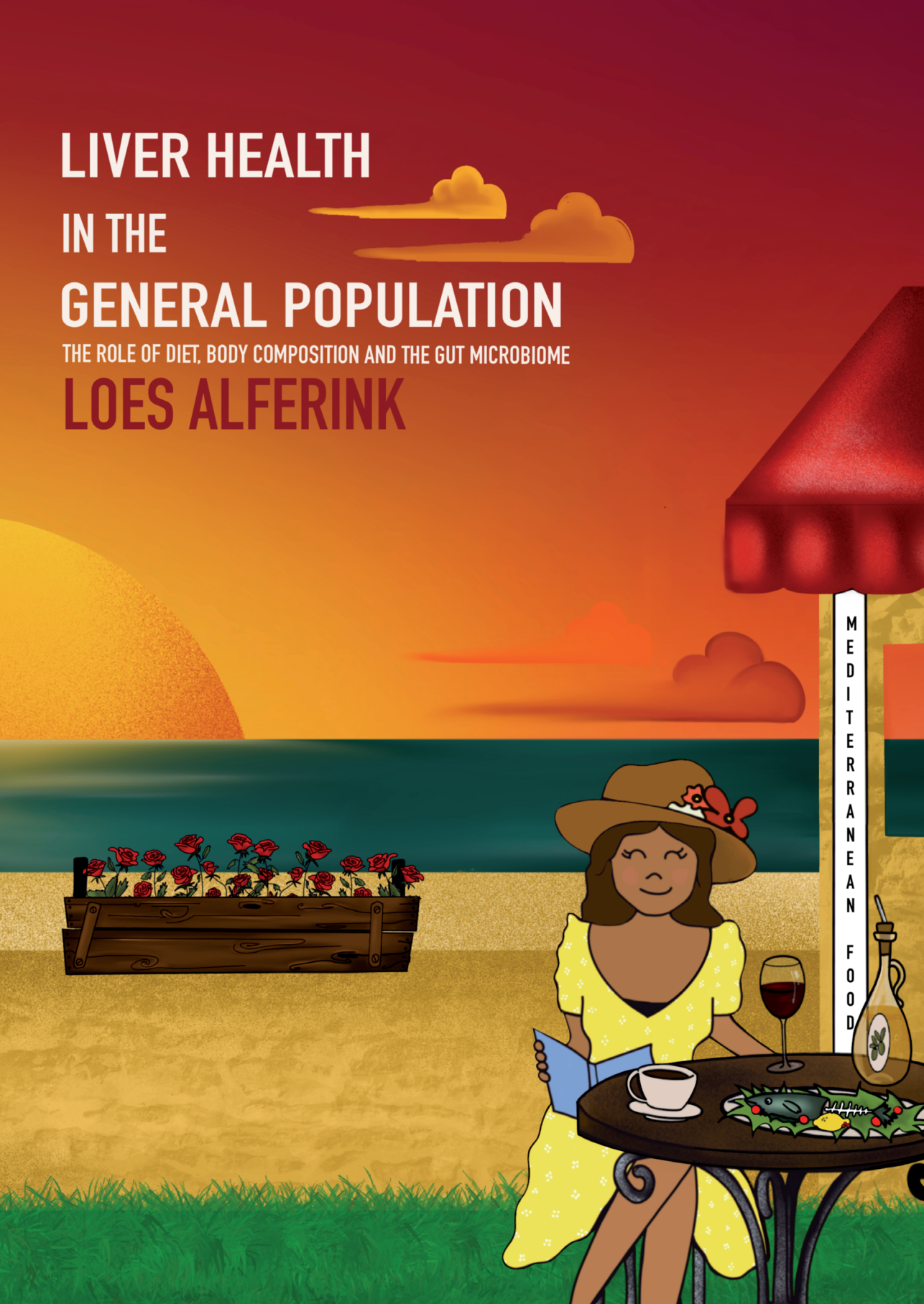
LIVER HEALTH IN THE GENERAL POPULATION

THE ROLE OF DIET, BODY COMPOSITION AND THE GUT MICROBIOME

LOES ALFERINK



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Liver Health in the General Population

the Role of Diet, Body Composition and the Gut Microbiome

leverziekten in de algemene populatie

de rol van dieet, lichaamsbouw en darmflora

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Part I

INTRODUCTION

Chapter 1

Introduction

General introduction liver disease

The liver is the largest solid organ in the human body.[1] In Latin, the word for liver is *hepar*, hence *hepatology* literally means study of the liver. Hepatology is not an old study. It was not until the late 19th century that the first functional description of the liver was given in Dutch literature.[2] In this description, it was concluded that the most important function of the liver was the production of bile. At present, we know that there is much more to the function of the liver than only the production of bile. The liver, for example plays an important role in the production of blood products that are necessary for coagulation and in the production of transport protein albumin. Moreover, the liver plays an important role in the metabolism and detoxification of potential toxins from the gut and blood stream.[3]

At first, the pathophysiology of liver diseases was poorly understood. Clinicians therefore classified and treated according to the presentation of the disease. At least, as much as treatment was available at that time.[4] It was only in the second half of the 20th century that clinicians realized that treatment according to aetiology (instead of presentation of the liver disease) led to much better results. Today we still subdivide the study of the liver in 1) the presentation of liver disease (e.g. acute or chronic), 2) the stage of liver disease (e.g. simple steatosis or cirrhosis), and 3) the aetiology of liver disease (e.g. infectious, auto-immunological, or toxicological).

The most important staging parameter of the liver is the extent of scarring. An internal (e.g. auto-immunological) or external (e.g. toxicological) trigger causes liver damage and inflammation, after which the cells of the liver, the hepatocytes, die. This cell death leads to scarring which we call fibrosis. The end-stage of fibrosis is referred to as cirrhosis, in this stage the liver is basically atrophic. If the function of the liver is still intact we call this stage compensated liver cirrhosis, whereas if the liver fails to function we call this stage decompensated liver cirrhosis. In some cases fibrosis is preceded by a fatty liver, which we refer to as hepatic steatosis. Finally, a last presentation of liver disease, most often co-occurring in cirrhosis, could be liver cancer or hepatocellular carcinoma (HCC).[5]

As the name of this dissertation implies "lifestyle and liver disease in the general population", this handling will be focussed on the rather mild spectrum of liver disease. Because of improved, cheaper, and more readily available diagnostic tools to identify liver disease, we learned to know that liver disease is much more common in the general population than we used to think.[6] Questions, however, remain. Why do these people develop liver disease? And who –of all those people– will develop clinically significant liver disease? Who do we need to treat? And how do we need to treat these people?

Burden of liver disease worldwide

In 2015, there were 2 million deaths due to liver disease, which is 3.5% of global mortality.[7] Roughly half of these deaths is due to decompensated liver cirrhosis, and the other half is due to HCC. This number is higher than death due to human immunodeficiency virus or tuberculosis. Liver cirrhosis was indeed the 11th and HCC the 16th cause of death worldwide. In addition, liver transplantation is the second most common solid organ transplantation globally. Sadly, less than 10% of the global transplantation needs are met.[7]

The highest relative mortality due to liver disease is to be found in Latin America & the Caribbean, the Middle East, and North Africa, whereas the highest absolute mortality due to liver disease is in South and East Asia. Together, India and China comprise almost one third of the worldwide liver disease burden.[8] Aetiology varies per region as well. In Western countries the major causes of liver disease are non-alcoholic and alcoholic fatty liver disease, whereas in Asia, the major cause is viral hepatitis.[9] In this introduction we will concisely set forth these three major causes of liver disease.

Non-alcoholic fatty liver disease or NAFLD is currently the most common liver disease worldwide with an estimated prevalence of 25%. Prevalence of NAFLD continues to increase along with the increasing numbers of the major risk-factors of obesity and diabetes mellitus.[7] Prevalence is much higher in obese individuals (up to 90%)[10] and in patients with diabetes mellitus (up to 70%).[11] Notwithstanding, NAFLD can also occur in non-overweight individuals, which is particularly common in Asia.[12] NAFLD is a term that covers a broad clinical spectrum ranging from simple steatosis, steatohepatitis, fibrosis, cirrhosis and HCC. Progression of disease, however, is generally slower than in other diseases such as alcoholic fatty liver disease.[13]

Almost 50% of the global cirrhosis-related mortality is related to alcohol. Particularly amongst adolescents, alcohol is the leading risk factor of death and decrease in quality of life. Age-standardised heavy drinking is most prevalent in Europe, in particular in Germany, the Netherlands, and France.[14] Rate of progression from alcoholic hepatitis to cirrhosis is 3–12%. However, co-existence with other liver disease aetiologies such as viral hepatitis or non-alcoholic fatty liver disease could speed up progression rate.[15]

Viral hepatitis is mainly prevalent in low and middle-income countries. In 2015, 1.3 million people died of viral hepatitis-related disease. Viral hepatitis is the main cause of acute hepatitis. In addition, hepatitis B (66%) and C (30%) may lead to chronic disease. And besides cirrhosis-related complications, hepatitis B and C are also risk factors for the devel-

opment of HCC or cholangiocarcinoma.[7] The estimated prevalence of worldwide chronic hepatitis B is 3.5% and of chronic hepatitis C is 1%.[16]

Non-alcoholic fatty liver disease

NAFLD is the most prevalent liver aetiology in both adults and children.[17,18] And as NALFD is a new kid on the block, relatively little is known about its treatment and natural history. A large epidemiological study cohort, such as the Rotterdam Study, is therefore ideally suited to study this prevalent liver disease. This main theme of this thesis is NAFLD and therefore this aetiology will be described in more detail below.

Natural history

The definition "NAFLD" is a continuum of liver abnormalities with as common denominator the presence of steatosis in absence of classical risk factors of liver disease. This spectrum begins with simple steatosis, which is defined as the presence of fat droplets within at least 5% of the hepatocytes. It sometimes co-occurs with either mild lobular inflammation or ballooning of hepatocytes.[19] Simple steatosis is reversible in approximately one third of the patients, often upon lifestyle modification.[20] However, simple steatosis can also progress to steatosis with co-occurring portal inflammation and, more importantly, fibrosis. The latter progressed stage of NAFLD is commonly referred to as non-alcoholic steatohepatitis (NASH) and is prevalent is an estimated 1–5% of the Western population.[7,13] The progression to cirrhosis in NAFLD is uncommon and slow.[21] Thus, even though prevalence of NAFLD itself is great, NASH-related cirrhosis is present in less than 1% of the general population. Nevertheless, NASH as indication for liver transplantation is increasing and already the second most common indication for transplantation in the United States.[22,23]

The difficulty is that the natural history of NAFLD is highly variable. On average, fibrosis progresses 1 stage per 14 years in individuals with simple steatosis, whereas this is 1 stage per 7 years in NASH.[20] Initially, it was believed that steatosis alone was benign without risk of future fibrosis. However, this belief has recently been re-challenged by a longitudinal study. In this study 108 patients were biopsied and at follow-up after a median time of 6.6 years, 22% patients with initial simple steatosis progressed to stage 3 fibrosis and 44% developed NASH.[24] In addition, another study identified that approximately one fifth of patients are so-called 'rapid progressors', hence patients that progress quickly from simple steatosis to NASH. Unfortunately, it is unknown how to distinguish this group of patients in advance.[20]

Recent studies show that in particular the presence of fibrosis is important for the prognosis of patients with NAFLD.[21] Interestingly, it is not only liver disease that NAFLD patients are at risk for. In fact, patients with NAFLD are twice as likely to die from cardiovascular disease than from liver disease.[17]

Lastly, as already mentioned above, having NAFLD is also a risk factor for the development of HCC, and possibly also for other malignancies such as colorectal cancer.[25] Interestingly, however, is that over one third of the patients with NAFLD-related HCC did not have co-occurring cirrhosis, whereas this was rare amongst other liver aetiologies such as viral hepatitis.[26]

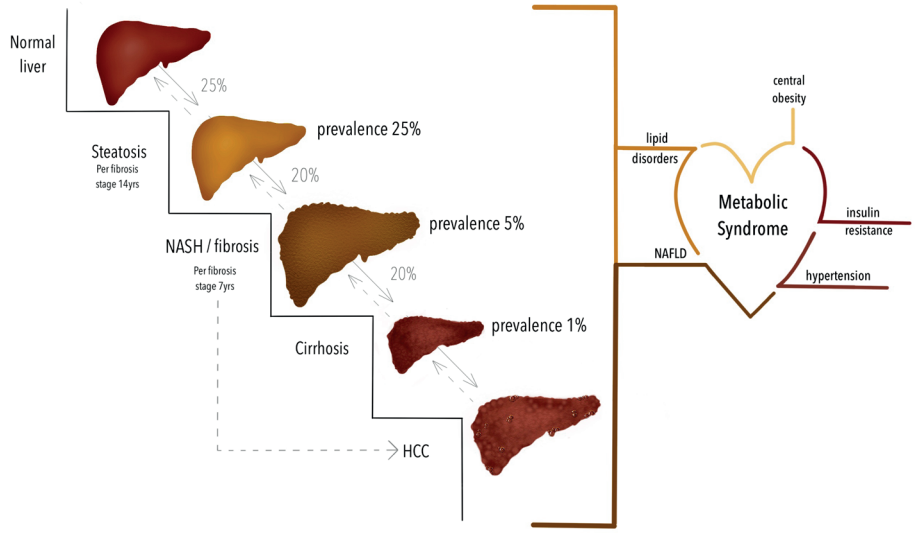

Figure 1: Natural History of NAFLD

Risk factors

NAFLD is defined as hepatic steatosis without the presence of well-known risk factors of steatosis, such as alcohol misuse, steatogenic drug use, or viral hepatitis. As all classical risk factors for liver disease are excluded, NAFLD is basically a diagnosis *per exclusionum*. However, it can be argued whether the presence of diabetes mellitus and/or adiposity could be regarded as new-generation risk factors for liver disease.

Indeed, prevalence of NAFLD increases with BMI. Also, NAFLD prevalence seems to be higher in overweight men than in overweight women, as men with adiposity tend to have more abdominal fat (another risk factor for steatosis). In addition, risk of NAFLD is also dependent on ethnicity. Latin- Americans for example have higher NAFLD prevalence than

Caucasians.[27] It has been thought that part of this difference can be explained by cultural habits, but also by body composition, which differs between ethnicities. Asian people have a higher fat mass for a given BMI than Caucasians, and Caucasians have higher fat mass than Negroid and Polynesian people.[12]

In general, the presence of the metabolic syndrome is regarded as most prominent risk factor for NAFLD. The diagnosis of the metabolic syndrome can be made if three out of the following five features are present: 1) central obesity, 2) elevated fasting glucose, 3) elevated blood pressure, 4) elevated triglycerides, and 5) lowered high density lipoprotein (HDL).[28] Also, individuals with NAFLD are likely to develop DM.[11,18] Interestingly, from a biological point of view there is a tight link between NAFLD and DM as well, as insulin resistance is key in both phenotypes.[17] In addition, patients with combined hypertension and NAFLD are at higher risk of fibrosis progression than NAFLD alone.[29] But the relation between the metabolic syndrome and NAFLD seems not to be a one-way relation. It has been proposed that NAFLD is also an independent risk factor to develop a metabolic syndrome or manifest diabetes mellitus (DM). Hence, the link seems bidirectional.[30] NAFLD is therefore also referred to as the "hepatic manifestation" of the metabolic syndrome.

