01$). According to Model 1, severe fat accumulation predicted the risk for mortality from all causes when age, gender and study group were added as covariates (HR 1.60, CI 1.05-2.43, $p < 0.05$). The significance disappeared when body mass index was added as a covariate (data not shown).

We performed all Cox regression analyses after excluding the men consuming more than 210 g alcohol and the women drinking more than 140 g alcohol per week. This exclusion did not have any effect on the results (data not shown).

We performed all Cox regression analyses after excluding patients with insulin treated diabetes mellitus (n=9), cortisone treatment at baseline (n=41) and previous diagnosis for liver disease (n=15) (e.g., virus, medications). This exclusion did not have any effect on the results (data not shown).

Discussion

The incidences of non-alcoholic fatty liver disease and cardiovascular disease are continuously increasing in the Western world. The question if NAFLD is only a marker or also an early mediator of cardiovascular disease is still largely unanswered. According to the results of the present study, which had an approximately 19-year follow-up fatty liver does

predict the future risk for death from all causes, death from cardiovascular disease and risk of cardiovascular events. Insulin sensitivity seems to play a more dominant role in the development of cardiovascular events.

Only a few studies have investigated the risk for future cardiovascular risk among subjects with ultrasound-diagnosed fatty liver [22, 23]. There are a few follow-up-studies examining whether the fatty liver increases the risk for total mortality [24, 25]. Larger follow-up studies with ultrasound-diagnosed fatty liver investigating non-fatal and fatal cardiovascular endpoints are needed. An association between NAFLD and CVD has been reported [2, 22, 23, 26] however several earlier studies have used self-reported CVD history which may not be totally reliable. Although earlier studies on the risk for future cardiovascular risk among subjects with fatty liver have performed some adjustments, the full range of well-known CVD risk factors have been rarely considered [27]. These studies have used biochemical, radiological and histological methodology for NAFLD diagnosis and staging, which leads to a challenging interpretation of the results [28, 29].

This study had an approximately 19-year follow-up time. When compared to earlier studies [27, 29] this study seems to be the first follow-up study with a large population-based randomly selected study group and a very long follow-up time and ultrasound-diagnosed fatty liver. The diagnosis of cardiovascular events was based on the registry of the National Institute for Health and Welfare and mortality data were obtained from the National Death Registry. The earlier verified FINRISK classification [18] was used to classify the events. Therefore, the reliability of event diagnosis data is accurate and the classification is systematic. All subjects who had myocardial infarction or stroke before baseline were excluded because a history of

myocardial infarction is known to increase the risk for recurrent myocardial infarction or cardiovascular death [30] and medication as well as lifestyle secondary prevention strategies are intensive [31].

In the present study, severe fatty liver predicted the risk for overall mortality of any causes when age, gender and study group were added covariates, a result in line with an earlier report [32]. In the published literature, NASH rather than simple steatosis has been stated to be linked with decreased overall survival [33] although one study with a large cohort found no association between NAFLD and overall mortality [25]. In our study, the association between severe fatty liver and total mortality disappeared after further adjustment for BMI which means that severe fatty liver is not a strong predictor for overall mortality. In earlier studies NAFLD, especially NASH, has been reported to increase the risk for cardiovascular death [27]. In the present study, severe fatty liver disease did predict the risk for cardiovascular death but the association seemed to be dependent on insulin sensitivity.

The molecular mechanisms linking fatty liver with CVD have been investigated [10, 34]. Enlarged visceral adipose tissue may explain why NAFLD associates with CVD [10]. In individuals with visceral obesity, insulin resistance may contribute to impaired non-esterified fatty acid (NEFA) metabolism [7] and the increasing NEFA flux to the liver may impair liver metabolism leading to increased glucose metabolism and liver dysfunction [6]. The liver is one of the targets of the resulting systemic abnormalities and the source of several proatherogenic factors [2], such as CRP, fibrinogen, plasminogen activator inhibitor-1 and other inflammatory cytokines [10]. Furthermore, visceral adipose tissue and ectopic fat overexpress factors involved

in atherogenesis [10] such as NEFAs and proinflammatory cytokines, for instance interleukin-6 and tumor necrosis factor-α [7] leading to chronic systemic inflammation. In addition, hepatic steatosis leads to overproduction of cholesterol-rich remnant particles [3].

One limitation in this study may be that the grade of liver brightness was measured by ultrasound. The invasive diagnostic technique of liver biopsy is regarded as the golden standard, especially for the diagnosis of NASH [35]. Real time ultrasound using a combination of sonographic findings does have a high specificity but it underestimates the prevalence of hepatic steatosis when there is less than 20 % fat [36]. Nonetheless, the noninvasive ultrasound method was chosen because taking liver biopsies from large groups of symptomless subjects would have been ethically unjustifiable.

The OPERA study group consists of subjects with drug-treated hypertension and randomly selected sex- and age-matched controls. Study group was added as a covariate to minimize any selection bias.

Conclusions

Severe liver fat accumulation increases the risk of a future cardiovascular event and mortality to cardiovascular disease over the long-term follow-up but it does seem to be dependent on insulin sensitivity. Fatty liver also predicts the risk for overall mortality. However, conventional cardiovascular disease risk factors seem to play a major role in developing death from all causes. It would be beneficial to investigate larger cohorts and follow-up studies in order to validate this result.

Figure legend

Title: Kaplan Meier cumulative survival rates censored for cardiovascular death in subjects with no fat in the liver, moderate fat accumulation and severe fat accumulation.

CVD was the cause of death in 3.6% of the subjects (26/720) with non-fatty liver and 8.1% of the subjects (10/124) with moderate liver fat accumulation, while 12.5% of the subjects with severe fatty liver (18/144). Cox regression analysis is used for adjustments. M1 (Model 1): adjusted for study group, age and gender. M2 (Model 2): further adjustments for smoking, alcohol consumption, systolic blood pressure, LDL-cholesterol level and body mass index. M3 (Model 3): further adjustment for QUICKI. CVD, cardiovascular disease, CI, confidence interval, HR, hazard ratio, QUICKI, quantitative insulin sensitivity check index. ** $p < 0.01$, * $p < 0.05$.

Acknowledgements

The authors thank Markku Päivänsalo, MD, PhD, for the expert liver ultrasound examinations and Ms Saija Kortetjärvi, Ms Liisa Mannermaa, and Ms Leena Ukkola for the excellent technical assistance. M.Sc. Elina Malo and M.Sc. Meiju Saukko are thanked for the cooperation in organizing cardiovascular event and mortality data.

References

1. Angulo P. Nonalcoholic fatty liver disease. *N Engl J Med* 2002; Apr 18;346(16):1221-31.

2. Targher G, Day CP, Bonora E. Risk of cardiovascular disease in patients with nonalcoholic fatty liver disease. *N Engl J Med* 2010; Sep 30;363(14):1341-50.

3. Targher G, Arcaro G. Non-alcoholic fatty liver disease and increased risk of cardiovascular disease. *Atherosclerosis* 2007; Apr;191(2):235-40.

4. Roger VL, Go AS, Lloyd-Jones DM, Adams RJ, Berry JD, Brown TM, et al. Heart disease and stroke statistics--2011 update: a report from the American Heart Association. *Circulation* 2011; Feb 1;123(4):e18-e209.

5. Allender S, Scarborough P, Peto V, Rayner M, Leal J, Luengo-Fernandez R, Gray A. European cardiovascular disease statistics, 2008 ed. European Heart Network; 2008.

6. Despres JP, Lemieux I. Abdominal obesity and metabolic syndrome. *Nature* 2006; Dec 14;444(7121):881-7.

7. Van Gaal LF, Mertens IL, De Block CE. Mechanisms linking obesity with cardiovascular disease. *Nature* 2006; Dec 14;444(7121):875-80.

8. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med* 2005; Apr 21;352(16):1685-95.

9. Loria P, Lonardo A, Targher G. Is liver fat detrimental to vessels?: intersections in the pathogenesis of NAFLD and atherosclerosis. *Clin Sci (Lond)* 2008; Jul;115(1):1-12.

10. Targher G, Marra F, Marchesini G. Increased risk of cardiovascular disease in non-alcoholic fatty liver disease: causal effect or epiphenomenon?. *Diabetologia* 2008; Nov;51(11):1947-53.

11. Targher G, Bertolini L, Scala L, Zoppini G, Zenari L, Falezza G. Non-alcoholic hepatic steatosis and its relation to increased plasma biomarkers of inflammation and endothelial dysfunction in non-diabetic men. Role of visceral adipose tissue. *Diabet Med* 2005; Oct;22(10):1354-8.

12. Wong VW, Wong GL, Yip GW, Lo AO, Limquiaco J, Chu WC, et al. Coronary artery disease and cardiovascular outcomes in patients with non-alcoholic fatty liver disease. *Gut* 2011; Dec;60(12):1721-7.

13. Brea A, Mosquera D, Martin E, Arizti A, Cordero JL, Ros E. Nonalcoholic fatty liver disease is associated with carotid atherosclerosis: a case-control study. *Arterioscler Thromb Vasc Biol* 2005; May;25(5):1045-50.

14. Sookoian S, Pirola CJ. Non-alcoholic fatty liver disease is strongly associated with carotid atherosclerosis: a systematic review. *J Hepatol* 2008; Oct;49(4):600-7.

15. Rantala AO, Kauma H, Lilja M, Savolainen MJ, Reunanen A, Kesaniemi YA. Prevalence of the metabolic syndrome in drug-treated hypertensive patients and control subjects. *J Intern Med* 1999; Feb;245(2):163-74.

16. Sampi M, Veneskoski M, Ukkola O, Kesaniemi YA, Horkko S. High plasma immunoglobulin (Ig) A and low IgG antibody titers to oxidized low-density lipoprotein are associated with markers of glucose metabolism. *J Clin Endocrinol Metab* 2010; May;95(5):2467-75.

17. Pisto P, Ukkola O, Santaniemi M, Kesaniemi YA. Plasma adiponectin--an independent indicator of liver fat accumulation. *Metabolism* 2011; Nov;60(11):1515-20.

18. Pajunen P, Jousilahti P, Borodulin K, Harald K, Tuomilehto J, Salomaa V. Body fat measured by a near-infrared interactance device as a predictor of cardiovascular events: the FINRISK'92 cohort. *Obesity (Silver Spring)* 2011; Apr;19(4):848-52.

19. Katz A, Nambi SS, Mather K, Baron AD, Follmann DA, Sullivan G, et al. Quantitative insulin sensitivity check index: a simple, accurate method for assessing insulin sensitivity in humans. *J Clin Endocrinol Metab* 2000; Jul;85(7):2402-10.

20. Kauma H, Savolainen MJ, Rantala AO, Lilja M, Kervinen K, Reunanen A, et al. Apolipoprotein E phenotype determines the effect of alcohol on blood pressure in middle-aged men. *Am J Hypertens* 1998; Nov;11(11 Pt 1):1334-43.

21. Bessembinders K, Wielders J, van de Wiel A. Severe hypertriglyceridemia influenced by alcohol (SHIBA). *Alcohol Alcohol* 2011; Mar-Apr;46(2):113-6.

22. Hamaguchi M, Kojima T, Takeda N, Nagata C, Takeda J, Sarui H, et al. Nonalcoholic fatty liver disease is a novel predictor of cardiovascular disease. *World J Gastroenterol* 2007; Mar 14;13(10):1579-84.

23. Stepanova M, Younossi ZM. Independent Association Between Nonalcoholic Fatty Liver Disease and Cardiovascular Disease in the US Population. *Clin Gastroenterol Hepatol* 2012; Jun;10(6):646-50.

24. Dam-Larsen S, Franzmann M, Andersen IB, Christoffersen P, Jensen LB, Sorensen TI, et al. Long term prognosis of fatty liver: risk of chronic liver disease and death. *Gut* 2004; May;53(5):750-5.

25. Lazo M, Hernaez R, Bonekamp S, Kamel IR, Brancati FL, Guallar E, et al. Non-alcoholic fatty liver disease and mortality among US adults: prospective cohort study. *BMJ* 2011; Nov 18;343:d6891.

26. Targher G, Bertolini L, Poli F, Rodella S, Scala L, Tessari R, et al. Nonalcoholic fatty liver disease and risk of future cardiovascular events among type 2 diabetic patients. *Diabetes* 2005; Dec;54(12):3541-6.

27. Ghouri N, Preiss D, Sattar N. Liver enzymes, nonalcoholic fatty liver disease, and incident cardiovascular disease: a narrative review and clinical perspective of prospective data. *Hepatology* 2010; Sep;52(3):1156-61.

28. Ruttmann E, Brant LJ, Concin H, Diem G, Rapp K, Ulmer H, et al. Gamma-glutamyltransferase as a risk factor for cardiovascular disease mortality: an epidemiological investigation in a cohort of 163,944 Austrian adults. *Circulation* 2005; Oct 4;112(14):2130-7.

29. Bhatia LS, Curzen NP, Calder PC, Byrne CD. Non-alcoholic fatty liver disease: a new and important cardiovascular risk factor?. *Eur Heart J* 2012; May;33(10):1190-200.

30. Beckman JA, Creager MA, Libby P. Diabetes and atherosclerosis: epidemiology, pathophysiology, and management. *JAMA* 2002; May 15;287(19):2570-81.

31. Joseph P, Teo K. Optimal medical therapy, lifestyle intervention, and secondary prevention strategies for cardiovascular event reduction in ischemic heart disease. *Curr Cardiol Rep* 2011; Aug;13(4):287-95.

32. Calori G, Lattuada G, Ragogna F, Garancini MP, Crosignani P, Villa M, et al. Fatty liver index and mortality: the Cremona study in the 15th year of follow-up. *Hepatology* 2011; Jul;54(1):145-52.

33. Soderberg C, Stal P, Askling J, Glaumann H, Lindberg G, Marmur J, et al. Decreased survival of subjects with elevated liver function tests during a 28-year follow-up. *Hepatology* 2010; Feb;51(2):595-602.

34. Bhatia LS, Curzen NP, Byrne CD. Nonalcoholic fatty liver disease and vascular risk. *Curr Opin Cardiol* 2012; Jul;27(4):420-8.

35. Joy D, Thava VR, Scott BB. Diagnosis of fatty liver disease: is biopsy necessary?. *Eur J Gastroenterol Hepatol* 2003; May;15(5):539-43.

36. Dasarathy S, Dasarathy J, Khiyami A, Joseph R, Lopez R, McCullough AJ. Validity of real time ultrasound in the diagnosis of hepatic steatosis: a prospective study. *J Hepatol* 2009; Dec;51(6):1061-7.

437

Grade of liver bightness	0 (n=720)	1 (n=124)	2 (n=144)	p	p (0-1)	p (1-2)	p (0-2)
Age (years)	50.9 (6.0)	51.9 (6.1)	51.5 (5.5)	NS	NS	NS	NS
Males	44.3 % (n=319)	65.3 % (n=81)	59.9 % (n=82)	< 0.001	-	-	-
Hypertensives	41.4 % (n=298)	66.1 % (n=82)	71.5 % (n=103)	< 0.001	-	-	-
BMI (kg/m²)	26.4 (3.9)	29.8 (5.0)	31.9 (4.9)	< 0.001	< 0.001	< 0.001	< 0.001
Waist circumference (cm)	86.8 (11.9)	97.7 (12.0)	102.3 (11.8)	< 0.001	< 0.001	< 0.01	< 0.001
Smoking (pack years)	10.6 (13.3)	14.3 (14.9)	14.0 (14.6)	< 0.05	NS	NS	NS
Alcohol consumption (g/week)	51.1 (83.0)	95.1 (117.0)	82.6 (105.1)	< 0.01	< 0.05	NS	NS
Total serum cholesterol (mmol/L)	5.6 (1.0)	5.8 (1.1)	5.8 (1.1)	NS	NS	NS	NS
LDL (mmol/L)	3.5 (0.9)	3.7 (1.1)	3.5 (0.9)	NS	NS	NS	NS
Triglycerides (mmol/L)	1.4 (0.8)	1.9 (0.8)	2.2 (1.4)	< 0.001	< 0.001	< 0.05	< 0.001
Systolic blood pressure	145.2 (21.5)	152.7 (20.3)	157.1 (22.2)	< 0.001	< 0.01	NS	< 0.001
Fasting insulin (mmol/L)	10.8 (7.7)	18.2 (10.3)	23.8 (17.6)	< 0.001	< 0.001	< 0.001	< 0.001

Fasting glucose (mmol/L)	4.4 (0.7)	5.0 (1.4)	6.1 (2.8)	< 0.001	< 0.001	< 0.001	< 0.001
QUICKI	0.6 (0.1)	0.6 (0.1)	0.5 (0.1)	< 0.001	< 0.001	< 0.001	< 0.001
hs-CRP (ng/mL)	3039.4 (6758.3)	3981.4 (6068.2)	6122.0 (6630.8)	< 0.001	< 0.001	< 0.01	< 0.001
ALT U/L	26.2 (15.5)	37.8 (17.1)	55.4 (37.7)	< 0.001	< 0.001	< 0.001	< 0.001
GGT U/L	35.1 (33.5)	69.7 (116.3)	76.8 (92.4)	< 0.001	< 0.001	< 0.01	< 0.001
Anti-hypertensive treatment	43.6% (n=314)	66.9% (n=83)	72.9% (n=105)	< 0.001	-	-	-
Lipid-lowering treatment	2.2% (n=16)	1.6% (n=2)	6.2% (n=9)	< 0.05	-	-	-
Hypoglycaemic drug	1.1% (n=8)	1.6% (n=2)	10.4% (n=15)	< 0.001	-	-	-
Type 2 diabetes	2.4% (n=17)	12.1% (n=15)	36.8% (n=53)	< 0.001	-	-	-

Table 1. Baseline characteristics of the study group as means (standard deviations) or percentages. N= number of subjects. ALT, alanine aminotransferase, BMI, body mass index, GGT, gamma-glutamyltransferase, hs-CRP, high-sensitivity C-reactive protein, LDL, low-density lipoprotein, QUICKI, quantitative insulin sensitivity check index.

443

	Model 1	Model 2	Model 3	Model 4	Model 5
Moderate fat accumulation	1.51 (0.99-2.29)	1.44 (0.93-2.23)	1.31 (0.84-2.05)	1.30 (0.84-2.01)	1.49 (0.99-2.26)
Severe fat accumulation	1.92 (1.32-2.80)**	1.74 (1.16-2.63) **	1.49 (0.97-2.30)	1.43 (0.93-2.18)	1.76 (1.21- 2.56) **
Study group	1.34 (0.98-1.85)	1.29 (0.92-1.80)	1.28 (0.92-1.78)		
Age	1.06 (1.03-1.09)***	1.05(1.02-1.08)**	1.05 (1.02-1.08)**	1.05 (1.02-1.07)**	1.05 (1.02-1.08) **
Gender	2.39 (1.71-3.34)*	1.91 (1.34-2.71)***	1.80 (1.26-2.57)**	1.83 (1.29-2.60) **	1.92 (1.36-2.72) ***
LDL-cholesterol		1.17 (0.99-1.39)	1.15 (0.97-1.37)		
Smoking (pack-years)		1.02 (1.01-1.03)***	1.02 (1.01-1.03)***	1.02 (1.01-1.03)***	1.02 (1.01-1.03) ***
Alcohol consumption (gr1)		0.93 (0.59-1.45)	0.92(0.59-1.44)		
Alcohol consumption (gr2)		0.84 (0.44-1.60)	0.81(0.42-1.54)		
Systolic blood pressure		1.01 (1.00-1.02)**	1.01 (1.00-1.02)*	1.01 (1.00-1.02)**	1.01 (1.00-1.02) **
Body mass index		0.99 (0.96-1.03)	0.97 (0.93-1.01)		
QUICKI			0.12 (0.02-0.90)*	0.16 (0.03-0.99)*	

444

445

Table 2. Multivariate analysis for cardiovascular events with different degrees of adjustments (Cox regression analysis). CVD event occurred in 13.5% of the subjects with no fat in the liver (97/720), 24.2% (30/124) of subjects having moderate liver fat accumulation and 29.2% (42/144) of the subjects having severe fatty liver. Hazard ratios with 95% confidence interval with different degrees of adjustments are presented. Alcohol consumption was divided into groups (reference group: less than 1g/week in men and women, group 1: less than 210g/week in men and less than 140 g/week in women, group 2: more than 210g/week in men and more than 140g/week in women). Model 1: adjustment for study group, age and gender. Model 2: further adjustments for LDL-cholesterol, smoking, alcohol consumption, systolic blood pressure and body mass index. Model 3: further adjustment for QUICKI. Model 4: adjustments with statistically significant covariates. Model 5: adjustments with statistically significant covariates without QUICKI. LDL, low-density lipoprotein, QUICKI, quantitative insulin sensitivity check index. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Final model	Cardiovascular event c-index (95% CI)	Binary R^2
Model 3	0.729 (0.706-0.776)	0.153
Model 4	0.720 (0.689-0.763)	0.144
Model 5	0.717 (0.686-0.758)	0.138
Model 1	0.698 (0.656-0.742)	0.133

Table 3. Multivariate analysis for cardiovascular events (logistic regression analysis). Cardiovascular disease risk factors have been removed from the models step by step. Model 3 included liver brightness, study group, age, gender, smoking, alcohol consumption, systolic blood pressure, LDL-cholesterol level, body mass index and QUICKI. Model 4 included liver brightness, age, gender, smoking, blood pressure and QUICKI. Model 5 included liver brightness, age, gender, smoking, blood pressure. Model 1 included liver brightness, study group, age and gender. C-index with confidence intervals obtained from 250 bootstrap resamplings and binary R^2 was used. LDL, low-density lipoprotein, QUICKI, quantitative insulin sensitivity check index.

Contributor statement: All authors fulfill all three of the ICMJE guidelines for authorship

Pauliina Pisto: Data acquisition, statistical analysis and interpretation of data, manuscript writing, final approval of the version to be published

Merja Santaniemi: Data acquisition, statistical analysis and data interpretation, critical revision of the manuscript, final approval of the version to be published

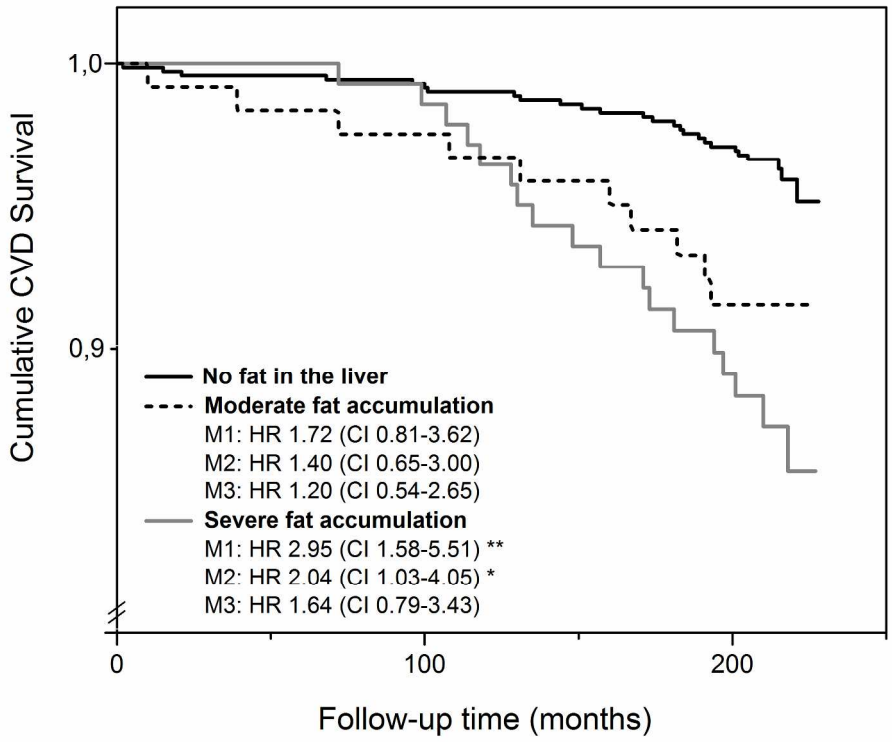
Risto Bloigu: Data analysis, interpretation of data, critical revision of the manuscript, final approval of the version to be published

Olavi Ukkola: Study design, data acquisition, data interpretation, critical revision of the manuscript, final approval of the version to be published

Y.A Kesäniemi: Study design, data acquisition, data interpretation, critical revision of the manuscript, final approval of the version to be published

Data sharing statement: Extra data is available by emailing pauliina.pisto(at)oulu.fi

497



Kaplan Meier cumulative survival rates censored for cardiovascular death in subjects with no fat in the liver, moderate fat accumulation and severe fat accumulation.
CVD was the cause of death in 3.6% of the subjects (26/720) with non-fatty liver and 8.1% of the subjects (10/124) with moderate liver fat accumulation, while 12.5% of the subjects with severe fatty liver (18/144). Cox regression analysis is used for adjustments. M1 (Model 1): adjusted for study group, age and gender. M2 (Model 2): further adjustments for smoking, alcohol consumption, systolic blood pressure, LDL-cholesterol level and body mass index. M3 (Model 3): further adjustment for QUICKI. CVD, cardiovascular disease, CI, confidence interval, HR, hazard ratio, QUICKI, quantitative insulin sensitivity check index. ** $p < 0.01$, * $p < 0.05$.

247x209mm (300 x 300 DPI)

STROBE Statement—Items to be included when reporting observational studies in a conference abstract

Item	Recommendation
Title	Indicate the study's design with a commonly used term in the title (e.g cohort, case-control, cross sectional)
Authors	Contact details for the corresponding author
Study design	Description of the study design (e.g cohort, case-control, cross sectional)
Objective	Specific objectives or hypothesis
Methods	
Setting	Description of setting, follow-up dates or dates at which the outcome events occurred or at which the outcomes were present, as well as any points or ranges on other time scales for the outcomes (e.g., prevalence at age 18, 1998-2007).
Participants	*Cohort study*—Give the most important eligibility criteria, and the most important sources and methods of selection of participants. Describe briefly the methods of follow-up *Case-control study*—Give the major eligibility criteria, and the major sources and methods of case ascertainment and control selection *Cross-sectional study*—Give the eligibility criteria, and the major sources and methods of selection of participants
	Cohort study—For matched studies, give matching and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case
Variables	Clearly define primary outcome for this report.
Statistical methods	Describe statistical methods, including those used to control for confounding
Results	
Participants	Report Number of participants at the beginning and end of the study
Main results	Report estimates of associations. If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period Report appropriate measures of variability and uncertainty (e.g., odds ratios with confidence intervals
Conclusions	General interpretation of study results

BMJ open

Fatty liver predicts the risk for cardiovascular events in middle-aged population: a population-based cohort study

Journal:	*BMJ Open*
Manuscript ID:	bmjopen-2013-003202.R1
Article Type:	Research
Date Submitted by the Author:	12-Aug-2013
Complete List of Authors:	Pisto, Pauliina; Institute of Clinical Medicine, Department of Internal Medicine Santaniemi, Merja; Institute of Clinical Medicine, Department of Internal Medicine Bloigu, Risto; Medical Informatics and Statistics Research Group, Ukkola, Olavi; Institute of Clinical Medicine, Department of Internal Medicine Kesäniemi, Antero; Institute of Clinical Medicine, Department of Internal Medicine
Primary Subject Heading:	Cardiovascular medicine
Secondary Subject Heading:	Cardiovascular medicine, Diabetes and endocrinology
Keywords:	Coronary heart disease < CARDIOLOGY, Myocardial infarction < CARDIOLOGY, General diabetes < DIABETES & ENDOCRINOLOGY, Hepatology < INTERNAL MEDICINE

Fatty liver predicts the risk for cardiovascular events in middle-aged population: a population-based cohort study

Pauliina Pisto[1], Merja Santaniemi[1], Risto Bloigu[2], Olavi Ukkola[1], Y. Antero Kesäniemi[1]

[1]Institute of Clinical Medicine, Department of Internal Medicine and Biocenter Oulu, University of Oulu, and Clinical Research Center, Oulu University Hospital, Oulu, Finland, P.O. Box 20, 90029 OYS, Finland

[2] Medical Informatics and Statistics Research Group, University of Oulu, Aapistie 7, P.O. Box 5000, 90014 Oulu, Finland

Contact information: Pauliina Pisto, corresponding author

University of Oulu

Institute of Clinical Medicine

Department of Internal medicine

P.O. Box 5000, 90014 Oulu, Finland

Tel.: +35885376310

Fax: +35885376318

E-mail: pauliina.pisto@oulu.fi

Word count: 5093

Keywords: coronary disease, fatty liver, insulin resistance, risk factors, stroke

Financial support: This study was supported by the Finnish Foundation for Cardiovascular Research, dated 16 Apr, 2012.

Disclosure summary: Authors report no conflict of interests.

ABSTRACT

Objective: We investigated if the differences in liver fat content would predict the development of non-fatal and fatal atherosclerotic endpoints (coronary heart disease and stroke).

Design, setting and participants: Our study group is a population-based, randomly recruited cohort (OPERA), initiated in 1991. The cohort consisted of 988 middle-aged Finnish subjects.

Intervention: Total mortality and hospital events were followed up to 2009 based on the registry of the National Institute for Health and Welfare and the National death registry.

Main outcome measure: The severity of hepatic steatosis was measured by ultrasound and divided into three groups (0-2). Cox regression analysis was used in the statistical analysis.

Results: In the follow-up of years 1991-2009, 13.5% of the subjects with non-fatty liver, 24.2% of subjects having moderate liver fat content and 29.2% of the subjects having severe fatty liver experienced a cardiovascular event during the follow-up time ($p < 0.001$). Severe liver fat content predicted the risk for future risk of cardiovascular event even when adjusted for age, gender and study group (HR 1.92, CI 1.32-2.80, $p < 0.01$). When further adjustments for smoking, alcohol consumption, LDL-cholesterol, BMI and systolic blood pressure were conducted, the risk still remained statistically significant (HR 1.74, CI 1.16-2.63, $p < 0.01$). Statistical significance disappeared with further adjustment for QUICKI.

Conclusions: Liver fat content increases the risk of future cardiovascular disease event in long-term follow-up but it is seems to be dependent on insulin sensitivity.

Article focus

1 To investigate if the differences in liver fat content predict the risk for development of fatal or nonfatal atherosclerotic endpoints such as coronary heart disease and stroke.

