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Liver fat and cardiometabolic health: a population-based perspective

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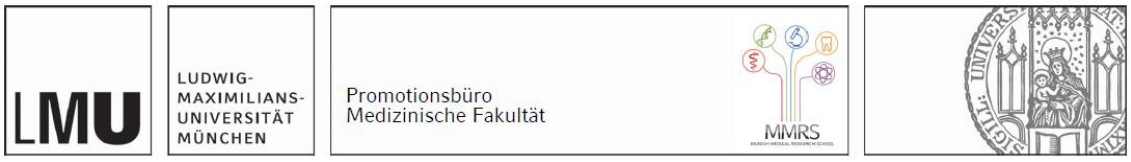

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For all the adventurers

Who see the problems in their live(r)s as

puzzles

Table of content

Summary

Fatty liver, a condition due to ectopic fat accumulation in the liver cells (>5%), is affecting around one fourth of the adult population worldwide. It is more prevalent in men than women. Sex differences in fatty liver can arise from different aspects, such as biological, behavioral and socioeconomical discrepancies between men and women. Accumulating evidence points out the potential role of sex hormones and sex hormone-binding globulin (SHBG) in the susceptibility to fat buildup in the liver. Excessive liver fat accumulation is not only related to a wide spectrum of liver disorders, ranging from simple steatosis to steatohepatitis with liver fibrosis and end stage liver diseases, but also adds substantially to extrahepatic clinical burden, such as chronic kidney disease (CKD) and cardiovascular diseases (CVD). However, the close relationship between liver fat accumulation and other metabolic derangements, such as diabetes and overall obesity, makes it difficult to delineate the independent association between sex hormones/SHBG and liver fat accumulation. For the same reason, it is still debatable whether excessive liver fat is an independent risk factor for CKD or CVD.

Using data from population-based studies, this thesis examined not only the potential etiological role of sex hormones and SHBG in liver fat accumulation in both men and women, but also dealt with the hypothesis that liver fat could be a target to prevent the development of CKD and CVD. Three research projects addressing these three aspects pertaining to the role of liver fat accumulation in cardiometabolic health are included in this thesis.

The first project examined the association between endogenous sex hormone and SHBG levels with excessive liver fat accumulation, estimated by the fatty liver index (FLI). We further investigated the potential causal role of these biomarkers in liver fat accumulation using a two-sample mendelian randomization approach based on genetic data from the largest publicly available genome-wide association studies with European ancestry. We found that endogenous sex hormones, including testosterone, free testosterone, dihydrotestosterone, progesterone and 17-alpha-hydroxyprogesterone, as well as SHBG were sex-specifically associated with FLI. However, we only found suggestive evidence that higher genetically determined SHBG could causally decrease liver fat content in women.

The second project investigated the association between liver fat accumulation, estimated by the FLI, and kidney function parameters as well as the risk of CKD. The potential joint mediating role of diabetes, hypertension, and C-reactive protein (CRP), in the association between the FLI and incident CKD was also examined. We observed that the associations between the FLI and kidney function parameters were not independent of cardiometabolic risk factors. Diabetes, hypertension, and CRP fully mediated the association between the FLI and incident CKD jointly.

The third project assessed the relation between liver fat content measured by magnetic resonance imaging and parameters of subclinical vascular disease, including carotid plaque and aortic diameters. Our results suggest that liver fat content is not independently associated with subclinical vascular disease, and the apparent associations are mainly confounded by overall obesity.

Altogether, this thesis suggests that the endogenous SHBG levels are highly relevant for liver fat regulation, especially in women. It also suggests that people with an elevated liver fat content may benefit from the treatment and monitoring of concomitant metabolic derangements, in order to reduce their risk of CKD or CVD.

List of abbreviations

17-OHP	17-alpha-hydroxyprogesterone
BMI	body mass index
cIMT	Carotid intima-media thickness
CKD	chronic kidney disease
CRP	C-reactive protein
CT	computed tomography
CVD	cardiovascular disease
DHEA	dehydroepiandrosterone
DHEAS	dehydroepiandrosterone-sulfate
DHT	dihydrotestosterone
E2	estradiol
eGFR	estimated glomerular filtration rate
eGFR-cC	estimated glomerular filtration rate based on serum cystatin C
eGFR-Cr	estimated glomerular filtration rate based on serum creatinine
FLI	fatty liver index
fDHT	free dihydrotestosterone
fT	free testosterone
GGT	gamma-glutamyl-transferase
GWAS	genome-wide association study
HDL-C	high-density lipoprotein-cholesterol
HSI	hepatic steatosis index
IVW	inverse-variance weighting
KORA	Cooperative Health Research in the Region of Augsburg
LDL-C	low-density lipoprotein
MONICA	Monitoring of Trends and Determinants in Cardiovascular Diseases
MR	Mendelian randomization
MRI	magnetic resonance imaging
NAFLD	non-alcoholic fatty liver disease
NAFLD-LFS	non-alcoholic fatty liver disease-liver fat score
NASH	non-alcoholic steatohepatitis
PDFF	proton density fat fraction
SBP	systolic blood pressure
SHBG	sex hormone-binding globulin
SHIP	Study of Health in Pomerania
T2D	type 2 diabetes
T	testosterone
UACR	urinary albumin-to-creatinine ratio

List of publications

The following articles form the foundation of this cumulative thesis.

Paper I (Published; This manuscript can be found in the Appendix)

X Cai, B Thorand, S Hohenester, C Prehn, A Cecil, J Adamski, T Zeller, A Dennis, R Banerjee, A Peters, H Yaghootkar, J Nano.

Association of Sex Hormones and Sex Hormone Binding Globulin with Liver Fat in Men and women: An Observational and Mendelian Randomization Study. Frontiers in Endocrinology (Impact factor: 5.2; ranked 36th out of 145 journals in the category Endocrinology & Metabolism) 2023, Oct 13:14:1223162. doi: 10.3389/fendo.2023.1223162.

Paper II (Published)

X Cai, B Thorand, S Hohenester, W Koenig, W Rathmann, A Peters, J Nano. Association between the Fatty Liver Index and Chronic Kidney Disease: the Population-based KORA Study. Nephrology Dialysis Transplantation (Impact factor: 7.19; ranked 10th out of 122 journals in the category Urology & Nephrology). 2023 May 4;38(5):1240-1248. doi: 10.1093/ndt/gfac266.

Paper III (Published)

X Cai, S Rospleszcz, B Mensel, U Schminke, JP Kühn, AA Aghdassi, C Storz, R Lorbeer, CL Schlett, W Rathmann, M Roden, S Hohenester, R Bülow, F Bamberg, A Peters, B Thorand, H Völzke, J Nano.

Association between Hepatic Fat and Subclinical Vascular Disease Burden in the General Population. BMJ Open Gastroenterology (Impact factor: 3.1; ranked 65th out of 139 journals in the category Gastroenterology & Hepatology) 2021 Sep;8(1):e000709.

Your contribution to the publications

For all three papers included in this thesis, I am the first author. I formulated the overarching research goals and aims and carried out the research mainly under the supervision of Dr. Jana Nano and Prof. Dr. Barbara Thorand.

For all papers, I conceptualized the study design and decided on the methodology to define study exposures and outcomes using state of the art measurements or otherwise the most feasible approaches, with feedbacks from my supervisors and collaborators throughout the research process.

I carried out the statistical analyses using the R program and I am responsible for the integrity of the data analyses. I interpreted the results, and I also presented the results at national and international conferences as posters or oral presentations. I prepared and revised the draft of all three publications, and carried out the submission procedure, until final publication of Paper II and III and until submission of Paper I.

1. Introductory summary

1.1 General introduction

1.1.1 Liver fat accumulation and non-alcoholic fatty liver disease

Fatty liver depicts a condition in which more than 5% of ectopic fat accumulates in hepatocytes. Non-alcoholic fatty liver disease (NAFLD) is a form of fatty liver that can be diagnosed when other causes such as excessive alcohol consumption, medications, or viral hepatic infections are absent (1). NAFLD has become the most common liver disease worldwide with the epidemic of high caloric diets and sedentary lifestyles (1, 2).

Sharing the common pathology of ectopic liver fat accumulation, NAFLD is a term covering a wide spectrum of histologically distinct non-alcoholic liver diseases. NAFLD at an early stage with simple fat buildup in the liver and no evidence of inflammation or injury is classified as non-alcoholic fatty liver or steatosis. A more severe stage of NAFLD is the non-alcoholic steatohepatitis (NASH), in which inflammation and hepatocyte injury with or without fibrosis coexist with hepatic steatosis. NASH can progress to end stage liver diseases, such as cirrhosis or even hepatocellular carcinoma (3).

1.1.2 Epidemiology

It is estimated that around 25% of the adult population worldwide is suffering from NAFLD. However, epidemiological data related to NAFLD vary by region and ethnic groups, with the highest prevalence reported in the Middle East (32%) and South America (31%) and the lowest in Africa (14%). The prevalence of NAFLD is reported to be the highest among people of Hispanic origin, followed by European, Asian and African. In Germany, the average prevalence of NAFLD is estimated to be 23% (4).

A higher prevalence of NAFLD in men than women has been constantly reported across studies (5). Sex differences of NAFLD arise from several aspects that are different between men and women, including eating habits and exercise levels as well as physiological effects of sex hormones (6, 7). Sex hormones are biologically synthesized from cholesterol and primarily inactivated in the liver (8, 9). Extensive research has pointed out that estrogen and androgen, for example, are highly

involved in regulating body fat distribution and modulating cardiovascular health (10). However, the effects of sex hormones in the development of NAFLD are still controversial and highly dependent on the sex (5, 11). Accordingly, estrogen deficiency in postmenopausal women and hyperestrogenism in men are both linked to higher risks of metabolic disorders and liver diseases (11, 12). An important factor that modulates the bioavailability of sex hormones to the targeted tissues is the sex hormone-binding globulin (SHBG). Evidence is accumulating that lower SHBG concentration is associated with a higher risk of metabolic syndrome and NAFLD on both sexes (13, 14). Nevertheless, longitudinal investigations on the sex-specific effects of sex hormones and SHBG on the risk of NAFLD independent of other known risk factors is still lacking. Therefore, sex differences in relation to sex hormones and SHBG as a risk factor for liver fat accumulation is one of the topics in this thesis.

1.1.3 Risk factors

NAFLD is a multifactorial disease. Male sex and older age are two main demographic risk factors for NAFLD (15). Apart from lifestyle factors, such as lack of physical activity or a western diet featured by the high intake of simple carbohydrates and unsaturated fatty acids, metabolic factors are also closely associated with NAFLD (3). Epidemiological data have shown that people with obesity or type 2 diabetes (T2D) are especially prone to NAFLD. The metabolic syndrome, a condition that encompasses several metabolic susceptibilities, such as larger waist circumference, hypertension, hyperglycemia, and dyslipidemia, is frequently observed among people with NAFLD (15). Moreover, accumulating evidence regarding the inter-individual variation of the susceptibility and severity of NAFLD suggests a potential role of genetic predisposition and gene-environment interaction in the manifestation of NAFLD. Since the discovery of the *PNPLA3* gene in the ethnical susceptibility of NAFLD, a growing body of genome-wide associations studies (GWAS) have embarked on revealing genetic variations involved in the development and progression of NAFLD (16-18).

1.1.4 Pathophysiology

Not only do dietary and lifestyle risk factors increase total and visceral fat deposition, but also, they can lead to hyperglycemia, β-cell dysfunction and insulin resistance. Hyperglycemia and hyperinsulinemia, in turn, provoke hepatic de-novo lipogenesis

and ectopic lipid storage in the liver. On one hand, increased adiposity tissues can secrete proinflammatory cytokines and infiltrate immune cells. The overflow of fatty acids to the liver, on the other hand, can induce lipotoxity. Together they initiate inflammation and exacerbate dysregulation of glucose and fat metabolism in the liver (19). Dysbiosis, a condition caused by disruption of gut microbiota due to imbalanced diet, also further contributes to fat accumulation in the liver and the inflammatory process, which further advances the stage of NAFLD (3). Genomic variants associated with NAFLD or hepatic fat content also provide evidence on potential pathological mechanisms of NAFLD, such as derangement of lipid metabolism in the liver (TM6SF2, PNPLA3, APOE), macrophage membrane remodeling and hyperactivation of inflammatory responses (MBOAT7), and upregulated glucose influx and de-novo lipogenesis in hepatocytes (GCKR) (17, 18, 20).

1.1.5 Diagnosis of excessive liver fat accumulation

Liver biopsy is the gold standard of diagnosing and histological staging of fatty liver disease. However, this invasive puncture method can often lead to sampling error and infectious complications, which hamper its usage in routine or repeated follow-up checkups (21). With the advancement in radiology, several medical imaging modalities emerge to non-invasively detect fatty liver and quantify liver fat content (22). Ultrasound is used widely in clinical practice, owing to its accessibility and relatively low cost. The detection of fatty liver with conventional ultrasound modality is based on qualitative examination of sonographic alterations. This leads to limited sensitivity in detecting fatty liver with moderate steatosis (<30% affected hepatocytes) as well as inter- and intra-observer biases. Although more advanced ultrasound modalities also allow for quantitative measurement of liver fat, they appear to be more cost-intensive and are still not widely accepted in routine practices (21, 23). Computed tomography (CT) quantifies liver fat content using absolute attenuation or relative liver-spleen attenuation values. Whilst allowing for more precise and objective determination of liver fat content, CT increases the risk of radiation hazard and allergic reactions to the contrast agent (22). Magnetic resonance imaging (MRI) quantifies liver fat content by calculating the proportion of proton density in fat molecules in relation to water. MRI provides the most accurate and precise non-invasive measurement of liver fat content to date, but its accessibility is limited due to its high cost and multitude of contraindications (22).

Meanwhile, a variety of liver indices, incorporating easy-to-access parameters, such as biomarkers and anthropometric measurements, have been developed to select patients for liver biopsy or radiological examinations in the clinical practice (24-27). Because of their non-invasiveness, high availability and cost-effectiveness, liver indices are also broadly used in epidemiological studies to predict steatosis. For example, based on body mass index (BMI), waist circumference, triglycerides and gamma-glutamyl-transferase (GGT), the fatty live index (FLI) opts in fatty liver with a score bigger than or equal to 60 and opts out fatty liver with a score smaller than 30 (24). FLI was originally developed and validated with ultrasound diagnosed fatty liver (24). Later on, the classification performance of FLI has been further validated by MRI data (28, 29). To address the limitations of ultrasound and also to consider ethnic differences, a number of alternative liver indices have been developed since then. The NAFLD liver fat score (NAFLD-LFS), for instance, was developed in a population with MRI diagnosed NAFLD (25), and the hepatic steatosis index (HSI) was developed to better suit the anthropometric traits of a Korean population (26). In spite of greater complexity and increased costs due to additional serological biomarkers, NAFLD-LFS and HSI do not seem to add predictive value substantially compared to FLI (30, 31).

1.1.6 Clinical burden

Data suggests that NASH substantially increases the risk of end stage liver diseases, such as cirrhosis, which constitutes the second most common indication for liver transplantation and is the 11th leading cause of mortality in the world (2, 32). However, the clinical burden caused by NAFLD is far beyond maladies of the liver. Concomitant with obesity and insulin resistance, NAFLD is associated with a myriad of extrahepatic diseases, including T2D, chronic kidney disease (CKD), cardiovascular disease (CVD), sleep apnea and osteoporosis (33).

Liver fat and chronic kidney disease

Patients with CKD can irreversibly progress to kidney failure and end stage kidney disease, which also present a pronounced risk for CVD morbidity and mortality (34, 35). Curtailing CKD at an early stage is therefore essential in improving the prognosis and life quality of the patients as well as reducing the CVD burden in the population. CKD refers to structural damage in the kidney, resulting in albuminuria defined as urinary albumin-to creatinine ratio (UACR) ≥ 30mg/g, or reduced glomerular filtration

rate (<60 ml/min/1.73 m^2) for more than three months (36). The development of CKD is often accompanied by several cardiometabolic risk factors, such as hypertension, hyperglycemia, dyslipidemia and inflammation, which largely contribute to the pathogenesis of NAFLD (34). Previous studies have provided consistent evidence on the elevated risk of CKD among patients with NAFLD using hospital or population-based data (37). Nevertheless, it remains controversial whether NAFLD represents an independent risk factor for CKD, which could potentially improve early recognition and risk assessment of CKD (38-40).

Liver fat and cardiovascular disease

Existing research has recognized an elevated risk of CVD events among patients with NAFLD (41, 42). Meanwhile, CVD is the primary cause of mortality among NAFLD patients (33). However, owing to the close relationship between NAFLD and cardiometabolic risk factors, it is still controversial if NAFLD acts as an independent risk factor for CVD or if it is merely the hepatic manifestation of the other shared metabolic risk factors.

Subclinical vascular outcomes refer to early pathological changes in blood vessels prior to the symptomatic onset of CVD, comprising subclinical atherosclerosis and aortic aneurysm (43). Studying the association between NAFLD and subclinical vascular outcomes can reveal insights into the role NAFLD in the etiology of CVD. One feature of subclinical atherosclerosis is the presence of plaque in the carotid artery. Carotid intima-media thickness (cIMT) is a surrogate marker for carotid plaque formation and adds to the risk assessment of CVD (44). Several attempts have been made to elucidate the association between NAFLD and carotid plaque. One meta-analysis showed that people with NAFLD are at higher risk of having carotid plaque (45). However, the discrepancies in diagnosing criteria, such as the use of different medical imaging modalities or different cut-off points of cIMT to determine carotid plaque, undermines the conclusiveness of the current results (45-47).

With the advancement and wide application of medical imaging, other under-researched vascular conditions, such as aortic aneurysm, can be detected and have attracted more research interest. Aortic aneurysm is the enlargement of aorta, which is defined when thoracic aortic diameter ≥ 5cm or abdominal aortic diameter ≥ 3cm (48). Due to its asymptomatic property, it is usually discovered only accidentally during the imaging examination due to other symptoms. However, if remains

untreated, it could develop into life-threatening events such as aortic dissection or rupture (48). Several risk factors for aortic aneurysm, such as obesity, age and hyperlipidemia also increase the risk of NAFLD. One case-control study found that patients with abdominal aortic aneurysm were more likely to have NAFLD, independent of other risk factors (49). However, due to highly-selective population based on hospital records and retrospective design, the results of the study are subject to scrutiny. Population-based prospective data are needed to access the link between NAFLD and aortic aneurysm.

1.1.7 Treatment

Early stage of NAFLD with simple steatosis can be reversed by lifestyle interventions, including dietary consultancy to restrict total energy and alcohol intake as well as increasing physical activity, aiming to reduce weight (1). NASH patients with advanced fibrosis or other progressing metabolic complications, such as T2D, can be advised to pharmaceutical treatments besides lifestyle modifications (1). Up to date, there is still no specific drugs approved for the treatment of NASH. Focus has been put on curtailing the cardiometabolic complications, such as insulin resistance, inflammation and dyslipidemia, to prevent from progressing to cirrhosis. Increasing clinical trials have shown improvement in serological markers of NASH after treatment with anti-diabetic agents (e.g. thiazolidinedione insulin sensitizers), antioxidants (e.g. vitamin E) and lipid lowering agents (e.g. statins) (1, 50). However, due to the invasiveness of liver biopsy, data from trials with repeated histological assessment of the liver to test the efficacy of potential treatments are limited.

1.2 Aims and outline of this thesis

The current thesis aimed to investigate how liver fat accumulation is related to cardiometabolic health in the general population. Three research questions addressed in the respective projects are schematically presented in Figure 1.

In Project I, we explored sex differences in fatty liver. Specifically, we examined whether endogenous sex hormones, including testosterone (T), free testosterone (fT), dehydroepiandrosterone (DHEA), dehydroepiandrosterone-sulfate (DHEAS), dihydrotestosterone (DHT), free dihydrotestosterone (fDHT), progesterone, 17-alpha-hydroxyprogesterone (17-OHP), and SHBG were associated with FLI in the population-based Cooperative Health Research in the Region of Augsburg (KORA)

study. Besides, we investigated whether there is potential causal relationship linking sex hormones and SHBG to liver fat content, using summary-level genetic data from large studies such as UK-Biobank and others.

In Project II, given the high extrahepatic clinical burden of fatty liver, we put our effort on investigating whether liver fat accumulation, estimated by FLI, is an independent risk factor for CKD, which is in turn, an important risk factor driving CVD mortality. We estimated the association between FLI and kidney function parameters, including estimated glomerular filtration rate (eGFR) and albuminuria, and prevalent CKD as well as incident CKD in the population-based KORA study. We also tested whether and to what extent metabolic risk factors, such as diabetes, hypertension and inflammation, jointly mediated the association between liver fat and incident CKD.

In Project III, we investigated the role of liver fat on subclinical vascular health in two population-based studies. Specifically, we sought to delineate the cross-sectional associations between MRI measured liver fat content and various subclinical vascular disease parameters, including occurrence of carotid plaque, plaque type and morphological measurements of the carotid artery as well as diameters of ascending, descending and infrarenal aorta.