Additional lifestyle risk factors have been described in association to NAFLD as well, such as smoking and low physical activity.[6] Also diet plays an important role in the risk of developing NAFLD as one of the main sources of fat deposition in NAFLD are dietary sugars turned into fat by *de novo* lipogenesis (DNL).[31]

And although these readily measurable risk factors explain a part of the risk of NAFLD and progression to advanced NASH, still some individuals progress without presence of the metabolic syndrome. Genetic risk factors might play a role in this particular group. Today, there are several polymorphisms (i.e. minor variations in our DNA) that have been described in relation to NAFLD: PNPLA3-I148M, TM6SF2 and HSD17B13.[32-34] Individuals with the PNPLA3 variant cannot degrade the lipid droplet formation in the liver as lipolysis is interfered. Therefore, this type of NAFLD does not co-occur with hypercholesterolemia.[32] Similarly, individuals that are TM6SF2 rs58542926-T carriers have an altered VLDL-excretion which causes increased fat storage in the liver at cost of fat storage in other organs.[33] And HSD17B13 loss of function is associated with less injury in hepatocytes and hence this variant is associated with a reduced risk from steatosis tot steatohepatitis.[34]

Pathophysiology of non-alcoholic fatty liver disease

For many years it has been believed that NAFLD began with simple steatosis (first hit) and that through risk factors such as oxidative stress (second hit) progression to NASH was

effectuated, this is the so-called two-hit hypothesis.[35] However, we know now that the pathophysiology of NAFLD is heterogeneous and multifactorial and not likely to be the same in every patient.[17]

Instead, NAFLD pathophysiology is complex, but can be simplified by thinking of the liver as a machine that processes fatty acids (FA) with a maximum work capacity.[17] If either the supply of FA is excessive, or the capacity of disposal is decreased, toxic lipids accumulate in the liver. These toxic lipids induce an inflammasome reaction, activate Kupffer cells and activate apoptotic pathways. Altogether this leads to hepatocellular (endoplasmic reticulum) stress, ultimately leading to fibrogenesis and genomic instability.

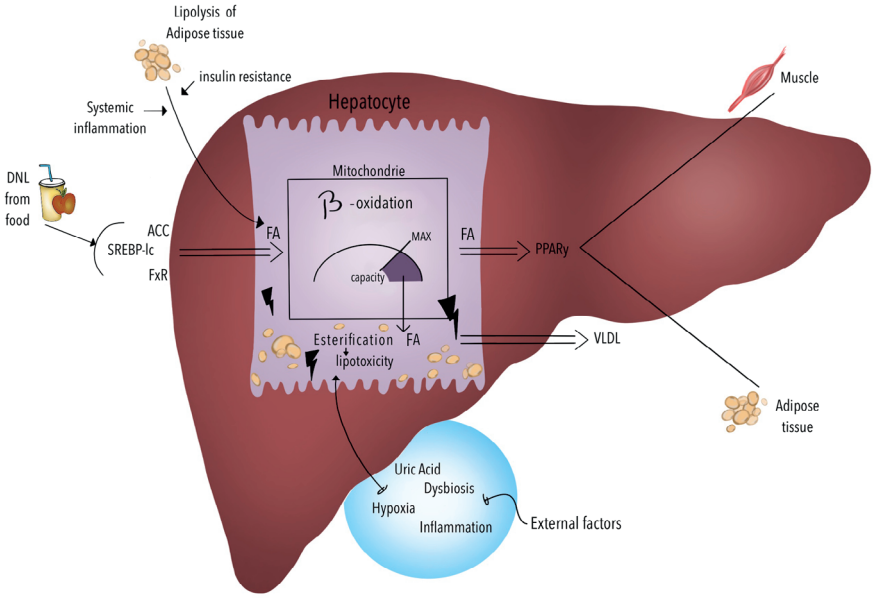

Figure 2: Pathophysiology of NAFLD

Sources of FA are various. Firstly, FA are freed from adipose tissue by lipolysis. In the case of insulin resistance this process is disturbed.[36] In addition, in case of systemic inflammation insulin sensitivity in the muscle is also decreased.[37] Secondly, FA can be extracted from glucose and fructose by DNL.[31] Nearly all the consumed fructose enters the liver via the portal vein. Increasing DNL depletes ATP and causes cell stress. Different transcriptional enzymes are involved in the process of DNL such as acetyl-CoA carboxylase (ACC), steroyl-CoA response element binding protein 1c (SREBP-1c) and farnesoid X receptor (FXR).[17]

Disposal of FA away from the liver into other tissues, such as skeletal muscle, peripheral adipose tissue or brown adipose tissue is regulated by peroxisome proliferator activated

receptor gamma (PPARγ).[38] In addition, consumption of FA can be promoted by activation of muscle or by thermogenesis via bile acid signalling that activates brown adipose tissue.[39]

The capacity of this FA 'machine' is binding FA to fatty acid-binding protein-1 (FABP-1) and metabolizing them by mitochondrial β-oxidation.[40] If this capacity has reached its maximum, esterification of FA into triglycerides is effectuated. These triglycerides can be exported from the liver to the blood as very large density lipoproteins or they can stay in the liver as lipid droplets in the hepatocytes (i.e. steatosis).[40] If these lipids are lipotoxic, it causes cell injury in the liver by inducing hepatocellular stress and by activating the inflammasome reaction (see above).

Additional external factors that can enhance hepatocellular stress[41] and injury are systemic inflammation (dysregulation of cytokines and adipokines),[42] but also uric acid toxicity[43], periodic hypoxia in the case of sleep apnea[44] and metabolites from gut microbial dysbiosis.[45]

Diagnosis

We do not systematically screen for NAFLD, and therefore, NAFLD is often found accidentally when an ultrasound of the abdomen is performed for various reasons. In addition, NAFLD is a diagnosis per exclusionum as other (more classical) risk factors for steatosis should be excluded. Viral hepatitis and auto-immune serology, medical history on chronic alcohol use, and more specific testing on rare liver diseases such as iron or copper accumulation need to be tested. In doubt, a liver biopsy can be performed to help diagnose the aetiology of liver disease histologically.

A liver biopsy is indeed the golden standard diagnostic tool for liver diseases. However, this technique has disadvantages as well. Sampling error is possible, therefore a sufficient specimen length is needed to limit this bias as much as possible.[46] One can imagine that in the case of focal non-steatosis –i.e. minor areas with less steatosis in the liver- a biopsy specimen may not necessarily be representative. In addition, liver biopsies are not without risk as severe haemorrhage can occur.[47] Nonetheless, a liver biopsy is the only way to actually diagnose NASH as it is a histological diagnosis. A scoring system for the histological severity of NASH is the NAFLD Activity Score (NAS).[48] It takes into account, steatosis, lobular inflammation, ballooning, and fibrosis. This score is particularly important in clinical trials to study the effect of drugs on NASH histology in a standardized manner.

Fortunately, there are plenty non-invasive tools to diagnose NAFLD and to proxy fibrosis. Firstly, there are the biomarker screening algorithms, such as the NAFLD Fibrosis Score (NFS), the BARD-score, and the Fatty Liver Index (FLI), that use clinical features (such as BMI,

age, and presence of diabetes) to estimate the presence of steatosis with or without co-occurring fibrosis.[49] Secondly, there are several imaging tools to diagnose steatosis of which hepatological ultrasound (US) is the most well-known and most used. The disadvantage of US is its poor sensitivity in grading steatosis severity.[50] But it is easy to perform, has good sensitivity and specificity for detecting presence/absence of steatosis, and is inexpensive. A relatively new diagnostic tool that still needs more validation is the Controlled Attenuation Parameter (CAP) that also uses ultrasound waves to diagnose severity of steatosis.[51] Also other imaging tools, like Magnetic Resonance Imaging and Computed Tomography are able to diagnose steatosis quite accurately.[52] Thirdly, there are imaging tools to proxy liver fibrosis, of which the transient elastography (Fibroscan) is the most commonly used.[53] It measures the velocity of a low-frequency elastic shear wave through the liver and with that it estimates the stiffness of the liver. The stiffer the liver, the more fibrosis. Although it is a widely used imaging tool in clinical practice, it has not yet been validated against liver biopsies in the general population. In addition, the presence of steatosis is known to influence the liver stiffness measurement.[54] Hence, optimal cut-off values to stage fibrosis in the general population still need to be determined. Other imaging tools for fibrosis are the acoustic radiation force impulse imaging (ARFI, a point shear wave elastography), 2D shear wave elastography, and MR elastography (which uses a modified phase-contrast method).[49] These modalities are, although promising, not widely available and costly.

Treatment

The treatment of NAFLD is diverse and dependent on the stage of the disease. Therefore, the different treatment modalities are given per category below.

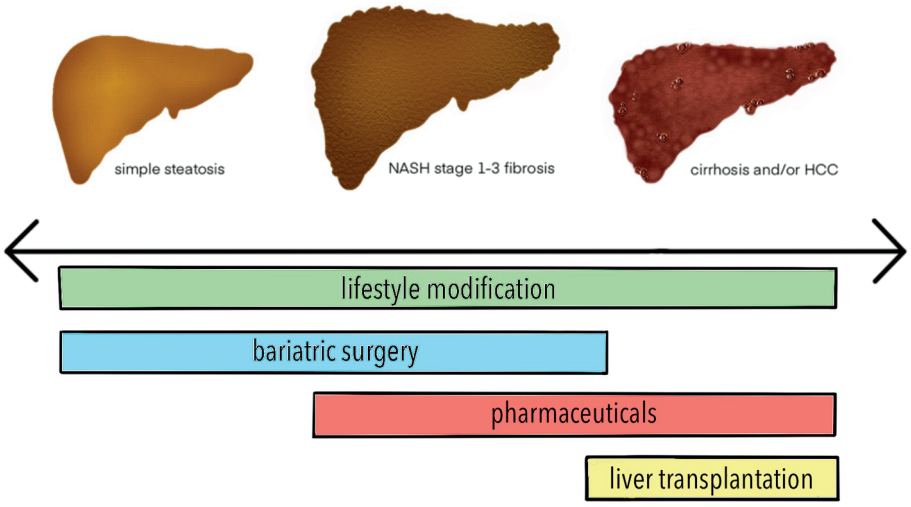

Figure 3: Treatment options for NAFLD

Lifestyle Modification

The general dietary advice for NAFLD at the moment is to lose 5–10% of body weight in order to reverse steatosis, but specific evidence-based guidelines for a healthy diet in NAFLD are lacking.[55] Consensus, however, on abolishing sugar-containing beverages (which have been repeatedly associated with NAFLD)[56,57] from the NAFLD diet has been reached. Furthermore, the Mediterranean diet (MD), which is rich in unsaturated fat and fibre and poor in red meat, has been shown to be beneficial in the prevention of cardiovascular diseases.[58] Also, there is modest evidence that this same diet has beneficial effects on liver fat as well.[59] This evidence however, originates from small studies (n=12–90 subjects) with suboptimal nutritional analyses or use of surrogate primary endpoints (i.e. liver transaminases) rather than imaging diagnosis of NAFLD. Currently, the dietary recommendations of the European Association for the Study of Liver diseases guidelines advice energy restriction, exclusion of NAFLD promoting components (processed food, and foods high in added fructose), caloric restriction, and a macronutrient composition according to the MD.[55]

Apart from dietary interventions, physical exercise has been shown to be beneficial for NAFLD as well. A meta-analysis on exercise in NAFLD including twenty original studies showed that intrahepatic fat improved upon exercise irrespective of weight change.[60] In addition, a recent systematic review concluded that exercise of at least 45 minutes, three times a week for three months was needed to improve hepatic steatosis. Type of exercise, aerobic or resistance training, were equally effective via different pathways.[61]

Bariatric surgery

As obesity and NAFLD are tightly connected and weight loss is a treatment goal in reducing NAFLD severity, bariatric surgery became a study topic of interest. A recent meta-analysis included 32 studies on bariatric surgery and NAFLD.[62] The results of this study showed that bariatric surgery led to a complete resolution of steatosis in 66% and of fibrosis in 40% of all patients. In addition, a mean reduction of 2.4 points on the NAS-score was found. However, a worrisome 12% of all patients had worsening of NAS features on histology after bariatric surgery. Of all methods, the Roux-and-Y gastric bypass is the technique of choice. Even though the results are promising, further randomized controlled trials are needed to further strengthen the body of evidence to perform bariatric surgery in obese patients with NASH.

Pharmaceuticals

The development of a new drug against NASH is well under way. There are hundreds of trials ongoing but most of them are in an early phase. Here we will highlight the most well-studied and promising drugs.

Amongst the PPAR ligands there are several promising drugs. At first Pioglitazone, a PPARγ agonist, was studied in the PIVENS trial.[63] A higher resolution of NASH, but not of fibrosis was found. Important side effects, however, were weight gain and an increased fracture risk. Another drug, Elafibranor, a PPARα and PPARδ agonist, regulates the mitondrial β-oxidation and has anti-inflammatory effects. The GOLDEN-505 trial included 276 patient of which 23% had NASH resolution against 17% in the placebo group.[64] This effect was not significant, but a histological improvement was seen in the more advanced fibrosis subgroup. Therefore, the RESOLVE-IT phase III trial is now ongoing.

Another mechanism of action is via the FXR agonist, Obeticholic acid, which has been thought to improve both glucose metabolism and peripheral insulin sensitivity, and to reduce lipogenesis. The FLINT-trial, a phase IIb trial in 283 NASH patients was prematurely stopped due to superiority.[65] All histological endpoints improved including the fibrosis score (35% in the treatment vs. 19% in the placebo group). An important side effect, however, was the increase in LDL-cholesterol and pruritus. A phase III trial, the REGENERATE trial is currently ongoing.

The intestinal hormone glucagon-like peptide 1 (GLP-1) stimulates insulin secretion and inhibits secretion of glucagon. Liraglutide, a GLP-1 agonist is a registered drug in type 2 diabetes mellitus and has therefore been studied in NASH in the LEAN trial including 52 patients.[66] NASH resolution occurred in 39% vs. 9% in the placebo group. However, it is unknown whether this effect was independent from weight loss. In addition, this drug needs to be injected daily which is burdensome. Semaglutide, another GLP-1 agonist that needs only weekly dosing, is now being investigated in NASH as well.

Another potential drug target is that of the C-chemokine receptor 2 and 5. Activation of this receptor has been shown to promote recruitment of macrophages upon liver injury and activate hepatic stellate cells and subsequent fibrogenesis. Cenicriviroc is an antagonist of this receptor and has been tested in a phase IIb trial, the CENTAUR trial.[67] The primary study aim, improvement of NASH was not met, but after 1 year 20% of the patients in the treatment group had improvement of fibrosis by at least 1 stage against 10% of the placebo group. However, this effect was not significant anymore after 2 years. But again,

the effect was greater in patients with more severe fibrosis at baseline. Therefore, at this moment, a phase III trial, the AURORA trial, is carried out.

Lastly, apoptosis signal-regulating kinase-1 inhibitor Selonsertib was tested in a phase II trial for the treatment of NASH as well.[68] This drug has been proposed to inhibit activation of stress response pathways that worsen hepatic inflammation, apoptosis, and fibrosis. Indeed, 43% of the patients with the highest treatment dose had a reduction of fibrosis. Paradoxically, one of the adverse events of Selonsertib was a transient increase in transaminases. But given the promising results, two phase III trials, STELLAR 3 and STELLAR 4, are currently being carried out in patients with advanced fibrosis and cirrhosis.

Liver transplantation

The ultimate treatment for patients with end-stage NAFLD is liver transplantation. End-stage NAFLD includes both NASH-related decompensated cirrhosis and NAFLD-related HCC. In Europe, 35% of all recorded HCC cases was due to NAFLD.[69] In the past 10 years, NAFLD as indication for transplantation has increased by 170%.[23] In addition, NAFLD accounted for a denoting increase in combined liver-kidney transplantation as well.[70] Nonetheless, the leading cause of liver transplantation is still alcoholic liver disease (20%) whereas cryptogenic cirrhosis or NAFLD accounted for only 4% from 1968 onwards in the European Liver Transplantation Registry.[71] Discrepancies in labelling of the aetiology in this registry may account for part of this difference.