Key messages

1 Subjects with ultrasound-diagnosed fatty liver have cardiovascular disease more often compared to the subjects without fat in the liver

2 Severe liver fat content increases the risk of a future cardiovascular event and mortality to cardiovascular disease over the long-term follow-up but it does seem to be dependent on insulin sensitivity

3 Severe fatty liver predicts the risk for overall mortality but the association is dependent on traditional metabolic risk factors

Strengths and limitations of the study

1 This is a follow-up study with a large population-based study group and a very long follow-up time

2 Official registers used in event diagnoses - data is accurate and the classification is systematic

3 Grade of liver brightness was measured by ultrasound, which has a high specificity but low sensitivity

Introduction

Non-alcoholic fatty liver disease (NAFLD) refers to liver disorders such as abnormal fat content, which exists in a spectrum ranging from steatosis with no inflammation to non-alcoholic steatohepatitis (NASH), which can ultimately lead to liver cirrhosis [1]. The prevalence of NAFLD is estimated to range from 20 to 30% of population in Western countries, being the leading cause of liver disorders [2, 3]. It is associated with obesity, type 2 diabetes mellitus (T2DM) and hyperlipidemia [1]. NAFLD is commonly regarded as a hepatic manifestation of the metabolic syndrome and both conditions share several risk factors for cardiovascular disease (CVD) [3, 4].

In 2008, the prevalence of CVD in adults (≥ 20 years) in United States was 36.2% [5]. Every year, 4.3 million subjects die for CVD in Europe causing nearly half of the all deaths (48%) [6]. So-called traditional risk factors for cardiovascular disease are age, gender, smoking, high low-density lipoprotein (LDL) cholesterol concentration, hypertension and diabetes [7]. In addition, total body fatness as well as abdominal fat accumulation increase independently the risk of CVD and insulin resistance is regarded to be an important factor linking visceral adiposity to cardiovascular risk [8]. Adipose tissue is now recognized as a significant endocrine organ as adipocytes and macrophages infiltrating adipocytes secrete a number of bioactive mediators [7]. Adipokines, proinflammatory cytokines and hypofibrinolytic markers may lead to oxidative stress and endothelial dysfunction, finally leading to atherosclerosis [9].

Hepatic steatosis has been discussed as a possible mechanism to explain CVD morbidity and mortality [10]. NAFLD patients have been reported to have higher coronary heart disease (CHD) risk than the general population of the same age and gender [11]. According to previous study,

liver dysfunction associated with CVD mortality in men [12] whereas another large study found no association between NAFLD and CVD in general population [13]. In addition, fatty liver did not predict CVD mortality and morbidity in patients with established coronary artery disease [14].

The NAFLD and CVD share several molecular mechanisms [15, 16]. Fatty liver might play a part in the pathogenesis of CVD through the overexpression and systemic release of several inflammatory, hemostatic [17] and oxidative-stress mediators or via contributing to whole-body insulin resistance and atherogenic dyslipidemia [3]. NAFLD has also been reported to be linked with circulatory endothelial dysfunction [4, 14]. Several investigators have reported that NAFLD is associated with coronary artery disease [4, 14] and increased carotid intima-media thickness [18, 19]. Increased gamma-glutamyltransferase (GGT), which may be a marker of NAFLD, has been reported to be associated with stroke [20].

It is known that subjects with fatty liver disease have an increased risk of suffering CVD [4], but whether NAFLD is an independent indicator of cardiovascular disease is still far from clear. Long-term follow-up studies are needed to clarify the correlation between fatty liver and CVD. The aim of our study was to investigate if fatty liver could predict independently the risk for total mortality as well as non-fatal and fatal cardiovascular endpoints with a 19-year follow-up after adjusting for all known conventional risk factors.

Materials and methods

Human subjects

OPERA (Oulu Project Elucidating Risk of Atherosclerosis) is a population-based, epidemiological prospective cohort study designed to address the risk factors and disease end points of atherosclerotic cardiovascular diseases. Selection criteria of the study subjects have been described earlier [21]. In short, a total of 520 men and 525 women participated: 259 control men, 261 hypertensive men, 267 control women and 258 hypertensive women aged 40-59. Hypertensive participants were randomly selected from the national register for reimbursement of the costs of antihypertensive medication. For each hypertensive subject, an age- and sex-matched control subject was randomly selected from the same register. Informed consent in writing was obtained from each patient. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and this study was approved by the Ethical Committee of the Faculty of Medicine, University of Oulu.

Determination of hepatic steatosis

The determination of hepatic steatosis was based on liver-kidney contrast measured with ultrasonography [22] by one trained radiologist with 10 years' experience in abdominal ultrasound examinations. The severity of hepatic steatosis was based on the brightness of the liver and it was classified into three groups ranging from 0 to 2 (0 = normal bright, indicating a non-fatty liver, 1 = medium bright, a moderate lipid content and 2 = clearly bright, a severe lipid content and fatty liver) [23].

Follow-up

Both the hypertensive and the control men were recruited during December 1990 to May 1992 and the women approximately one year later (n=1045). In total, 1023 subjects had a liver ultrasound result available at baseline. Mortality data were obtained from the National Death Registry and the diagnoses of cardiovascular events were based on the registry of the National Institute for Health and Welfare. The follow-up time ended December 31, 2009 or whenever the first event occurred. Cardiovascular events included fatal and non-fatal endpoints. Subjects with a previous hospital-diagnosed myocardial infarction or stroke (n=41) at baseline were excluded. In total, 988 subjects participated in this part of the study.

CVD included a major CHD event and stroke (excluding subarachnoid hemorrhage, SAH) - whichever of these happened first [24]. The evidence of CHD was based on the following diagnosis: I20.0, I21, I22 [ICD-10, International Statistical Classification of Diseases and Related Health Problems] / 410, 4110 [ICD-8/9] as the main diagnosis (symptom or cause) and I21, I22 [ICD-10] / 410 [ICD-8/9] as a first side diagnosis (symptom or cause) or second side diagnosis (symptom or cause) and third side diagnosis (ICD-8/9 only) or if a subject had undergone coronary artery bypass graft (CABG) surgery or angioplasty. CHD as a cause of death included I20–I25, I46, R96, R98 [ICD-10] / 410-414, 798 (not 7980A) [ICD-8/9] as the underlying cause of death or immediate cause of death and I21 or I22 [ICD-10] / 410 [ICD-8/9] as first to third contributing cause of death. Stroke (excluding SAH) included I61, I63 (not I636), I64 [ICD -10] / 431, 4330A, 4331A, 4339A, 4340A, 4341A, 4349A, 436 [ICD-9] / 431 (except 43101, 43191) 433, 434, 436 [ICD-8] as main diagnosis (symptom or cause) or as a first or second side diagnosis (symptom or cause) or as a third side diagnosis (ICD-8/9 only) or as an underlying cause of death or immediate cause of death or as a first to third contributing cause of death [25].

Laboratory analyses

Waist circumference, body mass index (BMI) and blood pressure were measured as described in previous study [21].

All the laboratory test samples were obtained after an overnight fast. Blood insulin and glucose concentrations were analyzed at 0, 60, and 120 min after administration of 75 g glucose [23]. Insulin sensitivity was assessed using fasting plasma insulin concentrations and a quantitative insulin sensitivity check index (QUICKI) {QUICKI=1/[log (fasting insulin)+log (fasting glucose)]}[26].

Very-low-density lipoprotein (VLDL), high-density lipoprotein (HDL), low-density lipoprotein (LDL) and hs-CRP concentrations [23] as well as alanine aminotransferase (ALT) and GGT levels were measured as described previously [22]. Alcohol consumption and smoking history were determined by validated questionnaires [27]. Alcohol consumption was divided into three groups: 0 (n=161) mean alcohol consumption less than 1g/week in men and women, 1 (n=767) mean consumption less than 210g/week in men and less than 140 g/week in women, 2 (n=76) mean alcohol consumption more than 210g/week in men and more than 140g/week in women. Group 2 designates large-scale alcohol consumers according to the guidelines [28].

Statistical analysis

Statistical analysis was performed by using IBM SPSS Statistics for Windows, Version 20.0 (Armonk, NY: IBM Corp.). Analysis of variance was used to compare the means of the variables measured. Post hoc tests were performed using the Tukey method. Statistical significances between percentages were measured by using χ^2 test. Cumulative survival rates were estimated using Kaplan-Meier method. Cox regression analysis was performed to investigate if liver brightness (fat) could predict the future risk for total mortality, cardiovascular death or hospital events. A p value < 0.05 was regarded as significant.

Skewed variables (smoking, alcohol consumption, fasting insulin, fasting glucose, triglyceride, ALT, GGT concentration, hs-CRP level) were logarithmically transformed to improve normality before analysis of variance. We used three models with progressive degrees of adjustments. Model 1 included study group (subjects with medicine-treated hypertension and their age- and sex-matched controls), age and gender. Model 2 included further adjustments for smoking, alcohol consumption, systolic blood pressure, LDL-cholesterol level and body mass index. Model 3 included further adjustment for QUICKI. We carried out sensitivity analyses: in the analyses of cardiovascular events, we added all covariates one by one and investigated if the hazard ratios (HR) changed or remained stable when further adjustment with one covariate was performed. Model 4 included variables which were stable and were statistically significant in intermediate phases. Model 5 included stable and significant covariates without QUICKI (Table 2).

C-index was calculated for the model 1, model 3, model 4 and model 5 to assess the discrimination of the risk markers. The analyses were performed in 250 bootstrap resamplings to obtain 95% CI for c-index of each model.

Results

The main baseline characteristics of the study group are shown in Table 1.

Table 1 about here

Incidence of cardiovascular disease

The median follow-up time was 212 (maximum 228) months. During the follow-up time, 13.5% of the subjects with no fat in the liver (97/720), 24.2% (30/124) of subjects having moderate liver fat content and 29.2% (42/144) of the subjects having severe fatty liver experienced a CVD event ($p < 0.001$). CVD was the cause of death in 3.6% of the subjects with non-fatty liver (26/720) and 8.1% of the subjects with moderate liver fat content (10/124), while 12.5% (18/144) of the subjects with severe fatty liver ($p < 0.001$).

Severe liver fat content predicted the risk for future risk of cardiovascular event when adjusted for age, gender and study group (Model 1: HR 1.92, CI 1.32-2.80, $p < 0.01$) (Table 2). When further adjustments were made for smoking, alcohol consumption, LDL-cholesterol, BMI and systolic blood pressure (Model 2: HR 1.74, CI 1.16-2.63), the risk still remained statistically significant ($p < 0.01$). Statistical significance disappeared when further adjustment for QUICKI was performed (Model 3: HR 1.49, CI 0.97-2.30, p=0.071). In the CVD event sensitivity analyses, all covariates were added one by one and it was examined

whether the hazard ratios would change or remain stable. After adjusting for the statistically significant variables (including quick index) in the sensitivity analyses, the association between severe fatty liver was no longer significant (Model 4: HR 1.43, CI 0.93-2.18, p=0.10). When QUICKI was not added into Model 5, severe fatty liver did predict the risk for future risk for CVD event (HR 1.76, CI 1.21- 2.56, p < 0.001) (Table 2). The c-index decreased when the risk factors were removed from the model (Table 3).

Tables 2and 3 about here

The future risk of death from CVD in participants with severe fat content was significant when age, gender and study group were added as covariates (Model 1: HR 2.95, CI 1.58-5.51, p < 0.01). Even after further adjustments with other conventional risk factors (Model 2: HR 2.04, CI 1.03-4.05), statistical significance remained (p < 0.05). When QUICKI was added as the covariate, then significance disappeared (Model 3: HR 1.64, CI 0.79-3.43, NS) (Fig 1.).

Figure 1 about here

Fatty liver and total mortality

In total, 11.9% of the participants not having fatty liver, 18.5% of the subjects having moderate fatty liver and 22.2% of the subjects with severe fatty liver died from all causes (p < 0.01). According to Model 1, severe fat content predicted the risk for mortality from all

causes when age, gender and study group were added as covariates (HR 1.60, CI 1.05-2.43, p < 0.05). The significance disappeared when body mass index was added as a covariate (data not shown).

We performed all Cox regression analyses after excluding the men consuming more than 210 g alcohol and the women drinking more than 140 g alcohol per week. This exclusion did not have any effect on the results (data not shown).

We performed all Cox regression analyses after excluding patients with insulin treated diabetes mellitus (n=9), cortisone treatment at baseline (n=41) and previous diagnosis for liver disease (n=15) (e.g., virus, medications). This exclusion did not have any effect on the results (data not shown).

Discussion

The incidences of non-alcoholic fatty liver disease and cardiovascular disease are continuously increasing in the Western world. The question if NAFLD is only a marker or also an early mediator of cardiovascular disease is still largely unanswered. According to the results of the present study, which had an approximately 19-year follow-up fatty liver does predict the future risk for death from all causes, death from cardiovascular disease and risk of cardiovascular events. Insulin sensitivity seems to play a more dominant role in the development of cardiovascular events.

Only a few studies have investigated the risk for future cardiovascular risk among subjects with ultrasound-diagnosed fatty liver [29, 30] and larger studies with longer follow-up times are needed. An association between NAFLD and CVD has been reported [3, 29-31] although contrary

results also exist [13, 32]. An association between ultrasound-diagnosed fatty liver and CVD has been reported in general population [29] and in subjects with T2DM [31]. Furthermore, liver dysfunction has been reported to associate with CVD mortality [33, 34] and CHD risk [11] in follow-up studies and especially survival of subjects with NASH is reported to be reduced [32, 35, 36]. In the present study, severe fatty liver disease did predict the risk for cardiovascular death but the association seemed to be dependent on insulin sensitivity.

Several earlier studies have used self-reported CVD history which may not be totally reliable. Although earlier studies on the risk for future cardiovascular risk among subjects with fatty liver have performed some adjustments, the full range of well-known CVD risk factors have been rarely considered [32]. We have performed adjustments with all so-called traditional risk factors for cardiovascular disease (i.e. age, gender, smoking, LDL concentration, hypertension, insulin resistance). Previous studies have used biochemical, radiological and histological methodology for NAFLD diagnosis and staging, which leads to a challenging interpretation of the results [34, 37].

This study had an approximately 19-year follow-up time, which is longer than in previous studies [11-14]. When compared to earlier studies [32, 37] this study seems to be the first follow-up study with a large population-based randomly selected study group and a very long follow-up time and ultrasound-diagnosed fatty liver. The diagnosis of cardiovascular events was based on the registry of the National Institute for Health and Welfare and mortality data were obtained from the National Death Registry. The earlier verified FINRISK classification [25] was used to classify the events. Therefore, the reliability of event diagnosis data is accurate and the classification is systematic. All subjects who had myocardial infarction or stroke before

baseline were excluded because a history of myocardial infarction is known to increase the risk for recurrent myocardial infarction or cardiovascular death [38] and medication as well as lifestyle secondary prevention strategies are intensive [39].

There are a few follow-up-studies examining whether the fatty liver increases the risk for total mortality [13, 40]. In the present study, severe fatty liver predicted the risk for overall mortality of any causes when age, gender and study group were added covariates, a result in line with an earlier report [41]. In the published literature, NASH rather than simple steatosis has been stated to be linked with decreased overall survival [35] although one study with a large cohort found no association between NAFLD and overall mortality [13]. In our study, the association between severe fatty liver and total mortality disappeared after further adjustment for BMI which means that severe fatty liver is not a strong predictor for overall mortality.

The molecular mechanisms linking fatty liver with CVD have been investigated [10, 16]. Enlarged visceral adipose tissue may explain why NAFLD associates with CVD [16]. In individuals with visceral obesity, insulin resistance may contribute to impaired non-esterified fatty acid (NEFA) metabolism [8] and the increasing NEFA flux to the liver may impair liver metabolism leading to increased glucose metabolism and liver dysfunction [7]. The liver is one of the targets of the resulting systemic abnormalities and the source of several proatherogenic factors [3], such as CRP, fibrinogen, plasminogen activator inhibitor-1 and other inflammatory cytokines [16]. Furthermore, visceral adipose tissue and ectopic fat overexpress factors involved in atherogenesis [16] such as NEFAs and proinflammatory cytokines, for instance interleukin-6

and tumor necrosis factor-α [8] leading to chronic systemic inflammation. In addition, hepatic steatosis leads to overproduction of cholesterol-rich remnant particles [4].

One limitation in this study is that the grade of liver brightness was measured by ultrasound. The invasive diagnostic technique of liver biopsy is regarded as the "golden standard", especially for the diagnosis of NASH [42]. Real time ultrasound using a combination of sonographic findings does have a high specificity but it underestimates the prevalence of hepatic steatosis when there is less than 20 % fat [43]. Today, magnetic resonance spectroscopy is regarded as the best method for the quantification of liver fat, but this method is limited due to its availability [44]. Nonetheless, the noninvasive ultrasound method was chosen because taking liver biopsies from large groups of symptomless subjects would have been ethically unjustifiable and magnetic resonance spectroscopy was not available at the baseline.

The OPERA study group consists of subjects with drug-treated hypertension and randomly selected sex- and age-matched controls. Study group was added as a covariate to minimize any selection bias.

Conclusions

Severe liver fat content increased the risk of a future cardiovascular event and mortality to cardiovascular disease over the long-term follow-up but it seemed to be dependent on insulin sensitivity. Fatty liver also predicted the risk for overall mortality. However, conventional cardiovascular disease risk factors seemed to play a major role in developing death from all causes. It would be beneficial to investigate larger cohorts and follow-up studies in order to validate this result.

Figure legend

Title: Kaplan Meier cumulative survival rates censored for cardiovascular death in subjects with no fat in the liver, moderate fat content and severe fat content.

CVD was the cause of death in 3.6% of the subjects (26/720) with non-fatty liver and 8.1% of the subjects (10/124) with moderate liver fat content, while 12.5% of the subjects with severe fatty liver (18/144). Cox regression analysis is used for adjustments. M1 (Model 1): adjusted for study group, age and gender. M2 (Model 2): further adjustments for smoking, alcohol consumption, systolic blood pressure, LDL-cholesterol level and body mass index. M3 (Model 3): further adjustment for QUICKI. CVD, cardiovascular disease, CI, confidence interval, HR, hazard ratio, QUICKI, quantitative insulin sensitivity check index. ** $p < 0.01$, * $p < 0.05$.

Acknowledgements

The authors thank Markku Päivänsalo, MD, PhD, for the expert liver ultrasound examinations and Ms Saija Kortetjärvi, Ms Liisa Mannermaa, and Ms Leena Ukkola for the excellent technical assistance. M.Sc. Elina Malo and M.Sc. Meiju Saukko are thanked for the cooperation in organizing cardiovascular event and mortality data.

Contributorship The authors thank Markku Päivänsalo, MD, PhD, for the expert liver ultrasound examinations and Ms Saija Kortetjärvi, Ms Liisa Mannermaa, and Ms Leena Ukkola for the excellent technical assistance. M.Sc. Elina Malo and M.Sc. Meiju Saukko are thanked for the cooperation in organizing cardiovascular event and mortality data.

Funding Finnish Foundation for Cardiovascular Research

Competing Inetersts None

References

1. Angulo P. Nonalcoholic fatty liver disease. *N Engl J Med* 2002; Apr 18;346(16):1221-31.

2. Armstrong MJ, Houlihan DD, Bentham L, Shaw JC, Cramb R, Olliff S, et al. Presence and severity of non-alcoholic fatty liver disease in a large prospective primary care cohort. *J Hepatol* 2012; Jan;56(1):234-40.

3. Targher G, Day CP, Bonora E. Risk of cardiovascular disease in patients with nonalcoholic fatty liver disease. *N Engl J Med* 2010; Sep 30;363(14):1341-50.

4. Targher G, Arcaro G. Non-alcoholic fatty liver disease and increased risk of cardiovascular disease. *Atherosclerosis* 2007; Apr;191(2):235-40.

5. Roger VL, Go AS, Lloyd-Jones DM, Adams RJ, Berry JD, Brown TM, et al. Heart disease and stroke statistics--2011 update: a report from the American Heart Association. *Circulation* 2011; Feb 1;123(4):e18-e209.

6. Allender S, Scarborough P, Peto V, Rayner M, Leal J, Luengo-Fernandez R, Gray A. European cardiovascular disease statistics, 2008 ed. European Heart Network; 2008.

7. Despres JP, Lemieux I. Abdominal obesity and metabolic syndrome. *Nature* 2006; Dec 14;444(7121):881-7.

8. Van Gaal LF, Mertens IL, De Block CE. Mechanisms linking obesity with cardiovascular disease. *Nature* 2006; Dec 14;444(7121):875-80.

9. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med* 2005; Apr 21;352(16):1685-95.

10. Bhatia LS, Curzen NP, Byrne CD. Nonalcoholic fatty liver disease and vascular risk. *Curr Opin Cardiol* 2012; Jul;27(4):420-8.

11. Treeprasertsuk S, Leverage S, Adams LA, Lindor KD, St Sauver J, Angulo P. The Framingham risk score and heart disease in nonalcoholic fatty liver disease. *Liver Int* 2012; Jul;32(6):945-50.

12. Haring R, Wallaschofski H, Nauck M, Dorr M, Baumeister SE, Volzke H. Ultrasonographic hepatic steatosis increases prediction of mortality risk from elevated serum gamma-glutamyl transpeptidase levels. *Hepatology* 2009; Nov;50(5):1403-11.

13. Lazo M, Hernaez R, Bonekamp S, Kamel IR, Brancati FL, Guallar E, et al. Non-alcoholic fatty liver disease and mortality among US adults: prospective cohort study. *BMJ* 2011; Nov 18;343:d6891.

14. Wong VW, Wong GL, Yip GW, Lo AO, Limquiaco J, Chu WC, et al. Coronary artery disease and cardiovascular outcomes in patients with non-alcoholic fatty liver disease. *Gut* 2011; Dec;60(12):1721-7.

15. Loria P, Lonardo A, Targher G. Is liver fat detrimental to vessels?: intersections in the pathogenesis of NAFLD and atherosclerosis. *Clin Sci (Lond)* 2008; Jul;115(1):1-12.

16. Targher G, Marra F, Marchesini G. Increased risk of cardiovascular disease in non-alcoholic fatty liver disease: causal effect or epiphenomenon?. *Diabetologia* 2008; Nov;51(11):1947-53.

17. Targher G, Bertolini L, Scala L, Zoppini G, Zenari L, Falezza G. Non-alcoholic hepatic steatosis and its relation to increased plasma biomarkers of inflammation and endothelial dysfunction in non-diabetic men. Role of visceral adipose tissue. ***Diabet Med*** **2005; Oct;22(10):1354-8.**

18. Brea A, Mosquera D, Martin E, Arizti A, Cordero JL, Ros E. Nonalcoholic fatty liver disease is associated with carotid atherosclerosis: a case-control study. ***Arterioscler Thromb Vasc Biol*** **2005; May;25(5):1045-50.**

19. Sookoian S, Pirola CJ. Non-alcoholic fatty liver disease is strongly associated with carotid atherosclerosis: a systematic review. ***J Hepatol*** **2008; Oct;49(4):600-7.**

20. Fraser A, Harris R, Sattar N, Ebrahim S, Smith GD, Lawlor DA. Gamma-glutamyltransferase is associated with incident vascular events independently of alcohol intake: analysis of the British Women's Heart and Health Study and Meta-Analysis. ***Arterioscler Thromb Vasc Biol*** **2007; Dec;27(12):2729-35.**

21. Rantala AO, Kauma H, Lilja M, Savolainen MJ, Reunanen A, Kesaniemi YA. Prevalence of the metabolic syndrome in drug-treated hypertensive patients and control subjects. ***J Intern Med*** **1999; Feb;245(2):163-74.**

22. Sampi M, Veneskoski M, Ukkola O, Kesaniemi YA, Horkko S. High plasma immunoglobulin (Ig) A and low IgG antibody titers to oxidized low-density lipoprotein are associated with markers of glucose metabolism. ***J Clin Endocrinol Metab*** **2010; May;95(5):2467-75.**

23. Pisto P, Ukkola O, Santaniemi M, Kesaniemi YA. Plasma adiponectin--an independent indicator of liver fat accumulation. ***Metabolism*** **2011; Nov;60(11):1515-20.**

24. Santaniemi M., Ukkola O., Malo E., Bloigu R., Kesaniemi YA. Metabolic syndrome in the prediction of cardiovascular events: The potential additive role of hsCRP and adiponectin. *Eur J Prev Cardiol* 2013; Jun 20.

25. Pajunen P, Jousilahti P, Borodulin K, Harald K, Tuomilehto J, Salomaa V. Body fat measured by a near-infrared interactance device as a predictor of cardiovascular events: the FINRISK'92 cohort. *Obesity (Silver Spring)* 2011; Apr;19(4):848-52.

26. Katz A, Nambi SS, Mather K, Baron AD, Follmann DA, Sullivan G, et al. Quantitative insulin sensitivity check index: a simple, accurate method for assessing insulin sensitivity in humans. *J Clin Endocrinol Metab* 2000; Jul;85(7):2402-10.

27. Kauma H, Savolainen MJ, Rantala AO, Lilja M, Kervinen K, Reunanen A, et al. Apolipoprotein E phenotype determines the effect of alcohol on blood pressure in middle-aged men. *Am J Hypertens* 1998; Nov;11(11 Pt 1):1334-43.

28. Bessembinders K, Wielders J, van de Wiel A. Severe hypertriglyceridemia influenced by alcohol (SHIBA). *Alcohol Alcohol* 2011; Mar-Apr;46(2):113-6.

29. Hamaguchi M, Kojima T, Takeda N, Nagata C, Takeda J, Sarui H, et al. Nonalcoholic fatty liver disease is a novel predictor of cardiovascular disease. *World J Gastroenterol* 2007; Mar 14;13(10):1579-84.

30. Stepanova M, Younossi ZM. Independent Association Between Nonalcoholic Fatty Liver Disease and Cardiovascular Disease in the US Population. *Clin Gastroenterol Hepatol* 2012; Jun;10(6):646-50.

31. Targher G, Bertolini L, Poli F, Rodella S, Scala L, Tessari R, et al. Nonalcoholic fatty liver disease and risk of future cardiovascular events among type 2 diabetic patients. ***Diabetes*** **2005; Dec;54(12):3541-6.**

32. Ghouri N, Preiss D, Sattar N. Liver enzymes, nonalcoholic fatty liver disease, and incident cardiovascular disease: a narrative review and clinical perspective of prospective data. ***Hepatology*** **2010; Sep;52(3):1156-61.**

33. Dunn W, Xu R, Wingard DL, Rogers C, Angulo P, Younossi ZM, et al. Suspected nonalcoholic fatty liver disease and mortality risk in a population-based cohort study. ***Am J Gastroenterol*** **2008; Sep;103(9):2263-71.**

34. Ruttmann E, Brant LJ, Concin H, Diem G, Rapp K, Ulmer H, et al. Gamma-glutamyltransferase as a risk factor for cardiovascular disease mortality: an epidemiological investigation in a cohort of 163,944 Austrian adults. ***Circulation*** **2005; Oct 4;112(14):2130-7.**

35. Soderberg C, Stal P, Askling J, Glaumann H, Lindberg G, Marmur J, et al. Decreased survival of subjects with elevated liver function tests during a 28-year follow-up. ***Hepatology*** **2010; Feb;51(2):595-602.**

36. Ekstedt M, Franzen LE, Mathiesen UL, Thorelius L, Holmqvist M, Bodemar G, et al. Long-term follow-up of patients with NAFLD and elevated liver enzymes. ***Hepatology*** **2006; Oct;44(4):865-73.**

37. Bhatia LS, Curzen NP, Calder PC, Byrne CD. Non-alcoholic fatty liver disease: a new and important cardiovascular risk factor?. ***Eur Heart J*** **2012; May;33(10):1190-200.**

38. Beckman JA, Creager MA, Libby P. Diabetes and atherosclerosis: epidemiology, pathophysiology, and management. *JAMA* 2002; May 15;287(19):2570-81.

39. Joseph P, Teo K. Optimal medical therapy, lifestyle intervention, and secondary prevention strategies for cardiovascular event reduction in ischemic heart disease. *Curr Cardiol Rep* 2011; Aug;13(4):287-95.

40. Dam-Larsen S, Franzmann M, Andersen IB, Christoffersen P, Jensen LB, Sorensen TI, et al. Long term prognosis of fatty liver: risk of chronic liver disease and death. *Gut* 2004; May;53(5):750-5.

41. Calori G, Lattuada G, Ragogna F, Garancini MP, Crosignani P, Villa M, et al. Fatty liver index and mortality: the Cremona study in the 15th year of follow-up. *Hepatology* 2011; Jul;54(1):145-52.

42. Joy D, Thava VR, Scott BB. Diagnosis of fatty liver disease: is biopsy necessary?. *Eur J Gastroenterol Hepatol* 2003; May;15(5):539-43.

43. Dasarathy S, Dasarathy J, Khiyami A, Joseph R, Lopez R, McCullough AJ. Validity of real time ultrasound in the diagnosis of hepatic steatosis: a prospective study. *J Hepatol* 2009; Dec;51(6):1061-7.

44. Szczepaniak LS, Nurenberg P, Leonard D, Browning JD, Reingold JS, Grundy S, et al. Magnetic resonance spectroscopy to measure hepatic triglyceride content: prevalence of hepatic steatosis in the general population. *Am J Physiol Endocrinol Metab* 2005; Feb;288(2):E462-8.