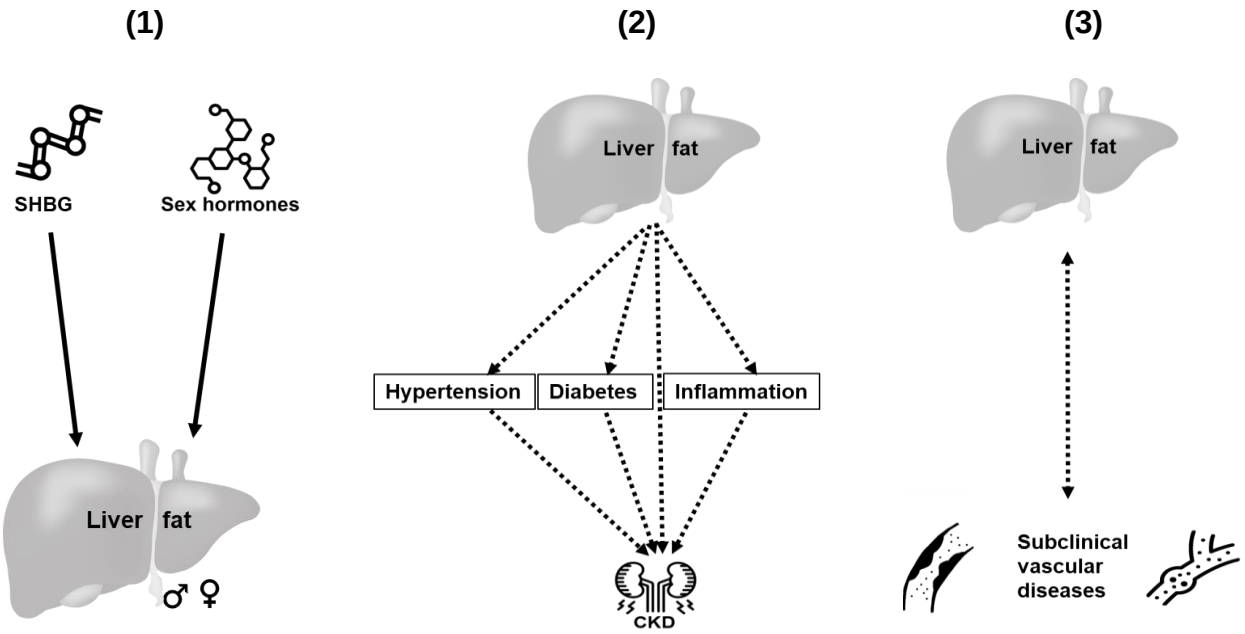

Figure 1. Schematic overview of the current thesis: liver fat and cardiometabolic health: a population-based perspective

The numbers are corresponding to the three projects included in the thesis. A dashed line with double arrows implies that an association was examined without specific direction. A dashed line with one arrow indicates that an association with assumed direction was examined. A solid line with one arrow indicates that a potential causal relationship with assumed direction was also investigated with Mendelian randomization analysis. The arrow points toward the outcome. Abbreviations: CKD, chronic kidney disease; SHBG, sex hormone-binding globulin.

1.3 Overview of methods of the thesis

1.3.1 Study design and population

All the projects of this thesis are based on the data provided by the prospective population-based KORA study (Project I, II, III) and the Study of Health in Pomerania (SHIP) (Project III) located in Germany. For the genetic analysis in Project I, other cohorts with European ancestry, such as UK Biobank, are used and listed in the supplementary material of Project I.

The prospective KORA F4 and FF4 study

The KORA study is a continuation of the MONICA project ("Monitoring of Trends and Determinants in Cardiovascular Diseases") initiated by the World Health Organization in 1984. Study participants were drawn randomly in a two-stage procedure from the city of Augsburg and two adjacent counties in south-eastern Germany. The KORA studies, among others, focus on diabetes and CVD research. The KORA F4 (conducted between 2006 and 2008) and FF4 (conducted between 2013 and 2014) studies are the first and second follow-up of the S4 survey conducted between 1999 and 2001.

The KORA FF4 MRI-substudy

The MRI-substudy was originally a case-control study within the KORA FF4 study, aiming to investigate subclinical disease burden in people with prediabetes (51). Participants from the KORA FF4 study were selected for a whole-body MRI scan, if they were younger than 72 years and did not have MRI contraindications, or history of CVD, such as stroke, central or peripheral artery disease. A sample of 400 participants, fitting in the category of either prediabetes (n=103) or diabetes (n=54) or matched normoglycemic controls (n=243), undertook the MRI examination (51).

The SHIP-TREND-0 study

In total, 4418 participants aged 20-79 years agreed to take part in the baseline examination of the SHIP-TREND study (SHIP-TREND-0), conducted between 2008 and 2012 after random selection of 8826 persons in West Pomerania, northeast Germany (52). A sub-sample of the SHIP-TREND-0 also underwent a whole body MRI scan, if they were willing to participate and they did not have MRI contraindications, such as cardiac pacemakers (53), leaving totally 1926 participants who completed the MRI examination.

1.3.2 Liver fat accumulation

In the prospective KORA F4 and FF4 study, the risk of excessive liver fat accumulation was estimated by the FLI, a score based on BMI, waist circumference, triglycerides, and GGT developed by Bedogni et al. (24). In Project I and II, the FLI was calculated with the following formula: FLI = ($e^{0.953*\log e(\text{triglycerides}) + 0.139*\text{BMI} + 0.718*\log e(\text{GGT}) + 0.053*\text{waist circumference} - 15.745}$) / ($1 + e^{0.953*\log e(\text{triglycerides}) + 0.139*\text{BMI} + 0.718*\log e(\text{GGT}) + 0.053*\text{waist circumference} - 15.745}$) * 100, with triglycerides measured in mmol/l, GGT in U/l, and waist circumference in cm. The resulting score ranges from 0 to 100, with an FLI < 30 opting out and an FLI ≥ 60 opting in fatty liver.

In the KORA FF4 MRI-substudy and SHIP-TREND-0 study, liver fat content was measured by MRI proton-density fat fraction (PDFF) at the level of portal vein. The existence of fatty liver was defined as a PDFF >5.6% (1). This measure was used in Project III.

1.3.3 Sex hormones and sex hormone-binding globulin

Serum concentrations of sex hormones, including T, DHEA, DHEAS, DHT, progesterone, and 17-OHP were quantified at the KORA F4 examination by Absolute*IDQ*™ Stero17 Kit and electrospray ionization liquid chromatography-mass spectrometry. Absolute quantification of SHBG was conducted using the chemiluminescent microparticle immunoassay ARCHITECT. The concentrations of fT and fDHT were calculated with mass action equations derived by Rinaldi et al. (54) accounting for the total sex hormone concentrations and their binding constants to serum SHBG and albumin. These measurements were used in Project I.

1.3.4 Selection of genetic instruments

GWAS aim to identify genetic variants that are statistically associated with a phenotype, such as a disease or a trait, in the entire genome of a large population (55). Summary-level genetic associations for all the sex hormones and SHBG were obtained from the largest up to date GWAS conducted in population with European ancestry for T (sex-specific), bioavailable T (bioT) (sex-specific), Estradiol (E2) (men), SHBG (sex-specific) (56), DHEAS (sex-combined) (57), progesterone (sex-specific), and 17-OHP (sex-specific) (58). Genetic instruments of E2 are not available in women, due to the small number of women with detectable E2 levels in the GWAS (56). We selected genome-wide significant genetic variants associated with sex

hormones and SHBG (p<5e-8). Genetic variants were clumped if they were in linkage disequilibrium (LD r^2>0.001). Afterwards, the gene-outcome associations were extracted from the largest up to date GWAS for MRI measured liver fat content conducted in the UK Biobank (18). Finally, we harmonized the gene-exposure and gene-outcome associations and dropped palindromic genetic variants. The genetic analysis was part of Project I.

1.3.5 Assessment of kidney function and chronic kidney disease

The eGFR and the UACR were used to estimate kidney function in KORA F4 and FF4 in Project II. The eGFR-Cr was calculated with serum creatinine accounting for age, race and sex, according to the equation established by the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) in 2009 (59). In addition, eGFR-cC based on the CKD-EPI 2012 cystatin C equation was also calculated as an alternative glomerular filtration marker, which is less affected by ethnicity and muscle mass (60). UACR was calculated by dividing the urinary albumin level by the urinary creatinine level and a UACR ≥ 30 mg/g was defined as albuminuria.

Prevalent CKD was defined, when a participant had an eGFR-Cr < 60 ml/min/1.73 m^2 at KORA F4 study. Incident CKD was determined, when a participant had an eGFR-Cr ≥ 60 ml/min/1.73 m2 at KORA F4 but an eGFR < 60 ml/min/1.73 m^2 at KORA FF4 examination. Sensitivity analysis was done using eGFR-cC to define CKD with the same threshold as eGFR-Cr.

1.3.6 Imaging measurement of subclinical vascular disease parameters

In the KORA FF4 MRI-substudy, the presence of carotid plaque was detected by MRI. The morphological features of carotid plaque, including wall thickness, lumen area and wall area, were determined and calculated for the left and right carotid artery, respectively. The classification of carotid plaque type was done according to the criteria of the America Heart Association, based on the presence of calcification, hemorrhage, wall thickness, and wall eccentricity (61). In the SHIP-TREND-0 study, the presence of carotid plaque was detected by ultrasound. The cIMT determined by ultrasound was used as a surrogate morphological marker for carotid plaque. In both the KORA FF4 MRI-substudy and the SHIP-TREND-0 study, the diameters of ascending, descending and infrarenal aorta were measured by MRI.

1.3.7 Statistical analysis

For Project I, II, III, cross-sectional and longitudinal analyses were conducted using multiple linear or logistic regression, adjusted for age, sex (except for Project I, where sex-stratified analysis was done), lifestyle (smoking, physical activity, alcohol consumption) and several cardiometabolic risk factors, such as systolic blood pressure (SBP), high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein (LDL-C), diabetes, antihypertensive medication, and lipid-lowering medication intake.

In Project I, in order to estimate the causal relationship between sex hormones or SHBG and liver fat accumulation, we further conducted two-sample Mendelian randomization (MR) analysis. Of note, the GWAS for T, bioT, E2 and SHBG was also conducted in the UK Biobank, which has an overlapping study population (<10%) with the outcome GWAS for MRI measured liver fat content (18, 56). The inverse-variance weighting (IVW) approach was used as the primary MR estimate. In addition, we also conducted additional analyses with three robust MR approaches, which provide valid and consistent MR estimates with different percentages of pleiotropic variants, including the weighted-median approach (up to 50% invalid instruments), the weighted mode approach (50% - 100% invalid instruments), and the MR-Egger approach (up to 100% invalid instruments). We considered causal evidence if the IVW estimate was significant and the estimates of the three robust MR approaches were in the congruent direction as the IVW estimate.

In Project II, the joint mediation effect of C-reactive protein (CRP), diabetes and hypertension was examined in regression models between FLI and incident CKD, adjusted for age, sex and lifestyle risk factors. The total, direct and indirect effects of FLI on incident CKD through mediators were estimated with the regression-based approach with multiple mediators (62, 63).

1.4 Summary of the main findings

Main finding Project I (Paper I in Appendix): Endogenous sex hormones, including T, fT, DHT, progesterone and 17-OHP, and SHBG were associated with FLI in a sex-specific manner. Higher genetically determined SHBG showed a suggestive causal role in decreasing liver fat content in women.

We analyzed data from 2239 participants (1328 to 1417 men and 667 to 762 postmenopausal women) from the KORA F4 study, among which 1505 participants

(941 to 1003 men and 408 to 468 postmenopausal women) also took part in the follow-up FF4 study after an average of 6.5 years. Endogenous T [(β-per standard deviation increase, 95%CI: -4.89 (-6.12, -3,66)], DHT [-2.97 (-4.20, -1.73)], progesterone [-2.75 (-4.02, -1.49)], and 17-OHP [-3.57 (-4.80, -2.34)] were inversely associated with the FLI in men, after adjustment for age, lifestyle (smoking, physical activity, alcohol consumption), and cardiometabolic risk factors, including SBP, HDL-C, LDL-C, diabetes, use of antihypertensive medication and lipid lowering medication. In postmenopausal women, fT [4.17 (1.35, 6.98)] was positively associated with the FLI. SHBG was inversely associated with the FLI in both sexes, although the effect of SHBG was stronger among postmenopausal women [-9.23 (-12.19, -6.28)] than men [-3.45 (-5.13, -1.78)].

We further explored the abovementioned associations between genetically determined sex hormones or SHBG with MRI measured liver fat by MR analysis. Using the summary level data from the largest to date GWAS with population of European ancestry for T, E2, SHBG, DHEAS, progesterone, 17-OHP and MRI hepatic PDFF respectively, our MR analyses showed a suggestive causal role of higher SHBG [-0.36 (-0.61, -0.12)] levels in decreasing liver fat content among women.

Main findings Project II (Paper II): The associations between FLI and incident CKD was not independent of cardiometabolic risk factors. Hypertension, diabetes and inflammation jointly completely mediated the relation between FLI and incident CKD.

Analyzing cross-sectional data from 2920 participants (1412 men; 1508 women) at KORA F4 study, we found that one SD increment in FLI was inversely associated with baseline eGFR-Cr, and positively associated with baseline UACR as well as higher odds of prevalent CKD based on eGFR-Cr, after adjusting for age, sex and lifestyle factors. All associations mentioned above were attenuated after further adjustment for cardiometabolic risk factors, especially CRP and hypertension. Of these baseline F4 participants, 1991 of them had complete follow-up data at the KORA FF4 study after an average of 6.5 years and were eligible for the longitudinal analysis. We observed that the baseline FLI was associated with a higher odds of incident CKD at follow-up. However, this association was not independent of cardiometabolic risk factors. Since the adjustment for cardiometabolic risk factors

substantially attenuated the association between the baseline FLI and incident CKD, we sought to investigate if diabetes, hypertension and CRP mediated this association. Due to the strong correlation between these variables, we examined the joint mediation effect of these potential mediators together. We found out that the association between FLI and incident CKD was fully mediated by these cardiometabolic factors jointly, with the proportion of mediation being 101.9% for the FLI as a continuous exposure and 92.9% for being in the highest category of the FLI (FLI ≥ 60) comparing to the lowest category of FLI (FLI < 30).

Main finding Project III (Paper III): Liver fat content measured by MRI was not independently associated with subclinical carotid plaque parameters or aortic diameters from two well characterized population-based studies.

Based on data from the SHIP-TREND-0 study for carotid plaque (n=1339), we found that higher liver fat content was associated with a higher odds of ultrasound-measured plaque presence and a greater cIMT in the age- and sex-adjusted models. However, adjusting for BMI undermined these associations. Multivariable regression analysis with data from the KORA FF4 MRI-substudy (n=367) showed that liver fat content was not associated with MRI measured plaque presence or plaque type. Neither was it associated with any morphological features related to the plaque, including wall thickness, lumen area, and wall area.

As for aortic diameters, in data from 1209 participants in the SHIP-TREND-0 study, we observed that higher MRI measured liver fat content was associated with larger ascending, descending and infrarenal aortic diameters in the age- and sex-adjusted model. Further adjustment for BMI substantially attenuated the associations and additional adjustment for cardiometabolic risk factors did not change the results. In the multivariable regression analysis with data from 367 participants in the KORA FF4 MRI-substudy with MRI measured aortic diameters, we did not observe any association between liver fat content and aortic diameters.

1.5 Discussion

This thesis investigated the effect of sex hormones and SHBG on liver fat accumulation and examined whether higher liver fat is an independent risk factor for more advanced cardiometabolic complications, such as CKD and subclinical vascular diseases.

The results show that derangement of sex hormones and SHBG is linked to the level of liver fat accumulation, and analysis using genetic instruments suggests that higher SHBG has a potential causal role in lower liver fat accumulation especially in women. The lack of association between genetically determined sex hormones and MRI measured liver fat content implies that the observational associations between sex hormones and FLI are most likely due to residual confounding or reverse causation.

The thesis also demonstrates that higher liver fat is related to worse kidney function and higher risk of developing CKD, but these associations are completely mediated by other closely related cardiometabolic disorders, such as hypertension and diabetes. Moreover, using MRI measured liver fat content from two population-based studies in Germany, it shows that higher liver fat is not independently associated with parameters of subclinical vascular disease parameters, including carotid plaque, its morphological features and aortic diameters. Their apparent associations are mainly confounded by overall adiposity and other cardiometabolic risk factors.

A more detailed discussion for the results of the analyses and their comparison with the existing literature are presented in the respective manuscripts of Project I – III. Next, some methodological considerations, clinical implications, and suggestions for future research are discussed as follows.

1.5.1 Methodological considerations

1.5.1.1 Study design and study population

We investigated the association between liver fat content and subclinical vascular diseases with a cross-sectional design in the first project. This study design allows for studying the association between exposure and outcome, but does not provide powerful evidence on the future risk of outcome attributable to the exposure, since exposure and outcome were ascertained at the same time point. Nevertheless, we found consistent results across two German population-based studies in fully adjusted models, which account for the regional differences of health status in Germany.

On the other hand, for investigating the association between sex hormones/SHBG and liver fat as well as the association between liver fat and kidney function, we implemented a prospective cohort study design, which inspects how the exposure contributes to the change in the outcome after a certain period of follow-up time.

Although prospective cohort studies allow the assessment of the risk of outcome in the future, due to its observational nature, it is still not free from residual confounding and reverse causality, which precludes causal inference.

In order to better address these limitations, we further used two-sample MR analysis with summary-level data from the largest up to date GWAS in populations of European ancestry for sex hormones, SHBG and MRI measured liver fat content to investigate the potential causal relationship between sex hormones/SHBG and liver fat accumulation. MR represents an epidemiological tool using gene-phenotype associations from GWAS to estimate potential causal relationship between exposure and outcome. Utilizing randomly inherited genetic variants as natural experiment to randomly allocate modifiable exposures, MR analysis is more robust to confounding. Ideally, two-sample MR analysis should use gene-exposure and gene-outcome associations from two GWAS without population overlap. However, for T, E2, and SHBG, gene-exposure and gene-outcome associations were both extracted from GWAS conducted with data from the UK Biobank (<10% population overlap). Two-sample MR with participant overlap is subject to “weak instrument bias”, which arises from unmeasured confounding in the overlapped sample, and increases with higher percentage of overlap and lower instrument strength. However, the sample overlap between the exposure and outcome GWAS was no more than 10% in our investigation. Due to relatively high variance (2% to 21%) of T, E2 and SHBG explained by the genetic variants and large sample size (n=178,782 to 230,454) of the exposure GWAS (56), weak instrument bias is expected to be neglectable.

1.5.1.2 Statistical considerations

In order to give an unbiased causal inference, genetic instruments used in MR should meet three key assumptions, including the relevance assumption, the independence assumption, and the exclusion restriction assumption (64). Whereby, the relevance assumption requires that genetic instruments are strongly associated with the exposure. The independence assumption requires that the associations between genetic instruments and outcome are not confounded. The exclusion restriction assumption requires that genetic instruments must not affect outcome through pathways other than the exposure of interest. The third assumption can be easily violated, especially in case of highly correlated metabolic phenotypes (65). To account for this, we used a variety of MR approaches allowing for different

percentages of pleiotropic genetic instruments in the analysis, in order to test the robustness of the MR estimates (66, 67).

Mediation analysis is another powerful tool to assess the effect of exposure attributable to a third variable and to investigate the potential mechanism underlying the association between exposure and outcome. One of the assumptions of mediation analysis requires that the confounders of mediator-outcome association should not be affected by the exposure (68). Due to the high correlation between diabetes, hypertension and inflammation, investigating the individual mediation effects of these factors would have probably violated this assumption, since the other two factors would confound the mediator-outcome association and they are affected by FLI. Therefore, we were not able to dissect the individual mediation effect of these factors. Regardless, we quantified the joint mediation effect of all these three factors in the association between FLI and incident CKD (62), adding to the literature that is predominantly of cross-sectional nature.

1.5.1.3 Data assessment

Extensive laboratory and physical examinations coupled with comprehensive health questionnaires enabled us to estimate liver fat content, ascertain cardiometabolic complications and other covariates longitudinally. Meanwhile, we were able to minimize the impact of confounding factors in the associations between liver fat and cardiometabolic health parameters by adjusting for a wide range of potential confounders or using genetic instruments fixed at conception.

MRI is the non-invasive golden standard for quantifying liver fat content (69). MRI measured liver fat content was used as exposure in the third project, which allowed us to investigate the associations of cardiometabolic parameters with the changes in liver fat accumulation more precisely. However, MRI examination is often not feasible due to its high cost. On the other hand, the FLI, consisting of serum biomarkers that are commonly available, is a cost-efficient alternative for MRI in large scale epidemiological studies. FLI can reliably predict the risk of ectopic liver fat accumulation, and its classification performance for fatty liver has been widely validated (28, 29).

For some of the sex hormones that we studied in the first project, such as progesterone and 17-OHP, we provided the first epidemiological evidence regarding their association with risk of liver fat accumulation. However, E2 measurements were

not available for the present analyses due to problems with the analyzing assay in our observational analysis, nor did we find eligible existing GWAS for E2 in women. Due to relatively high sex hormone fluctuation in premenopausal women (70), they are often not included in the investigations, because a single evaluation can only reflect the temporal hormone levels depending on the day of the menstruation cycle, and cannot be used as a precise measurement of average hormone levels.

1.5.2 Clinical implications

This thesis has given insights into the role of sex hormones and SHBG play in ectopic liver fat accumulation, as well as addressed the interplay between liver fat and other cardiometabolic risk factors in the development of more severe cardiometabolic complications.

1.5.2.1 SHBG as a potential therapeutic target to manage liver fat accumulation

SHBG is predominantly secreted by hepatocytes. Apart from its role in transporting sex hormones in the blood to their target organs, it also actively regulates metabolic homeostasis by interacting with the hepatic fatty acid receptor and insulin sensitivity (71, 72). We observed an inverse association between SHBG and FLI in our observational analysis in both men and women, which is supported by a recent meta-analysis with seven studies among men and women respectively (13). However, our MR analysis did not suggest a causal effect of SHBG on liver fat content among men. This could be a result of the physiological function of SHBG to bind and inactivate circulating T, whose reduction is associated with higher risk of NAFLD and higher liver fat among men (13).