But, with the increasing NAFLD prevalence (particularly amongst young adults), NAFLD is predicted to become the number one indication for liver transplantation in the coming years.[22] In the period preceding transplantation, NAFLD patients on the waiting list have a higher chance of being withdrawn from the list because of their (cardiovascular) comorbidities, physical performance (sarcopenia) and obesity.[72] Post-transplantation, it is known that (NAFLD-related) comorbidities such as obesity and diabetes are associated with poorer short and long-term outcomes.[73] But when correcting for these metabolic confounders, graft-survival appears similar in NAFLD compared to other etiologies.[74] Unfortunately, however, recurrent or NAFLD *de novo* is a frequent phenomenon after transplantation and occurs in approximately 30–60% of patients, both due to persistence of lifestyle habits, as well as universal weight gain post transplantation and side effects of immunosuppressive agents.[75] Treatment of recurrent NAFLD is primarily aimed at lifestyle modification and minimizing pro-steatotic agents as much as is tolerated.

Summary

In summary, a 3.5% liver-related mortality worldwide illustrates that the burden of liver disease is real. And whereas pharmaceutical options for viral hepatitis are increasingly successful, there is still no registered drug for NAFLD, which affects one quarter of the adult population and is therefore the most prevalent chronic liver disease. Amongst other metabolic and genetic risk factors, lifestyle plays a major role in both the development and treatment of NAFLD. However, universal evidence-based dietary and physical activity strategies are lacking. In addition, the natural history of NAFLD needs unravelling to greater extent to make adequate risk stratifications and follow-up strategies.

Chapter 2

Aims and outline of this thesis

Aims

The general aim of this thesis is to explore modifiable risk factors of liver disease in the general population. We examined the role of 1) dietary quality and composition, 2) body composition, and 3) the gut microbiome in relation to liver steatosis and liver stiffness.

Outline

As set forth in the introduction, diet plays a major role in the development and treatment of NAFLD. Furthermore, there is increasing evidence that it is not only dietary quantity, but also dietary quality that is important in promoting liver health. For instance, the consumption of nutraceuticals – foods with a particular health benefit – may promote liver health via various pathways. A well-known studied example of such a nutraceutical is coffee. Together with tea, it is the most consumed beverage worldwide and it has been suggested that coffee could prevent or even reverse hepatic fibrosis. However, studies in the general population are lacking. And although tea and coffee share certain features, such as the constituents caffeine and antioxidant polyphenols, the association between tea and liver health was not well-studied at all. We therefore examined whether coffee and tea were associated with hepatic steatosis and fibrosis in our large community-dwelling population in part II (**Chapter 3**). To further explore the mechanisms underlying the role of coffee in liver health, we conducted a systematic literature search in **Chapter 4**. Specifically, we reviewed experimental models studying the effect of coffee on hepatic steatogenesis, fibrogenesis, and carcinogenesis.

In part III, we studied the role of diet as a whole in relation to NAFLD. Lifestyle modification, generally aimed at weight reduction, is the cornerstone of treatment in NAFLD, but specific evidence-based recommendations on the optimal dietary composition for NAFLD lacked. Therefore, we studied the independent associations between macronutrients and NAFLD in **Chapter 5**. In this study we found in particular that excessive intake of animal protein was associated with higher NAFLD prevalence. To further examine the pathophysiological mechanisms behind this association we conducted a spin-off study on the association between diet-dependent acid load – which is known to be particularly high in animal protein rich diets – and NAFLD in **Chapter 6**. Lastly, to summarize the relation between diet and NAFLD, we conducted a study in which we longitudinally assessed the relation between well-known dietary quality indices, such as the Mediterranean Diet Score, the World Health Organization Score and the Dutch Dietary Guidelines, and risk of NAFLD. We additionally looked at population-specific dietary patterns and NAFLD (**Chapter 7**).

In part IV of this dissertation, we explored the in-depth association between body composition and NAFLD. We know that not every NAFLD patient is obese and that – vice versa – not every obese person has NAFLD. In addition, it is increasingly recognized that BMI is not an all-encompassing measure for adiposity. Asian studies have proposed that low skeletal muscle mass is an important risk factor for NAFLD as well. However, studies using validated tools to measure skeletal muscle mass performed in a Western population were lacking. In addition, the populations studied were rather overweight, so it was not clear whether the association found was an actual reflection of low skeletal muscle mass, or a mere reflection of excess fat mass. In **Chapter 8** we therefore studied the independent association of the different components of the body with NAFLD, stratified by sex and BMI. We were particularly interested in the association between NAFLD and skeletal muscle mass with (sarcopenia) or without (presarcopenia) loss of muscle function. In addition, we compared the various body parameters in relation to NAFLD prevalence. In **Chapter 9** we respond to an interesting prospective study on the lifestyle benefits in lean NAFLD patients. We discuss whether it is weight loss or something else about the lifestyle in this group that causes NAFLD to reverse. In addition, we plead against the use of FLI in the context of assessing body composition, based on additional data analyses performed in our population.

Over the last decade, the role of the gut microbiome in human health has gained global interest. The tight connection between the gut microbiome and the liver is referred to as the 'gut-liver-axis'. Indeed, microbiome-derived metabolites can reach the liver via the portal vein, which constitutes 70% of the hepatic blood inflow. It has even been proposed that ethanol-producing bacteria in the gut microbiome of children contribute to the pathogenesis of steatosis, mimicking alcoholic liver disease. In **Chapter 10** we therefore studied the association between microbial diversity, composition, predicted metagenomics and serum metabolomics with (non-alcoholic) steatosis and steatofibrosis.

Finally, part VI contains a compilation of all correspondence. **Chapter 11 to 13** concern replies to correspondences on studies from **Chapter 3 and 5**. These correspondences reflect the interest and discussion these publications triggered in the field.

Part II

COFFEE & LIVER HEALTH

MEDITERRANEAN FOOD

Chapter 3

Coffee and herbal tea consumption is associated with lower liver stiffness in the general population: The Rotterdam study

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Abstract

Introduction Coffee and tea have been proposed to limit progression of liver fibrosis in established liver disease, but it is unknown if this is also true for subclinical fibrosis. We therefore aimed to evaluate whether coffee and tea consumption are associated with liver stiffness in the general population.

Methods The Rotterdam Study is an ongoing prospective population-based cohort. We included participants who underwent transient elastography, ultrasound and completed a food frequency questionnaire. Coffee and tea consumption were categorized into no, moderate (>0–3), or frequent (≥3) intake (cups/day), and tea further into green, black and herbal tea (no/any). Significant fibrosis was defined as liver stiffness measurements (LSM) ≥8.0kPa. We performed regression analyses relating coffee and tea intake with fibrosis, steatosis and log-transformed LSM and adjusted for energy, sugar and creamer intake, age, gender, BMI, steatosis/LSM, HOMA-IR, ALT, alcohol, smoking, soda, healthy diet index and physical activity.

Results We included 2424 participants (age 66.5±7.4; 43% male) of whom 5.2% had LSM ≥8.0kPa and 34.6% steatosis. Proportion of LSM ≥8.0kPa decreased with higher coffee consumption (7.8%, 6.9% and 4.1% for no, moderate and frequent respectively; P_{trend}=0.006). This inverse association was confirmed in multivariable regression (OR_{mod} 0.75, 95%CI 0.33–1.67; OR_{freq} 0.39, 95%CI 0.18–0.86; *P*=0.005). Amongst tea consumers, only herbal tea consumers (36.3%) had lower log-transformed LSM after adjustment (Beta-0.05, 95%CI-0.08; –0.02, *P*=0.001). Subtypes of tea were associated with steatosis in univariate but not multivariable analysis.

Conclusion In the general population, frequent coffee and herbal tea consumption were inversely related with liver stiffness but not steatosis. Longitudinal analyses, as well as studies validating and unravelling underlying mechanisms are needed.

Introduction

Chronic liver diseases constitute a major public health problem. Liver cirrhosis was the 12th cause of death worldwide and the sixth cause of life-years lost in the adult population in developed countries in 2010.[76,77] Chronic liver diseases are often silent for over 20 years until cirrhosis develops. Indeed, several studies have suggested that liver fibrosis may be present within unselected individuals. Using transient elastography (TE) as diagnostic tool for liver fibrosis, a prevalence of 6–7% was found in the general population[78,79] and even up to 17% in those high-risk populations with metabolic syndrome and type 2 diabetes.[11] Lifestyle is an important factor in the pathogenesis of many liver diseases, examples of which include alcohol abuse in alcoholic liver disease and high caloric diet and inactivity in non-alcoholic fatty liver disease (NAFLD). At the same time, a healthy lifestyle, such as implementing a well-balanced diet or consumption of nutraceuticals, i.e. foods or nutrients with a health benefit, can prevent and even attenuate liver disease.[80]

Coffee and tea are the most consumed beverages worldwide and emerging as promising nutraceuticals for liver health.[81] Both beverages are part of well-rooted cultural traditions and also represent the second most traded commodity on world markets.[82] Consumption of these nutraceuticals has been associated with lower all-cause and cause-specific mortality, presumably through reducing risk of features of the metabolic syndrome.[83,84]

Coffee consumption was for the first time associated to liver health, that is lower liver enzymes, almost two decades ago.[85] Evidence supporting this protective effect of coffee on liver enzymes rapidly emerged henceforth.[86] Coffee consumption seemed to attenuate alcoholic liver disease[87] and studies in hepatitis C showed less severe fibrosis in coffee consumers.[88] Also in NAFLD patients, coffee consumption was inversely associated with fibrosis grade, but inconclusive concerning the relation to steatosis.[89-91] To date, only one study examined the relation between coffee and liver fibrosis in the general population, albeit using surrogate serum biomarker test as proxy for fibrosis, and found lower odds for fibrosis in frequent coffee consumers.[91]

The association between tea and liver health is less well established than that of coffee. All studies are either limited to Asian populations or include only serum transaminases as primary endpoint. In addition, results of these studies are inconclusive regarding the presumed health benefit of tea.[92-96]

To our knowledge, there are no studies examining whether coffee and tea consumption are associated with lower prevalence of steatosis and liver fibrosis, using reliable imaging techniques, in the general population. Hence, we conducted a cross-sectional analysis of individuals within a large prospective cohort study, who completed extensive dietary questionnaires, liver stiffness measurements (LSM), as proxy for fibrosis, and hepatic ultrasound (US) for the diagnosis of steatosis. Our aim was to determine whether coffee and tea consumption were associated with lower risk of liver fibrosis and steatosis in the general population.

Subjects and methods

Study population

This is a cross-sectional analysis of The Rotterdam Study, a large ongoing population-based cohort of participants aged 45 years and older living in a suburb of Rotterdam, The Netherlands. The rationale and design of this study have been described previously and a more detailed description of the design is added as *Supplementary Methods.*[97] For the purpose of our study, all participants visiting the research centre between January 2011 and September 2013 were included and underwent anthropometric assessment, abdominal US, TE and blood sampling. For all cohorts, this was the first hepatic examination in The Rotterdam Study. The Rotterdam Study has been approved by the institutional review board (Medical Ethics Committee) of the Erasmus MC University Medical Centre Rotterdam and by the review board of The Netherlands Ministry of Health, Welfare and Sports. Written informed consent was obtained from all participants.

Coffee and tea consumption

All participants completed an externally validated 389-item food frequency questionnaire (FFQ) developed for Dutch adults.[98,99] The questionnaire addressed type of food consumed over the last month, as well as frequency, portion size and preparation methods. Incomplete or unreliable FFQs, i.e. total energy intake less than 500 or more than 7500 kilocalories per day, were excluded. Questions regarding coffee and tea consumption included: "*How often did you drink coffee last month?*" and "*How often did you drink black / green / herbal tea (e.g. chamomile, red bush and nettle) last month?*" to which the possible answers were: "*1) not at all, 2)1 or 2–3 times, or 3)1; 2–3; 4–5 or 6–7 times a day*", and in case of daily consumption "*1–2; 3–4; 5–6; 7–8; 9–10 or 11 or more cups per day*". Coffee and tea consumption was thereafter categorized into no (0), moderate (>0–3) and frequent (≥3) consumption (in cups/day, one cup equals 150 grams). Tea consumption was further specified into herbal, green and black tea and subsequently dichotomized into no (0) and any (>0) consumption. Additionally, data on consumption of soda, alcohol, sugar and cream use in coffee or tea were obtained and used as covariates (in grams/day). Excessive alcohol consumption was defined as >14 units per week for women and >21 units per week for men (one unit equals 10 grams). Furthermore, to account for confounding by overall dietary quality, the Dutch Healthy Diet-index (DHDI) was added to multivariable analyses.[100]

Liver stiffness measurements and hepatic steatosis

LSM were performed using TE (Fibroscan®, EchoSens, Paris, France) by a single operator who had performed more than 1000 examinations before start of the study. Practical implementation of TE has been described previously.[78] The operator obtained 10 serial measurements of stiffness, using the M- or the XL-probe according to the manufacturer's instructions. Participants were excluded from our analyses if: 1) LSM did not meet the reliability criteria of Boursier et al., i.e. interquartile range (IQR)/ median LSM >0.30 with median LSM ≥7.1kPa;[101] 2) no LSM was obtained after at least 10 shots (defined as failure) and; 3) intra-cardiac devices or physical disabilities prohibited the use of TE.
Clinically relevant fibrosis was defined as LSM ≥8.0kPa and clinically relevant cirrhosis as LSM ≥13.0kPa. At these cut-off levels, previous studies showed high positive predictive values for presence of liver fibrosis and cirrhosis, respectively.[79,102,103]
Abdominal US was carried out by a certified and experienced technician on Hitachi HI VISION 900. Images were stored digitally and re-evaluated by a single hepatologist with more than 10 years of experience in US (PT). Diagnosis of steatosis was determined dichotomously according to the protocol of Hamaguchi et al.,[104] as presence or absence of a hyper echogenic liver parenchyma.

Biochemistry

Fasting blood samples were collected just before US and TE imaging. Blood lipids, platelet count, glucose, alanine aminotransferase (ALT), aspartate aminotransferase, gamma-glutamyltransferase (GGT), alkaline phosphatase and total bilirubin were measured using automatic enzyme procedures (Roche Diagnostic GmbH, Mannheim, DE). Insulin, hepatitis B surface antigen and anti-hepatitis C virus were measured by an automatic immunoassay (Roche Diagnostic GmbH). Patients with viral hepatitis were excluded from the analyses.