502

Grade of liver bightness	0 (n=720)	1 (n=124)	2 (n=144)	p	p (0-1)	p (1-2)	p (0-2)
Age (years)	50.9 (6.0)	51.9 (6.1)	51.5 (5.5)	NS	NS	NS	NS
Males	44.3 % (n=319)	65.3 % (n=81)	59.9 % (n=82)	< 0.001	-	-	-
Hypertensives	41.4 % (n=298)	66.1 % (n=82)	71.5 % (n=103)	< 0.001	-	-	-
BMI (kg/m²)	26.4 (3.9)	29.8 (5.0)	31.9 (4.9)	< 0.001	< 0.001	< 0.001	< 0.001
Waist circumference (cm)	86.8 (11.9)	97.7 (12.0)	102.3 (11.8)	< 0.001	< 0.001	< 0.01	< 0.001
Smoking (pack years)	10.6 (13.3)	14.3 (14.9)	14.0 (14.6)	< 0.05	NS	NS	NS
Alcohol consumption (g/week)	51.1 (83.0)	95.1 (117.0)	82.6 (105.1)	< 0.01	< 0.05	NS	NS
Total serum cholesterol (mmol/L)	5.6 (1.0)	5.8 (1.1)	5.8 (1.1)	NS	NS	NS	NS
LDL (mmol/L)	3.5 (0.9)	3.7 (1.1)	3.5 (0.9)	NS	NS	NS	NS
Triglycerides (mmol/L)	1.4 (0.8)	1.9 (0.8)	2.2 (1.4)	< 0.001	< 0.001	< 0.05	< 0.001
Systolic blood pressure	145.2 (21.5)	152.7 (20.3)	157.1 (22.2)	< 0.001	< 0.01	NS	< 0.001
Fasting insulin (mmol/L)	10.8 (7.7)	18.2 (10.3)	23.8 (17.6)	< 0.001	< 0.001	< 0.001	< 0.001

Fasting glucose (mmol/L)	4.4 (0.7)	5.0 (1.4)	6.1 (2.8)	< 0.001	< 0.001	< 0.001	< 0.001
QUICKI	0.6 (0.1)	0.6 (0.1)	0.5 (0.1)	< 0.001	< 0.001	< 0.001	< 0.001
hs-CRP (ng/mL)	3039.4 (6758.3)	3981.4 (6068.2)	6122.0 (6630.8)	< 0.001	< 0.001	< 0.01	< 0.001
ALT U/L	26.2 (15.5)	37.8 (17.1)	55.4 (37.7)	< 0.001	< 0.001	< 0.001	< 0.001
GGT U/L	35.1 (33.5)	69.7 (116.3)	76.8 (92.4)	< 0.001	< 0.001	< 0.01	< 0.001
Anti-hypertensive treatment	43.6% (n=314)	66.9% (n=83)	72.9% (n=105)	< 0.001	-	-	-
Lipid-lowering treatment	2.2% (n=16)	1.6% (n=2)	6.2% (n=9)	< 0.05	-	-	-
Hypoglycaemic drug	1.1% (n=8)	1.6% (n=2)	10.4% (n=15)	< 0.001	-	-	-
Type 2 diabetes	2.4% (n=17)	12.1% (n=15)	36.8% (n=53)	< 0.001	-	-	-

Table 1. Baseline characteristics of the study group as means (standard deviations) or percentages. N= number of subjects. ALT, alanine aminotransferase, BMI, body mass index, GGT, gamma-glutamyltransferase, hs-CRP, high-sensitivity C-reactive protein, LDL, low-density lipoprotein, QUICKI, quantitative insulin sensitivity check index.

	Model 1	Model 2	Model 3	Model 4	Model 5
Moderate fat content	1.51 (0.99-2.29)	1.44 (0.93-2.23)	1.31 (0.84-2.05)	1.30 (0.84-2.01)	1.49 (0.99-2.26)
Severe fat content	1.92 (1.32-2.80)**	1.74 (1.16-2.63) **	1.49 (0.97-2.30)	1.43 (0.93-2.18)	1.76 (1.21- 2.56) **
Study group	1.34 (0.98-1.85)	1.29 (0.92-1.80)	1.28 (0.92-1.78)		
Age	1.06 (1.03-1.09)***	1.05(1.02-1.08)**	1.05 (1.02-1.08)**	1.05 (1.02-1.07)**	1.05 (1.02-1.08) **
Gender	2.39 (1.71-3.34)*	1.91 (1.34-2.71)***	1.80 (1.26-2.57)**	1.83 (1.29-2.60) **	1.92 (1.36-2.72) ***
LDL-cholesterol		1.17 (0.99-1.39)	1.15 (0.97-1.37)		
Smoking (pack-years)		1.02 (1.01-1.03)***	1.02 (1.01-1.03)***	1.02 (1.01-1.03)***	1.02 (1.01-1.03) ***
Alcohol consumption (gr1)		0.93 (0.59-1.45)	0.92(0.59-1.44)		
Alcohol consumption (gr2)		0.84 (0.44-1.60)	0.81(0.42-1.54)		
Systolic blood pressure		1.01 (1.00-1.02)**	1.01 (1.00-1.02)*	1.01 (1.00-1.02)**	1.01 (1.00-1.02) **
Body mass index		0.99 (0.96-1.03)	0.97 (0.93-1.01)		
QUICKI			0.12 (0.02-0.90)*	0.16 (0.03-0.99)*	

Table 2. Multivariate analysis for cardiovascular events with different degrees of adjustments (Cox regression analysis). CVD event occurred in 13.5% of the subjects with no fat in the liver (97/720), 24.2% (30/124) of subjects having moderate liver fat content and 29.2% (42/144) of the subjects having severe fatty liver. Hazard ratios with 95% confidence interval with different degrees of adjustments are presented. Alcohol consumption was divided into groups (reference group: less than 1g/week in men and women, group 1: less than 210g/week in men and less than 140 g/week in women, group 2: more than 210g/week in men and more than 140g/week in women). Model 1: adjustment for study group, age and gender. Model 2: further adjustments for LDL-cholesterol, smoking, alcohol consumption, systolic blood pressure and body mass index. Model 3: further adjustment for QUICKI. Model 4: adjustments with statistically significant covariates. Model 5: adjustments with statistically significant covariates without QUICKI. LDL, low-density lipoprotein, QUICKI, quantitative insulin sensitivity check index. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Final model	Cardiovascular event c-index (95% CI)	Binary R^2
Model 3	0.729 (0.706-0.776)	0.153
Model 4	0.720 (0.689-0.763)	0.144
Model 5	0.717 (0.686-0.758)	0.138
Model 1	0.698 (0.656-0.742)	0.133

Table 3. Multivariate analysis for cardiovascular events (logistic regression analysis). Cardiovascular disease risk factors have been removed from the models step by step. Model 3 included liver brightness, study group, age, gender, smoking, alcohol consumption, systolic blood pressure, LDL-cholesterol level, body mass index and QUICKI. Model 4 included liver brightness, age, gender, smoking, blood pressure and QUICKI. Model 5 included liver brightness, age, gender, smoking, blood pressure. Model 1 included liver brightness, study group, age and gender. C-index with confidence intervals obtained from 250 bootstrap resamplings and binary R^2 was used. LDL, low-density lipoprotein, QUICKI, quantitative insulin sensitivity check index.

549

Fatty liver predicts the risk for cardiovascular events in middle-aged population: a population-based cohort study

Pauliina Pisto[1], Merja Santaniemi[1], Risto Bloigu[2], Olavi Ukkola[1], Y. Antero Kesäniemi[1]

[1]Institute of Clinical Medicine, Department of Internal Medicine and Biocenter Oulu, University of Oulu, and Clinical Research Center, Oulu University Hospital, Oulu, Finland, P.O. Box 20, 90029 OYS, Finland

[2] Medical Informatics and Statistics Research Group, University of Oulu, Aapistie 7, P.O. Box 5000, 90014 Oulu, Finland

Contact information: Pauliina Pisto, corresponding author

University of Oulu

Institute of Clinical Medicine

Department of Internal medicine

P.O. Box 5000, 90014 Oulu, Finland

Tel.: +35885376310

Fax: +35885376318

E-mail: pauliina.pisto@oulu.fi

Word count: 5093

Keywords: coronary disease, fatty liver, insulin resistance, risk factors, stroke

Financial support: This study was supported by the Finnish Foundation for Cardiovascular Research, dated 16 Apr, 2012.

Disclosure summary: Authors report no conflict of interests.

ABSTRACT

Objective: We investigated if the differences in liver fat content would predict the development of non-fatal and fatal atherosclerotic endpoints (coronary heart disease and stroke).

Design, setting and participants: Our study group is a population-based, randomly recruited cohort (OPERA), initiated in 1991. The cohort consisted of 988 middle-aged Finnish subjects.

Intervention: Total mortality and hospital events were followed up to 2009 based on the registry of the National Institute for Health and Welfare and the National death registry.

Main outcome measure: The severity of hepatic steatosis was measured by ultrasound and divided into three groups (0-2). Cox regression analysis was used in the statistical analysis.

Results: In the follow-up of years 1991-2009, 13.5% of the subjects with non-fatty liver, 24.2% of subjects having moderate liver fat content and 29.2% of the subjects having severe fatty liver experienced a cardiovascular event during the follow-up time ($p < 0.001$). Severe liver fat content predicted the risk for future risk of cardiovascular event even when adjusted for age, gender and study group (HR 1.92, CI 1.32-2.80, $p < 0.01$). When further adjustments for smoking, alcohol consumption, LDL-cholesterol, BMI and systolic blood pressure were conducted, the risk still remained statistically significant (HR 1.74, CI 1.16-2.63, $p < 0.01$). Statistical significance disappeared with further adjustment for QUICKI.

Conclusions: Liver fat content increases the risk of future cardiovascular disease event in long-term follow-up but it is seems to be dependent on insulin sensitivity.

Article focus

1 To investigate if the differences in liver fat content predict the risk for development of fatal or nonfatal atherosclerotic endpoints such as coronary heart disease and stroke.

Key messages

1 Subjects with ultrasound-diagnosed fatty liver have cardiovascular disease more often compared to the subjects without fat in the liver

2 Severe liver fat content increases the risk of a future cardiovascular event and mortality to cardiovascular disease over the long-term follow-up but it does seem to be dependent on insulin sensitivity

3 Severe fatty liver predicts the risk for overall mortality but the association is dependent on traditional metabolic risk factors

Strengths and limitations of the study

1 This is a follow-up study with a large population-based study group and a very long follow-up time

2 Official registers used in event diagnoses - data is accurate and the classification is systematic

3 Grade of liver brightness was measured by ultrasound, which has a high specificity but low sensitivity

Introduction

Non-alcoholic fatty liver disease (NAFLD) refers to liver disorders such as abnormal fat content, which exists in a spectrum ranging from steatosis with no inflammation to non-alcoholic steatohepatitis (NASH), which can ultimately lead to liver cirrhosis [1]. The prevalence of NAFLD is estimated to range from 20 to 30% of population in Western countries, being the leading cause of liver disorders [2, 3]. It is associated with obesity, type 2 diabetes mellitus (T2DM) and hyperlipidemia [1]. NAFLD is commonly regarded as a hepatic manifestation of the metabolic syndrome and both conditions share several risk factors for cardiovascular disease (CVD) [3, 4].

In 2008, the prevalence of CVD in adults (≥ 20 years) in United States was 36.2% [5]. Every year, 4.3 million subjects die for CVD in Europe causing nearly half of the all deaths (48%) [6]. So-called traditional risk factors for cardiovascular disease are age, gender, smoking, high low-density lipoprotein (LDL) cholesterol concentration, hypertension and diabetes [7]. In addition, total body fatness as well as abdominal fat accumulation increase independently the risk of CVD and insulin resistance is regarded to be an important factor linking visceral adiposity to cardiovascular risk [8]. Adipose tissue is now recognized as a significant endocrine organ as adipocytes and macrophages infiltrating adipocytes secrete a number of bioactive mediators [7]. Adipokines, proinflammatory cytokines and hypofibrinolytic markers may lead to oxidative stress and endothelial dysfunction, finally leading to atherosclerosis [9].

Hepatic steatosis has been discussed as a possible mechanism to explain CVD morbidity and mortality [10]. NAFLD patients have been reported to have higher coronary heart disease (CHD) risk than the general population of the same age and gender [11]. According to previous study,

liver dysfunction associated with CVD mortality in men [12] whereas another large study found no association between NAFLD and CVD in general population [13]. In addition, fatty liver did not predict CVD mortality and morbidity in patients with established coronary artery disease [14].

The NAFLD and CVD share several molecular mechanisms [15, 16]. Fatty liver might play a part in the pathogenesis of CVD through the overexpression and systemic release of several inflammatory, hemostatic [17] and oxidative-stress mediators or via contributing to whole-body insulin resistance and atherogenic dyslipidemia [3]. NAFLD has also been reported to be linked with circulatory endothelial dysfunction [4, 14]. Several investigators have reported that NAFLD is associated with coronary artery disease [4, 14] and increased carotid intima-media thickness [18, 19]. Increased gamma-glutamyltransferase (GGT), which may be a marker of NAFLD, has been reported to be associated with stroke [20].

It is known that subjects with fatty liver disease have an increased risk of suffering CVD [4], but whether NAFLD is an independent indicator of cardiovascular disease is still far from clear. Long-term follow-up studies are needed to clarify the correlation between fatty liver and CVD. The aim of our study was to investigate if fatty liver could predict independently the risk for total mortality as well as non-fatal and fatal cardiovascular endpoints with a 19-year follow-up after adjusting for all known conventional risk factors.

Materials and methods

Human subjects

OPERA (Oulu Project Elucidating Risk of Atherosclerosis) is a population-based, epidemiological prospective cohort study designed to address the risk factors and disease end points of atherosclerotic cardiovascular diseases. Selection criteria of the study subjects have been described earlier [21]. In short, a total of 520 men and 525 women participated: 259 control men, 261 hypertensive men, 267 control women and 258 hypertensive women aged 40-59. Hypertensive participants were randomly selected from the national register for reimbursement of the costs of antihypertensive medication. For each hypertensive subject, an age- and sex-matched control subject was randomly selected from the same register. Informed consent in writing was obtained from each patient. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and this study was approved by the Ethical Committee of the Faculty of Medicine, University of Oulu.

Determination of hepatic steatosis

The determination of hepatic steatosis was based on liver-kidney contrast measured with ultrasonography [22] by one trained radiologist with 10 years' experience in abdominal ultrasound examinations. The severity of hepatic steatosis was based on the brightness of the liver and it was classified into three groups ranging from 0 to 2 (0 = normal bright, indicating a non-fatty liver, 1 = medium bright, a moderate lipid content and 2 = clearly bright, a severe lipid content and fatty liver) [23].

Follow-up

Both the hypertensive and the control men were recruited during December 1990 to May 1992 and the women approximately one year later (n=1045). In total, 1023 subjects had a liver ultrasound result available at baseline. Mortality data were obtained from the National Death Registry and the diagnoses of cardiovascular events were based on the registry of the National Institute for Health and Welfare. The follow-up time ended December 31, 2009 or whenever the first event occurred. Cardiovascular events included fatal and non-fatal endpoints. Subjects with a previous hospital-diagnosed myocardial infarction or stroke (n=41) at baseline were excluded. In total, 988 subjects participated in this part of the study.

CVD included a major CHD event and stroke (excluding subarachnoid hemorrhage, SAH) - whichever of these happened first [24]. The evidence of CHD was based on the following diagnosis: I20.0, I21, I22 [ICD-10, International Statistical Classification of Diseases and Related Health Problems] / 410, 4110 [ICD-8/9] as the main diagnosis (symptom or cause) and I21, I22 [ICD-10] / 410 [ICD-8/9] as a first side diagnosis (symptom or cause) or second side diagnosis (symptom or cause) and third side diagnosis (ICD-8/9 only) or if a subject had undergone coronary artery bypass graft (CABG) surgery or angioplasty. CHD as a cause of death included I20–I25, I46, R96, R98 [ICD-10] / 410-414, 798 (not 7980A) [ICD-8/9] as the underlying cause of death or immediate cause of death and I21 or I22 [ICD-10] / 410 [ICD-8/9] as first to third contributing cause of death. Stroke (excluding SAH) included I61, I63 (not I636), I64 [ICD -10] / 431, 4330A, 4331A, 4339A, 4340A, 4341A, 4349A, 436 [ICD-9] / 431 (except 43101, 43191) 433, 434, 436 [ICD-8] as main diagnosis (symptom or cause) or as a first or second side diagnosis (symptom or cause) or as a third side diagnosis (ICD-8/9 only) or as an underlying cause of death or immediate cause of death or as a first to third contributing cause of death [25].

Laboratory analyses

Waist circumference, body mass index (BMI) and blood pressure were measured as described in previous study [21].

All the laboratory test samples were obtained after an overnight fast. Blood insulin and glucose concentrations were analyzed at 0, 60, and 120 min after administration of 75 g glucose [23]. Insulin sensitivity was assessed using fasting plasma insulin concentrations and a quantitative insulin sensitivity check index (QUICKI) {QUICKI=1/[log (fasting insulin)+log (fasting glucose)]}[26].

Very-low-density lipoprotein (VLDL), high-density lipoprotein (HDL), low-density lipoprotein (LDL) and hs-CRP concentrations [23] as well as alanine aminotransferase (ALT) and GGT levels were measured as described previously [22]. Alcohol consumption and smoking history were determined by validated questionnaires [27]. Alcohol consumption was divided into three groups: 0 (n=161) mean alcohol consumption less than 1g/week in men and women, 1 (n=767) mean consumption less than 210g/week in men and less than 140 g/week in women, 2 (n=76) mean alcohol consumption more than 210g/week in men and more than 140g/week in women. Group 2 designates large-scale alcohol consumers according to the guidelines [28].

Statistical analysis

Statistical analysis was performed by using IBM SPSS Statistics for Windows, Version 20.0 (Armonk, NY: IBM Corp.). Analysis of variance was used to compare the means of the variables measured. Post hoc tests were performed using the Tukey method. Statistical significances between percentages were measured by using χ^2 test. Cumulative survival rates were estimated using Kaplan-Meier method. Cox regression analysis was performed to investigate if liver brightness (fat) could predict the future risk for total mortality, cardiovascular death or hospital events. A p value < 0.05 was regarded as significant.

Skewed variables (smoking, alcohol consumption, fasting insulin, fasting glucose, triglyceride, ALT, GGT concentration, hs-CRP level) were logarithmically transformed to improve normality before analysis of variance. We used three models with progressive degrees of adjustments. Model 1 included study group (subjects with medicine-treated hypertension and their age- and sex-matched controls), age and gender. Model 2 included further adjustments for smoking, alcohol consumption, systolic blood pressure, LDL-cholesterol level and body mass index. Model 3 included further adjustment for QUICKI. We carried out sensitivity analyses: in the analyses of cardiovascular events, we added all covariates one by one and investigated if the hazard ratios (HR) changed or remained stable when further adjustment with one covariate was performed. Model 4 included variables which were stable and were statistically significant in intermediate phases. Model 5 included stable and significant covariates without QUICKI (Table 2).

C-index was calculated for the model 1, model 3, model 4 and model 5 to assess the discrimination of the risk markers. The analyses were performed in 250 bootstrap resamplings to obtain 95% CI for c-index of each model.

Results

The main baseline characteristics of the study group are shown in Table 1.

Table 1 about here

Incidence of cardiovascular disease

The median follow-up time was 212 (maximum 228) months. During the follow-up time, 13.5% of the subjects with no fat in the liver (97/720), 24.2% (30/124) of subjects having moderate liver fat content and 29.2% (42/144) of the subjects having severe fatty liver experienced a CVD event ($p < 0.001$). CVD was the cause of death in 3.6% of the subjects with non-fatty liver (26/720) and 8.1% of the subjects with moderate liver fat content (10/124), while 12.5% (18/144) of the subjects with severe fatty liver ($p < 0.001$).

Severe liver fat content predicted the risk for future risk of cardiovascular event when adjusted for age, gender and study group (Model 1: HR 1.92, CI 1.32-2.80, $p < 0.01$) (Table 2). When further adjustments were made for smoking, alcohol consumption, LDL-cholesterol, BMI and systolic blood pressure (Model 2: HR 1.74, CI 1.16-2.63), the risk still remained statistically significant ($p < 0.01$). Statistical significance disappeared when further adjustment for QUICKI was performed (Model 3: HR 1.49, CI 0.97-2.30, p=0.071). In the CVD event sensitivity analyses, all covariates were added one by one and it was examined

whether the hazard ratios would change or remain stable. After adjusting for the statistically significant variables (including quick index) in the sensitivity analyses, the association between severe fatty liver was no longer significant (Model 4: HR 1.43, CI 0.93-2.18, p=0.10). When QUICKI was not added into Model 5, severe fatty liver did predict the risk for future risk for CVD event (HR 1.76, CI 1.21- 2.56, p < 0.001) (Table 2). The c-index decreased when the risk factors were removed from the model (Table 3).

Tables 2and 3 about here

The future risk of death from CVD in participants with severe fat content was significant when age, gender and study group were added as covariates (Model 1: HR 2.95, CI 1.58-5.51, p < 0.01). Even after further adjustments with other conventional risk factors (Model 2: HR 2.04, CI 1.03-4.05), statistical significance remained (p < 0.05). When QUICKI was added as the covariate, then significance disappeared (Model 3: HR 1.64, CI 0.79-3.43, NS) (Fig 1.).

Figure 1 about here

Fatty liver and total mortality

In total, 11.9% of the participants not having fatty liver, 18.5% of the subjects having moderate fatty liver and 22.2% of the subjects with severe fatty liver died from all causes (p < 0.01). According to Model 1, severe fat content predicted the risk for mortality from all

causes when age, gender and study group were added as covariates (HR 1.60, CI 1.05-2.43, p < 0.05). The significance disappeared when body mass index was added as a covariate (data not shown).

We performed all Cox regression analyses after excluding the men consuming more than 210 g alcohol and the women drinking more than 140 g alcohol per week. This exclusion did not have any effect on the results (data not shown).

We performed all Cox regression analyses after excluding patients with insulin treated diabetes mellitus (n=9), cortisone treatment at baseline (n=41) and previous diagnosis for liver disease (n=15) (e.g., virus, medications). This exclusion did not have any effect on the results (data not shown).

Discussion

The incidences of non-alcoholic fatty liver disease and cardiovascular disease are continuously increasing in the Western world. The question if NAFLD is only a marker or also an early mediator of cardiovascular disease is still largely unanswered. According to the results of the present study, which had an approximately 19-year follow-up fatty liver does predict the future risk for death from all causes, death from cardiovascular disease and risk of cardiovascular events. Insulin sensitivity seems to play a more dominant role in the development of cardiovascular events.

Only a few studies have investigated the risk for future cardiovascular risk among subjects with ultrasound-diagnosed fatty liver [29, 30] and larger studies with longer follow-up times are needed. An association between NAFLD and CVD has been reported [3, 29-31] although contrary

results also exist [13, 32]. An association between ultrasound-diagnosed fatty liver and CVD has been reported in general population [29] and in subjects with T2DM [31]. Furthermore, liver dysfunction has been reported to associate with CVD mortality [33, 34] and CHD risk [11] in follow-up studies and especially survival of subjects with NASH is reported to be reduced [32, 35, 36]. In the present study, severe fatty liver disease did predict the risk for cardiovascular death but the association seemed to be dependent on insulin sensitivity.

Several earlier studies have used self-reported CVD history which may not be totally reliable. Although earlier studies on the risk for future cardiovascular risk among subjects with fatty liver have performed some adjustments, the full range of well-known CVD risk factors have been rarely considered [32]. We have performed adjustments with all so-called traditional risk factors for cardiovascular disease (i.e. age, gender, smoking, LDL concentration, hypertension, insulin resistance). Previous studies have used biochemical, radiological and histological methodology for NAFLD diagnosis and staging, which leads to a challenging interpretation of the results [34, 37].

This study had an approximately 19-year follow-up time, which is longer than in previous studies [11-14]. When compared to earlier studies [32, 37] this study seems to be the first follow-up study with a large population-based randomly selected study group and a very long follow-up time and ultrasound-diagnosed fatty liver. The diagnosis of cardiovascular events was based on the registry of the National Institute for Health and Welfare and mortality data were obtained from the National Death Registry. The earlier verified FINRISK classification [25] was used to classify the events. Therefore, the reliability of event diagnosis data is accurate and the classification is systematic. All subjects who had myocardial infarction or stroke before

baseline were excluded because a history of myocardial infarction is known to increase the risk for recurrent myocardial infarction or cardiovascular death [38] and medication as well as lifestyle secondary prevention strategies are intensive [39].

There are a few follow-up-studies examining whether the fatty liver increases the risk for total mortality [13, 40]. In the present study, severe fatty liver predicted the risk for overall mortality of any causes when age, gender and study group were added covariates, a result in line with an earlier report [41]. In the published literature, NASH rather than simple steatosis has been stated to be linked with decreased overall survival [35] although one study with a large cohort found no association between NAFLD and overall mortality [13]. In our study, the association between severe fatty liver and total mortality disappeared after further adjustment for BMI which means that severe fatty liver is not a strong predictor for overall mortality.

The molecular mechanisms linking fatty liver with CVD have been investigated [10, 16]. Enlarged visceral adipose tissue may explain why NAFLD associates with CVD [16]. In individuals with visceral obesity, insulin resistance may contribute to impaired non-esterified fatty acid (NEFA) metabolism [8] and the increasing NEFA flux to the liver may impair liver metabolism leading to increased glucose metabolism and liver dysfunction [7]. The liver is one of the targets of the resulting systemic abnormalities and the source of several proatherogenic factors [3], such as CRP, fibrinogen, plasminogen activator inhibitor-1 and other inflammatory cytokines [16]. Furthermore, visceral adipose tissue and ectopic fat overexpress factors involved in atherogenesis [16] such as NEFAs and proinflammatory cytokines, for instance interleukin-6

and tumor necrosis factor-α [8] leading to chronic systemic inflammation. In addition, hepatic steatosis leads to overproduction of cholesterol-rich remnant particles [4].

One limitation in this study is that the grade of liver brightness was measured by ultrasound. The invasive diagnostic technique of liver biopsy is regarded as the "golden standard", especially for the diagnosis of NASH [42]. Real time ultrasound using a combination of sonographic findings does have a high specificity but it underestimates the prevalence of hepatic steatosis when there is less than 20 % fat [43]. Today, magnetic resonance spectroscopy is regarded as the best method for the quantification of liver fat, but this method is limited due to its availability [44]. Nonetheless, the noninvasive ultrasound method was chosen because taking liver biopsies from large groups of symptomless subjects would have been ethically unjustifiable and magnetic resonance spectroscopy was not available at the baseline.

The OPERA study group consists of subjects with drug-treated hypertension and randomly selected sex- and age-matched controls. Study group was added as a covariate to minimize any selection bias.

Conclusions

Severe liver fat content increased the risk of a future cardiovascular event and mortality to cardiovascular disease over the long-term follow-up but it seemed to be dependent on insulin sensitivity. Fatty liver also predicted the risk for overall mortality. However, conventional cardiovascular disease risk factors seemed to play a major role in developing death from all causes. It would be beneficial to investigate larger cohorts and follow-up studies in order to validate this result.

Figure legend

Title: Kaplan Meier cumulative survival rates censored for cardiovascular death in subjects with no fat in the liver, moderate fat content and severe fat content.

CVD was the cause of death in 3.6% of the subjects (26/720) with non-fatty liver and 8.1% of the subjects (10/124) with moderate liver fat content, while 12.5% of the subjects with severe fatty liver (18/144). Cox regression analysis is used for adjustments. M1 (Model 1): adjusted for study group, age and gender. M2 (Model 2): further adjustments for smoking, alcohol consumption, systolic blood pressure, LDL-cholesterol level and body mass index. M3 (Model 3): further adjustment for QUICKI. CVD, cardiovascular disease, CI, confidence interval, HR, hazard ratio, QUICKI, quantitative insulin sensitivity check index. ** $p < 0.01$, * $p < 0.05$.

Acknowledgements

The authors thank Markku Päivänsalo, MD, PhD, for the expert liver ultrasound examinations and Ms Saija Kortetjärvi, Ms Liisa Mannermaa, and Ms Leena Ukkola for the excellent technical assistance. M.Sc. Elina Malo and M.Sc. Meiju Saukko are thanked for the cooperation in organizing cardiovascular event and mortality data.

References

1. Angulo P. Nonalcoholic fatty liver disease. *N Engl J Med* 2002; Apr 18;346(16):1221-31.

2. Armstrong MJ, Houlihan DD, Bentham L, Shaw JC, Cramb R, Olliff S, et al. Presence and severity of non-alcoholic fatty liver disease in a large prospective primary care cohort. *J Hepatol* 2012; Jan;56(1):234-40.

3. Targher G, Day CP, Bonora E. Risk of cardiovascular disease in patients with nonalcoholic fatty liver disease. *N Engl J Med* 2010; Sep 30;363(14):1341-50.

4. Targher G, Arcaro G. Non-alcoholic fatty liver disease and increased risk of cardiovascular disease. *Atherosclerosis* 2007; Apr;191(2):235-40.

5. Roger VL, Go AS, Lloyd-Jones DM, Adams RJ, Berry JD, Brown TM, et al. Heart disease and stroke statistics--2011 update: a report from the American Heart Association. *Circulation* 2011; Feb 1;123(4):e18-e209.

6. Allender S, Scarborough P, Peto V, Rayner M, Leal J, Luengo-Fernandez R, Gray A. European cardiovascular disease statistics, 2008 ed. European Heart Network; 2008.

7. Despres JP, Lemieux I. Abdominal obesity and metabolic syndrome. *Nature* 2006; Dec 14;444(7121):881-7.

8. Van Gaal LF, Mertens IL, De Block CE. Mechanisms linking obesity with cardiovascular disease. *Nature* 2006; Dec 14;444(7121):875-80.

9. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med* 2005; Apr 21;352(16):1685-95.

10. Bhatia LS, Curzen NP, Byrne CD. Nonalcoholic fatty liver disease and vascular risk. *Curr Opin Cardiol* 2012; Jul;27(4):420-8.

11. Treeprasertsuk S, Leverage S, Adams LA, Lindor KD, St Sauver J, Angulo P. The Framingham risk score and heart disease in nonalcoholic fatty liver disease. *Liver Int* 2012; Jul;32(6):945-50.

12. Haring R, Wallaschofski H, Nauck M, Dorr M, Baumeister SE, Volzke H. Ultrasonographic hepatic steatosis increases prediction of mortality risk from elevated serum gamma-glutamyl transpeptidase levels. *Hepatology* 2009; Nov;50(5):1403-11.

13. Lazo M, Hernaez R, Bonekamp S, Kamel IR, Brancati FL, Guallar E, et al. Non-alcoholic fatty liver disease and mortality among US adults: prospective cohort study. *BMJ* 2011; Nov 18;343:d6891.

14. Wong VW, Wong GL, Yip GW, Lo AO, Limquiaco J, Chu WC, et al. Coronary artery disease and cardiovascular outcomes in patients with non-alcoholic fatty liver disease. *Gut* 2011; Dec;60(12):1721-7.

15. Loria P, Lonardo A, Targher G. Is liver fat detrimental to vessels?: intersections in the pathogenesis of NAFLD and atherosclerosis. *Clin Sci (Lond)* 2008; Jul;115(1):1-12.

16. Targher G, Marra F, Marchesini G. Increased risk of cardiovascular disease in non-alcoholic fatty liver disease: causal effect or epiphenomenon?. *Diabetologia* 2008; Nov;51(11):1947-53.

17. Targher G, Bertolini L, Scala L, Zoppini G, Zenari L, Falezza G. Non-alcoholic hepatic steatosis and its relation to increased plasma biomarkers of inflammation and

endothelial dysfunction in non-diabetic men. Role of visceral adipose tissue. *Diabet Med* 2005; Oct;22(10):1354-8.