On the other hand, we have noticed a suggestive causal effect between higher SHBG and lower liver fat content among women. Apart from epidemiological evidence suggesting a link between lower circulating SHBG and a higher risk of NAFLD and liver fat (13, 73), existing studies have indicated a close relationship between derangement in SHBG and other metabolic disorders, such as obesity and T2D, which substantially increase the risk of NAFLD (56, 74). Furthermore, lower SHBG levels were also observed with higher pro-inflammatory cytokine levels among patients with chronic low-grade inflammation (75). Together with our findings, it can be speculated that increasing serum SHBG levels could be helpful in curtailing liver

fat buildup, especially in women. However, no clinical trial has been conducted to examine the effect of SHBG treatment in reducing liver fat. Thus, further research is needed to confirm the effectiveness of increasing SHBG in inhibiting liver fat accumulation.

1.5.2.2 Importance of monitoring cardiometabolic risk factors among people with non-alcoholic fatty liver disease and vice versa

NAFLD and CKD share several risk factors, including T2D and hypertension (33). This thesis suggests that liver fat increase may exacerbate cardiometabolic risk factors, such as diabetes, hypertension and inflammation, which in turn could lead to the development of CKD. Previous research on the association between NAFLD and CKD in European population was mainly restricted to cross-sectional data and highly selected hospital samples (39, 76-78). Longitudinal data regarding whether NAFLD independently contributes to incident CKD are controversial (38, 40). However, there is evidence that the coexistence of NAFLD with other cardiometabolic risk factors, especially T2D, is predictive of not only the progression of simple steatosis to liver fibrosis but also the development of more advanced cardiovascular complications, such as CKD and CVD (1, 79). On the other hand, many treatments for T2D, such as insulin sensitizers, have been shown to be effective in reducing liver fat and ameliorating inflammation in obese patients with NASH (50).

Although the results of this thesis do not justify the screening of NAFLD in the general population due to the invasiveness of biopsy and high cost of reliable non-invasive diagnostic methods, it does support the recommendation that people with metabolic derangements, especially hypertension, and T2D should be screened for NAFLD (1). On the other hand, it has been noticed that histological improvement of NASH was associated with improved kidney function and better insulin sensitivity (80). This suggests that people with NAFLD should also be monitored and treated for metabolic disorders, in order to prevent them from more advanced cardiovascular complications.

1.5.2.3 Weight loss as an indispensable approach to improve overall cardiometabolic health

It is recommended that people with early stage NAFLD without inflammation or fibrosis should receive lifestyle advices on caloric restriction and increasing physical

activity, both aiming for weight loss. Intervention studies have shown the effectiveness of weight loss in reducing liver fat, improving histological assessment of the liver as well as amelioration of NASH (1). Furthermore, weight loss intervention was reported to reduce oxidative stress markers of subclinical atherosclerosis and thus CVD risk (81).

The third project of the thesis revealed that BMI substantially attenuated the associations between liver fat content and subclinical vascular diseases, indicating that higher BMI, a marker for overall obesity, mainly drives these associations. Considering the close relationship between NAFLD and cardiometabolic risk factors, lifestyle modification can be beneficial on mitigating a wide spectrum of cardiometabolic disorders.

1.5.3 Future directions

In the first and the second projects of this thesis, we used the FLI as a surrogate marker to estimate the risk of excessive liver fat accumulation. The FLI is a widely validated surrogate marker to predict the presence of fatty liver in population-based studies, where biopsy and medical imaging modalities are not feasible due to budget. However, as the gold standard of non-invasive measurement of liver fat content, MRI should be recommended for more precise liver fat quantification in future studies.

NASH, the more advanced stage of NAFLD, and especially the progression of NASH fibrosis has been shown to more substantially increase the risk of developing end stage liver disease, such as cirrhosis and hepatocyte carcinoma, as well as cardiovascular complications than simple steatosis (33). We were not able to capture the occurrence of NASH in our investigations due to unavailability of relevant data. Although liver biopsy is still the gold standard for the histological diagnosis of NASH, advancement in medical imaging modalities, such as elastography with ultrasound or MRI, provides possibilities to non-invasively assess fibrosis and its progression (82). Future studies should examine the cardiometabolic impact of more advanced NAFLD using non-invasive measurements of NASH.

Since liver fat accumulation is a reversible process in the early stage, studies need to assess the progression or amelioration of NAFLD as well as the development of other cardiometabolic risk factors with repeated measurements at more frequent follow-up time points. This will allow us to better understand the chronological

interaction between the severity of liver fat accumulation and various cardiometabolic risk factors, and to better elucidate the underlying pathological mechanisms linking them together. By this means, we can conceptualize targeted strategies to prevent the progression of these metabolic disorders.

1.6 Conclusions

To understand the role of liver fat accumulation in the risk assessment of cardiometabolic diseases, it is important to evaluate the associations between liver fat content and cardiometabolic outcomes, such as CKD and CVD. Considering sex differences observed in the prevalence of NAFLD and in the related cardiometabolic profiles as well as the lack of targeting treatment for NAFLD, it is also valuable to elucidate the effect of sex hormones and SHBG on liver fat accumulation. This thesis suggests that increasing serum SHBG, within the therapeutic range to avoid causing other derangements, may be beneficial in preventing or reversing liver fat accumulation in women. It also suggests a link between liver fat accumulation and an elevated risk of various cardiometabolic disorders, including diabetes and hypertension, which could subsequently lead to the development of CKD. This highlights the importance of monitoring and treating cardiometabolic complications among people with NAFLD. Although the association between liver fat content and subclinical vascular diseases was found not to be independent of BMI, it indicates the pivotal role of obesity in the pathology of both liver fat accumulation and cardiovascular diseases. Considering the close relationship between NAFLD and other cardiometabolic derangements, people with NAFLD should be advised to lose weight in case of concomitant obesity and be screened for CVD outcomes.

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2. Paper II

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ORIGINAL ARTICLE

Association between the fatty liver index and chronic kidney disease: the population-based KORA study

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ABSTRACT

Background. We aimed to evaluate the relationship of fatty liver, estimated by the fatty liver index (FLI), with kidney function and chronic kidney disease (CKD) in a German cohort study, given the lack of prospective evidence in Europeans.

Methods. We included 2920 participants (51.6% women, mean age 56.1 years) from the KORA study, of which 1991 were followed up for an average of 6.5 years (± 0.3). Kidney function was assessed using the glomerular filtration rate estimated by creatinine (eGFR-Cr) or cystatin C (eGFR-cC). We used multiple logistic or linear regressions to evaluate the associations between the FLI, kidney function and CKD (eGFR < 60 ml/min/1.73 m^2) and mediation analysis to explore the mediation effects of metabolic factors.

Results. The prevalence of FLI ≥60 and CKD was 40.4% and 5.6% at baseline, respectively, and 182 participants developed CKD during the follow-up. Cross-sectionally, FLI was significantly inversely associated with eGFR-cC {$\beta = -1.14$ [95% confidence interval (CI) −1.81 to −0.47]} and prevalent CKD based on eGFR-cC [OR 1.28 (95% CI 1.01–1.61)], but not with other markers. After adjusting for lifestyle factors, we found a positive association between FLI and incident CKD defined by eGFR-cC or/eGFR-Cr, which was attenuated after controlling for metabolic risk factors. Mediation analysis showed that the association was completely mediated by inflammation, diabetes and hypertension jointly.

Conclusion. The positive association between FLI and CKD incidence was fully mediated by the joint effect of metabolic risk factors. Future longitudinal studies need to explore the chronological interplay between fatty liver, cardiometabolic risk factors and kidney function with repeated measurements.

Keywords: cardiometabolic risk factors, chronic kidney disease, European cohort, fatty liver index, mediation analysis

INTRODUCTION

Chronic kidney disease (CKD) affects 8–16% of the population in developed countries and its prevalence continues to increase worldwide, accelerated by the increase in metabolic risk factors such as diabetes, hypertension and obesity [1, 2]. Nevertheless, the management of traditional cardiometabolic risk factors has shown limited efficacy in curtailing the incidence of CKD [1]. Kidney function at its late stage represents an independent risk factor for cardiovascular morbidity, mortality and decreased quality of life, with a high burden on healthcare systems [2].

Fatty liver, a condition characterized by ectopic fat accumulation in the hepatic cells [3], is closely related to a spectrum of cardiometabolic risk factors involved in the pathophysiology of CKD and represents a potential novel modifiable risk factor for CKD [4]. Indeed, cross-sectional studies have shown a 2- to 10-fold increased prevalence of CKD among people with fatty liver compared with those without [5]. However, longitudinal evidence relating fatty liver to incident CKD in the general population is controversial and largely limited to Asian populations [6–10]. Due to genetic predisposition and environmental factors, discrepancies have arisen between populations with different ethnic backgrounds

KEY LEARNING POINTS

What is already known about this subject?

- People with fatty liver are at higher risk of developing chronic kidney disease (CKD), but it is still debatable if fatty liver constitutes an independent risk factor for CKD.
- Cardiometabolic conditions, such as diabetes and hypertension, are commonly involved in the pathogenesis of both fatty liver and CKD.
- The longitudinal evidence on the association between fatty liver and incident CKD has been contradictory and largely restricted to Asian populations.

What this study adds?

- In a large German cohort study, we found a positive association between fatty liver estimated by the fatty liver index (FLI) and CKD development after adjusting for lifestyle factors, but additional adjustment for cardiometabolic risk factors attenuated this association.
- The putative positive association between increased FLI and the risk of CKD was completely mediated by metabolic risk factors, i.e. diabetes, hypertension and inflammation, concomitant to fatty liver.

What impact this may have on practice or policy?

- Continuous clinical monitoring and management of accompanying comorbidities such as diabetes and hypertension in people with or at increased risk for fatty liver is recommended in order to prevent the development and progression of CKD.
- The use of easy and cost-effective indices (such as FLI) to estimate fatty liver risk in ambulatory or low-resource settings could help identify people who require close cardiometabolic monitoring as a measure for CKD prevention.

[11]. European population studies are limited by their low number of subjects and by their selective samples (e.g. hospitalized patients) [12–14]. Therefore, prospective studies investigating the association between fatty liver and CKD in general European populations are needed.

Unlike the gold standard diagnosis for fatty liver, i.e. liver biopsy, the fatty liver index (FLI) is a cost-effective and non-invasive tool to predict fatty liver in the general population [15, 16]. Based on body mass index (BMI), waist circumference, triglycerides (TGs) and gamma-glutamyl transferase (GGT), FLI has shown excellent performance in ruling in or ruling out fatty liver [15, 17–19].

In this prospective, population-based cohort study using FLI as a surrogate marker for fatty liver, we aimed to assess the association of FLI with kidney function and CKD development. Furthermore, we explored the potential joint-mediating role of the most important cardiometabolic risk factors, including diabetes, hypertension and inflammation, in this relationship.

MATERIALS AND METHODS

Population

The KORA (Cooperative Health Research in the Region of Augsburg) S4 survey was conducted between 1999 and 2001 and recruited 4261 participants ages 25–74 years from the general population. All participants underwent a standardized interview and a medical examination for the assessment of socio-economic and anthropometric measurements, lifestyle and physical health status [20–22]. The participants were followed up in a second visit between 2006 and 2008 (KORA F4, 3080 participants) and a third visit between 2013 and 2014 (KORA FF4, 2279 participants). The original aim of the S4/F4/FF4 study was to investigate the prevalence, trajectories and risk factors of cardiometabolic outcomes in the general population [20–22].

For the present analysis, KORA F4 was used as the baseline examination, since liver enzymes necessary for calculation of the FLI were lacking in S4. The study sample for the cross-sectional analyses included 2920 participants (1508 women, 1412 men) (see Fig. 1 for details). Of these, 2076 participated in the FF4 follow-up examination. After applying further exclusion criteria listed in Fig. 1, the final study population for the longitudinal analysis comprised 1991 participants (1018 women, 973 men) (Fig. 1).

All study participants provided written informed consent. The study was approved by the ethics committees of the Bavarian Chamber of Physicians (approval 06068), in adherence with the Declaration of Helsinki.

Laboratory and clinical measurements

After an overnight fast of at least 8 hours, a random spot urine sample and a blood sample without stasis were collected from each participant. Before blood sampling, participants were asked if they had a chronic infection with hepatitis B or C virus (HBV/HCV). Blood samples were kept at 4°C until centrifugation. Liver enzymes GGT, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were analysed using the Cobas system (Roche Diagnostics, Mannheim, Germany) according to the recommendations of the International Federation of Clinical Chemistry from 1983 (confirmed and extended in 2002) [23]. Serum total cholesterol (CHOL Flex), high-density lipoprotein cholesterol (HDL-C; AHDL Flex) and low-density lipoprotein cholesterol (LDL-C; ALDL Flex) concentrations were measured accord-

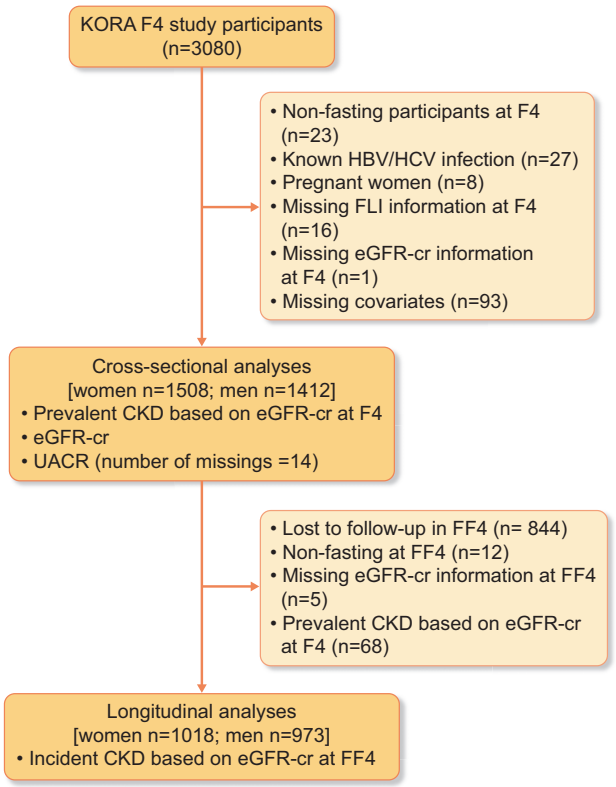

Figure 1: Flow chart of the study population. eGFR-Cr based on the equation established by the CKD-EPI (2009).

ing to the enzymatic methods (CHOD-PAP; Dade Behring, Marburg, Germany). TGs were measured by an enzymatic colour test (GPO-PAP method, TGL Flex; Dade Behring). Serum creatinine was assessed by a modified kinetic rate Jaffe method (Krea Flex; Dade Behring). High-sensitivity C-reactive protein (CRP) and serum cystatin C were determined by nephelometry on a BN II analyser (Siemens, Erlangen, Germany) from the frozen plasma and serum samples that were stored at −80°C until assaying. Urinary albumin and urinary creatinine concentrations were determined from the frozen urine samples that were stored at −80°C until assaying. Urinary creatinine was measured by a modified kinetic rate Jaffe method (CREATININ-JK, Greiner, Bahlingen, Germany) on a Cobas Mira analyser (Roche Diagnostics) [24] and urinary albumin was measured by nephelometry on a BN II analyser (Siemens).

Other clinical measurements, including oral glucose tolerance test, blood pressure and anthropometric measurements, and lifestyle ascertainment are described in the Supplementary Material [23, 25–28].

Definition of FLI

FLI was calculated based on BMI, waist circumference, TGs and GGT according to the algorithm developed by Bedogni *et al.* (15):

$$\text{FLI} = (e^{0.953*\log e(\text{TG}) + 0.139*\text{BMI} + 0.718*\log e(\text{GGT}) + 0.053*\text{waist circumference} - 15.745)} / (1 + e^{0.953*\log e(\text{TG}) + 0.139*\text{BMI} + 0.718*\log e(\text{GGT}) + 0.053*\text{waistcircumference} - 15.745)} * 100,$$

where TG is measured in milligrams per decilitre, GGT in units per litre and waist circumference in centimetres. The score ranges from 0 to 100, with an FLI <30 ruling out and an FLI ≥60 ruling in fatty liver.

Definition of estimated glomerular filtration rate (eGFR) and CKD

The eGFR was calculated from serum creatinine (eGFR-Cr), considering age, race and sex, in accordance with the equation established by the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) [29]. Serum cystatin C has been suggested to be an alternative glomerular filtration marker, which is less affected by ethnicity and muscle mass volume [30]. We also used serum cystatin C to calculate eGFR (eGFR-cC) based on the CKD-EPI 2012 cystatin C equation [31].

The level of eGFR-Cr was assessed both in the baseline F4 study and in the follow-up FF4 study for defining CKD-related outcomes. CKD was defined as an eGFR-Cr <60 ml/min/1.73 m^2. Incident CKD was defined as having an eGFR-Cr ≥60 ml/min/1.73 m^2 at the baseline and an eGFR-Cr <60 ml/min/1.73 m^2 at the follow-up visit. The same criteria were used when defining CKD based on eGFR-cC.

Urinary albumin:creatinine ratio (UACR)

The UACR reflects elevated urinary protein and is another marker of kidney function decline. The UACR was calculated by dividing the urinary albumin concentration (in milligrams) by the urinary creatinine concentration (in grams). Albuminuria was defined as a UACR ≥30 mg/g (32).

Statistical analysis

Baseline characteristics of the participants were compared among the categories of the FLI. Continuous variables are displayed as the arithmetic mean and standard deviation (SD) when normally distributed or the median and interquartile range (IQR) when non-normally distributed. For categorical variables, counts and percentages are shown. Differences in the baseline characteristics between the FLI categories were tested with analysis of variance (ANOVA) for continuous variables and chi-squared tests for categorical variables.

The FLI was *Z*-standardized prior to the subsequent analyses. We used linear regression to examine the association between the FLI and continuous outcomes (i.e. baseline eGFR and baseline UACR). Because the exact time of CKD diagnosis was not available, we could not calculate the time-to-event data of incident CKD, so we used logistic regression to examine the association between the FLI and binary outcomes (i.e. prevalent and incident CKD). Three models were constructed based on potential confounders and mediators from previous literature. Model 1 was adjusted for age and sex. Model 2 was further adjusted for lifestyle factors, including smoking status, physical activity and alcohol consumption. In order to investigate the effect of potential mediators in this relationship, we added individually one at a time metabolic risk factors

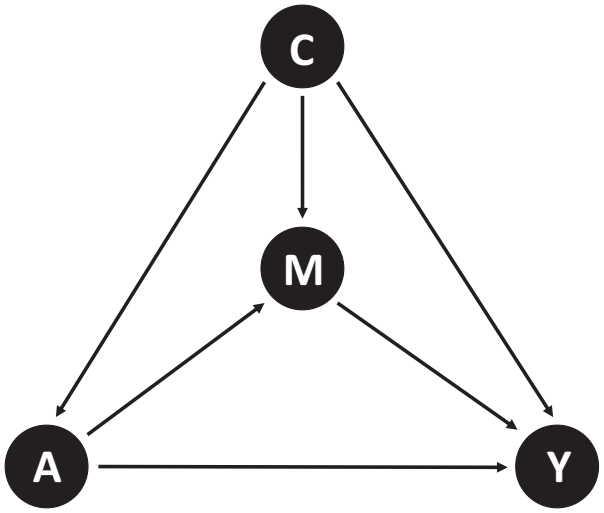

Figure 2: Directed acyclic graph of the variables used in the mediation analysis. **A** (exposure): FLI (continuous) or FLI ≥60 as a proxy for fatty liver; **M** (mediators): CRP (continuous), hypertension (yes/no), diabetes (yes/no); **Y** (outcome): incident CKD (yes/no); **C** (covariates not affected by the exposure): age, sex, smoking, physical activity, alcohol intake.

representing hyperlipidaemia (i.e. total cholesterol and HDL-C), hypertension (yes/no), inflammation (CRP) and diabetes (yes/no) to model 2. Model 3 was adjusted for all the above-mentioned metabolic risk factors simultaneously. For incident CKD, we calculated model 4, which was additionally adjusted for baseline eGFR.

Some investigations suggested that a more severe phenotype of fatty liver involving liver injury would be more detrimental to cardiometabolic health [33, 34]. Therefore we also examined incident CKD in relation to a more severe condition of fatty liver with liver injury, defined as an FLI ≥60 and elevated ALT levels (men: ≥500 nkat/l; women: ≥317 nkat/l) [35, 36].

Sensitivity analyses were done among participants without excessive alcohol intake (men <30 g/day, women <20 g/day) and intake of steatogenic drugs, including corticosteroid, tamoxifen and methotrexate. The interaction between the FLI and hypertension or diabetes was examined by entering a multiplication term (FLI × hypertension/diabetes) into the regression models. It has been implied that fatty liver could increase the risk of CKD, especially among diabetes patients, so we stratified our analysis according to the presence of diabetes at baseline. Since sex differences in fatty liver prevalence are observed in the general population, we also repeated the analysis within each sex stratum.

We performed causal mediation analysis to quantify the extent to which the association between the FLI and incident CKD was mediated by cardiometabolic risk factors (Fig. 2). Of note, because TGs, an important parameter of hyperlipidaemia, were included in the calculation of the FLI, we only considered hypertension, inflammation (measured through CRP) and diabetes to be potential mediators of the relationship between the FLI and incident CKD. Due to the high correlation between these factors, the mediation effects of the single factors were not exclusive of each other [37]. Therefore we assessed the effect mediated jointly by all three mediators together [37]. Covariates not affected by the exposure [38], including age, sex, smoking, physical activity and alcohol intake, were adjusted in the mediation analysis.