Additional Covariates

Data concerning demographics, education level, medical history, physical activity, comorbid conditions, smoking behaviour and drug use were obtained during an extensive home interview by trained interviewers. Detailed information on medication use was obtained from automated linkage to pharmacies with which 98% of the participants were registered. Anthropometric measurements were performed by well-trained research assistants. Body mass index (BMI) was calculated as weight (kg)/ height (m^2) and waist circumference (WC) in centimetres. Blood pressure measurements were taken as the average of two subsequent measurements on the same day in upright position. According to the Adult Treatment Panel III criteria,[105] metabolic syndrome was diagnosed if at least 3 of the follow-

ing 5 traits were present: 1) abdominal obesity, defined as WC >102 cm (40 inch) in men and >88 cm (35 inch) in women; 2) serum triglycerides ≥150 mg/dl (1.0 mmol/L) or drug treatment for elevated triglycerides; 3) serum high-density lipoprotein cholesterol (HDL-C) <40 mg/dl (1.0 mmol/L) in men and <50 mg/dl (1.3 mmol/L) in women or drug treatment for low HDL-C; 4) Blood pressure ≥130/85 mmHg or drug treatment for elevated Blood pressure; 5) fasting plasma glucose (FPG) ≥100 mg/dl (5.6 mmol/L) or drug treatment for elevated blood glucose. Homeostasis model assessment of insulin resistance (HOMA-IR) was used as proxy for insulin resistance and calculated by multiplying fasting glucose (mmol/dl) by fasting insulin (mU/L) divided by 22.5.[106]

Statistical analyses

Population characteristics were described using descriptive statistics. Continuous data were presented as mean ± standard deviation or median with interquartile range (IQR) according to the distribution of the variable. Chi-square test, Student's T-test or Wilcoxon Rank Sum test were used to assess significance of differences in distribution of categorical, normally distributed and not-normally distributed data, respectively. Univariate linear and logistic regression analyses were performed to examine the association between coffee, overall tea and its subtypes herbal, green and black tea consumption and LSM as continuous and dichotomous (LSM <8.0kPa vs. LSM ≥8.0kPa) variable. In all regression models, multivariable adjustments were made by including age, gender, energy intake, BMI, insulin resistance, steatosis, serum ALT, excessive alcohol intake, soda consumption, smoking status, cream use in coffee and sugar use in tea or coffee, DHDI, type of probe, physical activity and education level as potential covariates.[107] To evaluate whether there was interaction with gender, HOMA-IR, BMI and steatosis on the one hand and coffee and tea consumption on the other hand, we evaluated the interaction terms (e.g. coffee x gender) in the multivariable model. In case of $P<0.05$ stratified analyses were performed. Additionally, correlation and multicollinearity was tested between steatosis and liver stiffness, given their strong relation, i.e. simple steatosis is a well-known risk factor for fibrosis. Furthermore, predicted probabilities of having LSM ≥8.0kPa were calculated, using the fully adjusted multivariable logistic regression model with LSM ≥8.0kPa as dependent variable. Probabilities were graphically depicted for the different coffee categories and presence of steatosis, to give more insight in these possibly interrelated covariates. Data are expressed as mean (95%CI) percentage. Also, different cut-offs for significant fibrosis were tested as outcome variable in univariate logistic regression, to emphasize the robustness of our findings. Additionally, a cut-off of ≥6.2kPa was used, indicating significant fibrosis in participants with steatosis. This cut-off has been proposed to exhibit high sensitivity for staging F2 fibrosis in participants with NAFLD, recently.[108] Univariate and multivariable logistic regression analyses were carried out assessing the association between coffee, tea and the secondary outcome

of interest, hepatic steatosis. In addition to the previous mentioned potential covariates, LSM was added to the model as well.

Several sensitivity analyses were performed to test the robustness of our data. Firstly, subgroup analysis was done by stratifying participants who filled in FFQ data 5.8 years prior to liver imaging (RS III-2) and, participants who filled in FFQ simultaneously with liver imaging (RS I-5 & RS II-3). Because dietary data are known to be stable over time,[109] RS III-2 was included in the total study population. Secondly, to ensure that the study population is a general population with no known liver disease (even though participants with viral hepatitis were excluded based on serology and participants were asked to report liver comorbidities during home interviewing), a sensitivity analysis was performed, excluding abnormal levels of ALT (> 2x upper limit of normal, which is 80 U/L for men and 60 U/L for women). A *P*-value of <0.05 was considered statistically significant. All analyses were performed using SPSS 21.0 (SPSS Inc., Chicago, IL, USA).

Results

Study population

The flowchart of the study population is depicted in *Figure 1*. Of the 3439 participants visiting the research centre, participant failure of LSM was reported in 162 (4.8%) and unreliable results in 139 (4.2%) participants. In addition, 34 (1.1%) participants with unreliable FFQs and 566 (16.4%) with incomplete FFQs were excluded. Hence, the total study population was n=2424. Population characteristics are presented in *Table 1*. Fifty-seven percent of the participants were women, mean age was 66.5±7.4 years, mean BMI 27.2±4.0 kg/m² and median LSM was 4.7kPa (3.8–5.8). A total of 125 (5.2%) participants had LSM ≥8.0kPa, suggesting presence of significant fibrosis.

Data on coffee and tea consumption

Dietary data are shown in *Table 1*. In total, 2258 participants (93.2%) consumed coffee with a mean consumption of 2.6±1.7 cups/day. Frequent coffee consumers were proportionally more overweight or obese. Furthermore, it was associated with younger age, male gender, Caucasian ethnicity, higher education level, more current or past smoking, lower prevalence of lipid disorders and type 2 diabetes mellitus *(Supplementary Table 1)*. No difference was observed in serum ALT and HOMA-IR. Tea was consumed by 2052 (84.7%) participants, with a median of 1.2 (0.4–2.7) cups/day. Frequent tea consumption was associated with female gender, less excessive alcohol use, less smoking, lower BMI and less features of the metabolic syndrome *(Supplementary Table 1)*. Amongst subtypes of tea,

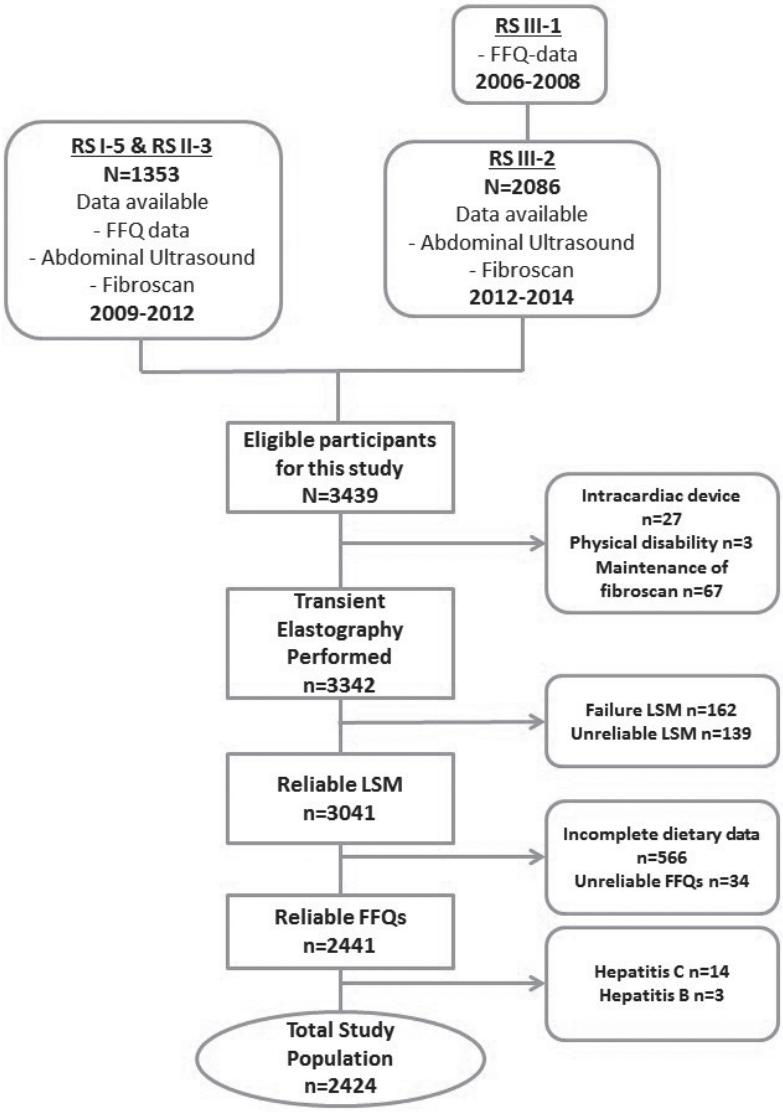


Figure 1: Flowchart of the study
Legend: RS= Rotterdam Study, I-III= number of cohort, 1–3 and 5= times cohort visited.

black tea was most commonly consumed (64.2%) followed by herbal (36.3%) and green tea (26.1%). Only 32 (1.3%) participants reported neither coffee nor tea consumption.

Table 1: Study Characteristics

	Total N=2424	LSM <8.0kPa N=2299	LSM ≥8.0kPa N=125	*P*-value
Demographics				
Age (years)	66.5±7.4	66.4±7.3	68.7±8.4	0.003
Female	56.6	57.7	35.2	<0.001
Caucasian	96.3	96.2	98.4	0.322
Low/Intermediate /High education	45.0/29.6 /25.4	45.2/29.4 /25.4	42.3/34.1 /23.6	0.531
Excessive alcohol use	15.9	15.9	16.0	1.000
Current/Past /Never smoker	11.3/52.9 /35.7	11.0/52.3 /36.6	16.8/63.9 /19.3	<0.001
Physical activity*	46 (19–85)	46 (19–85)	42 (18–85)	0.748
Physical examination				
BMI (kg/m^2)	27.2±4.0	27.1±3.9	28.8±5.3	0.001
-Normal	29.7	30.1	22.8	0.003
-Overweight	49.1	49.4	43.9	
-Obese	21.2	20.5	33.3	
WC (cm)				
-Men	99.1±10.2	98.9±9.9	101.1±13.1	0.152
-Women	89.0±11.5	88.7±11.3	96.6±15.7	0.002
Biochemistry¥				
Aspartate transaminase (U/L)	24 (21–28)	24 (21–28)	27 (24–37)	<0.001
ALT (U/L)	18 (14–24)	18 (14–23)	22 (16–33)	<0.001
Bilirubin (µmol/L)	8 (6–11)	8 (6–11)	9 (6–12)	0.252
GGT (U/L)	24 (17–35)	23 (17–34)	38 (26–71)	<0.001
Platelets (*10^9/L)	267.8±63.5	268.7±63.6	251.7±59.7	0.004
HOMA-IR	2.5 (1.7–3.9)	2.5 (1.7–3.8)	3.7 (2.1–7.1)	<0.001
Comorbidities				
Metabolic Syndrome	44.5	43.7	59.3	0.001
-WC >88cm (♀) or >120cm (♂)	44.1	43.5	53.7	0.032
-Triglycerides >150mg/dL	38.2	38.0	41.6	0.450
-HDL-C <40mg/dL (♂) or 50mg/dL (♀)	33.6	33.3	40.0	0.144
-Blood pressure ≥130/85mmHg¥	79.0	78.5	88.0	0.020
-FPG >100mg/dL	44.6	43.3	68.0	<0.001
Diabetes Mellitus	10.5	9.2	33.6	<0.001
Hypertension†	64.7	63.8	81.5	<0.001
Liver Imaging				
LSM (kPa)	4.7 (3.8–5.8)	4.6 (3.8–5.6)	9.1 (8.6–10.4)	<0.001
Steatosis	34.6	33.2	60.0	<0.001

Table 1 (*continued*)

	Total N=2424	LSM <8.0kPa N=2299	LSM ≥8.0kPa N=125	*P*-value
Dietary Data				
Coffee consumption (cups/day)	2.6±1.7	2.6±1.6	2.2±1.7	0.019
-None	6.8	6.7	10.4	0.006
-Moderate (>0–3)	28.8	28.3	38.4	
-Frequent (≥3)	64.4	65.1	51.2	
Tea consumption (cups/day)	1.2 (0.4–2.7)	1.2 (0.4–2.7)	1.2 (0.2–2.3)	0.211
-None	15.3	15.1	20.0	0.256
-Moderate (>0–3)	56.4	56.4	56.0	
-Frequent (≥3)	28.3	28.5	24.0	
Tea subtypes (%)				
Black	64.2	64.4	61.6	0.528
Green	26.1	26.3	21.6	0.242
Herbal	36.3	36.8	27.2	0.035
Other				
Alcohol (units/day)	0.8 (0.1–1.8)	0.8 (0.1–1.8)	0.9 (0.1–2.1)	0.334
Soda (%)	47.7	47.5	51.2	0.462
Kilocalorie intake	2189.7±740.3	2189.1±735.5	2199.7±826.2	0.877
Cream use	46.0	45.8	50.4	0.357
Sugar use	25.5	24.9	37.6	0.002

Data is presented as mean (±SD), median (IQR) or percentage. *P*-value is based on T-test, Wilcoxon rank sum test, Chi-square test or Fisher's exact test. *Physical activity in metabolic equivalent task hours/week. ¥Biochemistry data was available for 2320 and †BP data for 2051 participants.

Coffee consumption and liver stiffness

Proportion of participants with LSM ≥8.0kPa decreased with increasing coffee consumption (7.8% in no, 6.9% in moderate and 4.1% in frequent coffee consumption groups, P_{trend}=0.006). Using no coffee consumption as reference, univariate logistic regression showed an inverse association between coffee consumption and LSM ≥8.0kPa (OR_{mod} 0.87, 95%CI 0.46–1.64; OR_{freq} 0.50, 95%CI 0.27–0.94; *P*=0.007). This association enhanced after adjusting for energy intake, age, gender, BMI, steatosis, HOMA-IR, ALT, excessive alcohol use, current or former smoking, soda, DHDI, physical activity, total tea consumption and cream and sugar use in coffee (OR_{mod} 0.75, 95%CI 0.33–1.67; OR_{freq} 0.39, 95%CI 0.18–0.86; *P*=0.005) as shown in *Table 2*. Additional adjustment for education level, and type of probe did not affect this association (data not shown). Coffee consumption was also inversely related to log-transformed LSM as shown in a stepwise multivariable linear regression model in *Table 2* (P_{trend}=0.001). Interactions between covariables and coffee consumption were tested in all regression models, but found to be not significant.

Table 2: Association between coffee, tea and LSM by 1) stepwise linear regression modelling using log-transformed LSM as dependent variable and 2) stepwise logistic regression modelling using LSM ≥ 8 kPa as dependent variable. Non-consumers were used as reference.

	log-transformed LSM			LSM ≥ 8 kPa		
	Beta	**95%CI**	***P*-value**	**OR**	**95%CI**	***P*-value**
Model 1*						
Coffee						
No	0 (ref)		<0.001	1 (ref)		0.003
Moderate (>0–3)	–0.003	–0.058; 0.051		0.84	0.44; 1.61	
Frequent (≥3)	–0.055	–0.106; –0.003		0.46	0.24; 0.87	
Herbal Tea						
No	0 (ref)		<0.001	1 (ref)		0.024
Any	–0.100	–0.128; –0.071		0.61	0.40; 0.94	
Green Tea						
No	0 (ref)		0.076	1 (ref)		0.274
Any	–0.028	–0.059; 0.003		0.81	0.56; 1.18	
Black Tea						
No	0 (ref)		0.014	1 (ref)		0.476
Any	–0.034	–0.061; –0.007		0.85	0.54; 1.34	
Total Tea						
No	0 (ref)		<0.001	1 (ref)		0.116
Moderate (>0–3)	–0.073	–0.110; –0.035		0.74	0.46; 1.20	
Frequent (≥3)	–0.106	–0.147; –0.064		0.56	0.32; 0.97	
Model 2¥						
Coffee						
No	0 (ref)		0.001	1 (ref)		0.012
Moderate (>0–3)	–0.016	–0.069; 0.036		0.86	0.44; 1.70	
Frequent (≥3)	–0.060	–0.110; –0.010		0.50	0.26; 0.97	
Herbal Tea						
No	0 (ref)		<0.001	1 (ref)		0.446
Any	–0.051	–0.079; –0.023		0.84	0.54; 1.31	
Green Tea						
No	0 (ref)		0.304	1 (ref)		0.638
Any	0.016	–0.014; 0.046		1.12	0.70; 1.80	
Black Tea						
No	0 (ref)		0.131	1 (ref)		0.652
Any	–0.020	–0.046; 0.006		0.92	0.62; 1.35	
Total Tea						
No	0 (ref)		0.205	1 (ref)		0.945
Moderate (>0–3)	–0.035	–0.071; 0.001		0.95	0.58; 1.55	
Frequent (≥3)	–0.032	–0.073; 0.009		0.91	0.51; 1.62	

Table 2.(*continued*)

	log-transformed LSM			LSM ≥ 8 kPa		
	Beta	**95%CI**	***P*-value**	**OR**	**95%CI**	***P*-value**
Model 3[†]						
Coffee						
No	0 (ref)		0.001	1 (ref)		0.005
Moderate (>0–3)	–0.026	–0.083; 0.032		0.75	0.33; 1.67	
Frequent (≥3)	–0.067	–0.121; –0.013		0.39	0.18; 0.86	
Herbal Tea						
No	0 (ref)		0.001	1 (ref)		0.289
Any	–0.051	–0.081; –0.022		0.76	0.45; 1.27	
Green Tea						
No	0 (ref)		0.146	1 (ref)		0.424
Any	0.023	–0.008; 0.055		1.24	0.73; 2.10	
Black Tea						
No	0 (ref)		0.400	1 (ref)		0.615
Any	–0.012	–0.039; 0.016		0.88	0.52; 1.47	
Total Tea						
No	0 (ref)		0.514	1 (ref)		0.932
Moderate (>0–3)	–0.023	–0.061; 0.015		1.01	0.58; 1.77	
Frequent (≥3)	–0.063	–0.061; 0.024		1.11	0.58; 2.13	

[*]Model 1: adjusted for tea[‡] or coffee and energy intake
[¥]Model 2: adjusted for tea[‡] or coffee, energy intake, BMI, gender and age.
[†]Model 3: adjusted for tea[‡] or coffee, energy intake, BMI, gender, age, steatosis, ALT, excessive alcohol intake, current or former smoking and HOMA-IR, soda consumption, cream and sugar use, DHDI and physical activity.
[‡]All regression models either contain total tea or tea subtypes as covariate.