18. Brea A, Mosquera D, Martin E, Arizti A, Cordero JL, Ros E. Nonalcoholic fatty liver disease is associated with carotid atherosclerosis: a case-control study. *Arterioscler Thromb Vasc Biol* 2005; May;25(5):1045-50.

19. Sookoian S, Pirola CJ. Non-alcoholic fatty liver disease is strongly associated with carotid atherosclerosis: a systematic review. *J Hepatol* 2008; Oct;49(4):600-7.

20. Fraser A, Harris R, Sattar N, Ebrahim S, Smith GD, Lawlor DA. Gamma-glutamyltransferase is associated with incident vascular events independently of alcohol intake: analysis of the British Women's Heart and Health Study and Meta-Analysis. *Arterioscler Thromb Vasc Biol* 2007; Dec;27(12):2729-35.

21. Rantala AO, Kauma H, Lilja M, Savolainen MJ, Reunanen A, Kesaniemi YA. Prevalence of the metabolic syndrome in drug-treated hypertensive patients and control subjects. *J Intern Med* 1999; Feb;245(2):163-74.

22. Sampi M, Veneskoski M, Ukkola O, Kesaniemi YA, Horkko S. High plasma immunoglobulin (Ig) A and low IgG antibody titers to oxidized low-density lipoprotein are associated with markers of glucose metabolism. *J Clin Endocrinol Metab* 2010; May;95(5):2467-75.

23. Pisto P, Ukkola O, Santaniemi M, Kesaniemi YA. Plasma adiponectin--an independent indicator of liver fat accumulation. *Metabolism* 2011; Nov;60(11):1515-20.

24. Santaniemi M., Ukkola O., Malo E., Bloigu R., Kesaniemi YA. Metabolic syndrome in the prediction of cardiovascular events: The potential additive role of hsCRP and adiponectin. *Eur J Prev Cardiol* 2013; Jun 20.

25. Pajunen P, Jousilahti P, Borodulin K, Harald K, Tuomilehto J, Salomaa V. Body fat measured by a near-infrared interactance device as a predictor of cardiovascular events: the FINRISK'92 cohort. *Obesity (Silver Spring)* 2011; Apr;19(4):848-52.

26. Katz A, Nambi SS, Mather K, Baron AD, Follmann DA, Sullivan G, et al. Quantitative insulin sensitivity check index: a simple, accurate method for assessing insulin sensitivity in humans. *J Clin Endocrinol Metab* 2000; Jul;85(7):2402-10.

27. Kauma H, Savolainen MJ, Rantala AO, Lilja M, Kervinen K, Reunanen A, et al. Apolipoprotein E phenotype determines the effect of alcohol on blood pressure in middle-aged men. *Am J Hypertens* 1998; Nov;11(11 Pt 1):1334-43.

28. Bessembinders K, Wielders J, van de Wiel A. Severe hypertriglyceridemia influenced by alcohol (SHIBA). *Alcohol Alcohol* 2011; Mar-Apr;46(2):113-6.

29. Hamaguchi M, Kojima T, Takeda N, Nagata C, Takeda J, Sarui H, et al. Nonalcoholic fatty liver disease is a novel predictor of cardiovascular disease. *World J Gastroenterol* 2007; Mar 14;13(10):1579-84.

30. Stepanova M, Younossi ZM. Independent Association Between Nonalcoholic Fatty Liver Disease and Cardiovascular Disease in the US Population. *Clin Gastroenterol Hepatol* 2012; Jun;10(6):646-50.

31. Targher G, Bertolini L, Poli F, Rodella S, Scala L, Tessari R, et al. Nonalcoholic fatty liver disease and risk of future cardiovascular events among type 2 diabetic patients. *Diabetes* 2005; Dec;54(12):3541-6.

32. Ghouri N, Preiss D, Sattar N. Liver enzymes, nonalcoholic fatty liver disease, and incident cardiovascular disease: a narrative review and clinical perspective of prospective data. *Hepatology* 2010; Sep;52(3):1156-61.

33. Dunn W, Xu R, Wingard DL, Rogers C, Angulo P, Younossi ZM, et al. Suspected nonalcoholic fatty liver disease and mortality risk in a population-based cohort study. *Am J Gastroenterol* 2008; Sep;103(9):2263-71.

34. Ruttmann E, Brant LJ, Concin H, Diem G, Rapp K, Ulmer H, et al. Gamma-glutamyltransferase as a risk factor for cardiovascular disease mortality: an epidemiological investigation in a cohort of 163,944 Austrian adults. *Circulation* 2005; Oct 4;112(14):2130-7.

35. Soderberg C, Stal P, Askling J, Glaumann H, Lindberg G, Marmur J, et al. Decreased survival of subjects with elevated liver function tests during a 28-year follow-up. *Hepatology* 2010; Feb;51(2):595-602.

36. Ekstedt M, Franzen LE, Mathiesen UL, Thorelius L, Holmqvist M, Bodemar G, et al. Long-term follow-up of patients with NAFLD and elevated liver enzymes. *Hepatology* 2006; Oct;44(4):865-73.

37. Bhatia LS, Curzen NP, Calder PC, Byrne CD. Non-alcoholic fatty liver disease: a new and important cardiovascular risk factor?. *Eur Heart J* 2012; May;33(10):1190-200.

38. Beckman JA, Creager MA, Libby P. Diabetes and atherosclerosis: epidemiology, pathophysiology, and management. *JAMA* 2002; May 15;287(19):2570-81.

39. Joseph P, Teo K. Optimal medical therapy, lifestyle intervention, and secondary prevention strategies for cardiovascular event reduction in ischemic heart disease. *Curr Cardiol Rep* 2011; Aug;13(4):287-95.

40. Dam-Larsen S, Franzmann M, Andersen IB, Christoffersen P, Jensen LB, Sorensen TI, et al. Long term prognosis of fatty liver: risk of chronic liver disease and death. *Gut* 2004; May;53(5):750-5.

41. Calori G, Lattuada G, Ragogna F, Garancini MP, Crosignani P, Villa M, et al. Fatty liver index and mortality: the Cremona study in the 15th year of follow-up. *Hepatology* 2011; Jul;54(1):145-52.

42. Joy D, Thava VR, Scott BB. Diagnosis of fatty liver disease: is biopsy necessary?. *Eur J Gastroenterol Hepatol* 2003; May;15(5):539-43.

43. Dasarathy S, Dasarathy J, Khiyami A, Joseph R, Lopez R, McCullough AJ. Validity of real time ultrasound in the diagnosis of hepatic steatosis: a prospective study. *J Hepatol* 2009; Dec;51(6):1061-7.

44. Szczepaniak LS, Nurenberg P, Leonard D, Browning JD, Reingold JS, Grundy S, et al. Magnetic resonance spectroscopy to measure hepatic triglyceride content: prevalence of hepatic steatosis in the general population. *Am J Physiol Endocrinol Metab* 2005; Feb;288(2):E462-8.

483

Grade of liver bightness	0 (n=720)	1 (n=124)	2 (n=144)	p	p (0-1)	p (1-2)	p (0-2)
Age (years)	50.9 (6.0)	51.9 (6.1)	51.5 (5.5)	NS	NS	NS	NS
Males	44.3 % (n=319)	65.3 % (n=81)	59.9 % (n=82)	< 0.001	-	-	-
Hypertensives	41.4 % (n=298)	66.1 % (n=82)	71.5 % (n=103)	< 0.001	-	-	-
BMI (kg/m²)	26.4 (3.9)	29.8 (5.0)	31.9 (4.9)	< 0.001	< 0.001	< 0.001	< 0.001
Waist circumference (cm)	86.8 (11.9)	97.7 (12.0)	102.3 (11.8)	< 0.001	< 0.001	< 0.01	< 0.001
Smoking (pack years)	10.6 (13.3)	14.3 (14.9)	14.0 (14.6)	< 0.05	NS	NS	NS
Alcohol consumption (g/week)	51.1 (83.0)	95.1 (117.0)	82.6 (105.1)	< 0.01	< 0.05	NS	NS
Total serum cholesterol (mmol/L)	5.6 (1.0)	5.8 (1.1)	5.8 (1.1)	NS	NS	NS	NS
LDL (mmol/L)	3.5 (0.9)	3.7 (1.1)	3.5 (0.9)	NS	NS	NS	NS
Triglycerides (mmol/L)	1.4 (0.8)	1.9 (0.8)	2.2 (1.4)	< 0.001	< 0.001	< 0.05	< 0.001
Systolic blood pressure	145.2 (21.5)	152.7 (20.3)	157.1 (22.2)	< 0.001	< 0.01	NS	< 0.001
Fasting insulin (mmol/L)	10.8 (7.7)	18.2 (10.3)	23.8 (17.6)	< 0.001	< 0.001	< 0.001	< 0.001

Fasting glucose (mmol/L)	4.4 (0.7)	5.0 (1.4)	6.1 (2.8)	< 0.001	< 0.001	< 0.001	< 0.001
QUICKI	0.6 (0.1)	0.6 (0.1)	0.5 (0.1)	< 0.001	< 0.001	< 0.001	< 0.001
hs-CRP (ng/mL)	3039.4 (6758.3)	3981.4 (6068.2)	6122.0 (6630.8)	< 0.001	< 0.001	< 0.01	< 0.001
ALT U/L	26.2 (15.5)	37.8 (17.1)	55.4 (37.7)	< 0.001	< 0.001	< 0.001	< 0.001
GGT U/L	35.1 (33.5)	69.7 (116.3)	76.8 (92.4)	< 0.001	< 0.001	< 0.01	< 0.001
Anti-hypertensive treatment	43.6% (n=314)	66.9% (n=83)	72.9% (n=105)	< 0.001	-	-	-
Lipid-lowering treatment	2.2% (n=16)	1.6% (n=2)	6.2% (n=9)	< 0.05	-	-	-
Hypoglycaemic drug	1.1% (n=8)	1.6% (n=2)	10.4% (n=15)	< 0.001	-	-	-
Type 2 diabetes	2.4% (n=17)	12.1% (n=15)	36.8% (n=53)	< 0.001	-	-	-

Table 1. Baseline characteristics of the study group as means (standard deviations) or percentages. N= number of subjects. ALT, alanine aminotransferase, BMI, body mass index, GGT, gamma-glutamyltransferase, hs-CRP, high-sensitivity C-reactive protein, LDL, low-density lipoprotein, QUICKI, quantitative insulin sensitivity check index.

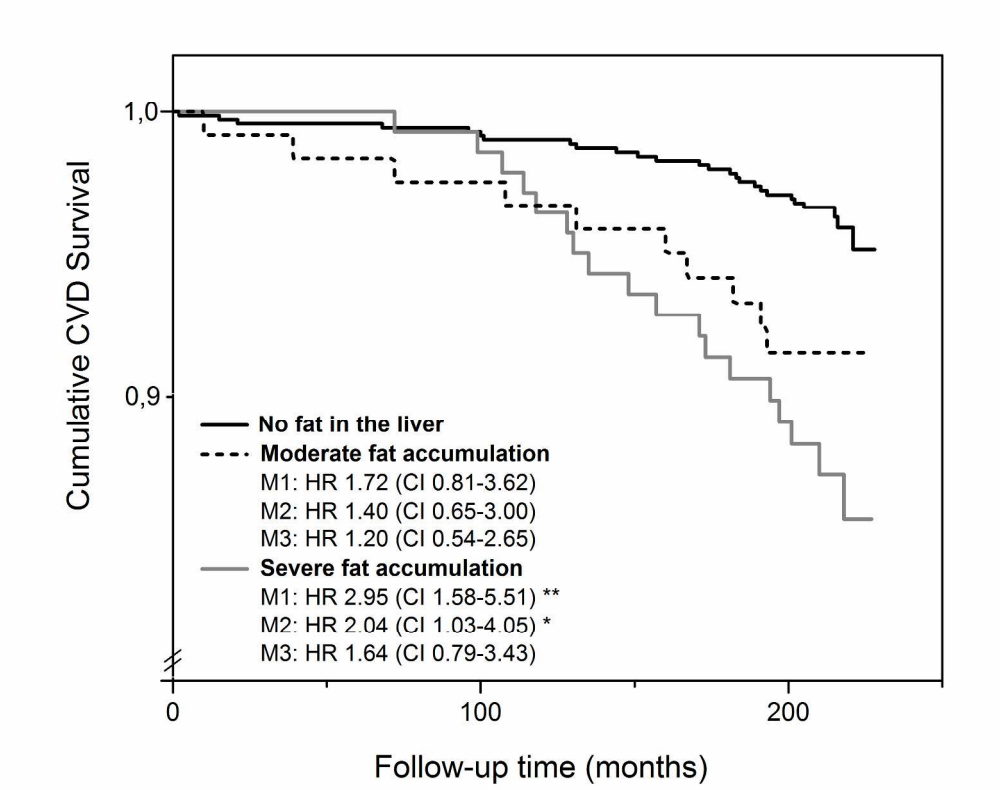
	Model 1	Model 2	Model 3	Model 4	Model 5
Moderate fat content	1.51 (0.99-2.29)	1.44 (0.93-2.23)	1.31 (0.84-2.05)	1.30 (0.84-2.01)	1.49 (0.99-2.26)
Severe fat content	1.92 (1.32-2.80)**	1.74 (1.16-2.63) **	1.49 (0.97-2.30)	1.43 (0.93-2.18)	1.76 (1.21- 2.56) **
Study group	1.34 (0.98-1.85)	1.29 (0.92-1.80)	1.28 (0.92-1.78)		
Age	1.06 (1.03-1.09)***	1.05(1.02-1.08)**	1.05 (1.02-1.08)**	1.05 (1.02-1.07)**	1.05 (1.02-1.08) **
Gender	2.39 (1.71-3.34)*	1.91 (1.34-2.71)***	1.80 (1.26-2.57)**	1.83 (1.29-2.60) **	1.92 (1.36-2.72) ***
LDL-cholesterol		1.17 (0.99-1.39)	1.15 (0.97-1.37)		
Smoking (pack-years)		1.02 (1.01-1.03)***	1.02 (1.01-1.03)***	1.02 (1.01-1.03)***	1.02 (1.01-1.03) ***
Alcohol consumption (gr1)		0.93 (0.59-1.45)	0.92(0.59-1.44)		
Alcohol consumption (gr2)		0.84 (0.44-1.60)	0.81(0.42-1.54)		
Systolic blood pressure		1.01 (1.00-1.02)**	1.01 (1.00-1.02)*	1.01 (1.00-1.02)**	1.01 (1.00-1.02) **
Body mass index		0.99 (0.96-1.03)	0.97 (0.93-1.01)		
QUICKI			0.12 (0.02-0.90)*	0.16 (0.03-0.99)*	

Table 2. Multivariate analysis for cardiovascular events with different degrees of adjustments (Cox regression analysis). CVD event occurred in 13.5% of the subjects with no fat in the liver (97/720), 24.2% (30/124) of subjects having moderate liver fat content and 29.2% (42/144) of the subjects having severe fatty liver. Hazard ratios with 95% confidence interval with different degrees of adjustments are presented. Alcohol consumption was divided into groups (reference group: less than 1g/week in men and women, group 1: less than 210g/week in men and less than 140 g/week in women, group 2: more than 210g/week in men and more than 140g/week in women). Model 1: adjustment for study group, age and gender. Model 2: further adjustments for LDL-cholesterol, smoking, alcohol consumption, systolic blood pressure and body mass index. Model 3: further adjustment for QUICKI. Model 4: adjustments with statistically significant covariates. Model 5: adjustments with statistically significant covariates without QUICKI. LDL, low-density lipoprotein, QUICKI, quantitative insulin sensitivity check index. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Final model	Cardiovascular event c-index (95% CI)	Binary R^2
Model 3	0.729 (0.706-0.776)	0.153
Model 4	0.720 (0.689-0.763)	0.144
Model 5	0.717 (0.686-0.758)	0.138
Model 1	0.698 (0.656-0.742)	0.133

Table 3. Multivariate analysis for cardiovascular events (logistic regression analysis). Cardiovascular disease risk factors have been removed from the models step by step. Model 3 included liver brightness, study group, age, gender, smoking, alcohol consumption, systolic blood pressure, LDL-cholesterol level, body mass index and QUICKI. Model 4 included liver brightness, age, gender, smoking, blood pressure and QUICKI. Model 5 included liver brightness, age, gender, smoking, blood pressure. Model 1 included liver brightness, study group, age and gender. C-index with confidence intervals obtained from 250 bootstrap resamplings and binary R^2 was used. LDL, low-density lipoprotein, QUICKI, quantitative insulin sensitivity check index.

530



Kaplan Meier cumulative survival rates censored for cardiovascular death in subjects with no fat in the liver, moderate fat accumulation and severe fat accumulation.
CVD was the cause of death in 3.6% of the subjects (26/720) with non-fatty liver and 8.1% of the subjects (10/124) with moderate liver fat accumulation, while 12.5% of the subjects with severe fatty liver (18/144). Cox regression analysis is used for adjustments. M1 (Model 1): adjusted for study group, age and gender. M2 (Model 2): further adjustments for smoking, alcohol consumption, systolic blood pressure, LDL-cholesterol level and body mass index. M3 (Model 3): further adjustment for QUICKI. CVD, cardiovascular disease, CI, confidence interval, HR, hazard ratio, QUICKI, quantitative insulin sensitivity check index. ** $p < 0.01$, * $p < 0.05$.

247x209mm (300 x 300 DPI)

STROBE Statement—Items to be included when reporting observational studies in a conference abstract

Item	Recommendation
Title	Indicate the study's design with a commonly used term in the title (e.g cohort, case-control, cross sectional)
Authors	Contact details for the corresponding author
Study design	Description of the study design (e.g cohort, case-control, cross sectional)
Objective	Specific objectives or hypothesis
Methods	
Setting	Description of setting, follow-up dates or dates at which the outcome events occurred or at which the outcomes were present, as well as any points or ranges on other time scales for the outcomes (e.g., prevalence at age 18, 1998-2007).
Participants	*Cohort study*—Give the most important eligibility criteria, and the most important sources and methods of selection of participants. Describe briefly the methods of follow-up *Case-control study*—Give the major eligibility criteria, and the major sources and methods of case ascertainment and control selection *Cross-sectional study*—Give the eligibility criteria, and the major sources and methods of selection of participants
	Cohort study—For matched studies, give matching and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case
Variables	Clearly define primary outcome for this report.
Statistical methods	Describe statistical methods, including those used to control for confounding
Results	
Participants	Report Number of participants at the beginning and end of the study
Main results	Report estimates of associations. If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period Report appropriate measures of variability and uncertainty (e.g., odds ratios with confidence intervals
Conclusions	General interpretation of study results

Fatty liver predicts the risk for cardiovascular events in middle-aged population: a population-based cohort study

Journal:	*BMJ Open*
Manuscript ID:	bmjopen-2014-004973
Article Type:	Research
Date Submitted by the Author:	31-Jan-2014
Complete List of Authors:	Pisto, Pauliina; Clinical medicine, Internal medicine Santaniemi, Merja; Institute of Clinical Medicine, Department of Internal Medicine Bloigu, Risto; Medical Informatics and Statistics Research Group, Ukkola, Olavi; Institute of Clinical Medicine, Department of Internal Medicine Kesäniemi, Antero; Institute of Clinical Medicine, Department of Internal Medicine
Primary Subject Heading:	Diabetes and endocrinology
Secondary Subject Heading:	Cardiovascular medicine
Keywords:	Adult cardiology < CARDIOLOGY, Coronary heart disease < CARDIOLOGY, General diabetes < DIABETES & ENDOCRINOLOGY, Hepatology < INTERNAL MEDICINE

Fatty liver predicts the risk for cardiovascular events in middle-aged population: a population-based cohort study

Pauliina Pisto[1], Merja Santaniemi[1], Risto Bloigu[2], Olavi Ukkola[1], Y. Antero Kesäniemi[1]

[1]Institute of Clinical Medicine, Department of Internal Medicine and Biocenter Oulu, University of Oulu, and Clinical Research Center, Oulu University Hospital, Oulu, Finland, P.O. Box 20, 90029 OYS, Finland

[2] Medical Informatics and Statistics Research Group, University of Oulu, Aapistie 7, P.O. Box 5000, 90014 Oulu, Finland

Contact information: Pauliina Pisto, corresponding author

University of Oulu

Institute of Clinical Medicine

Department of Internal medicine

P.O. Box 5000, 90014 Oulu, Finland

Tel.: +35885376310

Fax: +35885376318

E-mail: pauliina.pisto@oulu.fi

Word count: 6093

Keywords: coronary disease, fatty liver, insulin resistance, risk factors, stroke

Financial support: This study was supported by the Finnish Foundation for Cardiovascular Research, dated 16 Apr, 2012.

Disclosure summary: Authors report no conflict of interests.

ABSTRACT

Objective: We investigated if the differences in liver fat content would predict the development of non-fatal and fatal atherosclerotic endpoints (coronary heart disease and stroke).

Design, setting and participants: Our study group is a population-based, randomly recruited cohort (OPERA), initiated in 1991. The cohort consisted of 988 middle-aged Finnish subjects.

Intervention: Total mortality and hospital events were followed up to 2009 based on the registry of the National Institute for Health and Welfare and the National death registry.

Main outcome measure: The severity of hepatic steatosis was measured by ultrasound and divided into three groups (0-2). Cox regression analysis was used in the statistical analysis.

Results: In the follow-up of years 1991-2009, 13.5% of the subjects with non-fatty liver, 24.2% of subjects having moderate liver fat content and 29.2% of the subjects having severe fatty liver experienced a cardiovascular event during the follow-up time ($p < 0.001$). Severe liver fat content predicted the risk for future risk of cardiovascular event even when adjusted for age, gender and study group (HR 1.92, CI 1.32-2.80, $p < 0.01$). When further adjustments for smoking, alcohol consumption, LDL-cholesterol, BMI and systolic blood pressure were conducted, the risk still remained statistically significant (HR 1.74, CI 1.16-2.63, $p < 0.01$). Statistical significance disappeared with further adjustment for QUICKI.

Conclusions: Liver fat content increases the risk of future cardiovascular disease event in long-term follow-up but it is seems to be dependent on insulin sensitivity.

Article focus

1 To investigate if the differences in liver fat content predict the risk for development of fatal or nonfatal atherosclerotic endpoints such as coronary heart disease and stroke.

Key messages

1 Subjects with ultrasound-diagnosed fatty liver have cardiovascular disease more often compared to the subjects without fat in the liver

2 Severe liver fat content increases the risk of a future cardiovascular event and mortality to cardiovascular disease over the long-term follow-up but it does seem to be dependent on insulin sensitivity

3 Severe fatty liver predicts the risk for overall mortality but the association is dependent on traditional metabolic risk factors

Strengths and limitations of the study

1 This is a follow-up study with a large population-based study group and a very long follow-up time

2 Official registers used in event diagnoses - data is accurate and the classification is systematic

3 Grade of liver brightness was measured by ultrasound, which has a high specificity but low sensitivity

Introduction

Non-alcoholic fatty liver disease (NAFLD) refers to liver disorders such as abnormal fat content, which exists in a spectrum ranging from steatosis with no inflammation to non-alcoholic steatohepatitis (NASH), which can ultimately lead to liver cirrhosis [1]. The prevalence of NAFLD is estimated to range from 20 to 30% of population in Western countries, being the leading cause of liver disorders [2, 3]. It is associated with obesity, type 2 diabetes mellitus (T2DM) and hyperlipidemia [1]. NAFLD is commonly regarded as a hepatic manifestation of the metabolic syndrome and both conditions share several risk factors for cardiovascular disease (CVD) [3, 4].

In 2008, the prevalence of CVD in adults (≥ 20 years) in United States was 36.2% [5]. Every year, 4.3 million subjects die for CVD in Europe causing nearly half of the all deaths (48%) [6]. So-called traditional risk factors for cardiovascular disease are age, gender, smoking, high low-density lipoprotein (LDL) cholesterol concentration, hypertension and diabetes [7]. In addition, total body fatness as well as abdominal fat accumulation increase independently the risk of CVD and insulin resistance is regarded to be an important factor linking visceral adiposity to cardiovascular risk [8]. Adipose tissue is now recognized as a significant endocrine organ as adipocytes and macrophages infiltrating adipocytes secrete a number of bioactive mediators [7]. Adipokines, proinflammatory cytokines and hypofibrinolytic markers may lead to oxidative stress and endothelial dysfunction, finally leading to atherosclerosis [9].

Hepatic steatosis has been discussed as a possible mechanism to explain CVD morbidity and mortality [10]. NAFLD patients have been reported to have higher coronary heart disease (CHD) risk than the general population of the same age and gender [11]. According to previous study,

liver dysfunction associated with CVD mortality in men [12] whereas another large study found no association between NAFLD and CVD in general population [13]. In addition, fatty liver did not predict CVD mortality and morbidity in patients with established coronary artery disease [14].

The NAFLD and CVD share several molecular mechanisms [15, 16]. Fatty liver might play a part in the pathogenesis of CVD through the overexpression and systemic release of several inflammatory, hemostatic [17] and oxidative-stress mediators or via contributing to whole-body insulin resistance and atherogenic dyslipidemia [3]. NAFLD has also been reported to be linked with circulatory endothelial dysfunction [4, 14]. Several investigators have reported that NAFLD is associated with coronary artery disease [4, 14] and increased carotid intima-media thickness [18, 19]. Increased gamma-glutamyltransferase (GGT), which may be a marker of NAFLD, has been reported to be associated with stroke [20].

It is known that subjects with fatty liver disease have an increased risk of suffering CVD [4], but whether NAFLD is an independent indicator of cardiovascular disease is still far from clear. Long-term follow-up studies are needed to clarify the correlation between fatty liver and CVD. The aim of our study was to investigate if fatty liver could predict independently the risk for total mortality as well as non-fatal and fatal cardiovascular endpoints with a 19-year follow-up after adjusting for all known conventional risk factors.

Materials and methods

Human subjects

OPERA (Oulu Project Elucidating Risk of Atherosclerosis) is a population-based, epidemiological prospective cohort study designed to address the risk factors and disease end points of atherosclerotic cardiovascular diseases. Selection criteria of the study subjects have been described earlier [21]. In short, a total of 520 men and 525 women participated: 259 control men, 261 hypertensive men, 267 control women and 258 hypertensive women aged 40-59. Hypertensive participants were randomly selected from the national register for reimbursement of the costs of antihypertensive medication. For each hypertensive subject, an age- and sex-matched control subject was randomly selected from the same register. Informed consent in writing was obtained from each patient. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and this study was approved by the Ethical Committee of the Faculty of Medicine, University of Oulu.

Determination of hepatic steatosis

The determination of hepatic steatosis was based on liver-kidney contrast [22] measured with ultrasonography [23] by one trained radiologist with 10 years' experience in abdominal ultrasound examinations. Normal liver parenchyma should be slightly more echogenic (brighter) than the kidney parenchyma. In a case of increased liver echogenicity an ultrasound diagnosis of bright liver was settled. The severity of hepatic steatosis was based on the brightness of the liver and it was classified into three groups ranging from 0 to 2 (0 = normal bright, indicating a non-fatty liver, 1 = medium bright, a moderate lipid content and 2 = clearly bright, a severe lipid content and fatty liver) [24].

Follow-up

Both the hypertensive and the control men were recruited during December 1990 to May 1992 and the women approximately one year later (n=1045). In total, 1023 subjects had a liver ultrasound result available at baseline. Mortality data were obtained from the National Death Registry and the diagnoses of cardiovascular events were based on the registry of the National Institute for Health and Welfare. The follow-up time ended December 31, 2009 or whenever the first event occurred. Cardiovascular events included fatal and non-fatal endpoints. Subjects with a previous hospital-diagnosed myocardial infarction or stroke (n=41) at baseline were excluded. In total, 988 subjects participated in this part of the study.

CVD included a major CHD event and stroke (excluding subarachnoid hemorrhage, SAH) - whichever of these happened first [25]. The evidence of CHD was based on the following diagnosis: I20.0, I21, I22 [ICD-10, International Statistical Classification of Diseases and Related Health Problems] / 410, 4110 [ICD-8/9] as the main diagnosis (symptom or cause) and I21, I22 [ICD-10] / 410 [ICD-8/9] as a first side diagnosis (symptom or cause) or second side diagnosis (symptom or cause) and third side diagnosis (ICD-8/9 only) or if a subject had undergone coronary artery bypass graft (CABG) surgery or angioplasty. CHD as a cause of death included I20–I25, I46, R96, R98 [ICD-10] / 410-414, 798 (not 7980A) [ICD-8/9] as the underlying cause of death or immediate cause of death and I21 or I22 [ICD-10] / 410 [ICD-8/9] as first to third contributing cause of death. Stroke (excluding SAH) included I61, I63 (not I636), I64 [ICD -10] / 431, 4330A, 4331A, 4339A, 4340A, 4341A, 4349A, 436 [ICD-9] / 431 (except 43101, 43191) 433, 434, 436 [ICD-8] as main diagnosis (symptom or cause) or as a first or second side diagnosis (symptom or cause) or as a third side diagnosis (ICD-8/9 only)

or as an underlying cause of death or immediate cause of death or as a first to third contributing cause of death [26].

Laboratory analyses

Waist circumference, body mass index (BMI) and blood pressure were measured as described in previous study [21].

All the laboratory test samples were obtained after an overnight fast. Blood insulin and glucose concentrations were analyzed at 0, 60, and 120 min after administration of 75 g glucose [24]. Insulin sensitivity was assessed using fasting plasma insulin concentrations and a quantitative insulin sensitivity check index (QUICKI) {QUICKI=1/[log (fasting insulin)+log (fasting glucose)]}[27].

Very-low-density lipoprotein (VLDL), high-density lipoprotein (HDL), low-density lipoprotein (LDL) and hs-CRP concentrations [24] as well as alanine aminotransferase (ALT) and GGT levels were measured as described previously [23]. Alcohol consumption and smoking history were determined by validated questionnaires [28]. Alcohol consumption was divided into three groups: 0 (n=161) mean alcohol consumption less than 1g/week in men and women, 1 (n=767) mean consumption less than 210g/week in men and less than 140 g/week in women, 2 (n=76) mean alcohol consumption more than 210g/week in men and more than 140g/week in women. Group 2 designates large-scale alcohol consumers according to the guidelines [29].

Statistical analysis

Statistical analysis was performed by using IBM SPSS Statistics for Windows, Version 20.0 (Armonk, NY: IBM Corp.). Analysis of variance was used to compare the means of the variables measured. Post hoc tests were performed using the Tukey method. Statistical significances between percentages were measured by using χ^2 test. Cumulative survival rates were estimated using Kaplan-Meier method. Cox regression analysis was performed to investigate if liver brightness (fat) could predict the future risk for total mortality, cardiovascular death or hospital events. A p value < 0.05 was regarded as significant.