The mediation analysis was based on the counterfactual framework introduced by Robins and Greenland [39] and Pearl [40]. The total effect (TE) of the FLI on CKD can be decomposed into a direct effect (DE) and an indirect effect (IE), whereby the DE depicts the effect of the exposure on the outcome that is independent of the mediators. The IE depicts the effect of the exposure on the outcome that could be explained by the mediators. The proportion of the association explained by the mediators [IE/(DE + IE)] was estimated to quantify the magnitude of mediation. The TE, DE and IE were estimated using the regression-based approach proposed by Valeri *et al.* [41] and VanderWeele *et al.* [37], which allows for multiple correlated mediators to be considered jointly. The R package 'CMAverse' (R Foundation for Statistical Computing, Vienna, Austria) was used for the mediation analyses. Direct counterfactual imputation was used to obtain the mediation effects. Standard errors of the mediation effects were estimated by bootstrapping 200 times.

A *P*-value <.05 was set as the significance level. All analyses were performed with R version 4.1.0 (R Foundation for Statistical Computing).

RESULTS

Cross-sectional analyses

Among 2920 participants eligible for the cross-sectional analyses, 1181 (40.4%) had an FLI ≥60 and 163 (5.6%) had prevalent CKD (based on eGFR-Cr). The participants were on average 56 years old and there were slightly more women [1058 (51.6%)] than men [1412 (48.4%)]. Most of them were overweight, with an average BMI of ∼28 kg/m^2. Table 1 shows the baseline characteristics of the participants according to the FLI categories. Participants in higher FLI categories were older and more likely to be men. They had higher BMIs and larger waist circumferences. They had an unfavourable lifestyle as well as a worse metabolic profile, such as suffering more frequently from hyperlipidaemia, hypertension and diabetes. Meanwhile, higher CRP concentrations, lower baseline eGFR-Cr/eGFR-cC levels and higher CKD prevalence were observed among them. Participants in the highest FLI category had higher UACRs and suffered more frequently from albuminuria.

A 1 SD increase of the FLI was significantly associated with a lower eGFR-Cr at baseline only in models 1 and 2. Further adjustment for metabolic risk factors, especially the inclusion of hypertension and CRP, substantially attenuated the associations {$\beta = -0.43$ [95% confidence interval (CI) 1.09–0.23]}. Accordingly, a higher FLI was significantly associated with higher odds of prevalent CKD defined by eGFR-Cr in models 1 and 2. However, adjustment for metabolic risk factors substantially attenuated the associations [odds ratio (OR) 1.23 (95% CI 0.95–1.58)] (Table 2).

In contrast, the association between a higher FLI and lower baseline eGFR-cC and higher odds of prevalent CKD defined by eGFR-cC remained significant even after metabolic risk

Table 1: Baseline characteristics of participants according to the cut-off points of the FLI.

Characteristics	FLI <30 ($n = 1006$)	FLI ≥30–<60 ($n = 733$)	FLI ≥60 ($n = 1181$)	Total ($N = 2920$)	*P*-value
Age (years)	50.6 (12.2)	57.5 (13.5)	59.8 (12.2)	56.1 (13.2)	**<.001**
Women, *n* (%)	751 (74.7)	335 (45.7)	422 (35.7)	1508 (51.6%)	**<.001**
BMI (kg/m^2)	23.5 (2.3)	27.0 (2.3)	31.6 (4.3)	27.6 (4.8)	**<.001**
Waist circumference (cm)	80.1 (7.2)	92.9 (5.5)	106.0 (10.3)	93.8 (14.0)	**<.001**
Smoking, *n* (%)					**<.001**
Never smoker	454 (45.1)	320 (43.7)	447 (37.8)	1221 (41.8%)	
Ex-smoker	353 (35.1)	265 (36.2)	564 (47.8)	1182 (40.5%)	
Smoker	199 (19.8)	148 (20.2)	170 (14.4)	517 (17.7)	
Physically active, *n* (%)	630 (62.6)	417 (56.9)	554 (46.9)	1601 (54.8%)	**<.001**
Alcohol consumption, *n* (%)					**<.001**
None	308 (30.6)	213 (29.1)	351 (29.7)	872 (29.9)	
Moderate	529 (52.6)	381 (52.0)	546 (46.2)	1456 (49.9%)	
Excessive	169 (16.8)	139 (19.0)	284 (24.0)	592 (20.3)	
Systolic blood pressure (mmHg)	113.8 (16.3)	123.2 (17.9)	128.7 (17.9)	122.2 (18.5)	**<.001**
Diastolic blood pressure (mmHg)	71.8 (8.7)	75.2 (9.5)	78.0 (10.5)	75.1 (10.0)	**<.001**
Hypertension, *n* (%)	154 (15.3)	285 (38.9)	674 (57.1)	1113 (38.1%)	**<.001**
Total cholesterol (mg/dl)	207.4 (36.8)	217.6 (38.0)	221.8 (41.1)	215.8 (39.4)	**<.001**
HDL-C (mg/dl)	64.2 (14.1)	55.1 (12.8)	49.5 (11.9)	56.0 (14.4)	**<.001**
LDL-C (mg/dl)	125.2 (32.7)	140.8 (33.0)	142.2 (35.5)	136.0 (34.8)	**<.001**
TGs (mg/dl), median (IQR)	68.0 (53.0–92.8)	104.0 (78.0–133.0)	149.0 (110.0–207.0)	104.0 (71.0–149.0)	**<.001**
ALT (μkat/l)	0.3 (0.2)	0.4 (0.2)	0.5 (0.3)	0.4 (0.3)	**<.001**
AST (μkat/l)	0.4 (0.1)	0.4 (0.2)	0.5 (0.3)	0.4 (0.2)	**<.001**
GGT (U/l), median (IQR)	21.0 (17.0–26.0)	28.0 (22.0–37.0)	40.0 (29.0–62.0)	28.0 (21.0–43.0)	**<.001**
CRP (mg/l), median (IQR)	0.7 (0.3–1.3)	1.2 (0.6–2.5)	1.9 (1.0–3.8)	1.2 (0.6–2.6)	**<.001**
Diabetes, *n* (%)	15 (1.5)	66 (9.0)	247 (20.9)	328 (11.2)	**<.001**
Antihypertensive medication, *n* (%)	120 (11.9)	221 (30.2)	548 (46.4)	889 (30.4)	**<.001**
eGFR-Cr (ml/min/1.73 m^2)	93.5 (14.8)	87.1 (16.9)	83.5 (16.8)	87.8 (16.7)	**<.001**
eGFR-cC (ml/min/1.73 m^2)	100.6 (16.4)	90.8 (20.3)	85.9 (20.7)	92.2 (20.2)	**<.001**
Prevalent CKD (eGFR-Cr <60), *n* (%)	17 (1.7)	45 (6.1)	101 (8.6)	163 (5.6)	**<.001**
Prevalent CKD (eGFR-cC <60), *n* (%)	21 (2.1)	63 (8.6)	142 (12.0)	226 (7.7)	**<.001**
Albuminuria, *n* (%)	54 (5.4)	50 (6.8)	158 (13.5)	262 (9.0)	**<.001**
UACR (mg/g), median (IQR)	5.5 (3.7–9.8)	5.2 (3.4–9.9)	6.8 (3.9–14.7)	5.9 (3.7–11.5)	**<.001**
FLI at baseline	14.3 (8.1)	44.6 (8.8)	81.2 (11.4)	49.0 (30.6)	**<.001**

Values are presented as mean (SD) unless stated otherwise.
P-values were generated by ANOVA for continuous variables and chi-squared test for categorical variables. *P*-values <.05 are shown in bold.
eGFR-Cr was based on the equation established by the CKD-EPI (2009). eGFR-cC was on the equation established by the CKD-EPI (2012).
Excessive alcohol consumption was defined as men with an alcohol intake ≥30 g/day and women ≥20 g/day.
Number of missing values for eGFR-cC was 1.
Number of missing values for albuminuria was 14.

Table 2: Association of the FLI with kidney function and prevalent CKD in the KORA F4 study.

Variable	*n*	Model 1	*P*-value	Model 2	*P*-value	Model 3	*P*-value
		β (95% CI)		β (95% CI)		β (95% CI)	
eGFR-Cr	2920	−1.73 (−2.25 to −1.21)	<.001	−1.81 (−2.33 to −1.28)	<.001	−0.43 (−1.09–0.23)	.201
eGFR-cC	2919	−3.31 (−3.85 to −2.76)	<.001	−3.20 (−3.74 to −2.65)	<.001	−1.14 (−1.81 to −0.47)	.001
UACR	2906	0.09 (0.05–0.13)	<.001	0.08 (0.04–0.12)	<.001	−0.02 (−0.08–0.03)	.351
		OR (95% CI)		OR (95% CI)		OR (95% CI)	
Prevalent CKD based on eGFR-Cr	2920	1.57 (1.27–1.94)	<.001	1.61 (1.30–2.00)	<.001	1.23 (0.95–1.58)	.117
Prevalent CKD based on eGFR-cC	2919	1.72 (1.41–2.08)	<.001	1.70 (1.40–2.07)	<.001	1.28 (1.01–1.61)	.039

Model 1 was adjusted for age and sex.
Model 2: model 1 + smoking, physical activity and alcohol consumption.
Model 3: model 2 + total cholesterol, HDL-C, CRP, diabetes and hypertension.
The FLI was standardized prior to the analysis. The coefficient estimates represent the change of the outcomes corresponding to a 1 SD increase of the FLI.
Prevalent CKD was defined as eGFR-Cr or eGFR-cC <60 ml/min/1.73 m^2 at the baseline F4 study.
eGFR-Cr was based on the equation established by the CKD-EPI (2009). eGFR-cC was based on the equation established by the CKD-EPI (2012).

factor adjustments in model 3 [eGFR-cC: $\beta = -1.14$ (95% CI −1.81 to −0.47); CKD: OR 1.28 (95% CI 1.01–1.61)]. A higher FLI was not associated with baseline UACR after adjustment for metabolic risk factors [$\beta = -0.02$ (95% CI −0.08–0.03)] (Table 2).

Longitudinal analyses

During a mean follow-up of 6.5 years (SD 0.3), 182 (9.1%) participants newly developed CKD (based on eGFR-Cr), with half of the incident cases among participants with a baseline FLI ≥60. In the regression analyses, a 1 SD increase in the

Table 3: Association of the FLI or severe phenotype of fatty liver with liver injury and incident CKD (based on eGFR-Cr/cC) in the KORA F4-FF4 study.

	FLI		Fatty liver with liver injury	
Model	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
Incident CKD based on eGFR-Cr ($n = 1991$)				
Model 1	1.26 (1.03–1.53)	.023	1.16 (0.77–1.74)	.476
Model 2	1.24 (1.02–1.51)	.035	1.12 (0.74–1.69)	.590
Model 2 + total cholesterol and HDL-C	1.13 (0.90–1.42)	.282	0.94 (0.61–1.45)	.784
Model 2 + CRP	1.16 (0.94–1.43)	.168	1.04 (0.68–1.57)	.866
Model 2 + diabetes	1.18 (0.96–1.45)	.121	1.01 (0.66–1.55)	.961
Model 2 + hypertension	1.10 (0.89–1.35)	.387	0.96 (0.63–1.46)	.842
Model 3	0.91 (0.70–1.17)	.446	0.77 (0.49–1.20)	.242
Model 4	0.85 (0.65–1.12)	.247	0.70 (0.43–1.14)	.151
Incident CKD based on eGFR-cC ($n = 1927$)				
Model 1	1.66 (1.35–2.04)	<.001	1.85 (1.24–2.75)	.003
Model 2	1.64 (1.33–2.02)	<.001	1.84 (1.23–2.76)	.003
Model 2 + total cholesterol and HDL-C	1.59 (1.26–2.01)	<.001	1.60 (1.05–2.44)	.030
Model 2 + CRP	1.38 (1.10–1.72)	.005	1.54 (1.02–2.32)	.040
Model 2 + diabetes	1.71 (1.38–2.12)	<.001	1.90 (1.26–2.87)	.003
Model 2 + hypertension	1.51 (1.22–1.87)	<.001	1.64 (1.09–2.46)	.018
Model 3	1.27 (0.98–1.65)	.076	1.35 (0.87–2.10)	.177
Model 4	1.11 (0.83–1.47)	.485	1.29 (0.78–2.12)	.319

Model 1 was adjusted for age and sex.
Model 2: model 1 + smoking, physical activity and alcohol consumption.
Model 3: model 2 + total cholesterol, HDL-C, CRP, diabetes and hypertension.
Model 4: model 3 + baseline eGFR-Cr/cC.
The FLI was standardized prior to the analysis. The coefficients represent the OR of incident CKD according to a 1 SD increase of the FLI.
Fatty liver with liver injury was defined as a FLI ≥60 and elevated ALT levels (men: ≥500 nkat/l; women: ≥317 nkat/l).
Incident CKD was defined as an eGFR-Cr/cC <60 ml/min/1.73 m^2 at the follow-up FF4 study and eGFR-Cr/cC ≥60 ml/min/1.73 m^2 at the baseline F4 study.
eGFR-Cr was based on the equation established by the CKD-EPI (2009). eGFR-cC was based on the equation established by the CKD-EPI (2012).

FLI was significantly associated with higher odds of developing CKD after age, sex and lifestyle adjustment [model 2: OR 1.24 (95% CI 1.02–1.51)]. However, further adjustment for metabolic risk factors evidently undermined the associations [model 3: OR 0.91 (95% CI 0.70–1.17)] (Table 3). Moreover, fatty liver with liver injury (FLI ≥60 with elevated ALT levels) was not associated with incident CKD in any of the models [model 3: OR 0.77 (95% CI 0.49–1.20)] (Table 3). Analyses with incident CKD defined by eGFR-cC showed that a 1 SD increase in the FLI was associated with higher odds of incident CKD in models 1 and 2 [model 2: OR 1.64 (95% CI 1.33–2.02)]. However, further adjustment for metabolic risk factors attenuated the association [model 3: 1.27 (95% CI 0.98–1.65)] (Table 3). Similarly, fatty liver with liver injury was only associated with incident CKD based on eGFR-cC in models 1 and 2 [model 2: 1.84 (95% CI 1.23–2.76)], but not after adjustment for all metabolic risk factors [model 3: 1.35 (95% CI 0.87–2.10)] (Table 3).

Sensitivity analyses

After excluding participants with excessive alcohol intake or steatogenic medication intake, the regression analyses yielded similar results for both cross-sectional and longitudinal analyses (Supplementary Tables 1 and 3). We found significant interaction between the FLI and diabetes for the association between the FLI and baseline eGFR-Cr (*P* for interaction = .002). In the subgroup analysis we found that among participants with diabetes their FLI was significantly associated with lower baseline eGFR-Cr [β −3.81 (95% CI −6.32 to −1.31)] as well as higher odds of prevalent CKD based on eGFR-Cr [OR = 1.95 (95% CI 1.09–3.49)] in the full model, whereas in the non-diabetic group, we did not find any significant association (Supplementary Table 2). Longitudinally, we found that the FLI was not associated with incident CKD in the full model in either subgroup (Supplementary Table 2). We did not observe any interaction for the FLI with hypertension in the association analyses. In the sex-stratified analysis, effect estimates were similar in men and women and they did not reach statistical significance (Supplementary Table 4).

Mediation analysis

When CRP, diabetes and hypertension were examined together for their joint mediation effects, a 1 SD increase in the FLI indirectly increased the odds of developing incident CKD through these three mediators [OR 1.21 (95% CI 1.08–1.32)]. When the regression was conditional on all three potential mediators, the FLI had a non-significant inverse direct effect on incident CKD [0.995 (95% CI 0.84–1.18)]. Consequently, the proportion mediated by all three potential mediators jointly exceeded 100% (101.9%; $P = .02$) (Table 4). Of note, the proportion mediated exceeding 100% represents a mathematical result accounting for the directional change of the association between the FLI and incident CKD after adjusting for all three mediators in the model. To help with the intuitive understanding, we ran the mediation analysis comparing the highest FLI category (FLI ≥60) to the lowest (FLI <30) and also found an indirect increase in incident CKD through the mediators [1.52 (95% CI 1.21–1.79)]. The proportion mediated through CRP, diabetes and hypertension was 92.9% (Table 4). These results suggest that the effect of the FLI on incident CKD was completely mediated by inflammation, diabetes and hypertension jointly. The sensitivity analysis with CKD

Table 4: Mediation analysis for the association between the FLI and CKD (based on eGFR-Cr) development mediated through the joint effect of diabetes, inflammation and hypertension.

Variable	Multiple mediators					
	FLI (1 SD increase)		FLI ≥30–<60 (*ref* FLI<30)		FLI ≥60 (*ref* FLI<30)	
	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
Direct effect	0.996 (0.84–1.18)	.95	1.18 (0.83–1.78)	.43	1.04 (0.67–1.57)	.77
Indirect effect	1.21 (1.08–1.32)	**<.001**	1.24 (1.09–1.33)	**<.001**	1.52 (1.21–1.79)	**<.001**
Total effect	1.20 (1.03–1.38)	**.02**	1.47 (1.04–2.16)	**.02**	1.59 (1.05–2.23)	**.04**
Proportion mediated (%)	101.9	**.02**	60.8	**.02**	92.9	**.04**

Incident CKD was defined as an eGFR-Cr <60 ml/min/1.73 m^2 at the follow-up FF4 study and eGFR-Cr ≥60 ml/min/1.73 m^2 at the baseline F4 study.
Total, direct and indirect effects were estimated with age, sex, smoking, physical activity and alcohol intake as covariates not affected by the exposure. Effect estimates with *P*-values <.05 were shown in bold.
Multiple mediators included CRP (continuous), diabetes (yes/no) and hypertension (yes/no). The causal effects were estimated by considering all three potential mediators jointly in the mediation analysis.
eGFR-Cr was based on the equation established by the CKD-EPI (2009).

based on eGFR-cC showed similar results (Supplementary Table 5).

DISCUSSION

In this population of middle-aged and older German participants, we found that a higher FLI was associated with lower eGFR and increased risk of CKD development during 6.5 years of follow-up, independent of lifestyle risk factors. However, further cardiometabolic adjustments substantially undermined the associations. Mediation analysis indicated that the putative association between the FLI/fatty liver and the risk of developing CKD was completely jointly mediated by diabetes, hypertension and inflammation.

Accumulating evidence has shown that individuals with fatty liver had a higher risk of developing CKD [4]. However, it is still highly debatable if fatty liver constitutes an independent risk factor for CKD. Although extensive research efforts have been focused on detangling the relation between fatty liver and CKD, the majority of these studies have taken place in Asian populations [42]. Contradictory results have been observed in the existing evidence found among Caucasian populations [10, 12–14, 43]. Two large longitudinal studies found that people with fatty liver were 50% more likely to develop CKD than those without, matched on age, sex and other cardiorenal risk factors [12, 43]. Nevertheless, their retrospective design and inclusion of only people with physician visits subject these studies to misclassification and selection bias. On the other hand, a prospective study in the general European population could not confirm that fatty liver diagnosed by computed tomography (CT) or the elevation of GGT independently increased the incidence of CKD [14]. Accordingly, a mendelian randomization study using genetic instrumental variables identified for CT-measured fatty liver in a population with European ancestry found no evidence that fatty liver causally impaired renal function [9]. Therefore it is likely that the observed positive associations in the literature could be explained by reverse causation or residual confounding [6, 12, 43, 44].

Most existing studies have diagnosed fatty liver by ultrasound [6,13,44], which shows only moderate diagnostic sensitivity when lipid content of the hepatocytes is <30% [45]. Consequently, only fatty liver with a higher fat content could have been diagnosed with ultrasound. The positive associations found in these studies suggest that fatty liver in a more advanced stage might be more relevant to the pathogenesis of CKD, possibly driven by the accompanying cardiometabolic risk factors [4, 46]. In line with our results, data from the population-based Framingham study comprised predominantly of individuals of European descent, suggested that neither increased liver fat quantified by CT nor fatty liver with liver injury, was independently associated with CKD risk [10].

Previous research has shown a close relationship between fatty liver and diabetes, and fatty liver seems to particularly increase the risk of developing CKD among diabetes patients [13, 47]. However, in our subgroup analysis, we found that the FLI was not associated with the risk of incident CKD in those with and without diabetes. On the other hand, people with fatty liver very often exhibit other components of metabolic syndrome, such as atherogenic dyslipidaemia and hypertension, suggesting that the association between fatty liver and CKD could be mediated by these cardiometabolic risk factors [4, 47]. In our mediation analysis, we found that the increased risk of developing CKD due to an increase in the FLI or being in the highest category of the FLI (FLI ≥60) was completely mediated by the joint effect of diabetes, inflammation and hypertension. These results show that cardiometabolic risk factors may be the main drivers for CKD development among people with increased liver fat content and fatty liver patients should be evaluated for components of metabolic syndrome in order to mitigate the development of cardiorenal complications [28].

Until now, most of the existing studies have used the Modification of Diet in Renal Disease creatinine model to estimate GFR, which tends to underestimate renal function, especially in Caucasian women [48]. We used the CKD-EPI equation for eGFR-Cr, which could better categorize renal function with regard to adverse clinical outcomes [48]. However, although serum creatinine is widely used in clinical practice to estimate GFR, evidence shows that it can be influenced by muscle mass, advanced liver disease and other factors such as age, diet and race [30, 49], as opposed to serum cystatin C [30]. In our analysis, the discrepancy between prevalent CKD defined by eGFR-Cr and eGFR-cC in relation to the FLI could be due to the high proportion (40.4%) of participants with high fatty liver risk (FLI ≥60) and overweight in our study population, among whom serum creatinine is likely to overestimate and misclassify renal function [49, 50].