Additionally, steatosis and log-transformed liver stiffness were significantly correlated (r=0.152, *P*<0.001) but there was no multicollinearity (VIF 1.33). Nevertheless, steatosis remains an important well-known risk factor for the development of fibrosis. We therefore graphically depicted the adjusted predicted probabilities of having LSM ≥8.0kPa for the different coffee categories and presence of steatosis in Figure 2. As expected, the probability of having LSM ≥8.0kPa is higher for participants with steatosis than for those without (*P*<0.001). Within both the no steatosis and steatosis groups, the probability of LSM ≥8.0kPa for frequent coffee consumption is lower compared to no consumption (within no steatosis: 2.5% in frequent vs. 4.4% in no coffee consumption; $P_{\text{freq vs.no}}$=0.218 and within steatosis: 6.9% in frequent vs. 13.1% in no coffee consumption; $P_{\text{freq vs.no}}$=0.036).

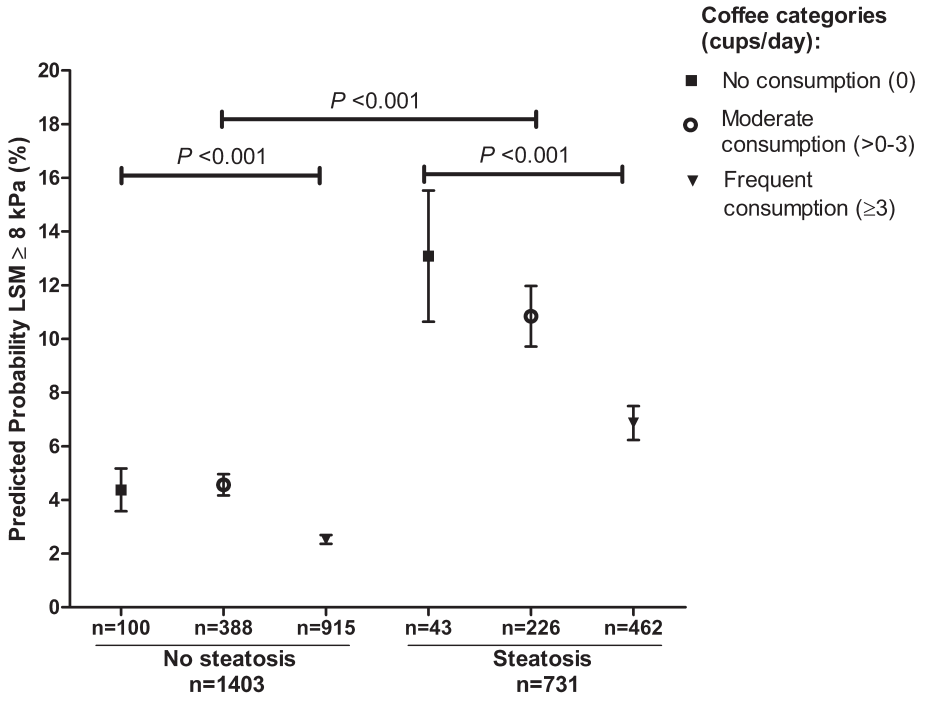


Figure 2: Predicted probabilities (%) of having LSM ≥8.0kPa for the different coffee categories amongst participants with and without steatosis

Y-axis: predicted probabilities of LSM ≥8.0kPa in percentages. Data are expressed as mean (95%CI) prediction. X-axis: different coffee consumption categories for participants with and without steatosis. Probabilities are adjusted for energy intake, age, gender, BMI, steatosis, HOMA-IR, ALT, excessive alcohol use, current or former smoking, soda, DHDI, physical activity, total tea consumption, cream and sugar use in coffee. The Ptrend is derived from the logistic regression analysis stratified for steatosis with the following results: in participants without steatosis: ORmod 1.19, 95%CI 0.35–4.06; ORfreq 0.47, 95%CI 0.14–1.57; P=0.025; Ptrend 0.017; and in participants with steatosis: ORmod 0.45, 95%CI 0.15–1.40; ORfreq 0.31, 95%CI 0.11–0.92; P=0.082; Ptrend 0.034. Interaction term was tested and found to be non-significant (Pinteraction=0.527 for steatosis x coffee). The comparison of predicted probability between the non-steatosis and steatosis group was based on the independent t-test (P<0.001).

Tea consumption and liver stiffness

Overall tea consumption was similar in participants with versus without LSM ≥8.0kPa *(Table 1).* Likewise, tea was not associated with log-transformed LSM in linear regression or with LSM ≥8.0Kpa in logistic regression when correcting for potential confounders *(Table 2).* Looking at subtypes of tea, only herbal tea was less frequently consumed by participants with LSM ≥8.0kPa compared to participants with normal LSM (*Table 1*), this finding remained when adjusted for energy intake and coffee consumption (OR 0.61, 95%CI 0.40–0.94; *P*=0.024). After further adjustment for lifestyle and metabolic traits herbal tea was not independently associated with LSM ≥8.0kPa, however, it remained

inversely related to log-transformed LSM values in a multivariable linear regression model as shown in *Table 2* (*P*=0.001). Interactions between covariables and herbal tea consumption were tested and not significant.

Tea, coffee consumption and steatosis

Steatosis was found in 34.6% of the study population and was higher amongst participants with LSM ≥8.0kPa (60% vs. 33.2%; *P*<0.001). Although proportion of steatosis was similar amongst coffee consumption groups (*P*=0.656), proportion differed significantly in tea consumers (42.7%, 35.4% en 28.6% for no, moderate and tea frequent consumers; *P*<0.001). Likewise, tea consumption was associated with steatosis in logistic regression analysis, in contrast to consumption of coffee. However, when correcting for potential confounders in a multivariable logistic regression model, this association was no longer significant, as shown in *Table 3*.

Table 3: Stepwise logistic regression models with steatosis as dependent variable using non-consumers as reference

	OR	95%CI	*P*-value
Model 1*			
Coffee			
No	1 (ref)		0.239
Moderate (>0–3)	1.32	0.92; 1.91	
Frequent (≥3)	1.17	0.82; 1.66	
Herbal Tea			
No	1 (ref)		0.039
Any	0.82	0.68; 0.99	
Green Tea			
No	1 (ref)		0.018
Any	0.78	0.63; 0.96	
Black Tea			
No	1 (ref)		0.006
Any	0.78	0.66; 0.93	
Total Tea			
No	1 (ref)		<0.001
Moderate (>0–3)	0.72	0.57; 0.92	
Frequent (≥3)	0.53	0.41; 0.69	
Model 2¥			
Coffee			
No	1 (ref)		0.186
Moderate (>0–3)	1.31	0.87; 1.98	

Table 3 (*continued*)

	OR	95%CI	*P*-value
Frequent (≥3)	1.09	0.74; 1.62	
Herbal Tea			
No (0)	1 (ref)		0.674
Any	0.96	0.77; 1.18	
Green Tea			
No	1 (ref)		0.297
Any	0.89	0.70; 1.11	
Black Tea			
No	1 (ref)		0.086
Any	0.84	0.69; 1.03	
Total Tea			
No	1 (ref)		0.064
Moderate (>0–3)	0.85	0.65; 1.10	
Frequent (≥3)	0.70	0.52; 0.95	
Model 3†			
Coffee			
No	1 (ref)		0.192
Moderate (>0–3)	1.38	0.88; 2.16	
Frequent (≥3)	1.15	0.75; 1.77	
Herbal Tea			
No	1 (ref)		0.779
Any	1.03	0.82; 1.29	
Green Tea			
No	1 (ref)		0.425
Any	0.91	0.71; 1.15	
Black Tea			
No	1 (ref)		0.169
Any	0.86	0.70; 1.06	
Total Tea			
No	1 (ref)		0.197
Moderate (>0–3)	0.86	0.65; 1.14	
Frequent (≥3)	0.74	0.54; 1.03	

* Model 1: adjusted for tea‡ or coffee and energy intake

¥ Model 2: adjusted for tea‡ or coffee, energy intake, BMI, gender and age.

† Model 3: adjusted for tea‡ or coffee, energy intake, BMI, gender, age, HOMA-IR, excessive alcohol intake, current or former smoking. Additional adjustment for LSM did not affect this association.

‡All regression models either contain total tea or tea subtypes as covariate.

Sensitivity analyses

In 1360 participants (56.1%), FFQs were completed 5.8 years prior to TE examination. As can be seen from this sensitivity analysis presented in *Supplementary Table 2,* results in both subgroups were consistent to that of the total study population, underlining the stable character of dietary data. Furthermore, 13 (0.5%) participants had LSM ≥13.0kPa, suggestive of cirrhosis. As expected, the association between coffee and LSM ≥13.0kPa was also found to be significant (OR_{mod} 0.35, 95%CI 0.10–1.26; OR_{freq} 0.08, 95%CI 0.02–0.35; *P*=0.004). The inverse, univariate relationship between different cut-offs of LSM and coffee consumption is graphically presented in *Figure 3*. Moreover, when applying a more stringent cut-off for significant fibrosis, i.e. LSM ≥6.2kPa, in participants with steatosis, similar results were found, albeit slightly attenuated and statistically not significant (OR_{mod} 0.62, 95%CI 0.28–1.38 and OR_{freq} 0.47, 95%CI 0.22–1.02; *P*=0.096). Lastly, exclusion of abnormal ALT levels from all analyses, to ensure results are generalizable for the general population without known liver disease, did not alter the results (data not shown).

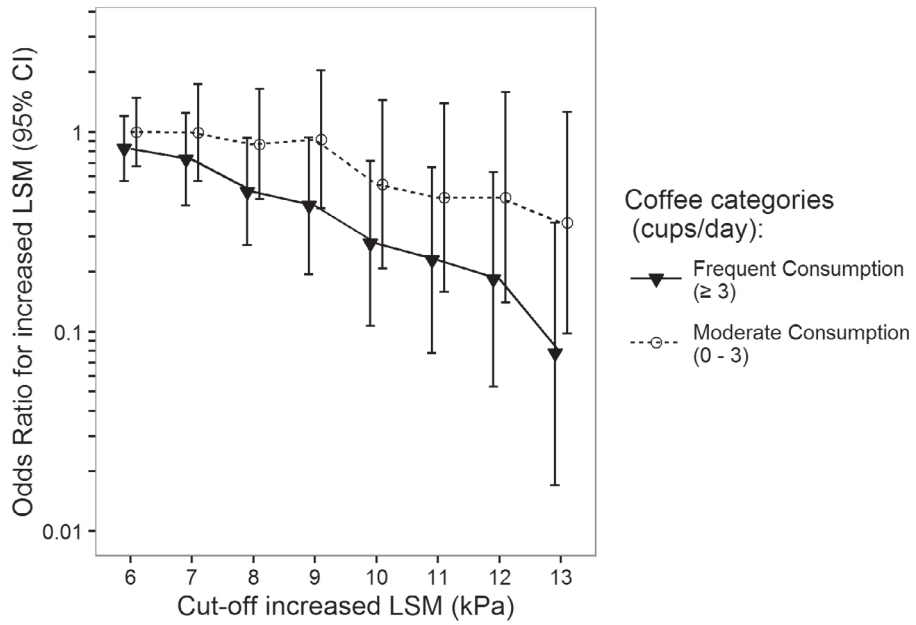


Figure 3: Estimated odds ratio for moderate and frequent coffee consumers versus no coffee consumers for a grid of different cut-off values of LSM

Results of the univariate logistic regression analyses of the effect of coffee consumption for the endpoint of LSM greater than a specific cut-point (yes/no). Y-axis: univariate OR (95%CI) on log-scale. X-axis: different cut-off values for LSM.

Discussion

To the best of our knowledge, this is the first study to determine whether coffee and tea consumption are associated with subclinical fibrosis and steatosis, using reliable imaging techniques, in the general population. This large population-based cohort study shows an inverse and independent association between coffee and herbal tea consumption and log-transformed LSM. Frequent coffee consumption was also independently associated with lower odds and probability of significant liver fibrosis (defined as LSM ≥8.0kPa) compared to non-consumers, independent of steatosis presence and a great number of metabolic and environmental traits.

A recent meta-analysis showed that, amongst liver disease patients, coffee consumers were less likely to develop liver fibrosis and cirrhosis than no-coffee consumers.[110] Yet, all studies included in this meta-analysis concerned specific, non-generalizable populations, i.e. patients with cirrhosis, other chronic liver disease or morbid obesity.[88,89,111] In addition, a study from a NASH consortium showed that coffee consumption was independently associated with lower odds (OR 0.64) of advanced histology-proven fibrosis in subjects with low insulin resistance.[90] Another study found that NASH patients with advanced fibrosis, i.e. fibrosis score 2–4, consumed less coffee than patients with fibrosis score 0–1.[112] But to date, a study of Zelber-Sagi et al. is the only one on the association between coffee and liver fibrosis in the general population, albeit using Fibrotest (i.e. a commercial biomarker test combining serum markers age and gender) rather than biopsy or TE, to assess fibrosis.[91] In line with our data, an association between prevalence of NAFLD and coffee consumption was not found. The authors found a lower proportion of significant fibrosis (defined as Fibrotest score ≥2, borderline F1-F2 included) in participants consuming 3 or more coffee cups/day (OR 0.49), which is also comparable to our data. However, the authors did not include a clear reference group, i.e. non-consumers, in their analyses and the association was only significant in univariate fashion. In contrast, we found that this association in fact enhanced after adjustment for several potential metabolic and environmental confounders. We hypothesize that this can be explained by the relatively unhealthy lifestyle of coffee consumers, as they were more often heavier drinkers, current or former smokers and obese.