Skewed variables (smoking, alcohol consumption, fasting insulin, fasting glucose, triglyceride, ALT, GGT concentration, hs-CRP level) were logarithmically transformed to improve normality before analysis of variance. We used three models with progressive degrees of adjustments. Model 1 included study group (subjects with medicine-treated hypertension and their age- and sex-matched controls), age and gender. Model 2 included further adjustments for smoking, alcohol consumption, systolic blood pressure, LDL-cholesterol level and body mass index. Model 3 included further adjustment for QUICKI. We carried out sensitivity analyses: in the analyses of cardiovascular events, we added all covariates one by one and investigated if the hazard ratios (HR) changed or remained stable when further adjustment with one covariate was performed. Model 4 included variables which were stable and were statistically significant in intermediate phases. Model 5 included stable and significant covariates without QUICKI (Table 2).

C-index was calculated for the model 1, model 3, model 4 and model 5 to assess the discrimination of the risk markers. The analyses were performed in 250 bootstrap resamplings to obtain 95% CI for c-index of each model.

Results

The main baseline characteristics of the study group are shown in Table 1.

Table 1 about here

Incidence of cardiovascular disease

The median follow-up time was 212 (maximum 228) months. During the follow-up time, 13.5% of the subjects with no fat in the liver (97/720), 24.2% (30/124) of subjects having moderate liver fat content and 29.2% (42/144) of the subjects having severe fatty liver experienced a CVD event ($p < 0.001$). CVD was the cause of death in 3.6% of the subjects with non-fatty liver (26/720) and 8.1% of the subjects with moderate liver fat content (10/124), while 12.5% (18/144) of the subjects with severe fatty liver ($p < 0.001$) (Table 3).

Severe liver fat content predicted the risk for future risk of cardiovascular event when adjusted for age, gender and study group (Model 1: HR 1.92, CI 1.32-2.80, $p < 0.01$) (Table 2). When further adjustments were made for smoking, alcohol consumption, LDL-cholesterol, BMI and systolic blood pressure (Model 2: HR 1.74, CI 1.16-2.63), the risk still remained

statistically significant ($p < 0.01$). Statistical significance disappeared when further adjustment for QUICKI was performed (Model 3: HR 1.49, CI 0.97-2.30, p=0.071). In the CVD event sensitivity analyses, all covariates were added one by one and it was examined whether the hazard ratios would change or remain stable. After adjusting for the statistically significant variables (including quick index) in the sensitivity analyses, the association between severe fatty liver was no longer significant (Model 4: HR 1.43, CI 0.93-2.18, p=0.10). When QUICKI was not added into Model 5, severe fatty liver did predict the risk for future risk for CVD event (HR 1.76, CI 1.21- 2.56, $p < 0.001$) (Table 2). The c-index decreased when the risk factors were removed from the model (Table 4).

Tables 2, 3 and 4 about here

The future risk of death from CVD in participants with severe fat content was significant when age, gender and study group were added as covariates (Model 1: HR 2.95, CI 1.58-5.51, $p < 0.01$). Even after further adjustments with other conventional risk factors (Model 2: HR 2.04, CI 1.03-4.05), statistical significance remained ($p < 0.05$). When QUICKI was added as the covariate, then significance disappeared (Model 3: HR 1.64, CI 0.79-3.43, NS) (Fig 1.).

Figure 1 about here

Fatty liver and total mortality

In total, 11.9% of the participants not having fatty liver, 18.5% of the subjects having moderate fatty liver and 22.2% of the subjects with severe fatty liver died from all causes ($p < 0.01$). According to Model 1, severe fat content predicted the risk for mortality from all causes when age, gender and study group were added as covariates (HR 1.60, CI 1.05-2.43, $p < 0.05$). The significance disappeared when body mass index was added as a covariate (data not shown).

We performed all Cox regression analyses after excluding the men consuming more than 210 g alcohol and the women drinking more than 140 g alcohol per week. This exclusion did not have any effect on the results (data not shown).

We performed all Cox regression analyses after excluding patients with insulin treated diabetes mellitus (n=9), cortisone treatment at baseline (n=41) and previous diagnosis for liver disease (n=15) (e.g., virus, medications). This exclusion did not have any effect on the results (data not shown).

Discussion

The incidences of non-alcoholic fatty liver disease and cardiovascular disease are continuously increasing in the Western world. The question if NAFLD is only a marker or also an early mediator of cardiovascular disease is still largely unanswered. According to the results of the present study, which had an approximately 19-year follow-up fatty liver does predict the future risk for death from all causes, death from cardiovascular disease and risk of cardiovascular events. Insulin sensitivity seems to play a more dominant role in the development of cardiovascular events.

Only a few studies have investigated the risk for future cardiovascular risk among subjects with ultrasound-diagnosed fatty liver [30, 31] and larger studies with longer follow-up times are needed. An association between NAFLD and CVD has been reported [3, 30-32] although contrary results also exist [13, 33]. A previous large population-based prospective cohort study found no association between NAFLD and CVD, however they categorized the degree of steatosis as a two level variable: none to mild and moderate to severe [13]. An association between ultrasound-diagnosed fatty liver and CVD has been reported in general population [30] and in subjects with T2DM [32]. Furthermore, liver dysfunction has been reported to associate with CVD mortality [34, 35] and CHD risk [11] in follow-up studies and especially survival of subjects with NASH is reported to be reduced [33, 36, 37]. In the present study, severe fatty liver disease did predict the risk for cardiovascular death but the association seemed to be dependent on insulin sensitivity.

Several earlier studies have used self-reported CVD history which may not be totally reliable. Although earlier studies on the risk for future cardiovascular risk among subjects with fatty liver have performed some adjustments, the full range of well-known CVD risk factors have been rarely considered [33]. We have performed adjustments with all so-called traditional risk factors for cardiovascular disease (i.e. age, gender, smoking, LDL concentration, hypertension, insulin resistance). Previous studies have used biochemical, radiological and histological methodology for NAFLD diagnosis and staging, which leads to a challenging interpretation of the results [35, 38].

This study had an approximately 19-year follow-up time, which is longer than in previous studies [11-14]. When compared to earlier studies [33, 38] this study seems to be the first follow-up

study with a large population-based randomly selected study group and a very long follow-up time and ultrasound-diagnosed fatty liver. The diagnosis of cardiovascular events was based on the registry of the National Institute for Health and Welfare and mortality data were obtained from the National Death Registry. The earlier verified FINRISK classification [26] was used to classify the events. Therefore, the reliability of event diagnosis data is accurate and the classification is systematic. All subjects who had myocardial infarction or stroke before baseline were excluded because a history of myocardial infarction is known to increase the risk for recurrent myocardial infarction or cardiovascular death [39] and medication as well as lifestyle secondary prevention strategies are intensive [40].

There are a few follow-up-studies examining whether the fatty liver increases the risk for total mortality [13, 41]. In the present study, severe fatty liver predicted the risk for overall mortality of any causes when age, gender and study group were added covariates, a result in line with an earlier report [42]. In the published literature, NASH rather than simple steatosis has been stated to be linked with decreased overall survival [36] although one study with a large cohort found no association between NAFLD and overall mortality [13]. In our study, the association between severe fatty liver and total mortality disappeared after further adjustment for BMI which means that severe fatty liver is not a strong predictor for overall mortality.

The molecular mechanisms linking fatty liver with CVD have been investigated [10, 16]. Enlarged visceral adipose tissue may explain why NAFLD associates with CVD [16]. In individuals with visceral obesity, insulin resistance may contribute to impaired non-esterified fatty acid (NEFA) metabolism [8] and the increasing NEFA flux to the liver may impair liver

metabolism leading to increased glucose metabolism and liver dysfunction [7]. The liver is one of the targets of the resulting systemic abnormalities and the source of several proatherogenic factors [3], such as CRP, fibrinogen, plasminogen activator inhibitor-1 and other inflammatory cytokines [16]. Furthermore, visceral adipose tissue and ectopic fat overexpress factors involved in atherogenesis [16] such as NEFAs and proinflammatory cytokines, for instance interleukin-6 and tumor necrosis factor-α [8] leading to chronic systemic inflammation. In addition, hepatic steatosis leads to overproduction of cholesterol-rich remnant particles [4].

One limitation in this study is that the grade of liver brightness was measured by ultrasound. The invasive diagnostic technique of liver biopsy is regarded as the "golden standard", especially for the diagnosis of NASH [43]. Real time ultrasound using a combination of sonographic findings does have a high specificity but it underestimates the prevalence of hepatic steatosis when there is less than 20 % fat [44]. Today, magnetic resonance spectroscopy is regarded as the best method for the quantification of liver fat, but this method is limited due to its availability [45]. Nonetheless, the noninvasive ultrasound method was chosen because taking liver biopsies from large groups of symptomless subjects would have been ethically unjustifiable and magnetic resonance spectroscopy was not available at the baseline.

The OPERA study group consists of subjects with drug-treated hypertension and randomly selected sex- and age-matched controls. Study group was added as a covariate to minimize any selection bias.

Conclusions

Severe liver fat content increased the risk of a future cardiovascular event and mortality to cardiovascular disease over the long-term follow-up but it seemed to be dependent on insulin sensitivity. Fatty liver also predicted the risk for overall mortality. However, conventional cardiovascular disease risk factors seemed to play a major role in developing death from all causes. It would be beneficial to investigate larger cohorts and follow-up studies in order to validate this result.

Figure legend

Title: Kaplan Meier cumulative survival rates censored for cardiovascular death in subjects with no fat in the liver, moderate fat content and severe fat content.

CVD was the cause of death in 3.6% of the subjects (26/720) with non-fatty liver and 8.1% of the subjects (10/124) with moderate liver fat content, while 12.5% of the subjects with severe fatty liver (18/144). Cox regression analysis is used for adjustments. M1 (Model 1): adjusted for study group, age and gender. M2 (Model 2): further adjustments for smoking, alcohol consumption, systolic blood pressure, LDL-cholesterol level and body mass index. M3 (Model 3): further adjustment for QUICKI. CVD, cardiovascular disease, CI, confidence interval, HR, hazard ratio, QUICKI, quantitative insulin sensitivity check index. ** $p < 0.01$, * $p < 0.05$.

Acknowledgements

The authors thank Markku Päivänsalo, MD, PhD, for the expert liver ultrasound examinations and Ms Saija Kortetjärvi, Ms Liisa Mannermaa, and Ms Leena Ukkola for the excellent

technical assistance. M.Sc. Elina Malo and M.Sc. Meiju Saukko are thanked for the cooperation in organizing cardiovascular event and mortality data.

Contributor statement: All authors fulfill all three of the ICMJE guidelines for authorship

Pauliina Pisto: Data acquisition, statistical analysis and interpretation of data, manuscript writing, final approval of the version to be published

Merja Santaniemi: Data acquisition, statistical analysis and data interpretation, critical revision of the manuscript, final approval of the version to be published

Risto Bloigu: Data analysis, interpretation of data, critical revision of the manuscript, final approval of the version to be published

Olavi Ukkola: Study design, data acquisition, data interpretation, critical revision of the manuscript, final approval of the version to be published

Y.A Kesäniemi: Study design, data acquisition, data interpretation, critical revision of the manuscript, final approval of the version to be published

Data sharing statement: Extra data is available by emailing pauliina.pisto(at)oulu.fi

Competing Interests: None

References

1. Angulo P. Nonalcoholic fatty liver disease. *N Engl J Med* 2002; Apr 18;346(16):1221-31.

2. Armstrong MJ, Houlihan DD, Bentham L, Shaw JC, Cramb R, Olliff S, et al. Presence and severity of non-alcoholic fatty liver disease in a large prospective primary care cohort. *J Hepatol* 2012; Jan;56(1):234-40.

3. Targher G, Day CP, Bonora E. Risk of cardiovascular disease in patients with nonalcoholic fatty liver disease. *N Engl J Med* 2010; Sep 30;363(14):1341-50.

4. Targher G, Arcaro G. Non-alcoholic fatty liver disease and increased risk of cardiovascular disease. *Atherosclerosis* 2007; Apr;191(2):235-40.

5. Roger VL, Go AS, Lloyd-Jones DM, Adams RJ, Berry JD, Brown TM, et al. Heart disease and stroke statistics--2011 update: a report from the American Heart Association. *Circulation* 2011; Feb 1;123(4):e18-e209.

6. Allender S, Scarborough P, Peto V, Rayner M, Leal J, Luengo-Fernandez R, Gray A. European cardiovascular disease statistics, 2008 ed. European Heart Network; 2008.

7. Despres JP, Lemieux I. Abdominal obesity and metabolic syndrome. *Nature* 2006; Dec 14;444(7121):881-7.

8. Van Gaal LF, Mertens IL, De Block CE. Mechanisms linking obesity with cardiovascular disease. *Nature* 2006; Dec 14;444(7121):875-80.

9. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med* 2005; Apr 21;352(16):1685-95.

10. Bhatia LS, Curzen NP, Byrne CD. Nonalcoholic fatty liver disease and vascular risk. *Curr Opin Cardiol* 2012; Jul;27(4):420-8.

11. Treeprasertsuk S, Leverage S, Adams LA, Lindor KD, St Sauver J, Angulo P. The Framingham risk score and heart disease in nonalcoholic fatty liver disease. *Liver Int* 2012; Jul;32(6):945-50.

12. Haring R, Wallaschofski H, Nauck M, Dorr M, Baumeister SE, Volzke H. Ultrasonographic hepatic steatosis increases prediction of mortality risk from elevated serum gamma-glutamyl transpeptidase levels. *Hepatology* 2009; Nov;50(5):1403-11.

13. Lazo M, Hernaez R, Bonekamp S, Kamel IR, Brancati FL, Guallar E, et al. Non-alcoholic fatty liver disease and mortality among US adults: prospective cohort study. *BMJ* 2011; Nov 18;343:d6891.

14. Wong VW, Wong GL, Yip GW, Lo AO, Limquiaco J, Chu WC, et al. Coronary artery disease and cardiovascular outcomes in patients with non-alcoholic fatty liver disease. *Gut* 2011; Dec;60(12):1721-7.

15. Loria P, Lonardo A, Targher G. Is liver fat detrimental to vessels?: intersections in the pathogenesis of NAFLD and atherosclerosis. *Clin Sci (Lond)* 2008; Jul;115(1):1-12.

16. Targher G, Marra F, Marchesini G. Increased risk of cardiovascular disease in non-alcoholic fatty liver disease: causal effect or epiphenomenon?. *Diabetologia* 2008; Nov;51(11):1947-53.

17. Targher G, Bertolini L, Scala L, Zoppini G, Zenari L, Falezza G. Non-alcoholic hepatic steatosis and its relation to increased plasma biomarkers of inflammation and

endothelial dysfunction in non-diabetic men. Role of visceral adipose tissue. *Diabet Med* 2005; Oct;22(10):1354-8.

18. Brea A, Mosquera D, Martin E, Arizti A, Cordero JL, Ros E. Nonalcoholic fatty liver disease is associated with carotid atherosclerosis: a case-control study. *Arterioscler Thromb Vasc Biol* 2005; May;25(5):1045-50.

19. Sookoian S, Pirola CJ. Non-alcoholic fatty liver disease is strongly associated with carotid atherosclerosis: a systematic review. *J Hepatol* 2008; Oct;49(4):600-7.

20. Fraser A, Harris R, Sattar N, Ebrahim S, Smith GD, Lawlor DA. Gamma-glutamyltransferase is associated with incident vascular events independently of alcohol intake: analysis of the British Women's Heart and Health Study and Meta-Analysis. *Arterioscler Thromb Vasc Biol* 2007; Dec;27(12):2729-35.

21. Rantala AO, Kauma H, Lilja M, Savolainen MJ, Reunanen A, Kesaniemi YA. Prevalence of the metabolic syndrome in drug-treated hypertensive patients and control subjects. *J Intern Med* 1999; Feb;245(2):163-74.

22. Yajima Y, Ohta K, Narui T, Abe R, Suzuki H, Ohtsuki M. Ultrasonographical diagnosis of fatty liver: significance of the liver-kidney contrast. *Tohoku J Exp Med* 1983; Jan;139(1):43-50.

23. Sampi M, Veneskoski M, Ukkola O, Kesaniemi YA, Horkko S. High plasma immunoglobulin (Ig) A and low IgG antibody titers to oxidized low-density lipoprotein are associated with markers of glucose metabolism. *J Clin Endocrinol Metab* 2010; May;95(5):2467-75.

24. Pisto P, Ukkola O, Santaniemi M, Kesaniemi YA. Plasma adiponectin--an independent indicator of liver fat accumulation. *Metabolism* 2011; Nov;60(11):1515-20.

25. Santaniemi M., Ukkola O., Malo E., Bloigu R., Kesaniemi YA. Metabolic syndrome in the prediction of cardiovascular events: The potential additive role of hsCRP and adiponectin. *Eur J Prev Cardiol* 2013; Jun;20.

26. Pajunen P, Jousilahti P, Borodulin K, Harald K, Tuomilehto J, Salomaa V. Body fat measured by a near-infrared interactance device as a predictor of cardiovascular events: the FINRISK'92 cohort. *Obesity (Silver Spring)* 2011; Apr;19(4):848-52.

27. Katz A, Nambi SS, Mather K, Baron AD, Follmann DA, Sullivan G, et al. Quantitative insulin sensitivity check index: a simple, accurate method for assessing insulin sensitivity in humans. *J Clin Endocrinol Metab* 2000; Jul;85(7):2402-10.

28. Kauma H, Savolainen MJ, Rantala AO, Lilja M, Kervinen K, Reunanen A, et al. Apolipoprotein E phenotype determines the effect of alcohol on blood pressure in middle-aged men. *Am J Hypertens* 1998; Nov;11(11 Pt 1):1334-43.

29. Bessembinders K, Wielders J, van de Wiel A. Severe hypertriglyceridemia influenced by alcohol (SHIBA). *Alcohol Alcohol* 2011; Mar-Apr;46(2):113-6.

30. Hamaguchi M, Kojima T, Takeda N, Nagata C, Takeda J, Sarui H, et al. Nonalcoholic fatty liver disease is a novel predictor of cardiovascular disease. *World J Gastroenterol* 2007; Mar 14;13(10):1579-84.

31. Stepanova M, Younossi ZM. Independent Association Between Nonalcoholic Fatty Liver Disease and Cardiovascular Disease in the US Population. *Clin Gastroenterol Hepatol* 2012; Jun;10(6):646-50.

32. Targher G, Bertolini L, Poli F, Rodella S, Scala L, Tessari R, et al. Nonalcoholic fatty liver disease and risk of future cardiovascular events among type 2 diabetic patients. ***Diabetes*** **2005; Dec;54(12):3541-6.**

33. Ghouri N, Preiss D, Sattar N. Liver enzymes, nonalcoholic fatty liver disease, and incident cardiovascular disease: a narrative review and clinical perspective of prospective data. ***Hepatology*** **2010; Sep;52(3):1156-61.**

34. Dunn W, Xu R, Wingard DL, Rogers C, Angulo P, Younossi ZM, et al. Suspected nonalcoholic fatty liver disease and mortality risk in a population-based cohort study. ***Am J Gastroenterol*** **2008; Sep;103(9):2263-71.**

35. Ruttmann E, Brant LJ, Concin H, Diem G, Rapp K, Ulmer H, et al. Gamma-glutamyltransferase as a risk factor for cardiovascular disease mortality: an epidemiological investigation in a cohort of 163,944 Austrian adults. ***Circulation*** **2005; Oct 4;112(14):2130-7.**

36. Soderberg C, Stal P, Askling J, Glaumann H, Lindberg G, Marmur J, et al. Decreased survival of subjects with elevated liver function tests during a 28-year follow-up. ***Hepatology*** **2010; Feb;51(2):595-602.**

37. Ekstedt M, Franzen LE, Mathiesen UL, Thorelius L, Holmqvist M, Bodemar G, et al. Long-term follow-up of patients with NAFLD and elevated liver enzymes. ***Hepatology*** **2006; Oct;44(4):865-73.**

38. Bhatia LS, Curzen NP, Calder PC, Byrne CD. Non-alcoholic fatty liver disease: a new and important cardiovascular risk factor?. ***Eur Heart J*** **2012; May;33(10):1190-200.**

39. Beckman JA, Creager MA, Libby P. Diabetes and atherosclerosis: epidemiology, pathophysiology, and management. *JAMA* 2002; May 15;287(19):2570-81.

40. Joseph P, Teo K. Optimal medical therapy, lifestyle intervention, and secondary prevention strategies for cardiovascular event reduction in ischemic heart disease. *Curr Cardiol Rep* 2011; Aug;13(4):287-95.

41. Dam-Larsen S, Franzmann M, Andersen IB, Christoffersen P, Jensen LB, Sorensen TI, et al. Long term prognosis of fatty liver: risk of chronic liver disease and death. *Gut* 2004; May;53(5):750-5.

42. Calori G, Lattuada G, Ragogna F, Garancini MP, Crosignani P, Villa M, et al. Fatty liver index and mortality: the Cremona study in the 15th year of follow-up. *Hepatology* 2011; Jul;54(1):145-52.

43. Joy D, Thava VR, Scott BB. Diagnosis of fatty liver disease: is biopsy necessary?. *Eur J Gastroenterol Hepatol* 2003; May;15(5):539-43.

44. Dasarathy S, Dasarathy J, Khiyami A, Joseph R, Lopez R, McCullough AJ. Validity of real time ultrasound in the diagnosis of hepatic steatosis: a prospective study. *J Hepatol* 2009; Dec;51(6):1061-7.

45. Szczepaniak LS, Nurenberg P, Leonard D, Browning JD, Reingold JS, Grundy S, et al. Magnetic resonance spectroscopy to measure hepatic triglyceride content: prevalence of hepatic steatosis in the general population. *Am J Physiol Endocrinol Metab* 2005; Feb;288(2):E462-8.

503

Grade of liver bightness	0 (n=720)	1 (n=124)	2 (n=144)	p	p (0-1)	p (1-2)	p (0-2)
Age (years)	50.9 (6.0)	51.9 (6.1)	51.5 (5.5)	NS	NS	NS	NS
Males	44.3 % (n=319)	65.3 % (n=81)	59.9 % (n=82)	< 0.001	-	-	-
Hypertensives	41.4 % (n=298)	66.1 % (n=82)	71.5 % (n=103)	< 0.001	-	-	-
BMI (kg/m²)	26.4 (3.9)	29.8 (5.0)	31.9 (4.9)	< 0.001	< 0.001	< 0.001	< 0.001
Waist circumference (cm)	86.8 (11.9)	97.7 (12.0)	102.3 (11.8)	< 0.001	< 0.001	< 0.01	< 0.001
Smoking (pack years)	10.6 (13.3)	14.3 (14.9)	14.0 (14.6)	< 0.05	NS	NS	NS
Alcohol consumption (g/week)	51.1 (83.0)	95.1 (117.0)	82.6 (105.1)	< 0.01	< 0.05	NS	NS
Total serum cholesterol (mmol/L)	5.6 (1.0)	5.8 (1.1)	5.8 (1.1)	NS	NS	NS	NS
LDL (mmol/L)	3.5 (0.9)	3.7 (1.1)	3.5 (0.9)	NS	NS	NS	NS
Triglycerides (mmol/L)	1.4 (0.8)	1.9 (0.8)	2.2 (1.4)	< 0.001	< 0.001	< 0.05	< 0.001
Systolic blood pressure	145.2 (21.5)	152.7 (20.3)	157.1 (22.2)	< 0.001	< 0.01	NS	< 0.001
Fasting insulin (mmol/L)	10.8 (7.7)	18.2 (10.3)	23.8 (17.6)	< 0.001	< 0.001	< 0.001	< 0.001

Fasting glucose (mmol/L)	4.4 (0.7)	5.0 (1.4)	6.1 (2.8)	< 0.001	< 0.001	< 0.001	< 0.001
QUICKI	0.6 (0.1)	0.6 (0.1)	0.5 (0.1)	< 0.001	< 0.001	< 0.001	< 0.001
hs-CRP (ng/mL)	3039.4 (6758.3)	3981.4 (6068.2)	6122.0 (6630.8)	< 0.001	< 0.001	< 0.01	< 0.001
ALT U/L	26.2 (15.5)	37.8 (17.1)	55.4 (37.7)	< 0.001	< 0.001	< 0.001	< 0.001
GGT U/L	35.1 (33.5)	69.7 (116.3)	76.8 (92.4)	< 0.001	< 0.001	< 0.01	< 0.001
Anti-hypertensive treatment	43.6% (n=314)	66.9% (n=83)	72.9% (n=105)	< 0.001	-	-	-
Lipid-lowering treatment	2.2% (n=16)	1.6% (n=2)	6.2% (n=9)	< 0.05	-	-	-
Hypoglycaemic drug	1.1% (n=8)	1.6% (n=2)	10.4% (n=15)	< 0.001	-	-	-
Type 2 diabetes	2.4% (n=17)	12.1% (n=15)	36.8% (n=53)	< 0.001	-	-	-

Table 1. Baseline characteristics of the study group as means (standard deviations) or percentages. N= number of subjects. ALT, alanine aminotransferase, BMI, body mass index, GGT, gamma-glutamyltransferase, hs-CRP, high-sensitivity C-reactive protein, LDL, low-density lipoprotein, QUICKI, quantitative insulin sensitivity check index.

	Model 1	Model 2	Model 3	Model 4	Model 5
Moderate fat content	1.51 (0.99-2.29)	1.44 (0.93-2.23)	1.31 (0.84-2.05)	1.30 (0.84-2.01)	1.49 (0.99-2.26)
Severe fat content	1.92 (1.32-2.80)**	1.74 (1.16-2.63) **	1.49 (0.97-2.30)	1.43 (0.93-2.18)	1.76 (1.21- 2.56) **
Study group	1.34 (0.98-1.85)	1.29 (0.92-1.80)	1.28 (0.92-1.78)		
Age	1.06 (1.03-1.09)***	1.05(1.02-1.08)**	1.05 (1.02-1.08)**	1.05 (1.02-1.07)**	1.05 (1.02-1.08) **
Gender	2.39 (1.71-3.34)*	1.91 (1.34-2.71)***	1.80 (1.26-2.57)**	1.83 (1.29-2.60) **	1.92 (1.36-2.72) ***
LDL-cholesterol		1.17 (0.99-1.39)	1.15 (0.97-1.37)		
Smoking (pack-years)		1.02 (1.01-1.03)***	1.02 (1.01-1.03)***	1.02 (1.01-1.03)***	1.02 (1.01-1.03) ***
Alcohol consumption (gr1)		0.93 (0.59-1.45)	0.92(0.59-1.44)		
Alcohol consumption (gr2)		0.84 (0.44-1.60)	0.81(0.42-1.54)		
Systolic blood pressure		1.01 (1.00-1.02)**	1.01 (1.00-1.02)*	1.01 (1.00-1.02)**	1.01 (1.00-1.02) **
Body mass index		0.99 (0.96-1.03)	0.97 (0.93-1.01)		
QUICKI			0.12 (0.02-0.90)*	0.16 (0.03-0.99)*	

Table 2. Multivariate analysis for cardiovascular events with different degrees of adjustments (Cox regression analysis). CVD event occurred in 13.5% of the subjects with no fat in the liver (97/720), 24.2% (30/124) of subjects having moderate liver fat content and 29.2% (42/144) of the subjects having severe fatty liver. Hazard ratios with 95% confidence interval with different degrees of adjustments are presented. Alcohol consumption was divided into groups (reference group: less than 1g/week in men and women, group 1: less than 210g/week in men and less than 140 g/week in women, group 2: more than 210g/week in men and more than 140g/week in women). Model 1: adjustment for study group, age and gender. Model 2: further adjustments for LDL-cholesterol, smoking, alcohol consumption, systolic blood pressure and body mass index. Model 3: further adjustment for QUICKI. Model 4: adjustments with statistically significant covariates. Model 5: adjustments with statistically significant covariates without QUICKI. LDL, low-density lipoprotein, QUICKI, quantitative insulin sensitivity check index. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

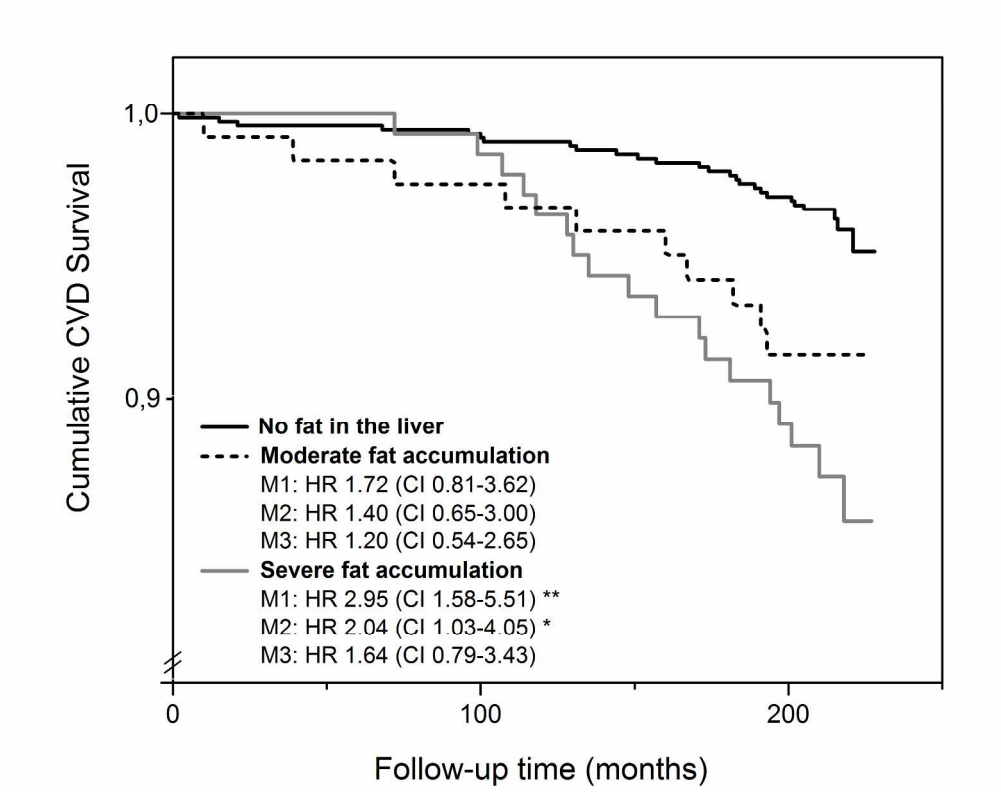
Grade of liver bightness	Total	0 (n=720)	1 (n=124)	2 (n=144)	p
Non-fatal events					
CVD	11.6% (115)	9.9% (71)	16.1% (20)	16.7% (24)	< 0.05
CHD	7.8% (77)	6.5% (47)	11.3% (14)	11.1% (16)	NS
Stroke	5.0% (49)	4.2% (30)	8.1% (10)	6.2% (9)	NS
Fatal events					
CVD	5.5% (54)	3.6% (26)	8.1% (10)	12.5% (18)	< 0.001
CHD	4.8% (47)	3.2% (23)	7.3% (9)	10.4% (15)	< 0.01
Stroke	0.8% (8)	0.6% (4)	0.8% (1)	2.1% (3)	NS

Table 3. CVD, CHD and stroke follow-up data of the study group as percentages (number of events). CVD included a major CHD event and stroke (excluding subarachnoid hemorrhage) - whichever of these happened first. N=number of subjects. CHD, coronary heart disease, CVD, cardiovascular disease.