Our study has several strengths. It is one of the few studies that has prospectively examined the association between fatty liver and incident CKD in a population-based cohort with European participants. A diverse set of cardiometabolic risk factors allowed us to adjust the models and rigorously perform mediation analysis. However, some limitations also need to be mentioned. The literature has indicated that the temporal directionality between fatty liver and cardiometabolic comorbidities could be reversed [51]. Therefore the results of the mediation analysis are only valid with the assumption that the pathway suggested in our analysis holds true. Due to the inclusion of TGs and BMI in the FLI calculation, to avoid collinearity we did not further adjust for these covariates in the regression models. Non-invasive imaging methods such as CT show higher sensitivity in assessing fatty liver. In particular, magnetic resonance spectroscopy and/or magnetic resonance imaging-derived proton density fat fraction are deemed the state-of-the-art methods for non-invasive quantification of hepatic fat. However, CT exerts potential radiation hazards and magnetic resonance imaging is still not commonly available due to high costs. In comparison, the FLI as a cost-effective tool has consistently demonstrated good accuracy for predicting the presence of fatty liver in several validation studies with imaging data, making it an adequate marker for population studies [17–19, 36].

CONCLUSION

We found that an increased FLI, a measure for fatty liver, was associated with an increased risk of developing CKD, independent of lifestyle factors in a general German population. However, the relationship was completely mediated by the joint effect of diabetes, inflammation and hypertension. People with an elevated FLI/fatty liver are recommended to undertake regular medical visits to monitor and manage their cardiometabolic health, including diabetes and hypertension, to prevent the progression of CKD. Future prospective studies need to investigate the chronological interaction and causal relationship of fatty liver, metabolic risk factors and kidney function with frequent follow-up visits.

SUPPLEMENTARY DATA

Supplementary data is available at *ndt* online.

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AUTHORS' CONTRIBUTIONS

X.C. designed the analyses, interpreted the data and drafted the manuscript. J.N. and B.T. contributed to the conception, design and interpretation of the data and approval of the manuscript. S.H., A.P., W.R. and W.K. contributed substantially to the interpretation of the data and critically revised the manuscript for important intellectual content.

DATA AVAILABILITY STATEMENT

The data underlying this article cannot be shared publicly due to data protection reasons. The data will be shared upon reasonable request to the corresponding author.

CONFLICT OF INTEREST STATEMENT

W.K. has received Grants and provision of reagents to institution from Singulex, Dr. Beckmann Pharma, Abbott, Roche Diagnostics, consulting fees from AstraZeneca, Novartis, Amgen, Pfizer, the Medicines Company, DalCor Pharmaceuticals, Kowa, Corvidia Therapeutics, OMEICOS, Daiichi Sankyo, Novo Nordisk, Esperion, LIB Therapeutics, NewAmsterdam Pharma, Genentech, and lecture fees from BristolMyers Squibb, Novartis, Amgen, Berlin-Chemie, Sanofi, AstraZeneca. W.R has received consulting fees for attending educational sessions or advisory boards from AstraZeneca, Boehringer Ingelheim and NovoNordisk. The authors have no conflicts of interest to declare that are relevant to the content of this article. The results presented in this article have not been published previously in whole or part, except in abstract format.

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3. Paper III

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Association between hepatic fat and subclinical vascular disease burden in the general population

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ABSTRACT

Objective It is still controversial if increased hepatic fat independently contributes to cardiovascular risk. We aimed to assess the association between hepatic fat quantified by MRI and various subclinical vascular disease parameters.

Design We included two cross-sectional investigations embedded in two independent population-based studies (Study of Health in Pomerania (SHIP): n=1341; Cooperative Health Research in the Region of Augsburg (KORA): n=386). The participants underwent a whole-body MRI examination. Hepatic fat content was quantified by proton-density fat fraction (PDFF). Aortic diameters in both studies and carotid plaque-related parameters in KORA were measured with MRI. In SHIP, carotid intima-media thickness (cIMT) and plaque were assessed by ultrasound. We used (ordered) logistic or linear regression to assess associations between hepatic fat and subclinical vascular disease.

Results The prevalence of fatty liver disease (FLD) (PDFF >5.6%) was 35% in SHIP and 43% in KORA. In SHIP, hepatic fat was positively associated with ascending (β, 95% CI 0.06 (0.04 to 0.08)), descending (0.05 (0.04 to 0.07)) and infrarenal (0.02 (0.01 to 0.03)) aortic diameters, as well as with higher odds of plaque presence (OR, 95% CI 1.22 (1.05 to 1.42)) and greater cIMT (β, 95% CI 0.01 (0.004 to 0.02)) in the age-adjusted and sex-adjusted model. However, further adjustment for additional cardiometabolic risk factors, particularly body mass index, attenuated these associations. In KORA, no significant associations were found.

Conclusions The relation between hepatic fat and subclinical vascular disease was not independent of overall adiposity. Given the close relation of FLD with cardiometabolic risk factors, people with FLD should still be prioritised for cardiovascular disease screening.

Summary box

What is already known about this subject?

► Fatty liver disease and subclinical vascular disease share several common cardiometabolic risk factors, such as type 2 diabetes and obesity. There is not yet population-based study to investigate the association between hepatic fat content and the expansion of aortic diameters. Epidemiological studies on the relation between fatty liver disease and atherosclerosis yielded controversial results.

What are the new findings?

► With data from the general population, we found that hepatic fat content measured with MRI was neither independently associated with greater aortic diameters nor the risk of carotid plaque, after adjusting for cardiometabolic risk factors, especially obesity.

How might it impact on clinical practice in the foreseeable future?

► Given that fatty liver disease is closely related to concurrent obesity, type 2 diabetes and other cardiometabolic disorders, people with fatty liver disease should still be recommended to monitor their cardiovascular risk factors and prioritised for cardiovascular disease screening.

INTRODUCTION

Fatty liver disease (FLD), defined as an ectopic fat accumulation (≥5%) in the hepatocytes, constitutes the leading cause of chronic liver disease worldwide.[1] With a growing prevalence of 2%–44% in the general population, clinical manifestations of FLD ranging from simple steatosis, steatohepatitis, fibrosis and eventually cirrhosis and hepatocellular carcinoma pose a substantial burden on healthcare systems.[2] In particular, cardiovascular disease (CVD), one of the extrahepatic repercussions of FLD, remains to be the largest contributor of mortality among people with FLD.[3]

Prior to the clinical manifestation of CVD, subclinical vascular disease, representing pathological changes of various blood vessels, can provide important aetiological insights into early detection of CVD development.[4] We have previously shown with our data

that hepatic fat was positively associated with subclinical vascular parameters such as left ventricular remodelling index.[5] Moreover, data from other population-based studies have also indicated a positive association between FLD and subclinical calcified plaque in different vessels.[6 7]

One understudied outcome of interest in the realm of subclinical vascular changes is aortic aneurysm, which represents a disproportionate dilation of aortic diameter (thoracic aorta diameter ≥5 cm or abdominal aorta diameter ≥3 cm) that could result in life-threatening events such as aortic rupture or dissection.[8] Several risk factors for the development of aortic aneurysm including age, increased body mass index (BMI) and hyperlipidaemia are mutually shared with FLD.[9] However, whether hepatic fat is associated with expanding aortic dimeters, measured along multiple locations of the aorta, has not been previously investigated in a population-based setting.

Furthermore, the evidence is still inconsistent regarding whether hepatic fat represents an independent modifiable risk factor for subclinical atherosclerosis, such as carotid plaque. A meta-analysis reported that compared with people without FLD those who with FLD were almost 80% more likely to have carotid plaque detected by ultrasound.[10] However, different criteria used for defining carotid plaque made their conclusions unconvincing. Ultrasound is less precise than CT or MRI in quantifying plaque calcification, which is particularly prone to progress to CVD events.[11] Interestingly, two well-powered population-based studies could not show a link between FLD and carotid calcification measured by CT.[12 13] On the other hand, most previous studies used either ultrasound or CT to define FLD, which are less sensitive than MRI when fat content is low.[14]

Therefore, we aimed to determine the association of hepatic fat quantified by dedicated whole-body MRI protocols with aortic diameters and carotid atherosclerosis measured by MRI or ultrasound in two well-characterised independent German studies.

METHODS

Study population

The Study of Health in Pomerania

The baseline examination of the Study of Health in Pomerania (SHIP)—TREND-0 (SHIP-TREND-0 abbreviated as SHIP) was conducted between 2008 and 2012 and included participants from West Pomerania, northeastern part of Germany. The study design has previously been described in detail.[15] Out of 8826 adults (20–79 years) randomly drawn from local population registries, a sample of 4420 eligible participants completed the baseline examination including personal interviews, laboratory measurements, ultrasonography and simple medical examinations.[15] After excluding participants with MRI contraindication or who refused to participate (n=2492), 1926 participants underwent a whole-body MRI examination. Exclusion criteria for MRI examination were described elsewhere.[16] Further exclusion criteria for the present analysis included not fasting at the time of medical examination, missing values for hepatic fat measurements or any covariate (n=585) leading to a total of 1341 participants for the final analyses (figure 1).

The Cooperative Health Research in the Region of Augsburg Study

Cooperative Health Research in the Region of Augsburg (KORA) FF4 study, conducted between 2013 and 2014, was the second follow-up study of the KORA S4 survey (conducted between 1999 and 2001). We used the data from a subpopulation of the KORA FF4 study, which was originally selected for a nested case–control study to detect subclinical CVD in individuals with pre-diabetes and diabetes compared with those with normal glucose tolerance. Of all 4261 participants of the S4 survey, 2279 participants also participated in the FF4 study. The inclusion and exclusion criteria of the KORA-FF4-MRI (abbreviated as KORA) protocol were described elsewhere.[17] A total of 400 participants underwent the whole-body MRI examination. They were free of overt CVD events, such as stroke, myocardial infarction and peripheral artery diseases. Finally, due to missing values in MRI-measured

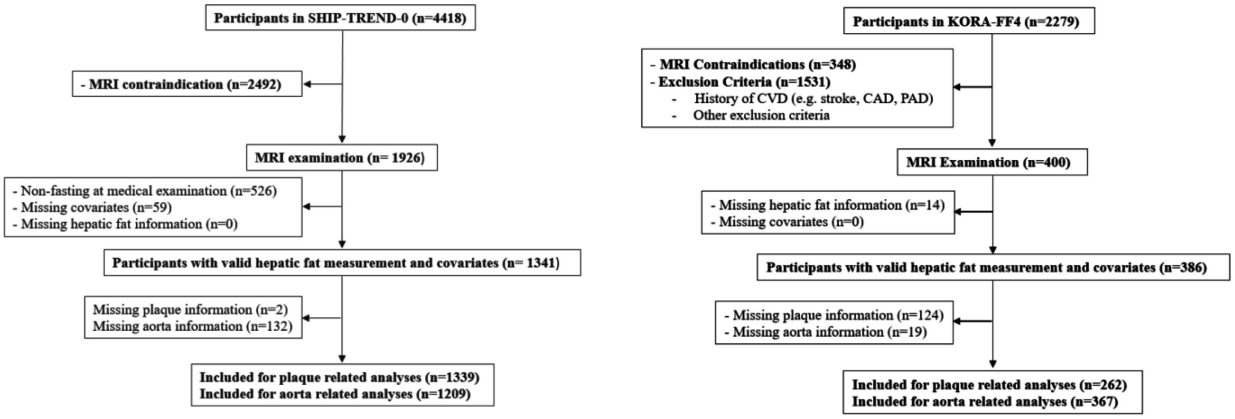

Figure 1 Flowchart of the study design. CAD, coronary artery disease; CVD, cardiovascular; KORA, Cooperative Health Research in the Region of Augsburg; PAD, peripherial artery disease; SHIP, Study of Health in Pomerania.

hepatic fat content (n=14), the final population included in the analyses comprised 386 participants (figure 1).

Both studies comply with the Declaration of Helsinki. The SHIP study was approved by the Ethics Committee of University of Greifswald (BB 39/08). The KORA study was approved by the institutional review board of the Medical Faculty of Ludwig-Maximilian University Munich (06068). All participants provided written informed consent.

Assessment of hepatic fat content and subclinical vascular parameters

Hepatic fat content was estimated by MRI proton-density fat fraction (PDFF, %) at the level of portal vein in both SHIP and KORA (see online supplemental materials 1 for the MRI device and sequence used). FLD was defined according to the Clinical Practice Guidelines for the management of non-alcoholic FLD[18]: No FLD (PDFF ≤5.6%) versus with FLD (PDFF >5.6%).

The diameters (cm) of the ascending, descending and infrarenal aorta were measured in axial plane both in SHIP and KORA with MRI. As for carotid injury, in SHIP, the carotid intima-media thickness (cIMT) (mm) was measured with ultrasound and averaged over the left carotid artery (LCA) and the right carotid artery (RCA). Carotid plaque presence was adjudicated if any of the following three criteria was met: any focal thickening of the intima-media complex protruding into the vessel lumen, a focal increase in echogenicity with a homogeneously hyperechoic echotexture within an otherwise hypoechoic intima-media complex, a uniformly increased cIMT (>1.3 mm) without focal thickening. In KORA, presence and morphological composition of carotid plaque were determined in the MRI protocol. Mean wall thickness (mm), lumen area (mm^2) and wall area (mm^2) were separately calculated for the LCA and the RCA. Normalised wall index (NWI), as calculated with wall area/(lumen area +wall area), describes the percentage of the wall surface in proportion to the total blood vessel surface. According to the presence of calcification and haemorrhage as well as wall thickness and wall eccentricity, plaque was differentiated and classified to type I, type III, type IV/V and type VI/VII with the criteria of the American Heart Association.[19] Participants with type III, type IV/V or type VI/VII plaque were considered as having carotid plaque.

Traditional cardiovascular risk factors and other covariates

Other covariates were assessed in both studies, including traditional cardiovascular risk factors—such as BMI (kg/m^2), waist circumference (cm), smoking status (smoker, ex-smoker, never smoker), physically active (yes, no), alcohol consumption (no intake, moderate intake, excessive intake: ≥20 g/day for women or ≥30 g/day for men), hypertension (yes, no), systolic blood pressure (SBP) (mm Hg), diastolic blood pressure (mm Hg), total cholesterol (mmol/L), high-density lipoprotein cholesterol (HDL-C) (mmol/L), low-density lipoprotein cholesterol (LDL-C) (mmol/L), triglycerides (mmol/L) and liver enzymes (aspartate aminotransferase (AST) (µkat/L), alanine aminotransferase (ALT) (µkat/L), gamma-glutamyl transferase (GGT) (µkat/L)). Visceral adipose tissue (VAT) in litre was also measured in the whole-body MRI protocols. Participants were divided into three groups according to their glucose tolerance status: normoglycaemic (fasting glucose <6.1 mmol/L and 2 hour glucose <7.8 mmol/L), pre-diabetes (2 hour glucose between 7.8 mmol/L and 11.0 mmol/L and normal fasting glucose or fasting glucose between 6.1 mmol/L and 6.9 mmol/L and normal 2 hour glucose) and diabetes (fasting glucose >6.9 mmol/L and/or 2 hour glucose >11.0 mmol/L) following each study protocols.[15 17] Medication use within the last 7 days prior to the interview, such as use of antihypertensive[20] and lipid-lowering medication, was ascertained in both studies. In SHIP, history of CVD included events of myocardial infarction, stroke and angina pectoris. A detailed description of the MRI device measurements, the sequence used for hepatic fat content and subclinical vascular disease parameters and the definitions of other covariates are provided in online supplemental materials 1.[15–27]

Statistical analyses

We calculated the descriptive variables separately for SHIP and KORA as well as for the groups: participants with FLD (PDFF >5.6%) versus participants without FLD (PDFF ≤5.6%) within each study. For continuous variables, we displayed mean (SD) if they were normally distributed, and median (IQR) if the distribution was not normal. We show categorical variables with counts (percentages, %). We used two-sample t-test for comparison of continuous variables and χ^2 test for comparison of categorical variables. We log-transformed the variables that did not follow a normal distribution including triglycerides, ALT, AST, GGT in both studies, wall thickness of RCA and LCA, lumen area of RCA and LCA and wall area of RCA and LCA in KORA.

The coefficient estimates represent the change in subclinical disease parameters corresponding to one SD increase in log-transformed hepatic fat content. In both studies, we conducted linear regressions to examine the associations between hepatic fat content and continuous outcomes and logistic regression for categorical outcomes. The following three models were constructed for both SHIP and KORA. In model 1, we adjusted for age and sex. In model 2, we additionally included BMI. In model 3, we further adjusted for smoking, physical activity, alcohol intake, SBP, HDL-C, LDL-C, triglycerides, glucose tolerance status, history of CVD (in SHIP), use of antihypertensive medication and use of lipid-lowering medication. Considering the potential collinearity among the cardiometabolic covariates in model 3, we also calculated variance inflation factor for each covariate,[28] which refuted the existence of strong collinearity among the covariates. The same models were conducted with FLD (yes vs no), as an important clinical endpoint of excessive

hepatic fat. The interactions between hepatic fat content (or FLD) and diabetes as well as obesity (BMI≥30 kg/m^2) were examined by entering a multiplicative term (PDFF ×diabetes or PDFF ×obesity) in the models. The interaction between hepatic fat and history of CVD (PDFF ×CVD) was only assessed in SHIP.

In addition, sensitivity analyses were conducted in both studies excluding participants with excessive alcohol intake, defined as a daily alcohol intake ≥30 g for men and ≥20 g for women.[18] Kühn *et al* have suggested another cut-off of PDFF (>5.1%) for FLD based on histopathologic calibrations.[21] We also conducted sensitivity analyses with FLD (yes vs no) defined by this cut-off in both studies. In order to examine the influence of visceral adiposity, we substituted BMI with VAT or waist circumference as a covariate in the models. Further sensitivity analyses excluding participants with a history of CVD were conducted in SHIP.

The significance level was set to a nominal p value <0.05. Data analysis was performed with R-Studio V.4.0.2.

RESULTS

Table 1 summarises the baseline characteristics for the participants from the SHIP and KORA studies. Participants with a valid PDFF measurement consisted of 612 (45.6%) men and 729 (54.4%) women in SHIP and 223 (57.8%) men and 163 (42.2%) women in KORA. Mean age was lower in SHIP (50.4±13.7 years) than in KORA (56.2±9.1 years). Moreover, participants in SHIP had lower waist circumference, were more physically active, suffered less from pre-diabetes and diabetes, but had higher prevalence of hypertension, compared with participants from KORA. Median PDFF and prevalence of FLD were lower in SHIP (469, 35.0%) than in KORA (166, 43.0%). We present demographic, anthropometric, lifestyle, cardiometabolic profiles stratified by FLD in both studies in online supplemental table 1.

Aortic diameters were greater among SHIP participants (ascending aorta: 3.32±0.46 cm; descending aorta: 2.46±0.35 cm; infrarenal aorta: 1.85±0.23 cm) than among KORA participants (ascending aorta: 2.96±0.41 cm; descending aorta: 2.09±0.31 cm; infrarenal aorta: 1.50±0.21 cm). The presence of carotid plaque was more frequent in SHIP (n=467, 34.9%) than in KORA (n=54, 20.6%). Morphological features of the plaque are listed in table 2. They are not comparable between the two studies due to different methods. Subclinical vascular parameters according to FLD in both studies are presented in online supplemental table 2.

Table 1 Characteristics of study participants in SHIP and KORA

	SHIP	KORA
	Total (N=1341)	Total (N=386)
Age (years)	50.4 (13.7)	56.2 (9.1)
Women	729 (54.4%)	163 (42.2%)
BMI (kg/m^2)	27.4 (4.4)	28.1 (4.9)
Waist circumference (cm)	88.9 (12.9)	98.5 (14.3)
Physically active	960 (71.6%)	230 (59.6%)
Smoking		
Smoker	289 (21.6%)	77 (19.9%)
Ex-smoker	486 (36.2%)	169 (43.8%)
Never smoker	566 (42.2%)	140 (36.3%)
Alcohol consumption		
No intake	164 (12.2%)	92 (23.8%)
Moderate intake	1091 (81.4%)	191 (49.5%)
Excessive intake	86 (6.4%)	103 (26.7%)
Systolic blood pressure (mm Hg)	125.1 (16.8)	120.6 (16.8)
Diastolic blood pressure (mm Hg)	76.6 (9.7)	75.3 (10.0)
Hypertension	539 (40.2%)	132 (34.2%)
Total cholesterol (mmol/L)	5.5 (1.1)	5.6 (0.9)
HDL-C (mmol/L)	1.5 (0.4)	1.6 (0.5)
LDL-C (mmol/L)	3.4 (0.9)	3.6 (0.9)
Triglycerides (mmol/L)	1.2 (0.9, 1.7)	1.2 (0.9, 1.8)
ALT (µkat/L)	0.4 (0.3, 0.5)	0.5 (0.3, 0.6)
AST (µkat/L)	0.3 (0.2, 0.4)	0.4 (0.3, 0.5)
GGT (µkat/L)	0.5 (0.4, 0.7)	0.5 (0.3, 0.7)
Glucose tolerance status		
Normoglycaemic	932 (69.5%)	239 (61.9%)
Pre-diabetes	281 (21.0%)	95 (24.6%)
Diabetes	128 (9.5%)	52 (13.5%)
PDFF (%)	3.85 (2.32, 7.74)	4.62 (2.63, 11,89)
FLD	469 (35.0%)	166 (43.0%)
Antihypertensive medication use	369 (27.5%)	98 (25.4%)
Lipid-lowering medication use	109 (8.1%)	41 (10.6%)
History of CVD	93 (6.9%)	NA

Values are expressed as the mean (SD) for normally distributed continuous variables or median (IQR) for non-normally distributed continuous variables, or n (%) for categorical variables.
Hepatic fat content was quantified on the level of portal vein by MRI PDFF. FLD (PDFF >5.6%) was defined according to the European Association for the Study of the Liver (EASL)-European Association for the Study of Diabetes (EASD)-European Association for the Study of Obesity (EASO) Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CVD, cardiovascular disease; FLD, fatty liver disease; GGT, gamma-glutamyl transferase; HDL-C, high-density lipoprotein cholesterol; KORA, Cooperative Health Research in the Region of Augsburg; LDL-C, low-density lipoprotein cholesterol; PDFF, proton density fat fraction; SHIP, Study of Health in Pomerania.