Only few studies have assessed the effect of overall tea consumption on liver disease and these show inconsistent results.[92,111,113] Moreover, five large Asian studies specifically examined green tea consumption in relation to liver health,[93,94] of which three found a significant beneficial association between green tea and serum transaminases.[95,96,114] However, this association was only seen in individuals consuming more than 700ml green tea per day. We could not detect a beneficial effect of green tea in our study. This may partly be explained by our predominantly elderly Caucasian population, in which drinking green tea is not customary, as reflected in the low consumption rate. We speculate that

the beneficial effects of tea could be dose-dependent and might only exert an independent effect on liver health when consumed above a certain threshold. Furthermore, considering the favourable lifestyle traits in frequent tea consumers, a univariate association of (green) tea with liver stiffness and steatosis could be confounded by a healthy lifestyle in general. Interestingly, we did find herbal tea to be independently associated with lower liver stiffness. To the best of our knowledge, no other study has addressed this association. Although herbal tea encompasses a broad spectrum of different types of herbs, such as *Chamomile* and *Calicotome villosa*, and methods of preparation, a study of Carlsen et al.[115] stated that, despite the great diversity, 'herbal plants' are amongst the most antioxidant-rich food items and supplements worldwide and contribute the most to antioxidant capacity as assessed by ferric-reducing ability of plasma. We therefore hypothesize that the antioxidant capacity of herbal tea exceeds that of other teas, displaying a more pronounced effect on liver health. In addition, it has been previously demonstrated that the ferric-reducing ability of plasma is reduced in patients with steatosis and NASH.[12] However, future studies are needed to confirm this observation.

Mechanisms through which coffee and herbal tea promote liver health are unclear. Also, it is not known if these two beverage act through similar pathways. However, our data suggest that both have inhibitory properties for fibrogenesis and not steatogenesis. Both coffee and tea consist of more than 100 individual components, of which few, i.e. polyphenols and caffeine, are shared. Shim et al. showed that caffeine inhibits hepatic stellate cell proliferation in cirrhotic rat models and subsequently counteracted fibrogenesis.[116] Interestingly, caffeine in coffee had a greater effect on insulin sensitivity than caffeine in water alone. Moreover, another animal study found that conventional coffee, decaffeinated coffee and caffeine alone all reduced serum levels of ALT and fibrosis scores. But, only rats fed with conventional coffee showed significantly less histologic injury from repeated administration of toxics.[117]

Since caffeine alone does not seem to fully explain the beneficial effect of coffee, other components are proposed to contribute. Both coffee and tea contain a substantial amount of polyphenols, which have been suggested to promote hepatoprotective effects independent of caffeine.[118] Animal studies showed that coffee-polyphenols, chlorogenic acid and tocopherols, attenuate obesity-related lipid accumulation in the liver[119] and have antioxidant properties. Furthermore, experimental animal data suggest that tea-polyphenols attenuate fibrosis, oxidative stress and inflammation through pivotal inflammatory transcription factor[120] and inhibition of hepatic stellate cell activation.[121] In our population study, we could not explore the underlying mechanistic pathway responsible for the observed effects.

A major strength of this study is the inclusion of a large number of subjects from an unselected population, correcting for a great number of environmental and metabolic traits and, using well-validated diagnostic tools. Furthermore, we performed several sensitivity analyses, which should be interpreted in light of decreased power, showing consistency in

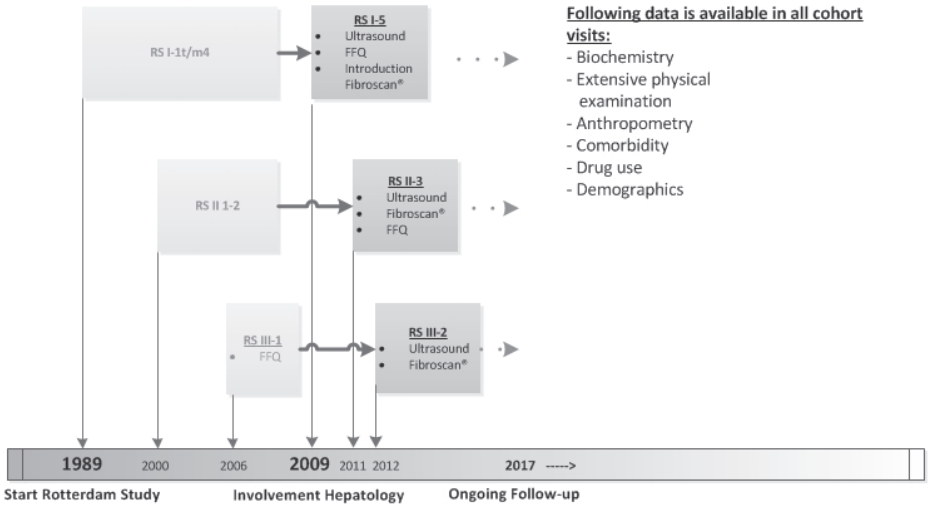
magnitude and direction of associations found in the overall analyses. However, some limitations need to be addressed. First, due to the cross-sectional design of this study, we are not able to draw conclusions regarding the cause-effect relationship of coffee, herbal tea and liver stiffness. Second, the golden standard for diagnosis of liver fibrosis remains a liver biopsy rather than TE. However, performing an invasive liver biopsy in presumed healthy participants, is unethical. TE strongly correlates with histologic stages of liver fibrosis[122] and hence, LSM appears to be a good surrogate marker for liver fibrosis in the general population.[11,78,79] Third, as with any questionnaire, data are subject to reporter- and recall-bias even though this FFQ has been extensively validated in previous studies[98,99] and unreliable FFQs were excluded. Also, none of the subjects were aware of the presence of fibrosis, since liver imaging has been conducted after dietary assessment. Fourth, unfortunately FFQs do not provide information on type of coffee or method of preparation. Therefore we could not further specify type of coffee consumption. However, previous studies have been inconclusive about whether type of coffee is of any influence. Fifth, in light of a healthy lifestyle, the association between herbal tea and liver stiffness could be affected by residual confounding even though we adjusted for a great number of lifestyle factors. Lastly, part of our study population completed the FFQs 5.8 years prior to liver imaging. Since dietary data are known to be globally stable, we assumed that drinking habits for coffee and tea did not change significantly over time in this group either.[109] This assumption is supported by our sensitivity analysis, showing that magnitude and direction of results in both groups (i.e. RS I-5 and RS II-3 vs. RS III-2) was very similar to the overall analyses (*supplementary results*).

In conclusion, we found a protective association between coffee and liver stiffness that not only occurs in disease-specific settings but appears also to be present in the general population with and without steatosis. Since coffee is an accessible and relatively inexpensive beverage, it could be further implemented as preventative strategy if future studies were to confirm our findings. Though we did not find an association with steatosis per se, coffee and tea consumption might still be useful to prevent progression to more advanced stages, such as inflammation and in particular, fibrosis. Prospective studies are needed to establish a cause-effect relation between coffee and liver fibrosis. Herbal tea consumption was also independently related to lower liver stiffness, even though consumed in small quantities. To date, very little is known about herbal tea and its effects on human health, let alone, its mechanisms of action in liver disease. Future studies are therefore needed to validate our data on the protective effect of herbal tea on liver stiffness.

Supplementary Files

Supplementary Methods: The Rotterdam Study

In short, since 1989, a total of three longitudinal cohorts (cohort RS I starting 1989; cohort RS II starting 2000, cohort RS III starting 2006) of inhabitants of Ommoord have undergone extensive home interviews and serial examinations (every 4–5 years) including, but not limited to, physical examination, laboratory assessment, cardiovascular and neurological imaging, neuropsychiatric analyses, and testing on ophthalmological, dermatological, metabolic and hepatic diseases. Liver assessments were only included into the core protocol of the Rotterdam Study from 2011 onwards. For the purpose of our study, all participants visiting the research centre between January 2011 and September 2013 (5th visit of the first cohort (RS I-5), 3rd visit of the second cohort RS II-3, and 2nd visit of the third cohort RS III-2) were included and underwent anthropometric assessment, abdominal ultrasound, transient elastography, and blood sampling. Food frequency questionnaires were available for (RS I-5, RS II-3 and RS III-1). Follow-up time between RS III-1 and III-2 is 5.8 (IQR 5.7–6.0) years.



Supplementary Figure: The Rotterdam Study Design and Timeline

Supplementary Table 1: Characteristics of low (no or moderate) vs. frequent coffee and tea consumers.

	<3 cups coffee/ day n=864	≥3cups coffee/ day n=1560	*P*-value	<3 cups tea/ day n=1738	≥3cups tea/ day n=686	*P*-value
Demographics						
Age (years)	68.2 ± 8.1	65.6 ± 6.7	<0.001	66.6 ± 7.2	66.3 ± 7.8	0.442
Female	63.0	53.0	<0.001	51.2	70.3	<0.001
Caucasian	92.8	98.2	<0.001	96.2	96.5	0.718
Low / Intermediate / High education	48.7 / 27.9 / 23.5	43.0 / 30.6 / 26.4	0.026	44.9 / 31.5 / 23.5	45.2 / 24.9 / 30.0	<0.001
Excessive alcohol	14.0	16.9	0.060	16.9	13.3	0.027
Current / Past / Never smoker	7.1 / 49.2 / 43.7	13.7 / 55.0 / 31.3	<0.001	13.3 / 53.7 / 33.0	6.3 / 50.9 / 42.7	<0.001
Physical activity*	46 (18–84)	46 (19–85)	0.540	46 (19–85)	46 (20–84)	0.852
Physical Examination						
BMI (kg/m^2)	27.0 ± 4.1	27.3 ± 3.9	0.055	27.4 ± 3.9	26.7 ± 4.1	<0.001
Normal	32.8	28.0	0.003	27.0	36.6	<0.001
Overweight	46.7	50.5		50.3	46.3	
Obese	20.5	21.5		22.7	17.2	
WC (cm)						
Men	99.7 ± 10.7	98.8 ± 9.9	0.209	99.7 ± 10.2	96.6 ± 9.7	<0.001
Women	88.4 ± 11.6	89.4 ± 11.4	0.118	89.5 ± 11.2	88.0 ± 12.0	0.022
Biochemistry						
AST (U/L)	25 (21–29)	24 (21–28)	0.007	24 (21–28)	24 (21–28)	0.268
ALT (U/L)	18 (15–23)	18 (14–24)	0.956	18 (14–24)	17 (14–23)	0.038
Bilirubin (μmol/L)	8 (7–11)	8 (6–10)	0.002	8 (6–11)	8 (6–10)	0.178
ALP (U/L)	68 (57–80)	66 (56–77)	0.011	67.7 ± 17.6	70.0 ± 17.8	0.005
GGT (U/L)	24 (16–34)	24 (17–35)	0.453	25 (18–35)	21.5 (16–32)	<0.001
Platelets (*109/L)	267.4 ± 64.1	268.0 ± 63.2	0.830	265.9 ± 64.0	272.6 ± 62.0	0.019
HOMA-IR	2.6 (1.7–4.8)	2.5 (1.7–3.8)	0.079	2.6 (1.7–4.1)	2.3 (1.6–3.4)	<0.001
Comorbidity						
Metabolic Syndrome	47.0	43.1	0.080	48.2	35.0	<0.001
- WC >88cm (♀) or >120cm (♂)	45.7	43.2	0.247	45.8	39.6	0.006
- Triglycerides >150mg/dL	41.1	36.6	0.032	40.2	32.9	0.001
- HDL-C <40mg/dL (♂) or 50mg/dL (♀)	36.7	31.9	0.019	35.9	27.8	<0.001
- Blood pressure ≥130/85mmHg¥	80.4	78.3	0.284	81.4	73.0	<0.001
- FPG >100mg/dL	45.5	44.1	0.548	47.3	37.7	<0.001

Supplementary Table 1 (*continued*)

	<3 cups coffee/ day n=864	≥3cups coffee/ day n=1560	*P*-value	<3 cups tea/ day n=1738	≥3cups tea/ day n=686	*P*-value
Diabetes Mellitus	13.2	9.1	0.002	11.3	8.6	0.054
Hypertension¥	66.5	63.7	0.213	67.5	57.6	<0.001
Liver Imaging						
LSM (kPa)	4.8 (3.8–5.9)	4.7 (3.8–5.7)	0.004	4.8 (3.8–5.9)	4. 6 (3.7–5.6)	0.008
Steatosis	35.2	34.2	0.656	36.9	28.6	<0.001

Data are presented as mean (±SD), median (IQR) or percentage. P-value is based on T-test, Wilcoxon rank sum test, Chi-square test or Fisher's exact test.
* Physical activity in metabolic equivalent task hours/week.
¥ Blood measurement data was available for 2051 participants.
Abbreviations: ALP, alkaline phosphatase; AST, aspartate transaminase.

Supplementary Table 2 A:Sensitivity analyses of RS-III cohort (n=1360) in which FFQs were completed 5.8 years previous to all other measurements, amongst them hepatological imaging.

Coffee	Odds Ratio[1]	95% Confidence Interval	P-value
Fully adjusted model*			0.122
No	1 (ref)		
Moderate (>0–3)	0.67	0.18–2.48	
Frequent (≥3)	0.37	0.11–1.27	
	β[2]	**95% Confidence Interval**	**P-value**
Fully adjusted model*			
Coffee			
No	0 (ref)		0.019
Moderate (>0–3)	0.006	–0.076 ; 0.088	
Frequent (≥3)	–0.045	–0.121 ; 0.031	
Herbal tea			
No	0 (ref)		0.018
Any	–0.046	–0.084 ; –0.008	
Green Tea			
No	0 (ref)		0.342
Any	0.019	–0.020 ; 0.058	
Black Tea			
No	0 (ref)		0.977
Any	0.001	–0.036 ; 0.037	
Total Tea			
No	0 (ref)		0.265
Moderate (>0–3)	–0.022	–0.075 ; 0.032	
Frequent (≥3)	–0.034	–0.094 ; 0.025	

Supplementary Table 2 B:Sensitivity analyses of RS-I and II cohort (n=1064) in which FFQs were completed at the same time as all other measurements.

Coffee	Odds Ratio[1]	95% Confidence Interval	P-value
Fully adjusted model*			0.110
No	1 (ref)		
Moderate (>0–3)	0.75	0.26–2.14	
Frequent (≥3)	0.43	0.15–1.23	
	β[2]	**95% Confidence Interval**	**P-value**
Fully adjusted model*			
Coffee			
No	0 (ref)		0.065
Moderate (>0–3)	–0.047	–0.130 ; 0.035	
Frequent (≥3)	–0.071	–0.150 ; 0.008	
Herbal tea			
No	0 (ref)		0.052
Any	–0.046	–0.092 ; 0.000	
Green Tea			
No	0 (ref)		0.298
Any	0.027	–0.024 ; 0.079	
Black Tea			
No	0 (ref)		0.260
Any	–0.024	–0.066 ; 0.018	
Total Tea			
No	0 (ref)		0.814
Moderate (>0–3)	–0.016	–0.070 ; –0.039	
Frequent (≥3)	0.004	–0.059 ; 0.067	

* Adjusted for tea[‡] or coffee, energy intake, BMI, gender, age, steatosis, ALT, excessive alcohol intake, current or former smoking and HOMA-IR, soda consumption, DHDI, physical activity, cream and sugar use.

[‡]All regression models either contain total tea or tea subtypes as covariate.

[1] Multivariable logistic regression models were used to determine the association between explanatory variables coffee and tea consumption with dependent variable LSM ≥8.0 kPa. No consumption is used as reference (OR=1).

[2] Multivariable linear regression models were used to determine the association between explanatory variables coffee and tea consumption with dependent variable log-transformed LSM. No consumption is used as reference (β=0).

MEDITERRANEAN FOOD

Chapter 4

Potential Mechanisms Underlying the Role of Coffee in Liver Health

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Semin Liver Dis. 2018 Aug;38(3):193–214.