Final model	Cardiovascular event c-index (95% CI)	Binary R^2
Model 3	0.729 (0.706-0.776)	0.153
Model 4	0.720 (0.689-0.763)	0.144
Model 5	0.717 (0.686-0.758)	0.138
Model 1	0.698 (0.656-0.742)	0.133

Table 4. Multivariate analysis for cardiovascular events (logistic regression analysis). Cardiovascular disease risk factors have been removed from the models step by step. Model 3 included liver brightness, study group, age, gender, smoking, alcohol consumption, systolic blood pressure, LDL-cholesterol level, body mass index and QUICKI. Model 4 included liver brightness, age, gender, smoking, blood pressure and QUICKI. Model 5 included liver brightness, age, gender, smoking, blood pressure. Model 1 included liver brightness, study group, age and gender. C-index with confidence intervals obtained from 250 bootstrap resamplings and binary R^2 was used. LDL, low-density lipoprotein, QUICKI, quantitative insulin sensitivity check index.

553



Title: Kaplan Meier cumulative survival rates censored for cardiovascular death in subjects with no fat in the liver, moderate fat content and severe fat content.

CVD was the cause of death in 3.6% of the subjects (26/720) with non-fatty liver and 8.1% of the subjects (10/124) with moderate liver fat content, while 12.5% of the subjects with severe fatty liver (18/144). Cox regression analysis is used for adjustments. M1 (Model 1): adjusted for study group, age and gender. M2 (Model 2): further adjustments for smoking, alcohol consumption, systolic blood pressure, LDL-cholesterol level and body mass index. M3 (Model 3): further adjustment for QUICKI. CVD, cardiovascular disease, CI, confidence interval, HR, hazard ratio, QUICKI, quantitative insulin sensitivity check index. ** $p < 0.01$, * $p < 0.05$.

247x209mm (300 x 300 DPI)

STROBE Statement—Items to be included when reporting observational studies in a conference abstract

Item	Recommendation
Title	Indicate the study's design with a commonly used term in the title (e.g cohort, case-control, cross sectional) **page 1**
Authors	Contact details for the corresponding author **page 1**
Study design	Description of the study design (e.g cohort, case-control, cross sectional) **page 6**
Objective	Specific objectives or hypothesis **page 5**
Methods	**page 5**
Setting	Description of setting, follow-up dates or dates at which the outcome events occurred or at which the outcomes were present, as well as any points or ranges on other time scales for the outcomes (e.g., prevalence at age 18, 1998-2007). **page 7**
Participants	*Cohort study*—Give the most important eligibility criteria, and the most important sources and methods of selection of participants. Describe briefly the methods of follow-up **page 6** *Case-control study*—Give the major eligibility criteria, and the major sources and methods of case ascertainment and control selection *Cross-sectional study*—Give the eligibility criteria, and the major sources and methods of selection of participants
	Cohort study—For matched studies, give matching and number of exposed and unexposed **page 7** *Case-control study*—For matched studies, give matching criteria and the number of controls per case
Variables	Clearly define primary outcome for this report. **page 10**
Statistical methods	Describe statistical methods, including those used to control for confounding **page 9**
Results	
Participants	Report Number of participants at the beginning and end of the study **page 7**
Main results	Report estimates of associations. If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period Report appropriate measures of variability and uncertainty (e.g., odds ratios with confidence intervals **page 10**
Conclusions	General interpretation of study results **page 12**

BMJ open

Fatty liver predicts the risk for cardiovascular events in middle-aged population: a population-based cohort study

Journal:	*BMJ Open*
Manuscript ID:	bmjopen-2014-004973.R1
Article Type:	Research
Date Submitted by the Author:	21-Feb-2014
Complete List of Authors:	Pisto, Pauliina; Clinical medicine, Internal medicine Santaniemi, Merja; Institute of Clinical Medicine, Department of Internal Medicine Bloigu, Risto; Medical Informatics and Statistics Research Group, Ukkola, Olavi; Institute of Clinical Medicine, Department of Internal Medicine Kesäniemi, Antero; Institute of Clinical Medicine, Department of Internal Medicine
Primary Subject Heading:	Diabetes and endocrinology
Secondary Subject Heading:	Cardiovascular medicine
Keywords:	Adult cardiology < CARDIOLOGY, Coronary heart disease < CARDIOLOGY, General diabetes < DIABETES & ENDOCRINOLOGY, Hepatology < INTERNAL MEDICINE

Fatty liver predicts the risk for cardiovascular events in middle-aged population: a population-based cohort study

Pauliina Pisto[1], Merja Santaniemi[1], Risto Bloigu[2], Olavi Ukkola[1], Y. Antero Kesäniemi[1]

[1]Institute of Clinical Medicine, Department of Internal Medicine and Biocenter Oulu, University of Oulu, and Clinical Research Center, Oulu University Hospital, Oulu, Finland, P.O. Box 20, 90029 OYS, Finland

[2] Medical Informatics and Statistics Research Group, University of Oulu, Aapistie 7, P.O. Box 5000, 90014 Oulu, Finland

Contact information: Pauliina Pisto, corresponding author

University of Oulu

Institute of Clinical Medicine

Department of Internal medicine

P.O. Box 5000, 90014 Oulu, Finland

Tel.: +35885376310

Fax: +35885376318

E-mail: pauliina.pisto@oulu.fi

Word count: 6142

Keywords: coronary disease, fatty liver, insulin resistance, risk factors, stroke

ABSTRACT

Objective: We investigated if the differences in liver fat content would predict the development of non-fatal and fatal atherosclerotic endpoints (coronary heart disease and stroke).

Design, setting and participants: Our study group is a population-based, randomly recruited cohort (OPERA), initiated in 1991. The cohort consisted of 988 middle-aged Finnish subjects.

Intervention: Total mortality and hospital events were followed up to 2009 based on the registry of the National Institute for Health and Welfare and the National death registry.

Main outcome measure: The severity of hepatic steatosis was measured by ultrasound and divided into three groups (0-2). Cox regression analysis was used in the statistical analysis.

Results: In the follow-up of years 1991-2009, 13.5% of the subjects with non-fatty liver, 24.2% of subjects having moderate liver fat content and 29.2% of the subjects having severe fatty liver experienced a cardiovascular event during the follow-up time ($p < 0.001$). Severe liver fat content predicted the risk for future risk of cardiovascular event even when adjusted for age, gender and study group (HR 1.92, CI 1.32-2.80, $p < 0.01$). When further adjustments for smoking, alcohol consumption, LDL-cholesterol, BMI and systolic blood pressure were conducted, the risk still remained statistically significant (HR 1.74, CI 1.16-2.63, $p < 0.01$). Statistical significance disappeared with further adjustment for QUICKI.

Conclusions: Liver fat content increases the risk of future cardiovascular disease event in long-term follow-up but it is seems to be dependent on insulin sensitivity.

Article focus

1 To investigate if the differences in liver fat content predict the risk for development of fatal or nonfatal atherosclerotic endpoints such as coronary heart disease and stroke.

Key messages

1 Subjects with ultrasound-diagnosed fatty liver have cardiovascular disease more often compared to the subjects without fat in the liver

2 Severe liver fat content increases the risk of a future cardiovascular event and mortality to cardiovascular disease over the long-term follow-up but it does seem to be dependent on insulin sensitivity

3 Severe fatty liver predicts the risk for overall mortality but the association is dependent on traditional metabolic risk factors

Strengths and limitations of the study

1 This is a follow-up study with a large population-based study group and a very long follow-up time

2 Official registers used in event diagnoses - data is accurate and the classification is systematic

3 Grade of liver brightness was measured by ultrasound, which has a high specificity but low sensitivity

Introduction

Non-alcoholic fatty liver disease (NAFLD) refers to liver disorders such as abnormal fat content, which exists in a spectrum ranging from steatosis with no inflammation to non-alcoholic steatohepatitis (NASH), which can ultimately lead to liver cirrhosis [1]. The prevalence of NAFLD is estimated to range from 20 to 30% of population in Western countries, being the leading cause of liver disorders [2, 3]. It is associated with obesity, type 2 diabetes mellitus (T2DM) and hyperlipidemia [1]. NAFLD is commonly regarded as a hepatic manifestation of the metabolic syndrome and both conditions share several risk factors for cardiovascular disease (CVD) [3, 4].

In 2008, the prevalence of CVD in adults (≥ 20 years) in United States was 36.2% [5]. Every year, 4.3 million subjects die for CVD in Europe causing nearly half of the all deaths (48%) [6]. So-called traditional risk factors for cardiovascular disease are age, gender, smoking, high low-density lipoprotein (LDL) cholesterol concentration, hypertension and diabetes [7]. In addition, total body fatness as well as abdominal fat accumulation increase independently the risk of CVD and insulin resistance is regarded to be an important factor linking visceral adiposity to cardiovascular risk [8]. Adipose tissue is now recognized as a significant endocrine organ as adipocytes and macrophages infiltrating adipocytes secrete a number of bioactive mediators [7]. Adipokines, proinflammatory cytokines and hypofibrinolytic markers may lead to oxidative stress and endothelial dysfunction, finally leading to atherosclerosis [9].

Hepatic steatosis has been discussed as a possible mechanism to explain CVD morbidity and mortality [10]. NAFLD patients have been reported to have higher coronary heart disease (CHD) risk than the general population of the same age and gender [11]. According to previous study, liver dysfunction associated with CVD mortality in men [12] whereas another large study found no association between NAFLD and CVD in general population [13]. In addition, fatty liver did not predict CVD mortality and morbidity in patients with established coronary artery disease [14].

The NAFLD and CVD share several molecular mechanisms [15, 16]. Fatty liver might play a part in the pathogenesis of CVD through the overexpression and systemic release of several inflammatory, hemostatic [17] and oxidative-stress mediators or via contributing to whole-body insulin resistance and atherogenic dyslipidemia [3]. NAFLD has also been reported to be linked with circulatory endothelial dysfunction [4, 14]. Several investigators have reported that NAFLD is associated with coronary artery disease [4, 14] and increased carotid intima-media thickness [18, 19]. Increased gamma-glutamyltransferase (GGT), which may be a marker of NAFLD, has been reported to be associated with stroke [20].

It is known that subjects with fatty liver disease have an increased risk of suffering CVD [4], but whether NAFLD is an independent indicator of cardiovascular disease is still far from clear. Long-term follow-up studies are needed to clarify the correlation between fatty liver and CVD. The aim of our study was to investigate if fatty liver could predict independently the risk for total mortality as well as non-fatal and fatal cardiovascular endpoints with a 19-year follow-up after adjusting for all known conventional risk factors.

Materials and methods

Human subjects

OPERA (Oulu Project Elucidating Risk of Atherosclerosis) is a population-based, epidemiological prospective cohort study designed to address the risk factors and disease end points of atherosclerotic cardiovascular diseases. Selection criteria of the study subjects have been described earlier [21]. In short, a total of 520 men and 525 women participated: 259 control men, 261 hypertensive men, 267 control women and 258 hypertensive women aged 40-59. Hypertensive participants were randomly selected from the national register for reimbursement of the costs of antihypertensive medication. For each hypertensive subject, an age- and sex-matched control subject was randomly selected from the same register. Informed consent in writing was obtained from each patient. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and this study was approved by the Ethical Committee of the Faculty of Medicine, University of Oulu.

Determination of hepatic steatosis

The determination of hepatic steatosis was based on liver-kidney contrast [22] measured with ultrasonography [23] by one trained radiologist with 10 years' experience in abdominal ultrasound examinations. Normal liver parenchyma should be slightly more echogenic (brighter) than the kidney parenchyma. In a case of increased liver echogenicity an ultrasound diagnosis of bright liver was settled. The severity of hepatic steatosis was based on the brightness of the liver and it was classified into three groups ranging from 0 to 2 (0 = normal bright, indicating a

non-fatty liver, 1 = medium bright, a moderate lipid content and 2 = clearly bright, a severe lipid content and fatty liver) [24].

Follow-up

Both the hypertensive and the control men were recruited during December 1990 to May 1992 and the women approximately one year later (n=1045). In total, 1023 subjects had a liver ultrasound result available at baseline. Mortality data were obtained from the National Death Registry and the diagnoses of cardiovascular events were based on the registry of the National Institute for Health and Welfare. The follow-up time ended December 31, 2009 or whenever the first event occurred. Cardiovascular events included fatal and non-fatal endpoints. Subjects with a previous hospital-diagnosed myocardial infarction or stroke (n=41) at baseline were excluded. In total, 988 subjects participated in this part of the study.

CVD included a major CHD event and stroke (excluding subarachnoid hemorrhage, SAH) - whichever of these happened first [25]. The evidence of CHD was based on the following diagnosis: I20.0, I21, I22 [ICD-10, International Statistical Classification of Diseases and Related Health Problems] / 410, 4110 [ICD-8/9] as the main diagnosis (symptom or cause) and I21, I22 [ICD-10] / 410 [ICD-8/9] as a first side diagnosis (symptom or cause) or second side diagnosis (symptom or cause) and third side diagnosis (ICD-8/9 only) or if a subject had undergone coronary artery bypass graft (CABG) surgery or angioplasty. CHD as a cause of death included I20–I25, I46, R96, R98 [ICD-10] / 410-414, 798 (not 7980A) [ICD-8/9] as the underlying cause of death or immediate cause of death and I21 or I22 [ICD-10] / 410 [ICD-8/9] as first to third contributing cause of death. Stroke (excluding SAH) included I61, I63

(not I636), I64 [ICD -10] / 431, 4330A, 4331A, 4339A, 4340A, 4341A, 4349A, 436 [ICD-9] / 431 (except 43101, 43191) 433, 434, 436 [ICD-8] as main diagnosis (symptom or cause) or as a first or second side diagnosis (symptom or cause) or as a third side diagnosis (ICD-8/9 only) or as an underlying cause of death or immediate cause of death or as a first to third contributing cause of death [26].

Laboratory analyses

Waist circumference, body mass index (BMI) and blood pressure were measured as described in previous study [21].

All the laboratory test samples were obtained after an overnight fast. Blood insulin and glucose concentrations were analyzed at 0, 60, and 120 min after administration of 75 g glucose [24]. Insulin sensitivity was assessed using fasting plasma insulin concentrations and a quantitative insulin sensitivity check index (QUICKI) {QUICKI=1/[log (fasting insulin)+log (fasting glucose)]}[27].

Very-low-density lipoprotein (VLDL), high-density lipoprotein (HDL), low-density lipoprotein (LDL) and hs-CRP concentrations [24] as well as alanine aminotransferase (ALT) and GGT levels were measured as described previously [23]. Alcohol consumption and smoking history were determined by validated questionnaires [28]. Alcohol consumption was divided into three groups: 0 (n=161) mean alcohol consumption less than 1g/week in men and women, 1 (n=767) mean consumption less than 210g/week in men and less than 140 g/week in women,

2 (n=76) mean alcohol consumption more than 210g/week in men and more than 140g/week in women. Group 2 designates large-scale alcohol consumers according to the guidelines [29].

Statistical analysis

Statistical analysis was performed by using IBM SPSS Statistics for Windows, Version 20.0 (Armonk, NY: IBM Corp.). Analysis of variance was used to compare the means of the variables measured. Post hoc tests were performed using the Tukey method. Statistical significances between percentages were measured by using χ^2 test. Cumulative survival rates were estimated using Kaplan-Meier method. Cox regression analysis was performed to investigate if liver brightness (fat) could predict the future risk for total mortality, cardiovascular death or hospital events. A p value < 0.05 was regarded as significant.

Skewed variables (smoking, alcohol consumption, fasting insulin, fasting glucose, triglyceride, ALT, GGT concentration, hs-CRP level) were logarithmically transformed to improve normality before analysis of variance. We used three models with progressive degrees of adjustments. Model 1 included study group (subjects with medicine-treated hypertension and their age- and sex-matched controls), age and gender. Model 2 included further adjustments for smoking, alcohol consumption, systolic blood pressure, LDL-cholesterol level and body mass index. Model 3 included further adjustment for QUICKI. We carried out sensitivity analyses: in the analyses of cardiovascular events, we added all covariates one by one and investigated if the hazard ratios (HR) changed or remained stable when further adjustment with one covariate was performed. Model 4 included variables which

were stable and were statistically significant in intermediate phases. Model 5 included stable and significant covariates without QUICKI (Table 2).

C-index was calculated for the model 1, model 3, model 4 and model 5 to assess the discrimination of the risk markers. The analyses were performed in 250 bootstrap resamplings to obtain 95% CI for c-index of each model.

Results

The main baseline characteristics of the study group are shown in Table 1.

Table 1 about here

Incidence of cardiovascular disease

The median follow-up time was 212 (maximum 228) months. During the follow-up time, 13.5% of the subjects with no fat in the liver (97/720), 24.2% (30/124) of subjects having moderate liver fat content and 29.2% (42/144) of the subjects having severe fatty liver experienced a CVD event ($p < 0.001$). CVD was the cause of death in 3.6% of the subjects with non-fatty liver (26/720) and 8.1% of the subjects with moderate liver fat content (10/124), while 12.5% (18/144) of the subjects with severe fatty liver ($p < 0.001$) (Table 3).

Severe liver fat content predicted the risk for future risk of cardiovascular event when adjusted for age, gender and study group (Model 1: HR 1.92, CI 1.32-2.80, $p < 0.01$) (Table 2). When further adjustments were made for smoking, alcohol consumption, LDL-cholesterol, BMI and systolic blood pressure (Model 2: HR 1.74, CI 1.16-2.63), the risk still remained statistically significant ($p < 0.01$). Statistical significance disappeared when further adjustment for QUICKI was performed (Model 3: HR 1.49, CI 0.97-2.30, p=0.071). In the CVD event sensitivity analyses, all covariates were added one by one and it was examined whether the hazard ratios would change or remain stable. After adjusting for the statistically significant variables (including quick index) in the sensitivity analyses, the association between severe fatty liver was no longer significant (Model 4: HR 1.43, CI 0.93-2.18, p=0.10). When QUICKI was not added into Model 5, severe fatty liver did predict the risk for future risk for CVD event (HR 1.76, CI 1.21- 2.56, $p < 0.001$) (Table 2). The c-index decreased when the risk factors were removed from the model (Table 4).

Tables 2, 3 and 4 about here

The future risk of death from CVD in participants with severe fat content was significant when age, gender and study group were added as covariates (Model 1: HR 2.95, CI 1.58-5.51, $p < 0.01$). Even after further adjustments with other conventional risk factors (Model 2: HR 2.04, CI 1.03-4.05), statistical significance remained ($p < 0.05$). When QUICKI was added as the covariate, then significance disappeared (Model 3: HR 1.64, CI 0.79-3.43, NS) (Fig 1.).

Figure 1 about here

Fatty liver and total mortality

In total, 11.9% of the participants not having fatty liver, 18.5% of the subjects having moderate fatty liver and 22.2% of the subjects with severe fatty liver died from all causes ($p < 0.01$). According to Model 1, severe fat content predicted the risk for mortality from all causes when age, gender and study group were added as covariates (HR 1.60, CI 1.05-2.43, $p < 0.05$). The significance disappeared when body mass index was added as a covariate (data not shown).

We performed all Cox regression analyses after excluding the men consuming more than 210 g alcohol and the women drinking more than 140 g alcohol per week. This exclusion did not have any effect on the results (data not shown).

We performed all Cox regression analyses after excluding patients with insulin treated diabetes mellitus (n=9), cortisone treatment at baseline (n=41) and previous diagnosis for liver disease (n=15) (e.g., virus, medications). This exclusion did not have any effect on the results (data not shown).

Discussion

The incidences of non-alcoholic fatty liver disease and cardiovascular disease are continuously increasing in the Western world. The question if NAFLD is only a marker or also an early mediator of cardiovascular disease is still largely unanswered. According to the results of the present study, which had an approximately 19-year follow-up fatty liver does predict the future risk for death from all causes, death from cardiovascular disease and risk of

cardiovascular events. Insulin sensitivity seems to play a more dominant role in the development of cardiovascular events.

Only a few studies have investigated the risk for future cardiovascular risk among subjects with ultrasound-diagnosed fatty liver [30, 31] and larger studies with longer follow-up times are needed. An association between NAFLD and CVD has been reported [3, 30-32] although contrary results also exist [13, 33]. A previous large population-based prospective cohort study found no association between NAFLD and CVD, however they categorized the degree of steatosis as a two level variable: none to mild and moderate to severe [13]. An association between ultrasound-diagnosed fatty liver and CVD has been reported in general population [30] and in subjects with T2DM [32]. Furthermore, liver dysfunction has been reported to associate with CVD mortality [34, 35] and CHD risk [11] in follow-up studies and especially survival of subjects with NASH is reported to be reduced [33, 36, 37]. In the present study, severe fatty liver disease did predict the risk for cardiovascular death but the association seemed to be dependent on insulin sensitivity.

Several earlier studies have used self-reported CVD history which may not be totally reliable. Although earlier studies on the risk for future cardiovascular risk among subjects with fatty liver have performed some adjustments, the full range of well-known CVD risk factors have been rarely considered [33]. We have performed adjustments with all so-called traditional risk factors for cardiovascular disease (i.e. age, gender, smoking, LDL concentration, hypertension, insulin resistance). Previous studies have used biochemical, radiological and histological methodology for NAFLD diagnosis and staging, which leads to a challenging interpretation of the results [35, 38].

This study had an approximately 19-year follow-up time, which is longer than in previous studies [11-14]. When compared to earlier studies [33, 38] this study seems to be the first follow-up study with a large population-based randomly selected study group and a very long follow-up time and ultrasound-diagnosed fatty liver. The diagnosis of cardiovascular events was based on the registry of the National Institute for Health and Welfare and mortality data were obtained from the National Death Registry. The earlier verified FINRISK classification [26] was used to classify the events. Therefore, the reliability of event diagnosis data is accurate and the classification is systematic. All subjects who had myocardial infarction or stroke before baseline were excluded because a history of myocardial infarction is known to increase the risk for recurrent myocardial infarction or cardiovascular death [39] and medication as well as lifestyle secondary prevention strategies are intensive [40].

There are a few follow-up-studies examining whether the fatty liver increases the risk for total mortality [13, 41]. In the present study, severe fatty liver predicted the risk for overall mortality of any causes when age, gender and study group were added covariates, a result in line with an earlier report [42]. In the published literature, NASH rather than simple steatosis has been stated to be linked with decreased overall survival [36] although one study with a large cohort found no association between NAFLD and overall mortality [13]. In our study, the association between severe fatty liver and total mortality disappeared after further adjustment for BMI which means that severe fatty liver is not a strong predictor for overall mortality.

The molecular mechanisms linking fatty liver with CVD have been investigated [10, 16]. Enlarged visceral adipose tissue may explain why NAFLD associates with CVD [16]. In individuals with visceral obesity, insulin resistance may contribute to impaired non-esterified fatty acid (NEFA) metabolism [8] and the increasing NEFA flux to the liver may impair liver metabolism leading to increased glucose metabolism and liver dysfunction [7]. The liver is one of the targets of the resulting systemic abnormalities and the source of several proatherogenic factors [3], such as CRP, fibrinogen, plasminogen activator inhibitor-1 and other inflammatory cytokines [16]. Furthermore, visceral adipose tissue and ectopic fat overexpress factors involved in atherogenesis [16] such as NEFAs and proinflammatory cytokines, for instance interleukin-6 and tumor necrosis factor-α [8] leading to chronic systemic inflammation. In addition, hepatic steatosis leads to overproduction of cholesterol-rich remnant particles [4].

One limitation in this study is that the grade of liver brightness was measured by ultrasound. The invasive diagnostic technique of liver biopsy is regarded as the "golden standard", especially for the diagnosis of NASH [43]. Real time ultrasound using a combination of sonographic findings does have a high specificity but it underestimates the prevalence of hepatic steatosis when there is less than 20 % fat [44]. Today, magnetic resonance spectroscopy is regarded as the best method for the quantification of liver fat, but this method is limited due to its availability [45]. Unfortunately quantitative measurement of liver fat by ultrasound is subject to several limitations compared to more validated and standardized methods for diagnosing NAFLD and the analysis of intra-observer reproducibility could have been more accurate in the present study. Nonetheless, the noninvasive ultrasound method was chosen because taking liver biopsies from large groups of symptomless subjects would have been ethically unjustifiable and magnetic resonance spectroscopy was not available at the baseline.

The OPERA study group consists of subjects with drug-treated hypertension and randomly selected sex- and age-matched controls. Study group was added as a covariate to minimize any selection bias.

Conclusions

Severe liver fat content increased the risk of a future cardiovascular event and mortality to cardiovascular disease over the long-term follow-up but it seemed to be dependent on insulin sensitivity. Fatty liver also predicted the risk for overall mortality. However, conventional cardiovascular disease risk factors seemed to play a major role in developing death from all causes. It would be beneficial to investigate larger cohorts and follow-up studies in order to validate this result.

Figure legend

Title: Kaplan Meier cumulative survival rates censored for cardiovascular death in subjects with no fat in the liver, moderate fat content and severe fat content.

CVD was the cause of death in 3.6% of the subjects (26/720) with non-fatty liver and 8.1% of the subjects (10/124) with moderate liver fat content, while 12.5% of the subjects with severe fatty liver (18/144). Cox regression analysis is used for adjustments. M1 (Model 1): adjusted for study group, age and gender. M2 (Model 2): further adjustments for smoking, alcohol consumption, systolic blood pressure, LDL-cholesterol level and body mass index. M3 (Model 3): further adjustment for QUICKI. CVD, cardiovascular disease, CI, confidence

interval, HR, hazard ratio, QUICKI, quantitative insulin sensitivity check index. ** $p < 0.01$, * $p < 0.05$.

Acknowledgements

The authors thank Markku Päivänsalo, MD, PhD, for the expert liver ultrasound examinations and Ms Saija Kortetjärvi, Ms Liisa Mannermaa, and Ms Leena Ukkola for the excellent technical assistance. M.Sc. Elina Malo and M.Sc. Meiju Saukko are thanked for the cooperation in organizing cardiovascular event and mortality data.

Financial support: This study was supported by the Finnish Foundation for Cardiovascular Research, dated 16 Apr, 2012.

Contributor statement: All authors fulfill all three of the ICMJE guidelines for authorship

Pauliina Pisto: Data acquisition, statistical analysis and interpretation of data, manuscript writing, final approval of the version to be published

Merja Santaniemi: Data acquisition, statistical analysis and data interpretation, critical revision of the manuscript, final approval of the version to be published

Risto Bloigu: Data analysis, interpretation of data, critical revision of the manuscript, final approval of the version to be published

Olavi Ukkola: Study design, data acquisition, data interpretation, critical revision of the manuscript, final approval of the version to be published

Y.A Kesäniemi: Study design, data acquisition, data interpretation, critical revision of the manuscript, final approval of the version to be published

Disclosure summary: Authors report no conflict of interests.

Data sharing statement: Extra data is available by emailing pauliina.pisto(at)oulu.fi

Competing Interests: None

References

1. Angulo P. Nonalcoholic fatty liver disease. *N Engl J Med* 2002; Apr 18;346(16):1221-31.

2. Armstrong MJ, Houlihan DD, Bentham L, et al. Presence and severity of non-alcoholic fatty liver disease in a large prospective primary care cohort. *J Hepatol* 2012; Jan;56(1):234-40.

3. Targher G, Day CP, Bonora E. Risk of cardiovascular disease in patients with nonalcoholic fatty liver disease. *N Engl J Med* 2010; Sep 30;363(14):1341-50.

4. Targher G, Arcaro G. Non-alcoholic fatty liver disease and increased risk of cardiovascular disease. *Atherosclerosis* 2007; Apr;191(2):235-40.

5. Roger VL, Go AS, Lloyd-Jones DM, et al. Heart disease and stroke statistics--2011 update: a report from the American Heart Association. *Circulation* 2011; Feb 1;123(4):e18-e209.

6. Allender S, Scarborough P, Peto V, et al. European cardiovascular disease statistics, 2008 ed. European Heart Network; 2008.

7. Despres JP, Lemieux I. Abdominal obesity and metabolic syndrome. *Nature* 2006; Dec 14;444(7121):881-7.

8. Van Gaal LF, Mertens IL, De Block CE. Mechanisms linking obesity with cardiovascular disease. *Nature* 2006; Dec 14;444(7121):875-80.

9. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med* 2005; Apr 21;352(16):1685-95.

10. Bhatia LS, Curzen NP, Byrne CD. Nonalcoholic fatty liver disease and vascular risk. *Curr Opin Cardiol* 2012; Jul;27(4):420-8.

11. Treeprasertsuk S, Leverage S, Adams LA, et al. The Framingham risk score and heart disease in nonalcoholic fatty liver disease. *Liver Int* 2012; Jul;32(6):945-50.

12. Haring R, Wallaschofski H, Nauck M, et al. Ultrasonographic hepatic steatosis increases prediction of mortality risk from elevated serum gamma-glutamyl transpeptidase levels. *Hepatology* 2009; Nov;50(5):1403-11.

13. Lazo M, Hernaez R, Bonekamp S, et al. Non-alcoholic fatty liver disease and mortality among US adults: prospective cohort study. *BMJ* 2011; Nov 18;343:d6891.

14. Wong VW, Wong GL, Yip GW, et al. Coronary artery disease and cardiovascular outcomes in patients with non-alcoholic fatty liver disease. *Gut* 2011; Dec;60(12):1721-7.

15. Loria P, Lonardo A, Targher G. Is liver fat detrimental to vessels?: intersections in the pathogenesis of NAFLD and atherosclerosis. *Clin Sci (Lond)* 2008; Jul;115(1):1-12.