Associations between hepatic fat content and aortic diameters

In SHIP, one SD increase in hepatic fat content was significantly associated with greater ascending (β, 95% CI 0.06 (0.04 to 0.08)), descending (0.05 (0.04 to 0.07)) and infrarenal (0.02 (0.01 to 0.03)) aortic diameters in model 1. Further adjustment for BMI (model 2) substantially attenuated the estimates. Adding other cardiometabolic risk factors in model 3 changed the results only marginally.

Table 2 Subclinical vascular disease parameters among participants in SHIP and KORA

	SHIP	KORA
	Total (N=1341)	Total (N=386)
Ascending aorta diameter (cm)	3.32 (0.46)	2.96 (0.41)
Descending aorta diameter (cm)	2.46 (0.35)	2.09 (0.31)
Infrarenal aorta diameter (cm)	1.85 (0.23)	1.50 (0.21)
Plaque presence	467 (34.9%)	54 (20.6%)
Type of plaque	NA	
AHA type I		208 (79.4%)
AHA type III		38 (14.5%)
AHA type V		10 (3.8%)
AHA type VI or VII		6 (2.3%)
Carotid intima-media thickness (mm)	0.60 (0.14)	NA
Wall thickness, LCA (mm)	NA	0.73 (0.69, 0.79)
Wall thickness, RCA (mm)	NA	0.73 (0.69, 0.81)
Lumen area, LCA (mm^2)	NA	17.54 (14.02, 21.61)
Lumen area, RCA (mm^2)	NA	16.40 (13.14, 20.68)
Wall area, LCA (mm^2)	NA	12.39 (10.72, 14.03)
Wall area, RCA (mm^2)	NA	12.18 (10.36, 14.06)
NWI, LCA	NA	0.44 (0.05)
NWI, RCA	NA	0.45 (0.05)

Values are expressed as the mean (SD) for continuous variables, or n (%) for categorical variables.
Hepatic fat content was quantified on the level of portal vein by MRI PDFF. Plaque was detected using ultrasound in SHIP and MRI in KORA. Number of missing values for each outcome variable is shown in online supplemental table 2.
AHA, American Heart Association; KORA, Cooperative Health Research in the Region of Augsburg; LCA, left carotid artery; NA, not applicable; NWI, normalised wall index, calculated as wall area/(lumen area + wall area); PDFF, proton density fat fraction; RCA, right carotid artery; SHIP, Study of Health in Pomerania.

In KORA, hepatic fat content and aortic diameters were not significantly associated in all three models (table 3) (model 3: ascending aorta: β, 95% CI −0.04 (−0.09 to 0.02); descending aorta: −0.03 (−0.07 to 0.01) and infrarenal aorta: −0.01 (−0.04 to 0.01)).

Associations between hepatic fat content and carotid plaque and related parameters

In SHIP, one SD increase of hepatic fat content was associated with higher odds of plaque presence (OR, 95% CI 1.22 (1.05 to 1.42)) and greater cIMT (β, 95% CI 0.01 (0.004 to 0.02)) in model 1. Further adjustment for BMI in model 2 attenuated these associations (plaque presence: OR, 95% CI 1.13 (0.95 to 1.33) and cIMT: β, 95% CI 0.002 (-0.01 to 0.01)). Further adjustment for other cardiometabolic risk factors (model 3) changed the results only marginally.

In KORA, we did not observe any significant associations between hepatic fat content and plaque-related outcomes including plaque presence (model 3: OR, 95% CI 0.80 (0.49 to 1.29)) and plaque type (0.73 (0.45 to 1.19)) (table 3). Regression analyses with the morphological features of plaque revealed non-significant estimates in all models (table 3).

Sensitivity analysis

The interaction terms between hepatic fat content and diabetes/obesity/CVD in SHIP were not significant. In KORA, we found significant interactions between hepatic fat content and diabetes for plaque presence ($p_{PDFF\times diabetes}$=0.031) and plaque type ($p_{PDFF\times diabetes}$=0.020). Due to the enrichment of participants with altered glucose metabolism (pre-diabetes and diabetes) in KORA, we did subgroup analyses stratified by glycaemic status (normoglycaemia vs pre-diabetes or diabetes). Among participants with altered glucose metabolism, carotid plaque presence (OR, 95% CI 0.44 (0.21 to 0.91)) and plaque type (0.39 (0.19 to 0.79)) were inversely associated with hepatic fat content after full adjustment (online supplemental table 3).

Considering the role of alcohol intake in the pathogenesis of FLD, we also conducted sensitivity analyses excluding participants with excessive alcohol intake.[18] The latter did not change the estimates except for NWI of the RCA in KORA, which was significantly inversely associated with hepatic fat only in model 3 (β, 95% CI –0.01 (–0.02 to –0.0004)). Very likely this represents a spurious findings due to the large number of tests (online supplemental table 4). Therefore, most likely we can generalise our results to the context of non-alcoholic FLD.

Regression analyses with FLD (yes vs no) as exposure, defined by either cut-off of PDFF (5.6% or 5.1%), showed similar non-significant coefficient estimates in both studies (online supplemental table 5). Replacing BMI with either VAT or waist circumference as a covariate did not influence the results in both studies (online supplemental table 5). In SHIP, sensitivity analyses excluding participants with a history of CVD hardly affected the results (online supplemental table 6).

DISCUSSION

In this investigation comprising two cross-sectional investigations embedded in two independent population-based studies, we found no association between increasing hepatic fat content measured by MRI and parameters of subclinical vascular disease, including (ascending, descending, infrarenal) aortic diameters and carotid plaque presence and its morphological features. The link between hepatic fat content and subclinical vascular parameters was mainly driven by general adiposity and other cardiometabolic risk factors, such as hyperlipidemia and hypertension, which very often coexist with FLD. This indicates that the role of hepatic fat on subclinical vascular burden might be rather a reflection of worsened cardiometabolic profile.

Hepatic fat content and aortic diameters

The present investigation is the first to report the association between increasing hepatic fat and aortic diameters in a population-based setting. A clinical study from

Table 3 Associations of hepatic fat content with subclinical vascular disease parameters in SHIP and KORA studies

	N	Model 1	P value	Model 2	P value	Model 3*	P value
SHIP							
Aortic diameters		**β, 95% CI**		**β, 95% CI**		**β, 95% CI**	
Ascending aorta (cm)	1209	**0.06 (0.04 to 0.08)**	**<0.001**	0.01 (–0.02 to 0.03)	0.469	0.001 (–0.02 to 0.03)	0.913
Descending aorta (cm)	1209	**0.05 (0.04 to 0.07)**	**<0.001**	0.01 (–0.01 to 0.02)	0.228	0.01 (–0.01 to 0.02)	0.533
Infrarenal aorta (cm)	1209	**0.02 (0.01 to 0.03)**	**<0.001**	–0.01 (–0.02 to 0.01)	0.282	–0.001 (–0.01 to 0.01)	0.837
Carotid plaque		**OR, 95% CI**		**OR, 95% CI**		**OR, 95% CI**	
Plaque presence	1339	**1.22 (1.05 to 1.42)**	**0.008**	1.13 (0.95 to 1.33)	0.178	1.03 (0.85 to 1.25)	0.753
		β, 95% CI		**β, 95% CI**		**β, 95% CI**	
Carotid intima-media-thickness (mm)	1339	**0.01 (0.004 to 0.02)**	**0.002**	0.002 (–0.01 to 0.01)	0.538	–0.003 (–0.01 to 0.004)	0.401
KORA							
Aortic diameters		**β, 95% CI**		**β, 95% CI**		**β, 95% CI**	
Ascending aorta (cm)	367	0.03 (–0.01 to 0.07)	0.166	–0.03 (–0.08 to 0.02)	0.202	–0.04 (–0.09 to 0.02)	0.168
Descending aorta (cm)	367	0.01 (–0.02 to 0.04)	0.385	–0.03 (–0.06 to 0.002)	0.070	–0.03 (–0.07 to 0.01)	0.110
Infrarenal aorta (cm)	367	0.02 (–0.004 to 0.03)	0.115	–0.01 (–0.03 to 0.02)	0.536	–0.01 (–0.04 to 0.01)	0.349
Carotid plaque		**OR, 95% CI**		**OR, 95% CI**		**OR, 95% CI**	
Plaque presence	262	0.93 (0.66 to 1.32)	0.676	0.84 (0.56 to 1.25)	0.390	0.80 (0.49 to 1.29)	0.354
Plaque type	262	0.93 (0.66 to 1.31)	0.692	0.80 (0.53 to 1.20)	0.274	0.73 (0.45 to 1.19)	0.206
		β, 95% CI		**β, 95% CI**		**β, 95% CI**	
Wall thickness, LCA (mm)	251	0.01 (–0.01 to 0.03)	0.474	–0.01 (–0.03 to 0.01)	0.186	–0.02 (–0.04 to 0.01)	0.179
Wall thickness, RCA (mm)	257	0.01 (–0.01 to 0.02)	0.534	–0.01 (–0.03 to 0.01)	0.193	–0.01 (–0.03 to 0.01)	0.287
Lumen area, LCA (mm^2)	255	0.02 (–0.03 to 0.06)	0.481	–0.01 (–0.06 to 0.05)	0.841	0.001 (–0.06 to 0.06)	0.968
Lumen area, RCA (mm^2)	262	0.03 (–0.02 to 0.08)	0.266	–0.003 (–0.06 to 0.05)	0.915	0.04 (–0.03 to 0.10)	0.271
Wall area, LCA (mm^2)	251	0.01 (–0.02 to 0.05)	0.414	–0.02 (–0.06 to 0.02)	0.288	–0.02 (–0.06 to 0.03)	0.448
Wall area, RCA (mm^2)	257	0.02 (–0.01 to 0.06)	0.173	–0.01 (–0.05 to 0.03)	0.537	–0.01 (–0.04 to 0.05)	0.827
NWI, LCA	251	0.001 (–0.01 to 0.01)	0.675	–0.002 (–0.01 to 0.01)	0.642	–0.003 (–0.01 to 0.01)	0.453
NWI, RCA	257	–0.002 (–0.01 to 0.004)	0.454	–0.003 (-0.01 to 0.004)	0.370	–0.01 (–0.02 to–0.00003)	0.061

Model 1: adjusted for age, sex.
Model 2: model 1+BMI
Model 3: model 2+smoking status, physical activity, alcohol intake, systolic blood pressure, HDL-C, LDL-C, triglycerides, glucose tolerance status, use of antihypertensive medication, use of lipid-lowering medication.
Results with p value<0.05 are shown in bold. The coefficient estimates represent the change in subclinical disease parameters with a SD increment of log-transformed hepatic fat content.
*Model 3 in SHIP was additionally adjusted for history of cardiovascular diseases.
β, β-estimates from linear regression; BMI, body mass index; HDL-C, high-density lipoprotein cholesterol; KORA, Cooperative Health Research in the Region of Augsburg; LCA, left carotid artery; LDL-C, low-density lipoprotein cholesterol; NWI, normalised wall index, calculated as wall area/(lumen area+wall area); RCA, right carotid artery; SHIP, Study of Health in Pomerania.

Mahamid *et al*, including data from 495 hospitalised patients with abdominal aortic aneurysm (AAA) diagnosed with either ultrasound or CT and 500 matched controls, reported a positive relation between FLD and AAA occurrence adjusted for age, smoking, BMI and metabolic syndrome.[9] However, these results should be interpreted with caution given the highly selective sample of hospitalised patients, who were diagnosed with a clinically relevant and more advanced stage of infrarenal aorta dilation (≥3 cm),[29] which could have biased the results.

FLD and AAA share common pathophysiological risk factors such as adiposity, insulin resistance and inflammation. In our study, the adjustment for BMI substantially attenuated the associations of hepatic fat with aortic diameters. Accordingly, previous animal studies have shown that leptin secreted by adipocytes could promote proinflammatory cytokines, for example, interleukin-18, binding to their receptors on aortic smooth muscle cells. Consequently, adipocytes could enhance aortic inflammation and exacerbate AAA development.[30] Because of the close relation between hepatic fat and central obesity, we also substituted BMI with better measurements of visceral adiposity such as waist circumference and VAT, the latter measured with MRI. The results did not change. This indicates that higher general or central adiposity, which often coexists with FLD, confounds the association between hepatic fat content and increasing aortic diameters. Therefore, an independent effect of hepatic fat in the aetiology of AAA could not be shown.

Hepatic fat content, carotid plaque and related parameters

Previous studies have yielded controversial results regarding the association between FLD and subclinical carotid atherosclerosis. The largest meta-analysis to date with studies assessing FLD and carotid plaque defined by

ultrasound revealed a positive association between these two parameters.[10] Although cIMT provides insight into continuous change of vessel walls over time, the clinical cut-off point of cIMT that distinguishes people with increased cardiovascular risk varied among the studies. Moreover, the meta-analysis included different methods for determining FLD, either by ultrasound or biopsy. Although biopsy remains the gold standard for diagnosing FLD, it is prone to sampling error.[31] Ultrasound has limited performance in detecting FLD at a milder stage.[31] Due to these heterogeneities in measuring methods, the conclusion of this meta-analysis is less convincing and remains a controversial.

On the other side, results from two population-based studies suggested that FLD is not an independent risk factor for subclinical carotid plaque.[12 13] In an investigation using data from the Rotterdam Study, Wolff *et al* found that higher hepatic fat measured by CT was related to increasing volumes of coronary artery, yet not to carotid artery calcification.[12] Similar results were found in another study using CT scans from Koo *et al*, where they showed that FLD was positively associated with calcification in thoracic aorta and coeliac trunk, but not carotid artery. The distinct atherogenic effects of FLD on different vascular beds may reflect different underlying mechanisms. The location of subclinical alterations with regards to FLD merits emphasis in future studies.

In line with previous literature in population-based setting,[12 13] we found no evidence of an independent role of hepatic fat on carotid plaque development. An observation from Di Costanzo *et al* found that cIMT only increased among people with FLD sharing other metabolic abnormalities, but not in people with FLD without metabolic abnormalities, compared with controls without FLD.[32] It might be plausible that FLD does not add to the burden of carotid atherosclerosis, unless it coexists with metabolic abnormalities. In addition, other studies in settings enriched with patients with type 2 diabetes found no independent association between FLD and subclinical carotid plaque, after adjustment for insulin resistance.[33–35] It has been indicated by previous studies that insulin resistance could be a major factor increasing cardiovascular risks of people with FLD.[36] In the Tübingen Diabetes Family Study with participants at higher risk of diabetes, researchers have shown that insulin resistance increased with higher hepatic fat content measured by MRI,[37] mainly caused by imbalance of adipocytokines.[38] Moreover, insulin resistance could promote inflammation and endothelial dysfunction, two major factors of an atherogenic environment.[39] These evidences pointed out that ectopic fat accumulation in liver might be regarded as a hepatic manifestation of metabolic dysfunction.

While certainly not wanting to overlook the clinical relevance of FLD, focus should be laid on assessing and treating metabolic dysfunction of FLD people as an attempt to reduce CVD risk. In light of the new definition for metabolic dysfunction-associated FLD, the level of cardiovascular risks among metabolically unhealthy people with FLD is equally high, regardless of obesity.[40 41] Although we did not find an independent role of hepatic fat on subclinical vascular disease that extends beyond overall adiposity and other cardiometabolic risk factors, we still think it is important for continue screening patients with FLD for CVD.

Strengths and limitations

This is the first study to investigate the association between hepatic fat content/FLD and subclinical vascular changes in both aorta and carotid artery by whole-body MRI examination. With data from two cross-sectional investigations embedded in population-based cohorts, we were able to show the consistency of the associations. Both studies collected data on a variety of cardiometabolic and lifestyle risk factors that were considered in the model adjustment and allowed for examining potential differences in subgroups in the sensitivity analyses. Moreover, MRI demonstrates the best overall performance in determining FLD,[42] and it is also highly sensitive and specific for carotid plaque imaging.[43]

Some limitations need to be addressed. The MRI protocols used in SHIP and KORA were not able to distinguish more advanced stage of FLD involving fibrosis (using, eg, elastography) from simple steatosis, which could potentially modify the association between hepatic fat and subclinical vascular diseases. Despite the two different measuring modalities for carotid plaque (ultrasound in SHIP and MRT in KORA), the consistent null result indicated that the lack of association is unlikely to be due to methodological discrepancy. Additionally, due to the cross-sectional design of our study, we could only capture the relationship between hepatic fat content and subclinical vascular parameters at one point of time. This design does not allow for interpretation on the directionality of associations. Given that fat accumulation in liver is modifiable,[38] whether the elevation/amelioration of hepatic fat could accelerate/reverse the development of subclinical vascular diseases over time warrants further investigation.

CONCLUSION

We found that the associations between hepatic fat measured with MRI and subclinical vascular disease such as aortic diameters and subclinical atherosclerosis parameters were not independent of overall adiposity and a worsened cardiometabolic risk profile. Given the close relation of hepatic fat to other cardiometabolic risk factors, such as obesity, dyslipidaemia, hypertension and diabetes, we cannot afford to overlook the role of FLD on CVD development. Therefore, people with FLD should still be strongly advised to modify their CVD risks, such as overall adiposity, which can be targeted with lifestyle interventions. Well-powered prospective cohort studies with state-of-the-art imaging modalities are needed to better understand the contribution of hepatic fat in subclinical vascular disease development.

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Appendix A: Paper I

Title:	Association of Sex Hormones and Sex Hormone-Binding Globulin with Liver Fat in Men and Women: An Observational and Mendelian Randomization Study
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Association of sex hormones and sex hormone-binding globulin with liver fat in men and women: an observational and Mendelian randomization study

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Background: Sex hormones and sex hormone-binding globulin (SHBG) may play a role in fatty liver development. We sought to examine the association of various endogenous sex hormones, including testosterone (T), and SHBG with liver fat using complementary observational and Mendelian randomization (MR) analyses.

Methods: The observational analysis included a total of 2,239 participants (mean age 60 years; 35% postmenopausal women) from the population-based KORA study (average follow-up time: 6.5 years). We conducted linear regression analysis to investigate the sex-specific associations of sex hormones and SHBG with liver fat, estimated by fatty liver index (FLI). For MR analyses, we selected genetic variants associated with sex hormones and SHBG and extracted their associations with magnetic resonance imaging measured liver fat from the largest up to date European genome-wide associations studies.

Results: In the observational analysis, T, dihydrotestosterone (DHT), progesterone and 17α-hydroxyprogesterone (17-OHP) were inversely associated with FLI in men, with beta estimates ranging from -4.23 to -2.30 [p-value <0.001 to 0.003]. Whereas in women, a positive association of free T with FLI (β = 4.17, 95%CI:

1.35, 6.98) was observed. SHBG was inversely associated with FLI across sexes [men: -3.45 (-5.13, -1.78); women: -9.23 (-12.19, -6.28)]. No causal association was found between genetically determined sex hormones and liver fat, but higher genetically determined SHBG was associated with lower liver fat in women (β = -0.36, 95% CI: -0.61, -0.12).

Conclusion: Our results provide suggestive evidence for a causal association between SHBG and liver fat in women, implicating the protective role of SHBG against liver fat accumulation.

KEYWORDS

sex hormones, sex hormone-binding globulin, fatty liver index, liver fat, Mendelian randomization, European cohort

Introduction

Fatty liver, a condition characterized by excessive ectopic fat accumulation in the liver (≥5%), is affecting one fourth of the world population. It is increasingly contributing to the global healthcare burden with the late stage of liver disease, liver cirrhosis, being the 11th most common cause of death (1).

Epidemiological evidence reported that fatty liver is more prevalent among men than women (2). Several mechanisms have been proposed to explain these differences focusing mainly on the role of sex hormones, namely androgens and estrogen, on glucose-, cholesterol- and lipid- metabolism in the liver (3). Endocrine diseases such as male hypogonadism, a condition defined by reduced sex hormone levels, or polycystic ovary syndrome (PCOS), a condition usually resulting in excessive androgen levels in women, have been consistently shown to be associated with higher fatty liver risk (3).

A recent meta-analysis of population-based studies found that higher serum testosterone (T), the major form of androgen, was associated with lower risk of fatty liver among men, but not in women (4). Other studies on precursors of T such as dehydroepiandrosterone (DHEA) and its sulfate form DHEA-sulfate (DHEAS), have consistently shown an involvement in metabolic disorders (5). For example, supplementation of DHEA improved insulin sensitivity and increased lean body mass in older adults (6, 7). However, whether DHEA or DHEAS modulate fatty liver risk remains controversial (4, 8). In peripheral tissues, such as skin, DHEA and T are converted into dihydrotestosterone (DHT), and the latter has been related to lower risk of diabetes among older men (9). Nevertheless, there is no population-based evidence directly linking DHT to fatty liver.

Postmenopausal women exhibited higher fatty liver risk compared to premenopausal women, highlighting the protective role of estrogens, such as estradiol (E2), in cardiometabolic health (10). Other important sex hormones, such as progesterone and its derivative, 17α-hydroxyprogesterone (17-OHP), have also been linked to metabolic derangements, such as insulin resistance, obesity and diabetes (11, 12), conditions closely related to fatty liver (1). Sex hormone-binding globulin (SHBG), on the other hand, a liver derived protein that transports sex hormones in the blood and affects their bioactivity (13), has been associated with lower odds of fatty liver in both men and women in a recent meta-analysis (4).

In this study, we firstly aimed to investigate the cross-sectional and longitudinal association of serum sex hormone levels (e.g. T, DHEA) and SHBG with the fatty liver index (FLI), a validated non-invasive and cost-efficient tool for the estimation of fatty liver in population-based studies (14, 15). Secondly, to investigate whether the observed associations are causal, we used genetic instruments to investigate the role of sex hormones and SHBG on liver fat by Mendelian randomization analysis using the largest up to date genome-wide association studies (GWAS) (16–19).