Abstract

Coffee, the most consumed hot beverage worldwide, is composed of many substances, of which polyphenols, caffeine, and diterpenoids are well studied. Evidence on potential effects of coffee on human health has been accumulating over the past decades. Specifically, coffee has been postulated to be hepatoprotective in several epidemiological and clinical studies. Several underlying molecular mechanisms as to why coffee influences liver health have been proposed. In this review, we summarized the evidence on potential mechanisms by which coffee affects liver steatosis, fibrosis, and hepatic carcinogenesis. The experimental models reviewed almost unanimously supported the theorem that coffee indeed may benefit the liver. Either whole coffee or its specific compounds appeared to decrease fatty acid synthesis (involved in steatogenesis), hepatic stellate activation (involved in fibrogenesis), and hepatic inflammation. Moreover, coffee was found to induce apoptosis and increased hepatic antioxidant capacity, which are involved in carcinogenesis.

Introduction

Coffee has never been such a timely topic as it is today. In earlier years, coffee was viewed negatively due to the cholesterol increasing capacity of unfiltered coffees,[123] and the positive confounding association between lifestyle factors, such as alcohol consumption and smoking, with caffeine consumption.[124,125] Today, there is a paradigm shift due to the emerging evidence favouring various health benefits of coffee. A recent umbrella review of Poole et al. explored the effect of coffee consumption on multiple health outcomes and found that coffee consumption seemed more likely to benefit than to harm human health.[125] This meta-analysis showed that coffee was associated with lower all-cause and cardiovascular mortality, lower incidence of cancer, and lower incidence of metabolic diseases. Interestingly, the authors of this umbrella review found a particular noticeable effect of coffee consumption on liver health outcomes. Indeed, coffee in relation to liver health has been well studied in both epidemiological and mechanistic research. In 1986, Arnesen and colleagues first described a beneficial association of coffee consumption with liver health, as assessed by gamma-glutamyltransferase (GGT) levels, in a large population-based study.[126] Subsequently, several other epidemiological studies confirmed the reduction in levels of transaminases and GGT in both whole[86,127] and decaffeinated coffee consumers.[128] Coffee was also found to be associated with less fibrosis, lower hospitalization rates, and lower mortality rate in alcoholic liver disease.[87,129] Moreover, our group[130] and others[131,132] have consistently shown that coffee was inversely associated with (severity of) fibrosis in population-based studies independent of important environmental and lifestyle traits. Coffee consumption has also been associated with improved response to antiviral therapy,[133] increased survival[134] and improved biochemistry[135] in patients with hepatitis C. On the other hand, although coffee was associated with less fibrosis in patients with steatosis,[130,136] clinical evidence on the protective effect of coffee on steatosis itself remains ambiguous.[91,137,138] And last but not least, there is evidence that coffee consumption is associated with a reduced incidence of hepatocellular carcinoma.[139,140] However, most of these epidemiological and clinical studies offer little pathological insights, inherent to the observational nature of such studies. In order to understand the mechanisms behind the effects of coffee on liver health, we aimed to summarize the existing experimental evidence on coffee associations with 1) hepatic steatosis, 2) hepatic fibrosis, and 3) hepatocellular carcinoma.

Methods

We performed an extensive search on Embase.com, Medline Ovid, Google Scholar, Cochrane Central and Web of Science on the 7th of March (see Supplementary Methods for

a full description). This search yielded 4568 articles, and 2498 articles after automatic de-duplication. One researcher selected on title/abstract, and excluded 2243 articles that did not had liver disease as study endpoint, remaining duplicates, non-English articles, articles on primarily caffeine metabolism, articles without full text, and articles on supplemental use of coffee substances for sport boosting purposes. All meta-analyses, reviews and non-experimental studies (n=178 studies) were excluded but used as background information for this review. The remaining 77 in vitro and in vivo studies were fully reviewed. An additional 10 articles were included based on cross-references of individual publications. Studies that focused merely on inflammation (without focus on either steatosis, fibrosis or carcinogenesis, n=7) or which were solely in vitro (n=16), were excluded. Hence, this review included 64 articles.

Coffee preparation and composition

Coffee is a complex chemical mixture that contains more than a thousand different compounds, such as lipids, vitamins, caffeine, melanoidins and more than 800 volatile compounds which are formed during the roasting process of coffee beans.[141] Coffee is derived from the coffee plant belonging to the genus Coffea. Amongst this genus, there are numerous species of which the Coffea Arabica and the Coffea Canephora (also known as Robusta), are the most important and well-known species. More than three quarters of the coffee bean production is that of Arabica which is superior in taste over the Robusta and contains less caffeine and polyphenols.[142] Today, Brazil is the leading country in cultivating coffee plants and exporting coffee beans, followed by Vietnam and Colombia. Meanwhile, the European continent is the largest consumer, followed by the United States and Brazil. From an economical perspective, coffee is the second largest traded commodity worldwide after petroleum (ICO 2018).

To create coffee, multiple preparations of the green Coffea beans are needed. This is important to take into account as the preparation method is highly responsible for the final composition of the coffee brew.[143,144] The roasting process, for example, incorporates the activation of the so-called Maillard reaction that causes an increase in antioxidant activity and degradation of polyphenols. The degradation of polyphenols itself causes a decrease in antioxidant activity, and it has therefore been postulated that the intensity of roasting (along with the intensity of the thermal process) are more important for the antioxidant activity of the brew than type of coffee bean alone.[142] Importantly, roasting at very high temperatures can cause the formation of acrylamide, a potential carcinogenic substance.[145] In addition, filtering can change coffee composition, e.g. paper-filtering can lead to a drastic decrease in lipid and vitamin content (USDA 2018). Lastly, the way of consumption can affect bioavailability of coffee compounds. For example, the addition of milk[146] or

non-dairy creamers,[147] have been shown to either impair or delay the bioavailability of polyphenols.

Although we often refer to polyphenols, the word 'polyphenol' is technically not a chemical term but merely a collective name for flavonoids, tannins, and phenolic acids.[148] The most common phenolic acid in coffee is 5-caffeoyl quinic acid, also known as chlorogenic acid (CGA). CGA is the ester of caffeic acid with quinic acid.[149] Polyphenols are regarded as healthy dietary components but despite extensive research, exact mechanism of action on human health remains an enigma.[148] Other well-known coffee constituents are the diterpenoids, i.e. cafestol and kahweol, which are naturally present in all oil-containing coffees, such as unfiltered or boiled coffees (i.e. Turkish and Scandinavian-style).[150] The diterpenoids have been blamed for the lipogenic effects of coffee,[151] while at the same time they have also been suggested to have anti-oxidant and anti-carcinogenic properties.[152] A group of less well-known substances of coffee are the melanoidins, defined as high-molecular weight nitrogenous and brown-coloured compounds. They have been proposed to exert beneficial effects on human health in terms of anti-oxidant and anti-inflammatory function, but as the exact structure of these components are largely unknown, exact health mechanisms remain to be elucidated.[153] Last but not least is caffeine (1,3,4-trimethylxanthine), the most well-studied coffee compound and probably the most widely-used stimulating drug in the world.[154] Caffeine is a methylxanthine, which contains three main metabolites, i.e. theobromine, paraxanthine, and theophylline. Caffeine remains stable during the roasting process. Some researchers ascribe the beneficial health effects of coffee solely to the caffeine.[155] Interestingly, twins studies have linked pharmacokinetic and pharmacodynamic responses to caffeine to certain single nuclear polymorphisms,[156] suggesting that response to and consumption patterns of caffeine may be influenced by genetic diversity. Moreover, it has been suggested that genes can modulate the effect of habitual caffeine consumption on health outcomes, and that tolerance to this psycho-active drug is different between individuals.[157] In addition, caffeine is almost exclusively metabolized in the liver, which has led to the question of reverse causality - perhaps patients with cirrhosis drink less coffee because of more pronounced side-effects.[158] However, it remains a topic of debate whether this fully explained the observed differences.

Mechanisms of coffee benefits

Liver steatosis

In contrast to human studies, many experimental studies found a beneficial effect of coffee on hepatic steatosis (*Table 1*). Building on previous evidence that CGA modulates the activity of glucose-6 phosphatase (G6Pase; a gluconeogenesis-inducing enzyme),[159]

Table 1: Experimental evidence on coffee effects in steatosis

Author	Coffee compound	Animal Model	Main study findings
Rodriguez de Sotillo, 2002[160]	CGA	(fa/fa) Sprague-Dawley Zucker rats treated with CGA or water 3 weeks	CGA ↓ total cholesterol and Tg CGA ↑ insulin sensitivity CGA 24% ↓ liver Tg
Ong, 2013[161]	CGA	In vivo Type II DM Leprdb/db mice +/– CGA, CGA + compound C or metformin treatment and lean control C57BL/6J mice 2 weeks In vitro HepG2 cells	In vivo CGA + metformin had ↑ adiponectin vs diabetic controls CGA + metformin ↓ G6Pase expression and activity vs diabetic controls CGA + metformin ↓ liver total cholesterol and Tg vs diabetic controls CGA + metformin ↑ expression + translocation of GLUT4 (↑ glucose transport in skeletal muscles) In vitro CGA ↓ glucose synthesis dose and time dependently CGA ↓ G6Pase expression and ↑ ACC and AMPK phosphorylation time and dose dependently CGA + metformin both ↓ FAS and formation of oil droplets and vacuole degeneration dose and time dependently AMPK knockdown mice (siRNA) completely blocked CGA-mediated ↓ in glucose production and FAS
Ma, 2015[162]	CGA	1. C57BL/6 mice on ND or HFD + CGA or placebo 15 weeks 2. 10 obese mice +/– CGA 6 weeks	HFD + CGA ↓ mRNA upregulation of macrophage specific marker genes (Cd68, Cd11b) in white adipocytes vs HFD HFD + CGA ↓ fat accumulation in liver vs HFD HFD + CGA ↓ genes hepatic fat accumulation: mRNA expression of PPAR-γ 1 and 2, Cd36, FABP4, MGAT1 genes vs HFD HFD + CGA ↑ fatty acid metabolism (mRNA of CPT 1a and 1b, FGF21) vs HFD HFD + CGA ↓ hepatic inflammation (mRNA Cd68, Cd11b and Cd11c) vs HFD CGA ↔ weight, ↓ liver weight and ↓ lipid accumulation liver vs obese CGA ↓ genes hepatic fat accumulation: mRNA expression of PPAR-γ 1 and 2, Cd36, FABP4, MGAT1 genes AND ↑ PPAR-α and its target genes ACOX1 and FGF21. ↔ mRNA CPT1 vs obese CGA ↓ expression of macrophage markers: CCR2, F4/80, Cd68, and TNF-α (also mRNA ↓ in adipose tissue) vs obese
Vitaglione, 2010[118]	DC Polyphenols Melanoidins	Wistar rats controls or HFD (3 months) treated with water, DC, Polyphenols or Melanoidin	HFD + DC or polyphenols or melanoidins ↓ lipid droplets, inflammatory infiltrate and fibrotic septa vs HFD + water HFD + DC or polyphenols ↑ IL6, IL10, IL4 and HFD + polyphenols ↓ IFNγ vs HFD + water HFD + polyphenols or melanoidins ↓ TNFα, and oxidized glutathione vs HFD + water HFD + DC or polyphenols ↓ TNFα and TGFβ, ↑ adipo-R2, PPARα expression in liver vs HFD + water
Shokouh, 2017[163]	Caffeic acid Trigonelline Cafestol	Sprague Dawley rats HFD +/– nutraceutical mix 12 weeks	HFD + Mix ↑ adiponectin vs HFD HFD + Mix ↓ semi quantitative scoring of severe steatosis and ↓ plasma ALT. Trend towards ↓ Liver Tg
Murase, 2011[164]	Coffee polyphenols	In vivo C57BL/6J mice on ND or HFD +/– Polyphenols 15 weeks In Vitro Hepa 1–6 cells	In vivo HFD + polyphenols 1% ↓ liver Tg and cholesterol vs HFD HFD + polyphenols 1% ↓ mRNA levels FAS, ACC1 and stearoyl-CoA desaturase HFD + polyphenols 1% ↓ mRNA level of SREBP-1c In vitro Polyphenols ↓ mRNA expression of ACC1-2, stearoyl-CoA desaturase, SREBP1 and FAS Polyphenols ↔ PPAR activation Polyphenols ↔ AMPKα and ACC phosphorylation

Table 1 (*continued*)

Author	Coffee compound	Animal Model	Main study findings
Panchal, 2012[166]	Colombian Coffee Extract	Wistar rats on ND or HFD (8 weeks) + half of the littermates Coffee (8 weeks more)	Coffee + HFD ↓ liver fat deposition and no inflammatory infiltration nor portal fibrosis vs HFD Coffee + HFD ↓ ALT, aspartate aminotransferase, alkaline phosphatase, and lactate dehydrogenase vs HFD
Salomone, 2014[167]	DC	Wistar rat on HFD or ND +/−DC 3 months	HFD + DC mild steatosis but ↓ ballooning, inflammatory infiltrate and fibrosis vs HFD HFD + DC ↓ isoprostanes and 8-deoxyguanosine (=↓ oxidative stress) HFD + DC modulates expression of cell chaperones involved in protein folding, autophagy, and immune activation
Watanabe, 2017[168]	CC DC	15 Tsumura-Suzuki obese/ diabetic mice treated with water, DC or CC 11 months	DC and CC had ↓ ballooning after 6 months (control developed NASH) vs obese ↔ NAS score between groups CC had ↓ fibrosis and DC had NO fibrosis vs obese All groups ↔ liver tumour frequency, but atypical tumours occurred ↓ in CC and DC vs obese
Takahashi, 2014[169]	CC DC Unroasted CC	C57BL/6J mice on ND or HFD +/− treatment of water, CC, DC and unroasted CC 9 weeks	HFD + CC or DC or unroasted CC ↓ liver weight vs. ND HFD + CC or DC or unroasted CC ↓ lipid metabolism-related genes regulated by PPAR-γ (i.e. complement factor D, FABP2, ACC, CD36 and cell death-inducing DFFA like effector A and perilipin) vs HFD HFD + CC or unroasted coffee ↓ PPAR-γ and CD36 mRNA levels ↓ hepatic PPAR-γ levels (most in unroasted CC) vs HFD
Yun, 2008[170]	Caffeine	C57BL/6J mice on ND or HFD (12 weeks) or HFD + caffeine (4+8 weeks)	HFD + caffeine ↓ large microvacuolar steatosis vs HFD HFD + caffeine ↑ adiponectin and CPT1 activity vs HFD HFD + caffeine ↑ expression of phosphorylated ACC only at week 2 vs HFD
Sinha, 2014[171]	Caffeine	In vivo C57BL6 mice "short term" caffeine and CQ 3 days "long term" ND (4 weeks) or HFD + caffeine (4+4 weeks) In vitro HepG2 cells	In vivo Short-term caffeine ↑ flux through β-oxidation pathway in liver, ↑ hepatic lipolysis Short-term caffeine ↓ levels of p62 and ↑LC3-II (↑ autophagic flux), ↓ mTOR signalling Short-term caffeine ↑ β-oxidation, dependent of the autophagy-lysosomal pathway (after autophagy inhibitor CQ) Short-term caffeine↑ ACC and CPT-1 were independent of the autophagy-lysosomal pathway HFD + caffeine ↓ intrahepatic lipid accumulation HFD + caffeine ↑ autophagic, lipolytic and fatty acid oxidation pathways in liver In vitro Caffeine ↑ LC3 expression ↔ lipase levels Caffeine ↑ autophagosomes resulting in ↑ proautophagic proteins (ATL 5 and 7, Beclin) Caffeine ↓ mTOR signalling ↑ autophagic flux ATL5 knockdown (siRNA) blocks caffeine-mediated steatosis and hence is autophagy dependent
Helal, 2017[172]	Caffeine	Wistar rats ND (8 weeks) or HFD + caffeine (8+8 weeks)	HFD + caffeine 20 and 30 ↓ transaminases and bilirubin vs HFD (even lower than control) HFD + caffeine partially ↓ lipid peroxidation (i.e., MDA) vs HFD HFD + caffeine 20 and 30 partially ↑ glutathione vs HFD HFD + caffeine dose-dependently ↓ mRNA expression of ACC and FAS vs HFD