16. Targher G, Marra F, Marchesini G. Increased risk of cardiovascular disease in non-alcoholic fatty liver disease: causal effect or epiphenomenon?. *Diabetologia* 2008; Nov;51(11):1947-53.

17. Targher G, Bertolini L, Scala L, et al. Non-alcoholic hepatic steatosis and its relation to increased plasma biomarkers of inflammation and endothelial dysfunction in non-diabetic men. Role of visceral adipose tissue. *Diabet Med* 2005; Oct;22(10):1354-8.

18. Brea A, Mosquera D, Martin E, et al. Nonalcoholic fatty liver disease is associated with carotid atherosclerosis: a case-control study. *Arterioscler Thromb Vasc Biol* 2005; May;25(5):1045-50.

19. Sookoian S, Pirola CJ. Non-alcoholic fatty liver disease is strongly associated with carotid atherosclerosis: a systematic review. *J Hepatol* 2008; Oct;49(4):600-7.

20. Fraser A, Harris R, Sattar N, et al. Gamma-glutamyltransferase is associated with incident vascular events independently of alcohol intake: analysis of the British Women's Heart and Health Study and Meta-Analysis. *Arterioscler Thromb Vasc Biol* 2007; Dec;27(12):2729-35.

21. Rantala AO, Kauma H, Lilja M, et al. Prevalence of the metabolic syndrome in drug-treated hypertensive patients and control subjects. *J Intern Med* 1999; Feb;245(2):163-74.

22. Yajima Y, Ohta K, Narui T, et al. Ultrasonographical diagnosis of fatty liver: significance of the liver-kidney contrast. *Tohoku J Exp Med* 1983; Jan;139(1):43-50.

23. Sampi M, Veneskoski M, Ukkola O, et al. High plasma immunoglobulin (Ig) A and low IgG antibody titers to oxidized low-density lipoprotein are associated with markers of glucose metabolism. *J Clin Endocrinol Metab* 2010; May;95(5):2467-75.

24. Pisto P, Ukkola O, Santaniemi M, et al. Plasma adiponectin--an independent indicator of liver fat accumulation. *Metabolism* 2011; Nov;60(11):1515-20.

25. Santaniemi M., Ukkola O., Malo E., et al. Metabolic syndrome in the prediction of cardiovascular events: The potential additive role of hsCRP and adiponectin. *Eur J Prev Cardiol* 2013; Jun;20.

26. Pajunen P, Jousilahti P, Borodulin K, et al. Body fat measured by a near-infrared interactance device as a predictor of cardiovascular events: the FINRISK'92 cohort. *Obesity (Silver Spring)* 2011; Apr;19(4):848-52.

27. Katz A, Nambi SS, Mather K, et al. Quantitative insulin sensitivity check index: a simple, accurate method for assessing insulin sensitivity in humans. *J Clin Endocrinol Metab* 2000; Jul;85(7):2402-10.

28. Kauma H, Savolainen MJ, Rantala AO, et al. Apolipoprotein E phenotype determines the effect of alcohol on blood pressure in middle-aged men. *Am J Hypertens* 1998; Nov;11(11 Pt 1):1334-43.

29. Bessembinders K, Wielders J, van de Wiel A. Severe hypertriglyceridemia influenced by alcohol (SHIBA). *Alcohol Alcohol* 2011; Mar-Apr;46(2):113-6.

30. Hamaguchi M, Kojima T, Takeda N, et al. Nonalcoholic fatty liver disease is a novel predictor of cardiovascular disease. *World J Gastroenterol* 2007; Mar 14;13(10):1579-84.

31. Stepanova M, Younossi ZM. Independent Association Between Nonalcoholic Fatty Liver Disease and Cardiovascular Disease in the US Population. *Clin Gastroenterol Hepatol* 2012; Jun;10(6):646-50.

32. Targher G, Bertolini L, Poli F, et al. Nonalcoholic fatty liver disease and risk of future cardiovascular events among type 2 diabetic patients. *Diabetes* 2005; Dec;54(12):3541-6.

33. Ghouri N, Preiss D, Sattar N. Liver enzymes, nonalcoholic fatty liver disease, and incident cardiovascular disease: a narrative review and clinical perspective of prospective data. *Hepatology* 2010; Sep;52(3):1156-61.

34. Dunn W, Xu R, Wingard DL, et al. Suspected nonalcoholic fatty liver disease and mortality risk in a population-based cohort study. *Am J Gastroenterol* 2008; Sep;103(9):2263-71.

35. Ruttmann E, Brant LJ, Concin H, et al. Gamma-glutamyltransferase as a risk factor for cardiovascular disease mortality: an epidemiological investigation in a cohort of 163,944 Austrian adults. *Circulation* 2005; Oct 4;112(14):2130-7.

36. Soderberg C, Stal P, Askling J, et al. Decreased survival of subjects with elevated liver function tests during a 28-year follow-up. *Hepatology* 2010; Feb;51(2):595-602.

37. Ekstedt M, Franzen LE, Mathiesen UL, et al. Long-term follow-up of patients with NAFLD and elevated liver enzymes. *Hepatology* 2006; Oct;44(4):865-73.

38. Bhatia LS, Curzen NP, Calder PC, et al. Non-alcoholic fatty liver disease: a new and important cardiovascular risk factor?. *Eur Heart J* 2012; May;33(10):1190-200.

39. Beckman JA, Creager MA, Libby P. Diabetes and atherosclerosis: epidemiology, pathophysiology, and management. *JAMA* 2002; May 15;287(19):2570-81.

40. Joseph P, Teo K. Optimal medical therapy, lifestyle intervention, and secondary prevention strategies for cardiovascular event reduction in ischemic heart disease. *Curr Cardiol Rep* 2011; Aug;13(4):287-95.

41. Dam-Larsen S, Franzmann M, Andersen IB, et al. Long term prognosis of fatty liver: risk of chronic liver disease and death. *Gut* 2004; May;53(5):750-5.

42. Calori G, Lattuada G, Ragogna F, et al. Fatty liver index and mortality: the Cremona study in the 15th year of follow-up. *Hepatology* 2011; Jul;54(1):145-52.

43. Joy D, Thava VR, Scott BB. Diagnosis of fatty liver disease: is biopsy necessary?. *Eur J Gastroenterol Hepatol* 2003; May;15(5):539-43.

44. Dasarathy S, Dasarathy J, Khiyami A, et al. Validity of real time ultrasound in the diagnosis of hepatic steatosis: a prospective study. *J Hepatol* 2009; Dec;51(6):1061-7.

45. Szczepaniak LS, Nurenberg P, Leonard D, et al. Magnetic resonance spectroscopy to measure hepatic triglyceride content: prevalence of hepatic steatosis in the general population. *Am J Physiol Endocrinol Metab* 2005; Feb;288(2):E462-8.

502

Grade of liver bightness	0 (n=720)	1 (n=124)	2 (n=144)	p	p (0-1)	p (1-2)	p (0-2)
Age (years)	50.9 (6.0)	51.9 (6.1)	51.5 (5.5)	NS	NS	NS	NS
Males	44.3 % (n=319)	65.3 % (n=81)	59.9 % (n=82)	< 0.001	-	-	-
Hypertensives	41.4 % (n=298)	66.1 % (n=82)	71.5 % (n=103)	< 0.001	-	-	-
BMI (kg/m²)	26.4 (3.9)	29.8 (5.0)	31.9 (4.9)	< 0.001	< 0.001	< 0.001	< 0.001
Waist circumference (cm)	86.8 (11.9)	97.7 (12.0)	102.3 (11.8)	< 0.001	< 0.001	< 0.01	< 0.001
Smoking (pack years)	10.6 (13.3)	14.3 (14.9)	14.0 (14.6)	< 0.05	NS	NS	NS
Alcohol consumption (g/week)	51.1 (83.0)	95.1 (117.0)	82.6 (105.1)	< 0.01	< 0.05	NS	NS
Total serum cholesterol (mmol/L)	5.6 (1.0)	5.8 (1.1)	5.8 (1.1)	NS	NS	NS	NS
LDL (mmol/L)	3.5 (0.9)	3.7 (1.1)	3.5 (0.9)	NS	NS	NS	NS
Triglycerides (mmol/L)	1.4 (0.8)	1.9 (0.8)	2.2 (1.4)	< 0.001	< 0.001	< 0.05	< 0.001
Systolic blood pressure	145.2 (21.5)	152.7 (20.3)	157.1 (22.2)	< 0.001	< 0.01	NS	< 0.001
Fasting insulin (mmol/L)	10.8 (7.7)	18.2 (10.3)	23.8 (17.6)	< 0.001	< 0.001	< 0.001	< 0.001

Fasting glucose (mmol/L)	4.4 (0.7)	5.0 (1.4)	6.1 (2.8)	< 0.001	< 0.001	< 0.001	< 0.001
QUICKI	0.6 (0.1)	0.6 (0.1)	0.5 (0.1)	< 0.001	< 0.001	< 0.001	< 0.001
hs-CRP (ng/mL)	3039.4 (6758.3)	3981.4 (6068.2)	6122.0 (6630.8)	< 0.001	< 0.001	< 0.01	< 0.001
ALT U/L	26.2 (15.5)	37.8 (17.1)	55.4 (37.7)	< 0.001	< 0.001	< 0.001	< 0.001
GGT U/L	35.1 (33.5)	69.7 (116.3)	76.8 (92.4)	< 0.001	< 0.001	< 0.01	< 0.001
Anti-hypertensive treatment	43.6% (n=314)	66.9% (n=83)	72.9% (n=105)	< 0.001	-	-	-
Lipid-lowering treatment	2.2% (n=16)	1.6% (n=2)	6.2% (n=9)	< 0.05	-	-	-
Hypoglycaemic drug	1.1% (n=8)	1.6% (n=2)	10.4% (n=15)	< 0.001	-	-	-
Type 2 diabetes	2.4% (n=17)	12.1% (n=15)	36.8% (n=53)	< 0.001	-	-	-

Table 1. Baseline characteristics of the study group as means (standard deviations) or percentages. N= number of subjects. ALT, alanine aminotransferase, BMI, body mass index, GGT, gamma-glutamyltransferase, hs-CRP, high-sensitivity C-reactive protein, LDL, low-density lipoprotein, QUICKI, quantitative insulin sensitivity check index.

508

	Model 1	Model 2	Model 3	Model 4	Model 5
Moderate fat content	1.51 (0.99-2.29)	1.44 (0.93-2.23)	1.31 (0.84-2.05)	1.30 (0.84-2.01)	1.49 (0.99-2.26)
Severe fat content	1.92 (1.32-2.80)**	1.74 (1.16-2.63) **	1.49 (0.97-2.30)	1.43 (0.93-2.18)	1.76 (1.21- 2.56) **
Study group	1.34 (0.98-1.85)	1.29 (0.92-1.80)	1.28 (0.92-1.78)		
Age	1.06 (1.03-1.09)***	1.05(1.02-1.08)**	1.05 (1.02-1.08)**	1.05 (1.02-1.07)**	1.05 (1.02-1.08) **
Gender	2.39 (1.71-3.34)*	1.91 (1.34-2.71)***	1.80 (1.26-2.57)**	1.83 (1.29-2.60) **	1.92 (1.36-2.72) ***
LDL-cholesterol		1.17 (0.99-1.39)	1.15 (0.97-1.37)		
Smoking (pack-years)		1.02 (1.01-1.03)***	1.02 (1.01-1.03)***	1.02 (1.01-1.03)***	1.02 (1.01-1.03) ***
Alcohol consumption (gr1)		0.93 (0.59-1.45)	0.92(0.59-1.44)		
Alcohol consumption (gr2)		0.84 (0.44-1.60)	0.81(0.42-1.54)		
Systolic blood pressure		1.01 (1.00-1.02)**	1.01 (1.00-1.02)*	1.01 (1.00-1.02)**	1.01 (1.00-1.02) **
Body mass index		0.99 (0.96-1.03)	0.97 (0.93-1.01)		
QUICKI			0.12 (0.02-0.90)*	0.16 (0.03-0.99)*	

509

510

Table 2. Multivariate analysis for cardiovascular events with different degrees of adjustments (Cox regression analysis). CVD event occurred in 13.5% of the subjects with no fat in the liver (97/720), 24.2% (30/124) of subjects having moderate liver fat content and 29.2% (42/144) of the subjects having severe fatty liver. Hazard ratios with 95% confidence interval with different degrees of adjustments are presented. Alcohol consumption was divided into groups (reference group: less than 1g/week in men and women, group 1: less than 210g/week in men and less than 140 g/week in women, group 2: more than 210g/week in men and more than 140g/week in women). Model 1: adjustment for study group, age and gender. Model 2: further adjustments for LDL-cholesterol, smoking, alcohol consumption, systolic blood pressure and body mass index. Model 3: further adjustment for QUICKI. Model 4: adjustments with statistically significant covariates. Model 5: adjustments with statistically significant covariates without QUICKI. LDL, low-density lipoprotein, QUICKI, quantitative insulin sensitivity check index. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Grade of liver bightness	Total	0 (n=720)	1 (n=124)	2 (n=144)	p
Non-fatal events					
CVD	11.6% (115)	9.9% (71)	16.1% (20)	16.7% (24)	< 0.05
CHD	7.8% (77)	6.5% (47)	11.3% (14)	11.1% (16)	NS
Stroke	5.0% (49)	4.2% (30)	8.1% (10)	6.2% (9)	NS
Fatal events					
CVD	5.5% (54)	3.6% (26)	8.1% (10)	12.5% (18)	< 0.001
CHD	4.8% (47)	3.2% (23)	7.3% (9)	10.4% (15)	< 0.01
Stroke	0.8% (8)	0.6% (4)	0.8% (1)	2.1% (3)	NS

Table 3. CVD, CHD and stroke follow-up data of the study group as percentages (number of events). Statistical significances between percentages were measured by using χ^2 test. CVD included a major CHD event and stroke (excluding subarachnoid hemorrhage) - whichever of these happened first. N=number of subjects. CHD, coronary heart disease, CVD, cardiovascular disease.

Final model	Cardiovascular event c-index (95% CI)	Binary R^2
Model 3	0.729 (0.706-0.776)	0.153
Model 4	0.720 (0.689-0.763)	0.144
Model 5	0.717 (0.686-0.758)	0.138
Model 1	0.698 (0.656-0.742)	0.133

Table 4. Multivariate analysis for cardiovascular events (logistic regression analysis). Cardiovascular disease risk factors have been removed from the models step by step. Model 3 included liver brightness, study group, age, gender, smoking, alcohol consumption, systolic blood pressure, LDL-cholesterol level, body mass index and QUICKI. Model 4 included liver brightness, age, gender, smoking, blood pressure and QUICKI. Model 5 included liver brightness, age, gender, smoking, blood pressure. Model 1 included liver brightness, study group, age and gender. C-index with confidence intervals obtained from 250 bootstrap resamplings and binary R^2 was used. LDL, low-density lipoprotein, QUICKI, quantitative insulin sensitivity check index.

553

Fatty liver predicts the risk for cardiovascular events in middle-aged population: a population-based cohort study

Pauliina Pisto[1], Merja Santaniemi[1], Risto Bloigu[2], Olavi Ukkola[1], Y. Antero Kesäniemi[1]

[1]Institute of Clinical Medicine, Department of Internal Medicine and Biocenter Oulu, University of Oulu, and Clinical Research Center, Oulu University Hospital, Oulu, Finland, P.O. Box 20, 90029 OYS, Finland

[2] Medical Informatics and Statistics Research Group, University of Oulu, Aapistie 7, P.O. Box 5000, 90014 Oulu, Finland

Contact information: Pauliina Pisto, corresponding author

University of Oulu

Institute of Clinical Medicine

Department of Internal medicine

P.O. Box 5000, 90014 Oulu, Finland

Tel.: +35885376310

Fax: +35885376318

E-mail: pauliina.pisto@oulu.fi

Word count: 6142

Keywords: coronary disease, fatty liver, insulin resistance, risk factors, stroke

Financial support: This study was supported by the Finnish Foundation for Cardiovascular Research, dated 16 Apr, 2012.

Disclosure summary: Authors report no conflict of interests.

ABSTRACT

Objective: We investigated if the differences in liver fat content would predict the development of non-fatal and fatal atherosclerotic endpoints (coronary heart disease and stroke).

Design, setting and participants: Our study group is a population-based, randomly recruited cohort (OPERA), initiated in 1991. The cohort consisted of 988 middle-aged Finnish subjects.

Intervention: Total mortality and hospital events were followed up to 2009 based on the registry of the National Institute for Health and Welfare and the National death registry.

Main outcome measure: The severity of hepatic steatosis was measured by ultrasound and divided into three groups (0-2). Cox regression analysis was used in the statistical analysis.

Results: In the follow-up of years 1991-2009, 13.5% of the subjects with non-fatty liver, 24.2% of subjects having moderate liver fat content and 29.2% of the subjects having severe fatty liver experienced a cardiovascular event during the follow-up time ($p < 0.001$). Severe liver fat content predicted the risk for future risk of cardiovascular event even when adjusted for age, gender and study group (HR 1.92, CI 1.32-2.80, $p < 0.01$). When further adjustments for smoking, alcohol consumption, LDL-cholesterol, BMI and systolic blood pressure were conducted, the risk still remained statistically significant (HR 1.74, CI 1.16-2.63, $p < 0.01$). Statistical significance disappeared with further adjustment for QUICKI.

Conclusions: Liver fat content increases the risk of future cardiovascular disease event in long-term follow-up but it is seems to be dependent on insulin sensitivity.

Article focus

1 To investigate if the differences in liver fat content predict the risk for development of fatal or nonfatal atherosclerotic endpoints such as coronary heart disease and stroke.

Key messages

1 Subjects with ultrasound-diagnosed fatty liver have cardiovascular disease more often compared to the subjects without fat in the liver

2 Severe liver fat content increases the risk of a future cardiovascular event and mortality to cardiovascular disease over the long-term follow-up but it does seem to be dependent on insulin sensitivity

3 Severe fatty liver predicts the risk for overall mortality but the association is dependent on traditional metabolic risk factors

Strengths and limitations of the study

1 This is a follow-up study with a large population-based study group and a very long follow-up time

2 Official registers used in event diagnoses - data is accurate and the classification is systematic

3 Grade of liver brightness was measured by ultrasound, which has a high specificity but low sensitivity

Introduction

Non-alcoholic fatty liver disease (NAFLD) refers to liver disorders such as abnormal fat content, which exists in a spectrum ranging from steatosis with no inflammation to non-alcoholic steatohepatitis (NASH), which can ultimately lead to liver cirrhosis [1]. The prevalence of NAFLD is estimated to range from 20 to 30% of population in Western countries, being the leading cause of liver disorders [2, 3]. It is associated with obesity, type 2 diabetes mellitus (T2DM) and hyperlipidemia [1]. NAFLD is commonly regarded as a hepatic manifestation of the metabolic syndrome and both conditions share several risk factors for cardiovascular disease (CVD) [3, 4].

In 2008, the prevalence of CVD in adults (≥ 20 years) in United States was 36.2% [5]. Every year, 4.3 million subjects die for CVD in Europe causing nearly half of the all deaths (48%) [6]. So-called traditional risk factors for cardiovascular disease are age, gender, smoking, high low-density lipoprotein (LDL) cholesterol concentration, hypertension and diabetes [7]. In addition, total body fatness as well as abdominal fat accumulation increase independently the risk of CVD and insulin resistance is regarded to be an important factor linking visceral adiposity to cardiovascular risk [8]. Adipose tissue is now recognized as a significant endocrine organ as adipocytes and macrophages infiltrating adipocytes secrete a number of bioactive mediators [7]. Adipokines, proinflammatory cytokines and hypofibrinolytic markers may lead to oxidative stress and endothelial dysfunction, finally leading to atherosclerosis [9].

Hepatic steatosis has been discussed as a possible mechanism to explain CVD morbidity and mortality [10]. NAFLD patients have been reported to have higher coronary heart disease (CHD) risk than the general population of the same age and gender [11]. According to previous study,

liver dysfunction associated with CVD mortality in men [12] whereas another large study found no association between NAFLD and CVD in general population [13]. In addition, fatty liver did not predict CVD mortality and morbidity in patients with established coronary artery disease [14].

The NAFLD and CVD share several molecular mechanisms [15, 16]. Fatty liver might play a part in the pathogenesis of CVD through the overexpression and systemic release of several inflammatory, hemostatic [17] and oxidative-stress mediators or via contributing to whole-body insulin resistance and atherogenic dyslipidemia [3]. NAFLD has also been reported to be linked with circulatory endothelial dysfunction [4, 14]. Several investigators have reported that NAFLD is associated with coronary artery disease [4, 14] and increased carotid intima-media thickness [18, 19]. Increased gamma-glutamyltransferase (GGT), which may be a marker of NAFLD, has been reported to be associated with stroke [20].

It is known that subjects with fatty liver disease have an increased risk of suffering CVD [4], but whether NAFLD is an independent indicator of cardiovascular disease is still far from clear. Long-term follow-up studies are needed to clarify the correlation between fatty liver and CVD. The aim of our study was to investigate if fatty liver could predict independently the risk for total mortality as well as non-fatal and fatal cardiovascular endpoints with a 19-year follow-up after adjusting for all known conventional risk factors.

Materials and methods

Human subjects

OPERA (Oulu Project Elucidating Risk of Atherosclerosis) is a population-based, epidemiological prospective cohort study designed to address the risk factors and disease end points of atherosclerotic cardiovascular diseases. Selection criteria of the study subjects have been described earlier [21]. In short, a total of 520 men and 525 women participated: 259 control men, 261 hypertensive men, 267 control women and 258 hypertensive women aged 40-59. Hypertensive participants were randomly selected from the national register for reimbursement of the costs of antihypertensive medication. For each hypertensive subject, an age- and sex-matched control subject was randomly selected from the same register. Informed consent in writing was obtained from each patient. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and this study was approved by the Ethical Committee of the Faculty of Medicine, University of Oulu.

Determination of hepatic steatosis

The determination of hepatic steatosis was based on liver-kidney contrast [22] measured with ultrasonography [23] by one trained radiologist with 10 years' experience in abdominal ultrasound examinations. Normal liver parenchyma should be slightly more echogenic (brighter) than the kidney parenchyma. In a case of increased liver echogenicity an ultrasound diagnosis of bright liver was settled. The severity of hepatic steatosis was based on the brightness of the liver and it was classified into three groups ranging from 0 to 2 (0 = normal bright, indicating a non-fatty liver, 1 = medium bright, a moderate lipid content and 2 = clearly bright, a severe lipid content and fatty liver) [24].

Follow-up

Both the hypertensive and the control men were recruited during December 1990 to May 1992 and the women approximately one year later (n=1045). In total, 1023 subjects had a liver ultrasound result available at baseline. Mortality data were obtained from the National Death Registry and the diagnoses of cardiovascular events were based on the registry of the National Institute for Health and Welfare. The follow-up time ended December 31, 2009 or whenever the first event occurred. Cardiovascular events included fatal and non-fatal endpoints. Subjects with a previous hospital-diagnosed myocardial infarction or stroke (n=41) at baseline were excluded. In total, 988 subjects participated in this part of the study.

CVD included a major CHD event and stroke (excluding subarachnoid hemorrhage, SAH) - whichever of these happened first [25]. The evidence of CHD was based on the following diagnosis: I20.0, I21, I22 [ICD-10, International Statistical Classification of Diseases and Related Health Problems] / 410, 4110 [ICD-8/9] as the main diagnosis (symptom or cause) and I21, I22 [ICD-10] / 410 [ICD-8/9] as a first side diagnosis (symptom or cause) or second side diagnosis (symptom or cause) and third side diagnosis (ICD-8/9 only) or if a subject had undergone coronary artery bypass graft (CABG) surgery or angioplasty. CHD as a cause of death included I20–I25, I46, R96, R98 [ICD-10] / 410-414, 798 (not 7980A) [ICD-8/9] as the underlying cause of death or immediate cause of death and I21 or I22 [ICD-10] / 410 [ICD-8/9] as first to third contributing cause of death. Stroke (excluding SAH) included I61, I63 (not I636), I64 [ICD -10] / 431, 4330A, 4331A, 4339A, 4340A, 4341A, 4349A, 436 [ICD-9] / 431 (except 43101, 43191) 433, 434, 436 [ICD-8] as main diagnosis (symptom or cause) or as a first or second side diagnosis (symptom or cause) or as a third side diagnosis (ICD-8/9 only)

or as an underlying cause of death or immediate cause of death or as a first to third contributing cause of death [26].

Laboratory analyses

Waist circumference, body mass index (BMI) and blood pressure were measured as described in previous study [21].

All the laboratory test samples were obtained after an overnight fast. Blood insulin and glucose concentrations were analyzed at 0, 60, and 120 min after administration of 75 g glucose [24]. Insulin sensitivity was assessed using fasting plasma insulin concentrations and a quantitative insulin sensitivity check index (QUICKI) {QUICKI=1/[log (fasting insulin)+log (fasting glucose)]}[27].

Very-low-density lipoprotein (VLDL), high-density lipoprotein (HDL), low-density lipoprotein (LDL) and hs-CRP concentrations [24] as well as alanine aminotransferase (ALT) and GGT levels were measured as described previously [23]. Alcohol consumption and smoking history were determined by validated questionnaires [28]. Alcohol consumption was divided into three groups: 0 (n=161) mean alcohol consumption less than 1g/week in men and women, 1 (n=767) mean consumption less than 210g/week in men and less than 140 g/week in women, 2 (n=76) mean alcohol consumption more than 210g/week in men and more than 140g/week in women. Group 2 designates large-scale alcohol consumers according to the guidelines [29].

Statistical analysis

Statistical analysis was performed by using IBM SPSS Statistics for Windows, Version 20.0 (Armonk, NY: IBM Corp.). Analysis of variance was used to compare the means of the variables measured. Post hoc tests were performed using the Tukey method. Statistical significances between percentages were measured by using χ^2 test. Cumulative survival rates were estimated using Kaplan-Meier method. Cox regression analysis was performed to investigate if liver brightness (fat) could predict the future risk for total mortality, cardiovascular death or hospital events. A p value < 0.05 was regarded as significant.

Skewed variables (smoking, alcohol consumption, fasting insulin, fasting glucose, triglyceride, ALT, GGT concentration, hs-CRP level) were logarithmically transformed to improve normality before analysis of variance. We used three models with progressive degrees of adjustments. Model 1 included study group (subjects with medicine-treated hypertension and their age- and sex-matched controls), age and gender. Model 2 included further adjustments for smoking, alcohol consumption, systolic blood pressure, LDL-cholesterol level and body mass index. Model 3 included further adjustment for QUICKI. We carried out sensitivity analyses: in the analyses of cardiovascular events, we added all covariates one by one and investigated if the hazard ratios (HR) changed or remained stable when further adjustment with one covariate was performed. Model 4 included variables which were stable and were statistically significant in intermediate phases. Model 5 included stable and significant covariates without QUICKI (Table 2).

C-index was calculated for the model 1, model 3, model 4 and model 5 to assess the discrimination of the risk markers. The analyses were performed in 250 bootstrap resamplings to obtain 95% CI for c-index of each model.

Results

The main baseline characteristics of the study group are shown in Table 1.

Table 1 about here

Incidence of cardiovascular disease

The median follow-up time was 212 (maximum 228) months. During the follow-up time, 13.5% of the subjects with no fat in the liver (97/720), 24.2% (30/124) of subjects having moderate liver fat content and 29.2% (42/144) of the subjects having severe fatty liver experienced a CVD event ($p < 0.001$). CVD was the cause of death in 3.6% of the subjects with non-fatty liver (26/720) and 8.1% of the subjects with moderate liver fat content (10/124), while 12.5% (18/144) of the subjects with severe fatty liver ($p < 0.001$) (Table 3).

Severe liver fat content predicted the risk for future risk of cardiovascular event when adjusted for age, gender and study group (Model 1: HR 1.92, CI 1.32-2.80, $p < 0.01$) (Table 2). When further adjustments were made for smoking, alcohol consumption, LDL-cholesterol, BMI and systolic blood pressure (Model 2: HR 1.74, CI 1.16-2.63), the risk still remained

statistically significant ($p < 0.01$). Statistical significance disappeared when further adjustment for QUICKI was performed (Model 3: HR 1.49, CI 0.97-2.30, p=0.071). In the CVD event sensitivity analyses, all covariates were added one by one and it was examined whether the hazard ratios would change or remain stable. After adjusting for the statistically significant variables (including quick index) in the sensitivity analyses, the association between severe fatty liver was no longer significant (Model 4: HR 1.43, CI 0.93-2.18, p=0.10). When QUICKI was not added into Model 5, severe fatty liver did predict the risk for future risk for CVD event (HR 1.76, CI 1.21- 2.56, $p < 0.001$) (Table 2). The c-index decreased when the risk factors were removed from the model (Table 4).

Tables 2, 3 and 4 about here

The future risk of death from CVD in participants with severe fat content was significant when age, gender and study group were added as covariates (Model 1: HR 2.95, CI 1.58-5.51, $p < 0.01$). Even after further adjustments with other conventional risk factors (Model 2: HR 2.04, CI 1.03-4.05), statistical significance remained ($p < 0.05$). When QUICKI was added as the covariate, then significance disappeared (Model 3: HR 1.64, CI 0.79-3.43, NS) (Fig 1.).

Figure 1 about here

Fatty liver and total mortality

In total, 11.9% of the participants not having fatty liver, 18.5% of the subjects having moderate fatty liver and 22.2% of the subjects with severe fatty liver died from all causes ($p < 0.01$). According to Model 1, severe fat content predicted the risk for mortality from all causes when age, gender and study group were added as covariates (HR 1.60, CI 1.05-2.43, $p < 0.05$). The significance disappeared when body mass index was added as a covariate (data not shown).

We performed all Cox regression analyses after excluding the men consuming more than 210 g alcohol and the women drinking more than 140 g alcohol per week. This exclusion did not have any effect on the results (data not shown).

We performed all Cox regression analyses after excluding patients with insulin treated diabetes mellitus (n=9), cortisone treatment at baseline (n=41) and previous diagnosis for liver disease (n=15) (e.g., virus, medications). This exclusion did not have any effect on the results (data not shown).

Discussion

The incidences of non-alcoholic fatty liver disease and cardiovascular disease are continuously increasing in the Western world. The question if NAFLD is only a marker or also an early mediator of cardiovascular disease is still largely unanswered. According to the results of the present study, which had an approximately 19-year follow-up fatty liver does predict the future risk for death from all causes, death from cardiovascular disease and risk of cardiovascular events. Insulin sensitivity seems to play a more dominant role in the development of cardiovascular events.