Methods

Population

The study was performed among participants of the prospective population-based Cooperative Health Research in the Region of Augsburg (KORA) study. A total of 4,261 adults, aged 25-74 years, were included at baseline between 1999 and 2001 (S4 visit) with the primary aim to assess health and disease in Southern Bavaria, Germany. Follow-up examinations were conducted after 7 years (F4 visit, 2006 -2008) and after 14 years (FF4 visit, 2013 - 2014) (20–22). All study participants have provided written informed consent. The study was approved by the Ethics Committees of the Bavarian Chamber of Physicians (Ethical Approval Number 06068) adhering to the declaration of Helsinki.

The present analysis includes data from the F4 visit as baseline and FF4 visit as follow-up (average follow-time: 6.5 years). Excluding premenopausal women (n = 602), women with hysterectomy or bleeding due to hormone replacement therapy and younger than 60 years (n = 188), women with missing menopausal status (n = 4), participants without valid FLI information at baseline (n = 47), a total of 2,239 participants (1,456 men and 783 postmenopausal women) were included in the cross-sectional analysis (Figure 1). Due to

missing sex hormones information at baseline (n = 60 to 244), the final number of participants for the regression analyses differed by sex hormone (1,328 to 1,417 men; 667 to 762 postmenopausal women) at baseline. For the longitudinal analysis, we further excluded participants lost to follow-up (n = 720) and those without FLI information (n = 14) at the FF4 visit, leaving a sample size of 1,505 participants (941 to 1,003 men; 408 to 468 postmenopausal women).

Details of laboratory, clinical and anthropometric measurements as well as interviews are provided in the Supplementary Materials.

Sex hormones and SHBG assessments

T, DHEA, DHEAS, DHT, progesterone, and 17-OHP were quantified in serum samples which were stored at -80°C until being assayed. The detailed assessment procedure has already been described in detail (23). Samples were prepared and sex hormones were quantified using the Absolute*IDQ*™ Stero17 Kit and electrospray ionization liquid chromatography-mass spectrometry (ESI-LC-MS/MS). The quantification method of the Absolute*IDQ*™ Stero17 Kit has been proved to follow the European Medicines Agency's Guideline on bioanalytical method validation (July 21st 2011) (24). Metabolite concentrations were calculated using internal standards and reported in nM or ng/ml. Missing values of sex hormones were imputed (11). Sex hormones were then normalized, and different batches were calibrated (11). SHBG was measured in serum using the chemiluminescent microparticle immunoassay ARCHITECT for the absolute quantification of SHBG (Abbott Diagnostics).

In order to be transported in blood, sex hormones are bounded to SHBG or weakly bounded to albumin. The free circulating sex hormones [e.g. free T (fT), free DHT (fDHT)] represent the bioactive hormones that target tissues. The sum of albumin-bound and free sex hormone is bioavailable sex hormone (e.g. bioavailable T). In the KORA study, fT and fDHT were calculated using mass action equations based on the concentrations of the total hormones and their binding constants to serum SHBG and albumin according to Rinaldi et al. (25).

Calculation of FLI

FLI was calculated from BMI, waist circumference, triglycerides (TG) and gamma-glutamyl-transferase (GGT) with the algorithm developed by Bedogni et al. (14):

$$FLI = (e^{0.953*\log e(TG) + 0.139*BMI + 0.718*\log e(GGT) + 0.053*\text{waist circumference} - 15.745})/(1 + e^{0.953*\log e(TG) + 0.139*BMI + 0.718*\log e(GGT) + 0.053*\text{waist circumference} - 15.745}) * 100$$

with TG measured in mmol/l, GGT in U/l, and waist circumference in cm, resulting in a score ranging from 0 to 100, with a FLI < 30 ruling out and a FLI ≥ 60 ruling in fatty liver.

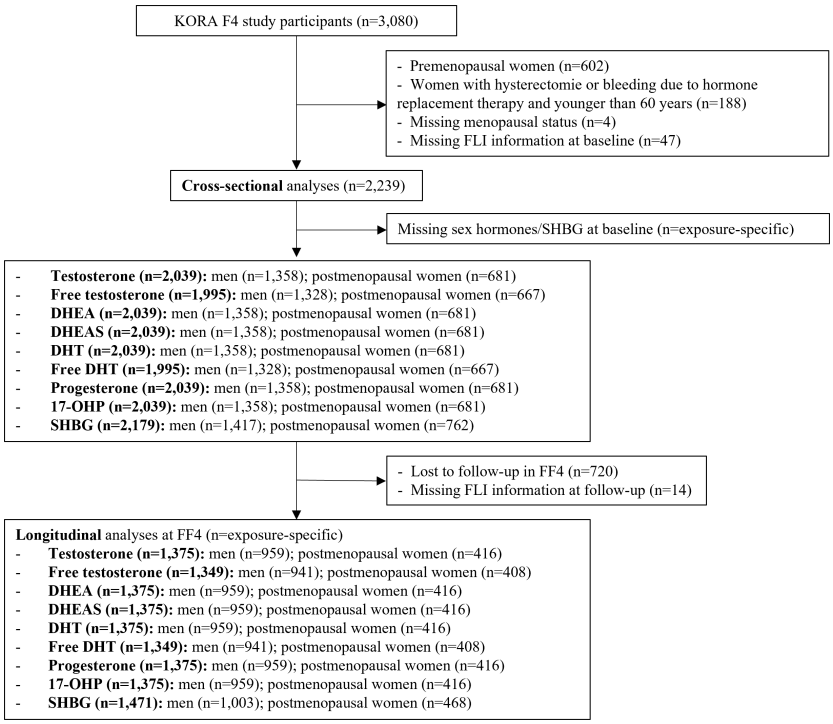

FIGURE 1
Flow chart of the KORA study population for the observational analysis. DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone-sulfate; DHT, dihydrotestosterone; SHBG, sex horhome-binding globulin; 17-OHP, 17α-hydroxyprogesterone.

Genetic instrumental variables

We searched the GWAS Catalogue using the full name of the sex hormones and identified the largest GWAS in the European population including total T, bioavailable T (bioT), E2, SHBG (18, 26), DHEAS (17), progesterone and 17-OHP (19). For DHT, the only GWAS in the European population was conducted in a study population at particular high risk of prostate cancer (men only, n= 3225) (27). One GWAS identified a SNP (rs34670419) associated with DHEA in a European population (n=1023); however, the association (p=2e-9) did not reach a genome-wide cut-off of p<5e-11 after multiple-testing adjustment (28). Therefore, we did not include DHT and DHEA in the MR analysis. Summary statistics for total T in men and women, bioT in men and women, E2 in men, SHBG in men and women (Ruth et al., 2020 (18)), DHEAS in men and women combined (Zhai et al., 2011 (17)), and progesterone in men and women, 17-OHP in men and women (Pott et al., 2021) (19) were obtained from the respective publications. Of note, a genetic instrument for E2 in women was not included, as in the GWAS of Ruth et al. most of the women were postmenopausal and showed E2 levels below the limit of detection (78%), which substantially reduced the power of analysis for genetic instruments of E2 and biased the associations towards loci associated with age at menopause (18).

After we included the genome-wide significant SNPs (p<5e-8) for sex hormones and SHBG, we clumped the SNPs if they were in linkage disequilibrium (LD) (LD r^2>0.001). The SNP-outcome associations were extracted from the largest GWAS available up to date for MRI measured hepatic proton-density fat fraction (PDFF) in the European population by Parisinos et al. using data from a subsample of UK Biobank (29) (Supplementary Table 1). We chose this study because MRI has been demonstrated as the most definitive non-invasive medical imaging to quantify liver fat content (30). Afterwards, we harmonized the SNP-exposure and SNP-outcome associations and excluded palindromic SNPs (Supplementary Figure 1). Three genetic instruments that could not be matched in the outcome dataset (rs543504257, rs2275560, rs78058190) were excluded from further MR analysis.

Statistical analyses

Baseline characteristics of the participants were compared among the FLI categories stratified by sex. For continuous variables, the arithmetic mean and standard deviation (SD) are shown if normally distributed or the median and interquartile (IQR) if non-normally distributed. For categorical variables, counts and percentages (%) were displayed. Analysis of variance (ANOVA) was used for continuous variables and chi-square test was used for categorical variables to test the differences between the groups.

Observational analysis in the KORA study

Sex-specific correlations of sex hormones were examined by Pearson's rho. Sex hormone concentrations were sex-specifically z-standardized. The associations between sex hormones and baseline FLI as well as FLI at the follow-up were investigated with linear regression stratified for men and postmenopausal women. Model adjustment was defined *a priori*. The main model was adjusted for age, conventional lifestyle and cardiometabolic risk factors for sex hormone derangement and ectopic fat accumulation, including smoking (never, ex-smoker, smoker), physical activity (active, inactive), alcohol consumption (no intake, moderate intake, excessive intake), systolic blood pressure (SBP), high-density lipoprotein-cholesterol (HDL-C), low-density lilpoprotein-cholesterol (LDL-C) (all continuous), clinically diagnosed diabetes (yes, no), use of antihypertensive medication (yes, no) and lipid lowering medication (yes, no) (Supplementary Material). The model for the longitudinal analysis was additionally adjusted for baseline FLI. In a sensitivity analysis, we further adjusted for continuous C-reactive protein (CRP), thyroid stimulating hormone (TSH), serum albumin and SHBG, which are either closely related to sex hormone derangement or determinant for bioavailable sex hormones. The significance level was set to p<0.0056 to account for multiple tests (9 exposures) using Bonferroni correction.

Mendelian randomization analysis

MR analysis was conducted to investigate the causal relationship between sex hormones/SHBG and hepatic PDFF. More detailed explanation of the methodology is provided in the Supplementary Material. Firstly, we conducted MR analysis with the inverse-variance weighting (IVW) approach. One of the MR assumptions (exclusion restriction) is that the association between the genetic instrument and the outcome goes only through the exposure (Supplementary Materials). The IVW approach is only valid if all genetic instruments fulfill the "exclusion restriction" assumption. In case of genetic pleiotropy where the "exclusion restriction" is violated and genetic variants are also associated with other risk factors of the outcome, other robust MR methods provide valid and consistent MR estimates. The weighted-median approach allows up to 50%, the weighted-mode approach 50% - 100% and the MR-Egger approach up to 100% for pleiotropic variants (31, 32). A statistically significant IVW result with directionally consistent MR estimates from all three sensitivity analyses was considered to be a potential causal effect (33). The existence of directional horizontal pleiotropy was defined if the intercept term of the MR-Egger regression significantly differed from zero (p for pleiotropy < 0.05).

For DHEAS, progesterone and 17-OHP, we conducted two-sample MR analysis. Whereas for total T, bioT, E2 and SHBG, MR analysis was carried out in a two-sample setting with population overlap (<10%), since summary statistics for both exposure and outcome were obtained from the UK Biobank. In large scale studies, the precision and bias of MR estimates (except for MR Egger approach) are similar in both two-sample or one-sample (with complete sample overlap) MR settings (34, 35).

In order to investigate the causal effect of T or E2 independent of SHBG, we conducted MR analysis using clusters of genetic instruments with primary effects on specific sex hormone (T or

E2 or SHBG) identified previously by Ruth and colleagues (Supplementary Table 1) (18). We also conducted sensitivity analysis excluding the SNPs with larger effects on the metabolic risk factors closely related to fatty liver, including fasting glucose, type 2 diabetes, coronary artery disease, HDL-C, LDL-C, triglycerides, total cholesterol, SBP, DBP, BMI and waist-to-hip ratio adjusted for BMI, than their effects on sex hormones identified by Steiger-Filtering previously (18). The Steiger test filters out SNPs that explain more variance in one phenotype (e.g. outcome/trait closely related to the outcome) than another (e.g. exposure), to reduce potential pleiotropic effects of these SNPs and avoid reverse causality (36).

All analyses were conducted using R statistical software, version 4.2.1, including the MR analyses for which we used the "TwoSampleMR" R package. We used multiple imputation with 5 imputed datasets for covariates with missing values less than 5% for the observational analysis. In the MR analysis, a $p<0.0071$ (0.05/7 exposure) was considered significant with Bonferroni correction for multiple testing.

Results

Observational analyses

Among 2,239 participants eligible for the cross-sectional analysis, the prevalence of FLI ≥ 60 was higher in men (54%, mean age 57 years) than in postmenopausal women (38%, mean age 66 years). For both men and women, participants with higher FLI were significantly older, had higher BMI, larger waist circumference and they were less physically active. They had higher blood pressure, higher blood lipid concentrations, higher CRP levels and higher liver enzyme levels. They also suffered more from diabetes, and were more likely to use antihypertensive or lipid lowering medication (Table 1, Supplementary Table 2). Among men, lower levels of sex hormones and SHBG were seen with higher FLI. Whereas among postmenopausal women, higher fT and lower DHEA, DHT and SHBG concentrations were observed with higher FLI (Table 2). A correlation matrix between the sex hormones and SHBG is shown in Supplementary Figure 2.

Multivariable adjusted regression analyses showed that among men, lower T [β, 95%CI: -4.89 (-6.12, -3.66)], DHT [-2.97 (-4.20, -1.73)], progesterone [-2.75 (-4.02, -1.49)], 17-OHP [-3.57 (-4.80, -2.34)] and SHBG [-4.64 (-5.89, -3.39)] were associated with higher FLI at baseline. Among postmenopausal women, higher fT [2.27 (0.77, 3.77)] and lower SHBG [-9.00 (-11.13, -6.87)] were associated with higher FLI at baseline (Figure 2 and Supplementary Table 3). In longitudinal analysis, similar trends followed for both men and women (Figure 2 and Supplementary Table 4). In the sensitivity analysis, additionally adjusting for CRP, TSH, serum albumin and SHBG hardly changed the associations (Supplementary Table 5).

All associations in the longitudinal analysis were attenuated after adjustment for baseline FLI (Supplementary Table 4), possibly due to reverse causation. However, baseline adjustment is only occasionally advantageous, and whether it eliminates or introduces bias depends crucially upon the causal structure relating the variables (37).

Mendelian randomization analysis

For sex hormones, the MR IVW estimates for total T [-0.09 (-0.16, -0.01)] in men and bioavailable T [0.13 (0.03, 0.23)] in women were nominally significant ($p<0.05$), but they did not pass the significance level of $p< 0.0071$ after Bonferroni correction. MR analyses with the IVW approach revealed that higher SHBG among women [-0.36 (-0.61, -0.12)] was associated with lower hepatic PDFF. Among men, the estimate was smaller [-0.19 (-0.33, -0.05)], and did not pass the Bonferroni threshold. Sensitivity analyses with weighted median, weighted mode and MR-Egger yielded estimates directionally consistent to the IVW estimates (Table 3). There was no indication of directional horizontal pleiotropy in the above MR analyses (p for pleiotropy from MR-Egger ≥ 0.05) (Table 3).

Due to genetic overlap between T, E2 and SHBG, we used clusters of instrumental SNPs with primary effects on T or E2 or SHBG to investigate the potential causal effect of T or E2 on hepatic PDFF independent of SHBG. MR analysis with clusters of T or E2 showed that there was no association between T and hepatic PDFF independent of SHBG in either men or women. Nor was there any association between E2 and hepatic PDFF independent of SHBG in men (Supplementary Table 6). The IVW estimates for both male SHBG cluster [-0.20 (-0.34, -0.06)] and female SHBG cluster [-0.43 (-0.61, -0.25)] reached statistical significance after Bonferroni correction. All three sensitivity analyses resulted in estimates in the same direction as the IVW estimates (Supplementary Table 6). The male SHBG cluster includes SNPs with primary SHBG increasing effect and secondary increasing effect on total T and decreasing effect on bioT as well as increasing effect on E2, and the female SHBG cluster includes SNPs with primary increasing effect on SHBG and secondary opposing effect on T and bioT. Taken together, this indicated that genetically determined higher SHBG has a decreasing effect on hepatic PDFF in both men and women, probably also through its effect on sex hormones (Table 3).

In order to minimize the pleiotropic effect of SNPs closely associated with metabolic risk factors, we further excluded them from the MR. The association between SHBG and hepatic PDFF attenuated, but maintained the same directionality (Supplementary Table 7).

Discussion

In this study, we investigated the observational and possible causal association of endogenous sex hormones and SHBG with liver fat combining evidence from a population-based study and summary-level data from the largest up to date GWAS. We observed that higher sex hormones, such as T, DHT, progesterone, 17-OHP, as well as SHBG were associated with lower FLI both at baseline and follow-up among men. Among postmenopausal women, lower fT and higher SHBG were both associated with lower FLI at baseline and follow-up. The MR

TABLE 1 Baseline characteristics of KORA F4 study participants among men and postmenopausal women.

	Men (n=1,456)				Postmenopausal women (n=783)			
	FLI<30 (N=264)	30 ≤ FLI < 60 (N=410)	FLI ≥ 60 (N=782)	*P* value	FLI<30 (N=278)	30 ≤ FLI < 60 (N=208)	FLI ≥ 60 (N=297)	*P* value
Age (years)	50.6 (13.0)	56.2 (14.1)	58.8 (12.5)	< **0.001**	62.7 (8.6)	67.1 (8.2)	66.9 (7.7)	< **0.001**
BMI (kg/m^2)	23.5 (1.8)	26.0 (1.8)	30.4 (3.9)	< **0.001**	24.1 (2.4)	27.7 (2.2)	33.3 (4.3)	< **0.001**
Waist Circumference (cm)	86.0 (5.5)	94.6 (5.3)	107.1 (10.5)	< **0.001**	80.2 (6.2)	90.7 (5.0)	103.7 (9.4)	< **0.001**
Smoking				< **0.001**				**0.002**
never smoker	105 (39.9%)	132 (32.3%)	212 (27.2%)		150 (54.0%)	137 (65.9%)	186 (62.6%)	
ex-smoker	98 (37.3%)	179 (43.8%)	436 (55.9%)		84 (30.2%)	51 (24.5%)	92 (31.0%)	
smoker	60 (22.8%)	98 (24.0%)	132 (16.9%)		44 (15.8%)	20 (9.6%)	19 (6.4%)	
Physically active	164 (62.4%)	243 (59.4%)	371 (47.6%)	< **0.001**	175 (62.9%)	106 (51.0%)	145 (48.8%)	**0.002**
Alcohol consumption				< **0.001**				**0.049**
no intake	55 (20.9%)	88 (21.5%)	155 (19.9%)		108 (38.8%)	73 (35.1%)	142 (47.8%)	
moderate intake	161 (61.2%)	231 (56.5%)	391 (50.1%)		125 (45.0%)	103 (49.5%)	117 (39.4%)	
excessive intake	47 (17.9%)	90 (22.0%)	234 (30.0%)		45 (16.2%)	32 (15.4%)	38 (12.8%)	
Systolic blood pressure (mmHg)	119.8 (15.9)	126.9 (16.9)	131.0 (17.5)	< **0.001**	119.0 (19.8)	122.7 (19.3)	125.9 (17.3)	< **0.001**
Diastolic blood pressure (mmHg)	73.5 (8.8)	76.4 (9.5)	79.4 (10.4)	< **0.001**	73.1 (9.3)	73.2 (9.4)	74.1 (9.3)	0.385
Hypertension	42 (16.0%)	160 (39.1%)	434 (55.6%)	< **0.001**	83 (29.9%)	104 (50.0%)	199 (67.2%)	< **0.001**
Total cholesterol (mmol/l)	5.1 (0.9)	5.5 (0.9)	5.6 (1.1)	< **0.001**	6.0 (0.9)	6.0 (1.0)	6.0 (1.1)	0.758
HDL-C (mmol/l)	1.5 (0.3)	1.3 (0.3)	1.2 (0.3)	< **0.001**	1.8 (0.4)	1.6 (0.3)	1.4 (0.3)	< **0.001**
LDL-C (mmol/l)	3.3 (0.8)	3.6 (0.8)	3.6 (0.9)	< **0.001**	3.6 (0.9)	3.8 (0.9)	3.8 (0.9)	**0.013**
Triglycerides (mmol/l)	0.8 (0.6, 1.1)	1.2 (0.9, 1.5)	1.8 (1.3, 2.5)	< **0.001**	0.9 (0.7, 1.1)	1.3 (1.0, 1.6)	1.6 (1.2, 2.2)	< **0.001**
ALT (ukat/l)	0.3 (0.3, 0.4)	0.4 (0.3, 0.5)	0.5 (0.4, 0.7)	< **0.001**	0.3 (0.2, 0.4)	0.3 (0.3, 0.4)	0.4 (0.3, 0.5)	< **0.001**
AST (ukat/l)	0.4 (0.4, 0.5)	0.4 (0.4, 0.5)	0.5 (0.4, 0.6)	< **0.001**	0.4 (0.3, 0.5)	0.4 (0.3, 0.5)	0.4 (0.3, 0.5)	< **0.001**
GGT (U/l)	24.0 (20.0, 29.0)	31.0 (25.0, 41.0)	46.0 (34.0, 71.8)	< **0.001**	21.0 (17.0, 26.0)	25.5 (20.0, 36.0)	31.0 (24.0, 48.0)	< **0.001**
C-reactive protein (mg/l)	0.5 (0.3, 1.1)	0.8 (0.5, 1.8)	1.5 (0.8, 2.9)	**0.008**	0.9 (0.5, 1.8)	1.5 (0.9, 3.0)	2.5 (1.4, 4.9)	< **0.001**
Diabetes	5 (2.0%)	43 (10.7%)	147 (19.2%)	< **0.001**	8 (2.9%)	19 (9.4%)	79 (26.9%)	< **0.001**
Antihypertensive medication	31 (11.8%)	111 (27.1%)	335 (42.8%)	< **0.001**	74 (26.6%)	95 (45.7%)	182 (61.3%)	< **0.001**
Lipid lowering medication	16 (6.1%)	53 (12.9%)	143 (18.3%)	< **0.001**	31 (11.2%)	42 (20.2%)	64 (21.5%)	**0.002**
Thyroid stimulating hormone (mIU/l)	1.2 (0.8, 1.8)	1.2 (0.9, 1.8)	1.3 (0.9, 1.9)	0.328	1.3 (0.8, 1.9)	1.1 (0.6, 1.7)	1.2 (0.8, 1.7)	0.155
Serum albumin (g/l)	45.6 (3.4)	45.3 (3.2)	45.3 (3.5)	0.363	44.0 (3.1)	43.6 (3.0)	43.5 (3.0)	0.102

Values are expressed as the mean (SD) for normally distributed continuous variables or median (interquartile range) for non-normally distributed continuous variables, or n (%) for categorical variables. P-values were generated by ANOVA for continuous variables and chi-square test for categorical variables. P-values< 0.05 are shown in bold.