Only a few studies have investigated the risk for future cardiovascular risk among subjects with ultrasound-diagnosed fatty liver [30, 31] and larger studies with longer follow-up times are needed. An association between NAFLD and CVD has been reported [3, 30-32] although contrary results also exist [13, 33]. A previous large population-based prospective cohort study found no association between NAFLD and CVD, however they categorized the degree of steatosis as a two level variable: none to mild and moderate to severe [13]. An association between ultrasound-diagnosed fatty liver and CVD has been reported in general population [30] and in subjects with T2DM [32]. Furthermore, liver dysfunction has been reported to associate with CVD mortality [34, 35] and CHD risk [11] in follow-up studies and especially survival of subjects with NASH is reported to be reduced [33, 36, 37]. In the present study, severe fatty liver disease did predict the risk for cardiovascular death but the association seemed to be dependent on insulin sensitivity.

Several earlier studies have used self-reported CVD history which may not be totally reliable. Although earlier studies on the risk for future cardiovascular risk among subjects with fatty liver have performed some adjustments, the full range of well-known CVD risk factors have been rarely considered [33]. We have performed adjustments with all so-called traditional risk factors for cardiovascular disease (i.e. age, gender, smoking, LDL concentration, hypertension, insulin resistance). Previous studies have used biochemical, radiological and histological methodology for NAFLD diagnosis and staging, which leads to a challenging interpretation of the results [35, 38].

This study had an approximately 19-year follow-up time, which is longer than in previous studies [11-14]. When compared to earlier studies [33, 38] this study seems to be the first follow-up

study with a large population-based randomly selected study group and a very long follow-up time and ultrasound-diagnosed fatty liver. The diagnosis of cardiovascular events was based on the registry of the National Institute for Health and Welfare and mortality data were obtained from the National Death Registry. The earlier verified FINRISK classification [26] was used to classify the events. Therefore, the reliability of event diagnosis data is accurate and the classification is systematic. All subjects who had myocardial infarction or stroke before baseline were excluded because a history of myocardial infarction is known to increase the risk for recurrent myocardial infarction or cardiovascular death [39] and medication as well as lifestyle secondary prevention strategies are intensive [40].

There are a few follow-up-studies examining whether the fatty liver increases the risk for total mortality [13, 41]. In the present study, severe fatty liver predicted the risk for overall mortality of any causes when age, gender and study group were added covariates, a result in line with an earlier report [42]. In the published literature, NASH rather than simple steatosis has been stated to be linked with decreased overall survival [36] although one study with a large cohort found no association between NAFLD and overall mortality [13]. In our study, the association between severe fatty liver and total mortality disappeared after further adjustment for BMI which means that severe fatty liver is not a strong predictor for overall mortality.

The molecular mechanisms linking fatty liver with CVD have been investigated [10, 16]. Enlarged visceral adipose tissue may explain why NAFLD associates with CVD [16]. In individuals with visceral obesity, insulin resistance may contribute to impaired non-esterified fatty acid (NEFA) metabolism [8] and the increasing NEFA flux to the liver may impair liver

metabolism leading to increased glucose metabolism and liver dysfunction [7]. The liver is one of the targets of the resulting systemic abnormalities and the source of several proatherogenic factors [3], such as CRP, fibrinogen, plasminogen activator inhibitor-1 and other inflammatory cytokines [16]. Furthermore, visceral adipose tissue and ectopic fat overexpress factors involved in atherogenesis [16] such as NEFAs and proinflammatory cytokines, for instance interleukin-6 and tumor necrosis factor-α [8] leading to chronic systemic inflammation. In addition, hepatic steatosis leads to overproduction of cholesterol-rich remnant particles [4].

One limitation in this study is that the grade of liver brightness was measured by ultrasound. The invasive diagnostic technique of liver biopsy is regarded as the "golden standard", especially for the diagnosis of NASH [43]. Real time ultrasound using a combination of sonographic findings does have a high specificity but it underestimates the prevalence of hepatic steatosis when there is less than 20 % fat [44]. Today, magnetic resonance spectroscopy is regarded as the best method for the quantification of liver fat, but this method is limited due to its availability [45]. Unfortunately quantitative measurement of liver fat by ultrasound is subject to several limitations compared to more validated and standardized methods for diagnosing NAFLD and the analysis of intra-observer reproducibility could have been more accurate in the present study. Nonetheless, the noninvasive ultrasound method was chosen because taking liver biopsies from large groups of symptomless subjects would have been ethically unjustifiable and magnetic resonance spectroscopy was not available at the baseline.

Comment [PP1]: Sentence added

The OPERA study group consists of subjects with drug-treated hypertension and randomly selected sex- and age-matched controls. Study group was added as a covariate to minimize any selection bias.

Conclusions

Severe liver fat content increased the risk of a future cardiovascular event and mortality to cardiovascular disease over the long-term follow-up but it seemed to be dependent on insulin sensitivity. Fatty liver also predicted the risk for overall mortality. However, conventional cardiovascular disease risk factors seemed to play a major role in developing death from all causes. It would be beneficial to investigate larger cohorts and follow-up studies in order to validate this result.

Figure legend

Title: Kaplan Meier cumulative survival rates censored for cardiovascular death in subjects with no fat in the liver, moderate fat content and severe fat content.

CVD was the cause of death in 3.6% of the subjects (26/720) with non-fatty liver and 8.1% of the subjects (10/124) with moderate liver fat content, while 12.5% of the subjects with severe fatty liver (18/144). Cox regression analysis is used for adjustments. M1 (Model 1): adjusted for study group, age and gender. M2 (Model 2): further adjustments for smoking, alcohol consumption, systolic blood pressure, LDL-cholesterol level and body mass index. M3 (Model 3): further adjustment for QUICKI. CVD, cardiovascular disease, CI, confidence interval, HR, hazard ratio, QUICKI, quantitative insulin sensitivity check index. ** $p < 0.01$, * $p < 0.05$.

Acknowledgements

The authors thank Markku Päivänsalo, MD, PhD, for the expert liver ultrasound examinations and Ms Saija Kortetjärvi, Ms Liisa Mannermaa, and Ms Leena Ukkola for the excellent technical assistance. M.Sc. Elina Malo and M.Sc. Meiju Saukko are thanked for the cooperation in organizing cardiovascular event and mortality data.

Contributor statement: All authors fulfill all three of the ICMJE guidelines for authorship

Pauliina Pisto: Data acquisition, statistical analysis and interpretation of data, manuscript writing, final approval of the version to be published

Merja Santaniemi: Data acquisition, statistical analysis and data interpretation, critical revision of the manuscript, final approval of the version to be published

Risto Bloigu: Data analysis, interpretation of data, critical revision of the manuscript, final approval of the version to be published

Olavi Ukkola: Study design, data acquisition, data interpretation, critical revision of the manuscript, final approval of the version to be published

Y.A Kesäniemi: Study design, data acquisition, data interpretation, critical revision of the manuscript, final approval of the version to be published

Data sharing statement: Extra data is available by emailing pauliina.pisto(at)oulu.fi

References

1. Angulo P. Nonalcoholic fatty liver disease. *N Engl J Med* 2002; Apr 18;346(16):1221-31.

2. Armstrong MJ, Houlihan DD, Bentham L, Shaw JC, Cramb R, Olliff S, et al. Presence and severity of non-alcoholic fatty liver disease in a large prospective primary care cohort. *J Hepatol* 2012; Jan;56(1):234-40.

3. Targher G, Day CP, Bonora E. Risk of cardiovascular disease in patients with nonalcoholic fatty liver disease. *N Engl J Med* 2010; Sep 30;363(14):1341-50.

4. Targher G, Arcaro G. Non-alcoholic fatty liver disease and increased risk of cardiovascular disease. *Atherosclerosis* 2007; Apr;191(2):235-40.

5. Roger VL, Go AS, Lloyd-Jones DM, Adams RJ, Berry JD, Brown TM, et al. Heart disease and stroke statistics--2011 update: a report from the American Heart Association. *Circulation* 2011; Feb 1;123(4):e18-e209.

6. Allender S, Scarborough P, Peto V, Rayner M, Leal J, Luengo-Fernandez R, Gray A. European cardiovascular disease statistics, 2008 ed. European Heart Network; 2008.

7. Despres JP, Lemieux I. Abdominal obesity and metabolic syndrome. *Nature* 2006; Dec 14;444(7121):881-7.

8. Van Gaal LF, Mertens IL, De Block CE. Mechanisms linking obesity with cardiovascular disease. *Nature* 2006; Dec 14;444(7121):875-80.

9. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med* 2005; Apr 21;352(16):1685-95.

10. Bhatia LS, Curzen NP, Byrne CD. Nonalcoholic fatty liver disease and vascular risk. *Curr Opin Cardiol* 2012; Jul;27(4):420-8.

11. Treeprasertsuk S, Leverage S, Adams LA, Lindor KD, St Sauver J, Angulo P. The Framingham risk score and heart disease in nonalcoholic fatty liver disease. *Liver Int* 2012; Jul;32(6):945-50.

12. Haring R, Wallaschofski H, Nauck M, Dorr M, Baumeister SE, Volzke H. Ultrasonographic hepatic steatosis increases prediction of mortality risk from elevated serum gamma-glutamyl transpeptidase levels. *Hepatology* 2009; Nov;50(5):1403-11.

13. Lazo M, Hernaez R, Bonekamp S, Kamel IR, Brancati FL, Guallar E, et al. Non-alcoholic fatty liver disease and mortality among US adults: prospective cohort study. *BMJ* 2011; Nov 18;343:d6891.

14. Wong VW, Wong GL, Yip GW, Lo AO, Limquiaco J, Chu WC, et al. Coronary artery disease and cardiovascular outcomes in patients with non-alcoholic fatty liver disease. *Gut* 2011; Dec;60(12):1721-7.

15. Loria P, Lonardo A, Targher G. Is liver fat detrimental to vessels?: intersections in the pathogenesis of NAFLD and atherosclerosis. *Clin Sci (Lond)* 2008; Jul;115(1):1-12.

16. Targher G, Marra F, Marchesini G. Increased risk of cardiovascular disease in non-alcoholic fatty liver disease: causal effect or epiphenomenon?. *Diabetologia* 2008; Nov;51(11):1947-53.

17. Targher G, Bertolini L, Scala L, Zoppini G, Zenari L, Falezza G. Non-alcoholic hepatic steatosis and its relation to increased plasma biomarkers of inflammation and endothelial dysfunction in non-diabetic men. Role of visceral adipose tissue. *Diabet Med* 2005; Oct;22(10):1354-8.

18. Brea A, Mosquera D, Martin E, Arizti A, Cordero JL, Ros E. Nonalcoholic fatty liver disease is associated with carotid atherosclerosis: a case-control study. *Arterioscler Thromb Vasc Biol* 2005; May;25(5):1045-50.

19. Sookoian S, Pirola CJ. Non-alcoholic fatty liver disease is strongly associated with carotid atherosclerosis: a systematic review. *J Hepatol* 2008; Oct;49(4):600-7.

20. Fraser A, Harris R, Sattar N, Ebrahim S, Smith GD, Lawlor DA. Gamma-glutamyltransferase is associated with incident vascular events independently of alcohol intake: analysis of the British Women's Heart and Health Study and Meta-Analysis. *Arterioscler Thromb Vasc Biol* 2007; Dec;27(12):2729-35.

21. Rantala AO, Kauma H, Lilja M, Savolainen MJ, Reunanen A, Kesaniemi YA. Prevalence of the metabolic syndrome in drug-treated hypertensive patients and control subjects. *J Intern Med* 1999; Feb;245(2):163-74.

22. Yajima Y, Ohta K, Narui T, Abe R, Suzuki H, Ohtsuki M. Ultrasonographical diagnosis of fatty liver: significance of the liver-kidney contrast. *Tohoku J Exp Med* 1983; Jan;139(1):43-50.

23. Sampi M, Veneskoski M, Ukkola O, Kesaniemi YA, Horkko S. High plasma immunoglobulin (Ig) A and low IgG antibody titers to oxidized low-density lipoprotein

are associated with markers of glucose metabolism. *J Clin Endocrinol Metab* 2010; May;95(5):2467-75.

24. Pisto P, Ukkola O, Santaniemi M, Kesaniemi YA. Plasma adiponectin--an independent indicator of liver fat accumulation. *Metabolism* 2011; Nov;60(11):1515-20.

25. Santaniemi M., Ukkola O., Malo E., Bloigu R., Kesaniemi YA. Metabolic syndrome in the prediction of cardiovascular events: The potential additive role of hsCRP and adiponectin. *Eur J Prev Cardiol* 2013; Jun;20.

26. Pajunen P, Jousilahti P, Borodulin K, Harald K, Tuomilehto J, Salomaa V. Body fat measured by a near-infrared interactance device as a predictor of cardiovascular events: the FINRISK'92 cohort. *Obesity (Silver Spring)* 2011; Apr;19(4):848-52.

27. Katz A, Nambi SS, Mather K, Baron AD, Follmann DA, Sullivan G, et al. Quantitative insulin sensitivity check index: a simple, accurate method for assessing insulin sensitivity in humans. *J Clin Endocrinol Metab* 2000; Jul;85(7):2402-10.

28. Kauma H, Savolainen MJ, Rantala AO, Lilja M, Kervinen K, Reunanen A, et al. Apolipoprotein E phenotype determines the effect of alcohol on blood pressure in middle-aged men. *Am J Hypertens* 1998; Nov;11(11 Pt 1):1334-43.

29. Bessembinders K, Wielders J, van de Wiel A. Severe hypertriglyceridemia influenced by alcohol (SHIBA). *Alcohol Alcohol* 2011; Mar-Apr;46(2):113-6.

30. Hamaguchi M, Kojima T, Takeda N, Nagata C, Takeda J, Sarui H, et al. Nonalcoholic fatty liver disease is a novel predictor of cardiovascular disease. *World J Gastroenterol* 2007; Mar 14;13(10):1579-84.

31. Stepanova M, Younossi ZM. Independent Association Between Nonalcoholic Fatty Liver Disease and Cardiovascular Disease in the US Population. *Clin Gastroenterol Hepatol* 2012; Jun;10(6):646-50.

32. Targher G, Bertolini L, Poli F, Rodella S, Scala L, Tessari R, et al. Nonalcoholic fatty liver disease and risk of future cardiovascular events among type 2 diabetic patients. *Diabetes* 2005; Dec;54(12):3541-6.

33. Ghouri N, Preiss D, Sattar N. Liver enzymes, nonalcoholic fatty liver disease, and incident cardiovascular disease: a narrative review and clinical perspective of prospective data. *Hepatology* 2010; Sep;52(3):1156-61.

34. Dunn W, Xu R, Wingard DL, Rogers C, Angulo P, Younossi ZM, et al. Suspected nonalcoholic fatty liver disease and mortality risk in a population-based cohort study. *Am J Gastroenterol* 2008; Sep;103(9):2263-71.

35. Ruttmann E, Brant LJ, Concin H, Diem G, Rapp K, Ulmer H, et al. Gamma-glutamyltransferase as a risk factor for cardiovascular disease mortality: an epidemiological investigation in a cohort of 163,944 Austrian adults. *Circulation* 2005; Oct 4;112(14):2130-7.

36. Soderberg C, Stal P, Askling J, Glaumann H, Lindberg G, Marmur J, et al. Decreased survival of subjects with elevated liver function tests during a 28-year follow-up. *Hepatology* 2010; Feb;51(2):595-602.

37. Ekstedt M, Franzen LE, Mathiesen UL, Thorelius L, Holmqvist M, Bodemar G, et al. Long-term follow-up of patients with NAFLD and elevated liver enzymes. *Hepatology* 2006; Oct;44(4):865-73.

38. Bhatia LS, Curzen NP, Calder PC, Byrne CD. Non-alcoholic fatty liver disease: a new and important cardiovascular risk factor?. *Eur Heart J* 2012; May;33(10):1190-200.

39. Beckman JA, Creager MA, Libby P. Diabetes and atherosclerosis: epidemiology, pathophysiology, and management. *JAMA* 2002; May 15;287(19):2570-81.

40. Joseph P, Teo K. Optimal medical therapy, lifestyle intervention, and secondary prevention strategies for cardiovascular event reduction in ischemic heart disease. *Curr Cardiol Rep* 2011; Aug;13(4):287-95.

41. Dam-Larsen S, Franzmann M, Andersen IB, Christoffersen P, Jensen LB, Sorensen TI, et al. Long term prognosis of fatty liver: risk of chronic liver disease and death. *Gut* 2004; May;53(5):750-5.

42. Calori G, Lattuada G, Ragogna F, Garancini MP, Crosignani P, Villa M, et al. Fatty liver index and mortality: the Cremona study in the 15th year of follow-up. *Hepatology* 2011; Jul;54(1):145-52.

43. Joy D, Thava VR, Scott BB. Diagnosis of fatty liver disease: is biopsy necessary?. *Eur J Gastroenterol Hepatol* 2003; May;15(5):539-43.

44. Dasarathy S, Dasarathy J, Khiyami A, Joseph R, Lopez R, McCullough AJ. Validity of real time ultrasound in the diagnosis of hepatic steatosis: a prospective study. *J Hepatol* 2009; Dec;51(6):1061-7.

45. Szczepaniak LS, Nurenberg P, Leonard D, Browning JD, Reingold JS, Grundy S, et al. Magnetic resonance spectroscopy to measure hepatic triglyceride content: prevalence of hepatic steatosis in the general population. *Am J Physiol Endocrinol Metab* 2005; Feb;288(2):E462-8.

505

506

Grade of liver bightness	0 (n=720)	1 (n=124)	2 (n=144)	p	p (0-1)	p (1-2)	p (0-2)
Age (years)	50.9 (6.0)	51.9 (6.1)	51.5 (5.5)	NS	NS	NS	NS
Males	44.3 % (n=319)	65.3 % (n=81)	59.9 % (n=82)	< 0.001	-	-	-
Hypertensives	41.4 % (n=298)	66.1 % (n=82)	71.5 % (n=103)	< 0.001	-	-	-
BMI (kg/m²)	26.4 (3.9)	29.8 (5.0)	31.9 (4.9)	< 0.001	< 0.001	< 0.001	< 0.001
Waist circumference (cm)	86.8 (11.9)	97.7 (12.0)	102.3 (11.8)	< 0.001	< 0.001	< 0.01	< 0.001
Smoking (pack years)	10.6 (13.3)	14.3 (14.9)	14.0 (14.6)	< 0.05	NS	NS	NS
Alcohol consumption (g/week)	51.1 (83.0)	95.1 (117.0)	82.6 (105.1)	< 0.01	< 0.05	NS	NS
Total serum cholesterol (mmol/L)	5.6 (1.0)	5.8 (1.1)	5.8 (1.1)	NS	NS	NS	NS
LDL (mmol/L)	3.5 (0.9)	3.7 (1.1)	3.5 (0.9)	NS	NS	NS	NS
Triglycerides (mmol/L)	1.4 (0.8)	1.9 (0.8)	2.2 (1.4)	< 0.001	< 0.001	< 0.05	< 0.001
Systolic blood pressure	145.2 (21.5)	152.7 (20.3)	157.1 (22.2)	< 0.001	< 0.01	NS	< 0.001
Fasting insulin (mmol/L)	10.8 (7.7)	18.2 (10.3)	23.8 (17.6)	< 0.001	< 0.001	< 0.001	< 0.001

Fasting glucose (mmol/L)	4.4 (0.7)	5.0 (1.4)	6.1 (2.8)	< 0.001	< 0.001	< 0.001	< 0.001
QUICKI	0.6 (0.1)	0.6 (0.1)	0.5 (0.1)	< 0.001	< 0.001	< 0.001	< 0.001
hs-CRP (ng/mL)	3039.4 (6758.3)	3981.4 (6068.2)	6122.0 (6630.8)	< 0.001	< 0.001	< 0.01	< 0.001
ALT U/L	26.2 (15.5)	37.8 (17.1)	55.4 (37.7)	< 0.001	< 0.001	< 0.001	< 0.001
GGT U/L	35.1 (33.5)	69.7 (116.3)	76.8 (92.4)	< 0.001	< 0.001	< 0.01	< 0.001
Anti-hypertensive treatment	43.6% (n=314)	66.9% (n=83)	72.9% (n=105)	< 0.001	-	-	-
Lipid-lowering treatment	2.2% (n=16)	1.6% (n=2)	6.2% (n=9)	< 0.05	-	-	-
Hypoglycaemic drug	1.1% (n=8)	1.6% (n=2)	10.4% (n=15)	< 0.001	-	-	-
Type 2 diabetes	2.4% (n=17)	12.1% (n=15)	36.8% (n=53)	< 0.001	-	-	-

Table 1. Baseline characteristics of the study group as means (standard deviations) or percentages. N= number of subjects. ALT, alanine aminotransferase, BMI, body mass index, GGT, gamma-glutamyltransferase, hs-CRP, high-sensitivity C-reactive protein, LDL, low-density lipoprotein, QUICKI, quantitative insulin sensitivity check index.

	Model 1	Model 2	Model 3	Model 4	Model 5
Moderate fat content	1.51 (0.99-2.29)	1.44 (0.93-2.23)	1.31 (0.84-2.05)	1.30 (0.84-2.01)	1.49 (0.99-2.26)
Severe fat content	1.92 (1.32-2.80)**	1.74 (1.16-2.63) **	1.49 (0.97-2.30)	1.43 (0.93-2.18)	1.76 (1.21- 2.56) **
Study group	1.34 (0.98-1.85)	1.29 (0.92-1.80)	1.28 (0.92-1.78)		
Age	1.06 (1.03-1.09)***	1.05(1.02-1.08)**	1.05 (1.02-1.08)**	1.05 (1.02-1.07)**	1.05 (1.02-1.08) **
Gender	2.39 (1.71-3.34)*	1.91 (1.34-2.71)***	1.80 (1.26-2.57)**	1.83 (1.29-2.60) **	1.92 (1.36-2.72) ***
LDL-cholesterol		1.17 (0.99-1.39)	1.15 (0.97-1.37)		
Smoking (pack-years)		1.02 (1.01-1.03)***	1.02 (1.01-1.03)***	1.02 (1.01-1.03)***	1.02 (1.01-1.03) ***
Alcohol consumption (gr1)		0.93 (0.59-1.45)	0.92(0.59-1.44)		
Alcohol consumption (gr2)		0.84 (0.44-1.60)	0.81(0.42-1.54)		
Systolic blood pressure		1.01 (1.00-1.02)**	1.01 (1.00-1.02)*	1.01 (1.00-1.02)**	1.01 (1.00-1.02) **
Body mass index		0.99 (0.96-1.03)	0.97 (0.93-1.01)		
QUICKI			0.12 (0.02-0.90)*	0.16 (0.03-0.99)*	

Table 2. Multivariate analysis for cardiovascular events with different degrees of adjustments (Cox regression analysis). CVD event occurred in 13.5% of the subjects with no fat in the liver (97/720), 24.2% (30/124) of subjects having moderate liver fat content and 29.2% (42/144) of the subjects having severe fatty liver. Hazard ratios with 95% confidence interval with different degrees of adjustments are presented. Alcohol consumption was divided into groups (reference group: less than 1g/week in men and women, group 1: less than 210g/week in men and less than 140 g/week in women, group 2: more than 210g/week in men and more than 140g/week in women). Model 1: adjustment for study group, age and gender. Model 2: further adjustments for LDL-cholesterol, smoking, alcohol consumption, systolic blood pressure and body mass index. Model 3: further adjustment for QUICKI. Model 4: adjustments with statistically significant covariates. Model 5: adjustments with statistically significant covariates without QUICKI. LDL, low-density lipoprotein, QUICKI, quantitative insulin sensitivity check index. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Grade of liver bightness	Total	0 (n=720)	1 (n=124)	2 (n=144)	p
Non-fatal events					
CVD	11.6% (115)	9.9% (71)	16.1% (20)	16.7% (24)	< 0.05
CHD	7.8% (77)	6.5% (47)	11.3% (14)	11.1% (16)	NS
Stroke	5.0% (49)	4.2% (30)	8.1% (10)	6.2% (9)	NS
Fatal events					
CVD	5.5% (54)	3.6% (26)	8.1% (10)	12.5% (18)	< 0.001
CHD	4.8% (47)	3.2% (23)	7.3% (9)	10.4% (15)	< 0.01
Stroke	0.8% (8)	0.6% (4)	0.8% (1)	2.1% (3)	NS

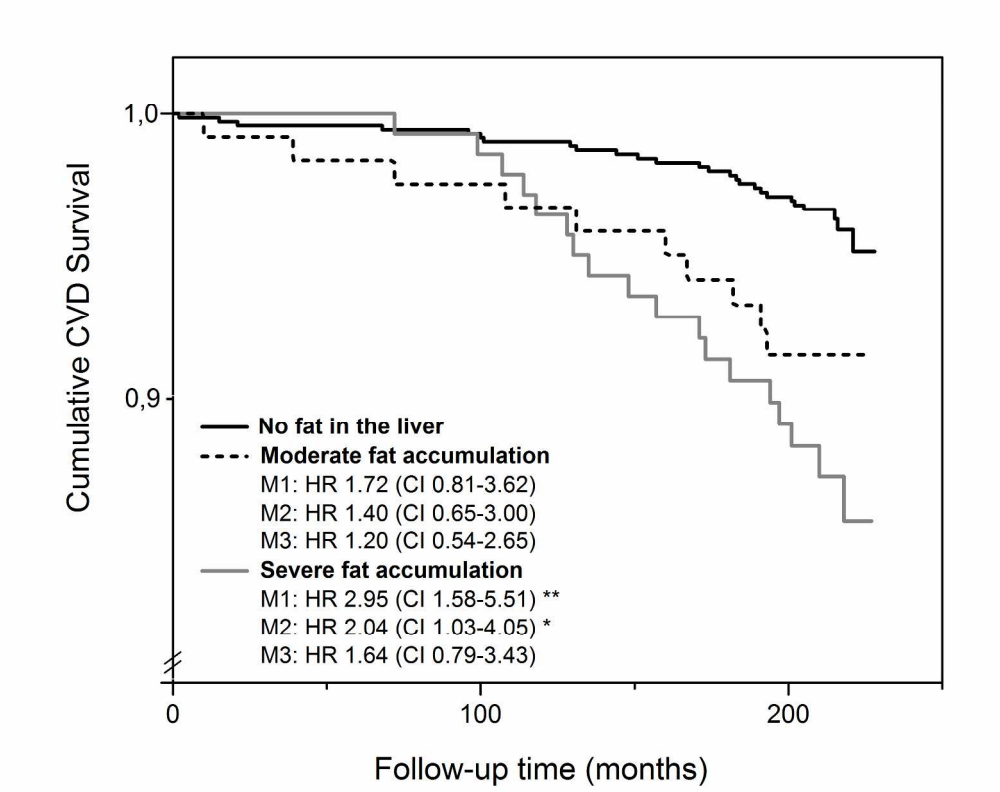
Table 3. CVD, CHD and stroke follow-up data of the study group as percentages (number of events). Statistical significances between percentages were measured by using χ^2 test. CVD included a major CHD event and stroke (excluding subarachnoid hemorrhage) - whichever of these happened first. N=number of subjects. CHD, coronary heart disease, CVD, cardiovascular disease.

Comment [PP2]: Sentence added

Final model	Cardiovascular event c-index (95% CI)	Binary R^2
Model 3	0.729 (0.706-0.776)	0.153
Model 4	0.720 (0.689-0.763)	0.144
Model 5	0.717 (0.686-0.758)	0.138
Model 1	0.698 (0.656-0.742)	0.133

Table 4. Multivariate analysis for cardiovascular events (logistic regression analysis). Cardiovascular disease risk factors have been removed from the models step by step. Model 3 included liver brightness, study group, age, gender, smoking, alcohol consumption, systolic blood pressure, LDL-cholesterol level, body mass index and QUICKI. Model 4 included liver brightness, age, gender, smoking, blood pressure and QUICKI. Model 5 included liver brightness, age, gender, smoking, blood pressure. Model 1 included liver brightness, study group, age and gender. C-index with confidence intervals obtained from 250 bootstrap resamplings and binary R^2 was used. LDL, low-density lipoprotein, QUICKI, quantitative insulin sensitivity check index.

557



Title: Kaplan Meier cumulative survival rates censored for cardiovascular death in subjects with no fat in the liver, moderate fat content and severe fat content.

CVD was the cause of death in 3.6% of the subjects (26/720) with non-fatty liver and 8.1% of the subjects (10/124) with moderate liver fat content, while 12.5% of the subjects with severe fatty liver (18/144). Cox regression analysis is used for adjustments. M1 (Model 1): adjusted for study group, age and gender. M2 (Model 2): further adjustments for smoking, alcohol consumption, systolic blood pressure, LDL-cholesterol level and body mass index. M3 (Model 3): further adjustment for QUICKI. CVD, cardiovascular disease, CI, confidence interval, HR, hazard ratio, QUICKI, quantitative insulin sensitivity check index. ** $p < 0.01$, * $p < 0.05$.

247x209mm (300 x 300 DPI)

STROBE Statement—Items to be included when reporting observational studies in a conference abstract

Item	Recommendation
Title	Indicate the study's design with a commonly used term in the title (e.g cohort, case-control, cross sectional) **page 1**
Authors	Contact details for the corresponding author **page 1**
Study design	Description of the study design (e.g cohort, case-control, cross sectional) **page 6**
Objective	Specific objectives or hypothesis **page 5**
Methods	**page 5**
Setting	Description of setting, follow-up dates or dates at which the outcome events occurred or at which the outcomes were present, as well as any points or ranges on other time scales for the outcomes (e.g., prevalence at age 18, 1998-2007). **page 7**
Participants	*Cohort study*—Give the most important eligibility criteria, and the most important sources and methods of selection of participants. Describe briefly the methods of follow-up **page 6** *Case-control study*—Give the major eligibility criteria, and the major sources and methods of case ascertainment and control selection *Cross-sectional study*—Give the eligibility criteria, and the major sources and methods of selection of participants
	Cohort study—For matched studies, give matching and number of exposed and unexposed **page 7** *Case-control study*—For matched studies, give matching criteria and the number of controls per case
Variables	Clearly define primary outcome for this report. **page 10**
Statistical methods	Describe statistical methods, including those used to control for confounding **page 9**
Results	
Participants	Report Number of participants at the beginning and end of the study **page 7**
Main results	Report estimates of associations. If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period Report appropriate measures of variability and uncertainty (e.g., odds ratios with confidence intervals **page 10**
Conclusions	General interpretation of study results **page 12**