Excessive alcohol consumption was defined as men with alcohol intake ≥ 30 g/day and women with alcohol intake ≥ 20 g/day.

FLI, fatty liver index; BMI, body mass index; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; ALT, Alanine Aminotransferase; AST, Aspartate Aminotransferase; GGT, Gamma-Glutamyl Transferase; SD, standard deviation; ANOVA, analysis of variance.

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TABLE 2 Baseline characteristics of KORA F4 study participants including sex hormones, SHBG and related variables among men and postmenopausal women.

	Men (n=1,456)				Postmenopausal women (n=783)				Missing
	FLI<30 (N=264)	30 ≤ FLI < 60 (N=410)	FLI ≥ 60 (N=782)	*P* value	FLI<30 (N=278)	30 ≤ FLI < 60 (N=208)	FLI ≥ 60 (N=297)	*P* value	
Testosterone (nmol/l)	18.00 (14.47, 21.54)	15.12 (12.06, 19.17)	13.41 (10.36, 16.80)	**< 0.001**	0.66 (0.48, 0.87)	0.58 (0.40, 0.90)	0.62 (0.43, 0.94)	0.325	200 (8.93%)
Free testosterone (pmol/l)	208.58 (179.71, 244.65)	194.17 (157.74, 239.79)	183.26 (143.29, 218.49)	**< 0.001**	5.47 (3.54, 8.10)	5.50 (3.92, 8.38)	7.39 (5.12, 10.93)	**< 0.001**	244 (10.90%)
DHEA (nmol/l)	10.84 (7.52, 16.04)	9.04 (5.51, 14.55)	7.89 (4.74, 13.13)	**< 0.001**	7.94 (4.58, 11.90)	6.41 (3.93, 9.11)	6.21 (4.17, 9.23)	**< 0.001**	200 (8.93%)
DHEAS (nmol/l)	3776.67 (2257.44, 5867.85)	3219.94 (1777.56, 5405.83)	2838.35 (1535.46, 4727.63)	**< 0.001**	1638.94 (940.49, 2429.63)	1267.93 (737.68, 2120.88)	1392.92 (735.54, 2201.20)	0.202	200 (8.93%)
DHT (nmol/l)	1.60 (1.17, 2.08)	1.36 (1.04, 1.78)	1.09 (0.76, 1.50)	**< 0.001**	0.21 (0.12, 0.34)	0.17 (0.09, 0.27)	0.15 (0.09, 0.24)	**< 0.001**	200 (8.93%)
free DHT (pmol/l)	13.37 (10.47, 17.33)	12.92 (9.72, 16.45)	11.11 (8.43, 14.38)	**< 0.001**	1.33 (0.65, 2.04)	1.15 (0.70, 1.95)	1.27 (0.72, 2.29)	0.399	244 (10.90%)
Progesterone (nmol/l)	0.24 (0.15, 0.37)	0.21 (0.11, 0.32)	0.17 (0.09, 0.29)	**< 0.001**	0.12 (0.05, 0.23)	0.12 (0.03, 0.18)	0.09 (0.04, 0.17)	0.569	200 (8.93%)
17-OHP (nmol/l)	3.19 (2.38, 4.31)	2.92 (2.29, 3.80)	2.47 (1.78, 3.50)	**< 0.001**	0.80 (0.51, 1.20)	0.79 (0.53, 1.21)	0.79 (0.54, 1.13)	0.545	200 (8.93%)
SHBG (nmol/l)	56.00 (41.05, 71.75)	49.45 (37.77, 67.12)	45.70 (31.63, 63.05)	**< 0.001**	88.50 (65.80, 112.55)	74.20 (53.70, 96.80)	53.35 (40.08, 73.65)	**< 0.001**	60 (2.68%)
Hormone replacement therapy	NA	NA	NA	NA	26 (9.4%)	11 (5.3%)	15 (5.1%)	0.077	

Values are expressed as the mean (SD) for normally distributed continuous variables or median (interquartile range) for non-normally distributed continuous variables, or n (%) for categorical variables. P-values were generated by ANOVA for continuous variables and chi-square test for categorical variables. P-values< 0.05 are shown in bold.
FLI, fatty liver index; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone-sulfate; DHT, dihydrotestosterone; SHBG, sex hormone-binding globulin; 17-OHP, 17α-hydroxyprogesterone; SD, standard deviation; ANOVA, analysis of variance; NA, not applicable.

analyses showed suggestive evidence for an inverse causal association of genetically determined SHBG on hepatic fat content in women, but no other potential causal effect was found for sex hormones on liver fat.

A recent meta-analysis including 16 studies found that higher T was associated with lower odds of fatty liver [0.59 (0.42, 0.76)] in men, but not in women. In KORA, we confirmed these results. Interestingly, although we did not find an association between T and FLI among women, higher fT was associated with higher FLI both cross-sectionally and longitudinally. Previous epidemiological evidence also suggested similar associations between fT or bioT levels and higher risk of fatty liver in women (38, 39). This indicates that not the total amount of circulating T but rather the amount of directly available T to the tissues is strongly related with fatty liver risk, especially in women. This could also be a secondary effect of SHBG, whose increase can reduce the levels of fT.

In a clinical trial, obese men treated with T had substantially increased muscle mass and improved insulin sensitivity as well as reduced liver fat, possibly owing to the protective role of T to regulate body composition and glucose metabolism in men (3, 40). However, T seems to exert a distinct metabolic effect in women, potentially due to decreased conversion of T to E2. Additionally, postmenopausal women are be at higher risk of fatty liver, as a result of weight gain, lipid dysregulation and unfavorable adipose distribution due to declining E2 levels (2, 10). In alignment, we found that fT was associated with FLI in opposite ways for men (inversely) and women (positively) in our study.

Although lower DHEAS levels were observed in the group of biopsy-proven more advanced fatty liver disease involving inflammation and fibrosis in a small study (8), we did not find any association between DHEA or DHEAS with FLI in our study sample. Our finding was supported by the null association in a population-based study comparing the risk of ultrasound diagnosed fatty liver in relation to DHEA and DHEAS levels (4). Our analysis also suggested inverse associations of DHT, progesterone and 17-OHP with FLI in men. Experimental studies have shown that DHT, progesterone and 17-OHP influence lipid and glucose metabolism and regulate inflammatory proteins, such as by interacting with insulin signaling in adipocytes or activating glucocorticoid receptor in the liver (12, 41, 42). However, there isn't yet consistent evidence from population-based studies linking these sex hormones to fatty liver. Further studies are needed to examine the role of these sex hormones and fatty liver risk longitudinally.

We noted that lower SHBG levels were associated with higher FLI in both men and women, which is consistent with the findings from a recent meta-analysis (4). Previous literature has shown that

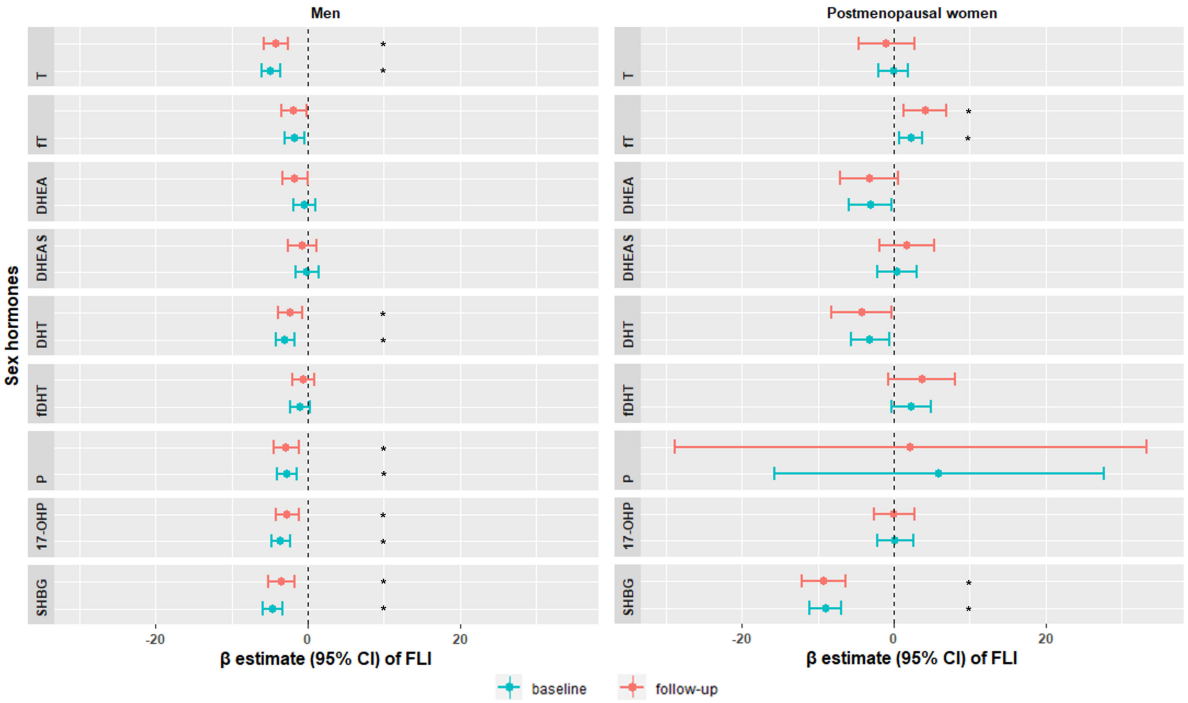

FIGURE 2
Sex-specific associations of sex hormones with fatty liver index at baseline KORA F4 study (blue) and at follow-up KORA FF4 study (red). Models were adjusted for age, smoking, physical activity, alcohol consumption, SBP, HDL-C, LDL-C, diabetes, antihypertensive medication and lipid lowering medication. Significant associations were labeled with *. T, testosterone; fT, free testosterone; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; DHT, dihydrotestosterone; fDHT, free dihydrotestosterone; P, progesterone; 17-OHP, 17α-hydroxyprogesterone; SHBG, sex hormone-binding globulin; FLI, fatty liver index; SBP, systolic blood pressure; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

lower endogenous SHBG level is associated with higher risk of cardiometabolic disorders and fatty liver, and this association is reported to be constant in both sexes across age groups (4, 43, 44). Moreover, lower SHBG has been associated with older age, obesity, and lifestyle risk factors, such as being physically inactive and alcohol consumption, all closely related to liver fat accumulation (45, 46). In our study, the association between SHBG and FLI remained significant after adjusting for all these factors. However, given the multifactorial nature of fatty liver, there might be other risk factors confounding the observed associations, which we were not able to correct for. Although the mechanism underlying the association between SHBG and liver fat regulation remains uncertain, animal experiments implied that increased SHBG level can downregulate the expression of the crucial enzymes involved in the hepatic lipogenesis, such as the adenosine triphosphate (ATP) citrate lyase (production of precursor for fatty acid), Acetyl-CoA-carboxylase and fatty acid synthase (further restriction of fatty acid synthesis) in the liver (47, 48), which could consequently reduce liver fat content. Meanwhile, *in vitro* experiments showed that SHBG can repress inflammatory cytokines, including interleukin-6 and tumor necrosis factor-alpha in adipocztes and macrophages, modulating inflammatory processes (48). Furthermore, SHBG may indirectly impact liver fat content by regulating the bioavailability and balance of sex hormones. On the other hand, liver cell function and other metabolic factors, such as insulin, can also regulate SHBG production (13). Additionally, the genetic determinants of SHBG overlap with those of other metabolic risk factors for fatty liver, as captured in our MR analysis. Therefore, the observational association between SHBG and risk of liver fat accumulation could be subject to residual confounding and reverse causation, which can be better addressed with MR analysis.

Previous MR studies have suggested the protective role of SHBG against the development of metabolic disorders, such as type 2 diabetes (18) and hypertension (49), both risk factors for fatty liver. Accordingly, we found that genetically determined circulating SHBG were inversely associated with liver fat content in women, consistent with the observational evidence. However, among men, this association was only nominal ($p<0.05$) but did not pass the Bonferroni correction threshold of $p<0.0071$. Although we did not detect any pleiotropy using a battery of robust MR methods, the associations between SHBG and hepatic PDFF should be interpreted with caution, since the association was attenuated after we excluded SNPs closely related to metabolic risk factors identified by Steiger-filtering in a previous study (18). This finding highlights the importance of carefully evaluating the assumptions underlying the MR analysis and employing appropriate methods to address potential confounding effects of metabolic risk factors, highly intermingled in fatty liver pathophysiology. We did not find implication regarding potential causal effect of sex hormones on liver fat.

This is the first study investigating the sex-specific role of a wide range of sex hormones in liver fat accumulation with both observational evidence from a well-characterized population-based

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TABLE 3 Mendelian randomization estimates of the relationship between sex hormones/SHBG on hepatic proton density fat fraction.

Exposure	Sex	N instruments	IVW		Weighted median		Weighted mode		MR-Egger		
			β (95% CI)	*P value*	β (95% CI)	*P value*	β (95% CI)	*P value*	β (95% CI)	*P value*	*P for* pleiotropy
Total testosterone	Men	104	-0.09 (-0.16, -0.01)	0.020	-0.05 (-0.13, 0.02)	0.157	-0.03 (-0.09, 0.03)	0.343	-0.02 (-0.14, 0.10)	0.713	0.163
	Women	124	-0.05 (-0.11, 0.01)	0.121	0.02 (-0.05, 0.09)	0.529	0.01 (-0.06, 0.08)	0.800	0.004 (-0.10, 0.11)	0.943	0.229
Bioavailable testosterone	Men	57	0.003 (-0.06, 0.06)	0.927	0.02 (-0.09, 0.12)	0.733	0.02 (-0.09, 0.13)	0.677	0.04 (-0.07, 0.14)	0.496	0.448
	Women	88	0.13 (0.03, 0.23)	0.012	0.13 (0.02, 0.24)	0.016	0.11 (0.01, 0.22)	0.036	0.10 (-0.09, 0.28)	0.312	0.678
Estradiol	Men	10	-0.35 (-1.16, 0.47)	0.400	-0.03 (-0.74, 0.68)	0.935	0.08 (-0.61, 0.77)	0.823	1.75 (-0.19, 3.69)	0.115	0.053
Progesterone	Women	3	0.004 (-0.06, 0.07)	0.910	-0.02 (-0.09, 0.05)	0.563	-0.03 (-0.11, 0.05)	0.531	0.19 (-0.48, 0.87)	0.678	0.681
17-OHP	Men	4	0.10 (-0.0003, 0.20)	0.051	0.04 (-0.04, 0.12)	0.290	0.05 (-0.04, 0.13)	0.351	0.01 (-0.18, 0.20)	0.912	0.404
	Women	2	0.01 (-0.01, 0.03)	0.247	NA	NA	NA	NA	NA	NA	NA
DHEAS	Sex-combined	4	0.01 (-0.16, 0.18)	0.916	0.03 (-0.11, 0.16)	0.694	0.07 (-0.05, 0.20)	0.341	0.26 (0.05, 0.47)	0.141	0.119
SHBG	Men	151	-0.19 (-0.33, -0.05)	0.0074	-0.09 (-0.22, 0.03)	0.151	-0.04 (-0.15, 0.06)	0.422	-0.14 (-0.34, 0.07)	0.190	0.479
	Women	160	**-0.36 (-0.61, -0.12)**	**0.004**	-0.18 (-0.32, -0.05)	0.008	-0.16 (-0.29, -0.02)	0.023	-0.14 (-0.53, 0.26)	0.503	0.150

Mendelian randomization analysis was carried out with the inverse-variance weighted approach as the main analysis, and robust methods such as weighted median, weighted mode and MR-Egger were carried out as sensitivity analyses. The robust methods allow for certain percentage of invalid (e.g. pleiotropic) instrumental SNPs in the Mendelian randomization analysis, and provide estimates of causal effect not subject to these violations. A statistically significant IVW result with directionally consistent Mendelian randomization estimates from all three sensitivity analyses was considered to be a potential causal effect.

P for pleiotropy is the p value to reject the null hypothesis that the intercept term of the MR Egger regression equals to zero. P for pleiotropy < 0.05 indicates the existence of directional pleiotropy. P<0.0071 (0.05/7) is considered significant with Bonferroni correction for multiple testing and was shown in bold.

SHBG, sex hormone-binding globulin; DHEAS, Dehydroepiandrosterone-sulfate; 17-OHP, 17α-hydroxyprogesterone; IVW, inverse-variance weighted; NA, Not applicable.

study as well as genetic data. Sex hormones were quantified by mass spectrometry, increasingly recognized as the gold standard, being more accurate and sensitive compared to the widely-used immunoassay (50). Using multiple genetic instruments and several MR sensitivity analyses, we could address the potential existence of horizontal pleiotropy and the robustness of the MR estimates. Nevertheless, our study also entails several limitations. We were unable to quantify the role of E2 in relation to liver fat. Although there is evidence indicating a protective role of E2 on liver injury and liver fat accumulation (2, 51), epidemiological studies comparing the risk of fatty liver related to the endogenous levels of E2 could not find a significant association between these two (4). Meanwhile, even though the administration of exogenous E2 has been shown to be associated with an increase in SHBG levels (52), we expect the effect of circulating E2 on SHBG to be neglectable in our study population of postmenopausal women and men since E2 levels are low and stable in this group (53). Nevertheless, future studies should focus on determining the impact of endogenous E2 levels and liver fat and, in particular, addressing the challenge of periodic fluctuations in E2 in premenopausal women. MRI has been deemed to be the gold standard for non-invasive measurement for liver fat content, but the high cost of MRI precludes it for large scale investigations. We did not use sex-specific genetic associations with hepatic PDFF for the MR analysis, but we don't expect large differences - a GWAS from the UK Biobank indicated no sex difference in the genetic signals for steatohepatitis (29). Up to date, the GWAS from UK Biobank include the highest number of genetic instruments for T, E2 and SHBG. Therefore, we employed the two-sample approach with sample overlap (<10%) for these exposures, which could bias the MR estimates towards the observational associations (weak instrument bias). However, in case of large study population, using strong genetic instruments (p < 5e-8) which explain high genetic heritability of the phenotypes (2% -21%), potential bias due to weak instruments is expected to be low (35).

Conclusion

Our complementary observational and MR results support suggestive causal associations between SHBG with liver fat, particularly in women, indicating that interventions targeting this pathway, along with management of accompanying risk factors, may help the prevention of fatty liver. Further observational studies are needed to examine the sex-specific associations between sex

hormones and liver fat accumulation quantified by MRI using population-based data.

Data availability statement

The data underlying this article cannot be shared publicly due to data protection reasons. The data will be shared on reasonable request to the corresponding author.

Ethics statement

The studies involving humans were approved by the Ethics Committees of the Bavarian Chamber of Physicians (Ethical Approval Number 06068). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

All persons listed as coauthors have significantly contributed to preparing the manuscript: XC has designed the analyses, interpreted the data and drafted the manuscript; JN and BT have contributed to the conception, design and interpretation of the data and approval of the manuscript; SH, CP, AC, JA, TZ, AD, RB, AP, HY have contributed substantially to the interpretation of the data and have critically revised the manuscript for important intellectual content. All authors contributed to the article and approved the submitted version.

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Conflict of interest

TZ is one of the shareholders of the ART.EMIS GmbH Hamburg. She is listed as co-inventor of an international patent on the use of a computing device to estimate the probability of myocardial infarction (International Publication Number WO2022043229A1).

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1223162/full#supplementary-material

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List of all scientific publications to date

1. Cai, X., Thorand, B., Hohenester, S., Prehn, C., Cecil, A., Adamski, J., Zeller, T., Dennis, A., Banerjee, R., Peters, A., Yaghootkar, H., Nano, J. (2023). Association of Sex Hormones and Sex Hormone Binding Globulin with Liver Fat in Men and women: An Observational and Mendelian Randomization Study. *Frontiers in Endocrinology*, Oct 13:14:1223162. doi: 10.3389/fendo.2023.1223162.
2. Cai, X., Thorand, B., Hohenester, S., Koenig, W., Rathmann, W., Peters, A., & Nano, J. (2023). Association between the fatty liver index and chronic kidney disease: the population-based KORA study. *Nephrology Dialysis Transplantation*. May 4;38(5):1240-1248. doi: 10.1093/ndt/gfac266.
3. Cai, X., Rospleszcz, S., Mensel, B., Schminke, U., Kühn, J. P., Aghdassi, A. A., ... & Nano, J. (2021). Association between hepatic fat and subclinical vascular disease burden in the general population. *BMJ Open Gastroenterology*, *8*(1), e000709.
4. Jung, A. Y.*, Cai, X.*, Thoene, K., Obi, N., Jaskulski, S., Behrens, S., ... & Chang-Claude, J. (2019). Antioxidant supplementation and breast cancer prognosis in postmenopausal women undergoing chemotherapy and radiation therapy. *The American journal of clinical nutrition*, *109*(1), 69-78. * Jung, A. Y. and Cai, X. contributed equally to this publication.