Table 1 (*continued*)

Author	Coffee compound	Animal Model	Main study findings
			HFD + caffeine 20 and 30 ↑ hepatic CPT-1 and PPAR-α mRNA expression vs HFD HFD + caffeine ↓ histologically assessed steatosis, inflammation, ballooning and mild fibrosis vs HFD
Sugiura, 2012[173]	Caffeine Catechin Epigallocatechin gallate	ICR mice treated with water, caffeine, catechin + caffeine or epigallocatechin gallate + caffeine 4 weeks	↔ liver Tg and total cholesterol Catechin + caffeine ↓ FAS activity, mRNA expression and protein levels in liver Catechin + caffeine ↑ ACC and CPT-II enzymatic activity, but ↔ ACC and CPT-II mRNA expression ↔ in PPAR-α, SREBP-1c mRNA expression in liver
Zheng, 2015[174]	Caffeine	Wild-type zebrafish larvae overfed steatosis or ND + caffeine 20 days	Caffeine 5–8% ↓ hepatic steatosis rate and liver Tg content vs steatosis Caffeine 2.5–8% ↓ amount and size of lipid droplets in liver vs steatosis Caffeine 5% ↓ level of gene expression of fatty acid transport protein (incl. fatty acid translocase/Cd36) vs steatosis Caffeine ↓ mRNA levels of UCP2 and SREBP1 vs steatosis Caffeine ↓ gene expression of key lipogenic enzymes involved in FAS including ACC-1 vs steatosis Caffeine 5% ↓ mRNA level of genes involved in ER stress vs steatosis Caffeine 5% ↓ expression level of IL1β and TNFα vs NAFLD (incl. protein level of IL1β and TNFα vs steatosis Caffeine 5% ↓ mRNA level of ATG12 and Beclin1 (involved in autophagy) vs steatosis

Abbreviations ACC: acetyl-CoA carboxylase, ACOX: acyl-coenzyme A oxidase, ALT: alanine transferase, AMPK: adenosine monophosphate-activated protein kinase, ATG: Autophagy-related protein, ATL: adult T-cell leukaemia, CC: conventional coffee, CCR: C-C chemokine receptor, CD: cluster differentiation, CGA: chlorogenic acid, CPT: carnitine palmitoyltransferase, CQ: chloroquine, DC: decaffeinated coffee, ER: endoplasmatic reticulum, FABP: fatty acid binding protein, FAS: fatty acid synthesis, FGF: fibroblast growth factor, G6Pase: glucose-6-phosphatase, GLUT: glucose transporter, HFD: high fat diet, IL: interleukin, LC: lipidation of microtubule-associated protein light-chain, MDA: malondialdehyde, MGAT: monoacylglycerol O-acyltransferase, mTOR: mammalian target of rapamycin, NAS(H): non-alcoholic steatosis (hepatitis), PPAR: peroxisome proliferator-activated receptor, SREBP: sterol element binding transcription factor, Tg: triglyceride, TGF: tissue growth factor, TNF: tumour necrosis factor, UCP: uncoupling protein.

Rodriguez de Sotillo and Hadley examined the effects of three weeks intravenous CGA administration in nine week-old, metabolic unhealthy rats. The authors showed that CGA administration prevented weight gain, attenuated triglyceride (Tg) accumulation in the liver, and improved lipid profile and insulin sensitivity.[160] These findings were confirmed by the study of Ong and colleagues who showed similar results upon that two weeks of oral CGA administration in genetically diabetic mice.[161] Additionally, in vitro analysis on HepG2 cells (a human hepatoma cell line) confirmed the inhibitory effect of CGA on G6Pase expression. Moreover, CGA completely inhibited fatty acid synthesis (FAS), conceivably by phosphorylation of adenosine monophosphate-activated protein kinase (AMPK) and subsequent downregulation of acetyl-CoA carboxylase (ACC), as AMPK knock-out cells did not show CGA-mediated inhibition of glucose production.[161] Finally, Ma et al. administered

CGA intraperitoneally in mice fed a high fat diet (HFD) or in already obese mice.[162] Both preventive and therapeutic CGA administration lowered steatosis in the liver. The authors found that preventive CGA lowered the mRNA expression of genes involved in I) hepatic fat accumulation, mediated by peroxisome proliferator-activated receptor (PPAR) γ1 and γ2, cluster of differentiation (CD) 36, fatty acid binding protein 4, and monoacylglycerol O-acyltransferase 1; II) hepatic inflammation (CD68, CD11b and 11c); and III) increased mRNA expression of fatty acid metabolism (including carnitine palmitoyltransferase (CPT)-1a and 1b, and fibroblast growth factor (FGF) 21). In addition, therapeutic CGA lowered mRNA expression of encoding inflammatory enzymes; C-C chemokine receptor 2 and tumour necrosis factor (TNF) α.[162]

In addition, three studies examined polyphenols in rodents with diet-induced steatosis, and found that coffee polyphenols could prevent,[163,164] as well as treat,[118] hepatic steatosis. It was suggested that the preventive effect of polyphenols worked via downregulation of mRNA lipogenic genes (i.e. FAS, ACC1, and stearoyl-CoA desaturase1), and sterol element binding transcription factor (SREBP) 1c, the main regulator of FAS.[164] An elegant study by Vitaglione et al. found that polyphenols, but also decaffeinated coffee (DC) and melanoidins, affected steatosis via I) modulation of inflammatory genes, i.e. interleukin (IL) 4, 6 and 10 increment, interferon (IFN) γ and TNFα decrement, II) increased fatty acid β oxidation, and III) reduced liver oxidative stress mediated by glutathione.[118] In this study, only polyphenols increased the ferric reducing ability of plasma, an index of antioxidant capacity.[165] The components examined did not contain caffeine or diterpenoids, suggesting that neither caffeine nor diterpenoids were responsible for this observed anti-steatogenic effect.[118] In contrast, another study on caffeine and diterpenoid-containing Colombian coffee extract in rats on a high carbohydrate/HFD, found less steatosis and inflammation rats.[166]

Salomone et al. found that DC administration to rats on a HFD partially prevented steatosis by mediating stress proteins in the endoplasmic reticulum, and via mitochondrial chaperones in the liver.[167] Two additional studies examined both conventional coffee (CC) and DC,[168,169] and found similar effects of both beverages. Of note, Watanabe and colleagues found less ballooning but no preventative effect of CC or DC on steatosis alone in genetically diabetic mice.[168] Takahashi et al. did not examine lipid content of the liver, but found lower lipid metabolism-related genes regulated by PPARγ (i.e. complement factor D, fatty acid binding protein 2, ACC, and CD36), in both CC and DC, albeit more pronounced in CC, implying an additive effect of caffeine.[169]

Five in vivo studies on caffeine and steatosis identified caffeine as anti-steatogenic.[170-174] Four of the studies were conducted in a (diet-induced) steatosis model and found less steatosis in caffeine-treated animals.[170-173] In addition, one of these studies found an increased fatty acid flux upon acute caffeine administration via the β-oxidation pathway in the liver. They postulated that the lipolytic effects of caffeine are mediated through autophagy, as they demonstrated a decreased effect of caffeine upon blocking autophagy.[171] In addition,

Helal et al., nominated CPT1, ACC and FAS expression levels as mediators in caffeine effects on steatosis. In addition, they also found an upregulation of glutathione (normally depleted in presence of oxidative stress), upon caffeine administration.[172] Another study examining caffeine in addition to catechin (a tea polyphenol) detected a decreased mRNA expression and enzyme activity of FAS and increased enzymatic activity of acyl-CoA oxidase and CPT2.[173] Lastly, a study using zebrafish larvae (which bear great similarity with the human genome) showed that overfed larvae had an upregulation of lipid β-oxidation, a downregulation of lipogenesis-associated genes (i.e. ACC1, CD36, uncoupling protein 2 and SREBP1), endoplasmic stress associated genes, and inflammatory cytokines (IL-1β and TNFα) upon exposure to moderate caffeine doses.[174]

Fibrosis

Coffee has been studied comprehensively in relation to fibrosis (*Table 2*). Polyphenols are amongst the most well-studied coffee components in fibrosis.[175-180] Caffeic acid, a specific 5-lipoxygenase inhibitor, was used therapeutically in carbon tetrachloride (CCl4) poisoned rats and led to lower liver enzymes, liver collagen content, and lipid peroxidation as measured by malondialdehyde (MDA; a marker for oxidative stress).[175] Other studies confirmed the transaminase-lowering capacity of caffeic acid using different toxins, such as acetaminophen.[176,178,179] Amongst them, two subsequent studies from Pang et al. assessed caffeic acid in vivo and in vitro.[178,179] Caffeic acid appeared to attenuate liver damage on histology and lowered MDA and reactive oxygen species formation in vivo. The authors ascribed these findings to the decreased Kelch-like ECH-associated protein (KEAP) 1 and increased nuclear factor erythroid 2-related factor (Nrf) 2 expression in vitro.[179] KEAP1 is an inhibitor of Nrf2 activation, and Nrf2 activates downstream phase II antioxidative enzymes such as nicotinamide adenine dinucleotide (phosphate) quinone oxidoreductase 1 and heme oxygenase 1. Hence, the KEAP1-Nrf2-cascade is thought to play a critical role in liver oxidative injury, which is in line with previous in vitro studies.[181] Therapeutically, caffeic acid reduced phosphorylation of extracellular signal-regulated protein kinase (ERK)1 and 2, which led to I) reduced mRNA expression of early growth response 1; II) reduced inflammatory cytokines IL6, IL1β and tissue factor; and III) reduced growth arrest and DNA damage inducible gene 45α mRNA expression (involved in growth arrest and apoptosis). Henceforth, caffeic acid led to restored cell viability in L-02 and HepG2 cell lines upon acetaminophen-intoxication.[178] Two additional studies examined CGA in presence of either CCl4 or arsenic toxic and found a transaminase-lowering effect of CGA.[177,180] One additionally showed that CGA lowered fibrosis histologically. Also, CGA treated rats showed decreased expression of α-Smooth Muscle Antigen (SMA; an actin isoform that can be used to identify activated hepatic stellate cells (HSCs)), and connective tissue growth factor (CTGF; an inhibitor of extracellular matrix deposition).[177] In addition, CGA reduced liver,

Table 2: Experimental studies on coffee effects in fibrosis

Author	Coffee compound	Animal Model	Main study findings
Reyes, 1995[175]	Caffeic acid	Wistar rats +/– CCl4 intraperitoneally and +/– caffeic acid 8 weeks	CCl4 + caffeic acid 50 partially ↓ serum activities of alkaline phosphatase, and glutamic pyruvic transaminase and completely ↓ GGT CCl4 + caffeic acid 20–50 ↓ lipid peroxidase (i.e. MDA) CCl4 + caffeic acid 20–50 (dose-dependently) ↓ liver collagen content
Janbaz, 2004[176]	Caffeic acid	1. Swiss mice with acetaminophen (1g/kg) +/– caffeic acid 2. Albino Wistar rats +/– acetaminophen (640mg/kg) or CCl4 +/– caffeic acid	Caffeic acid pre-treatment ↓ mortality 1g/kg acetaminophen 80% Caffeic acid pre-treatment ↓ transaminases rise by 640mg/kg acetaminophen and CCl4
Shi, 2013[177]	CGA	Sprague-Dawley rats +/– CCl4 +/– CGA 8 weeks	CCl4 + CGA ↓ transaminase rise and ↑ albumin vs CCl4 CCl4 + CGA ↓ degree of liver fibrogenesis and inflammatory cell infiltration vs CCl4 CCl4 + CGA ↓ αSMA and CTGF collagen I vs CCl4 CCl4 + CGA ↓ mRNA and protein levels of TLR4, MyD88, inducible NOS, COX2 rise and ↑ Bambi vs CCl4 CCl4 + CGA ↓ inflammatory cytokines expression and serum (TNFα, IL6 and IL1β) rise vs CCl4
Pang, 2016[179]	Caffeic acid preventive	*In vivo* C57BL/6 Mice +/– caffeic acid prior to +/– acetaminophen *In Vitro* L-02 cells, HepG2 cells +/– acetaminophen and CA 5–50uM	*In Vivo* Acetaminophen + caffeic acid 30 ↓ transaminase rise, ROS level and myeloperoxidase vs acetaminophen Acetaminophen + caffeic acid 10 and 30 ↑ liver glutathione vs acetaminophen Acetaminophen + caffeic acid 10 and 30 ↓ histology severity vs acetaminophen *In Vitro* Acetaminophen + caffeic acid 50 ↑ the reduced cell viability Acetaminophen + caffeic acid 10–50 ↓ ROS rise Acetaminophen + caffeic acid 50 ↑ Nrf2 expression and transcriptional activation Acetaminophen + caffeic acid ↔ on control knockout Nrf2 cells and viability Acetaminophen + caffeic acid 50 partially ↑ downstream antioxidative enzymes of Nrf2 (i.e. NADQ1, heme oxygenase1) Acetaminophen + caffeic acid 50 ↓ expression of KEAP1 (=inhibitor of Nrf2 activation) Acetaminophen + caffeic acid ↔ CYP2E1, CYP1A2 and CYP3A4 (=acetaminophen converters)
Pang, 2016[178]	Caffeic acid therapeutic	*In vivo* ICR mice +/– acetaminophen +/– caffeic acid 6 hours *In vitro* L-02 cells, HepG2 cells +/– acetaminophen and CA 5–50uM	*In vivo* Acetaminophen + caffeic acid 10–30 partially ↓ transaminase and MDA rise vs acetaminophen Acetaminophen + caffeic acid 10–30 partially ↓ severity of liver histology vs acetaminophen Acetaminophen + caffeic acid 10–30↓ tissue factor, IL1β and 6 and ↓ Egr1 expression vs acetaminophen Acetaminophen + caffeic acid 10 ↓ serpine1 level vs acetaminophen

Table 2 (*continued*)

Author	Coffee compound	Animal Model	Main study findings
			In Vitro Acetaminophen + caffeic acid 10–50 ↑ cell viability cells vs acetaminophen Acetaminophen + caffeic acid 20–50 ↓ mRNA expression rise in Egr1 vs acetaminophen Caffeic acid 10 ↓ GADD45α mRNA expression (=regulated by Egr1 leading to apoptosis) Acetaminophen ↔ cell viability in siRNA knockdown expression of Egr1 and GADD45α Acetaminophen + caffeic acid 50 ↓ ERK1 and 2 phosphorylation and C-jun N-terminal kinase in L-02 cells vs acetaminophen Acetaminophen + caffeic acid ↓ c-Raf & MEK1/2 phosphorylation (=upstream ERK signals) vs acetaminophen ERK1 and 2 inhibitors ↓ nuclear translocation of Egr1 but C-Jun N-terminal kinase inhibitors ↔ nuclear translocation of Egr1
Ghahhari, 2017[180]	CGA	Mice +/– CGA prior to +/– arsenic trioxide	Arsenic + CGA 10–100 ↓ transaminase rise vs arsenic Arsenic + CGA 10–100 ↑ glutathione
Lee, 2007[183]	Kahweol Cafestol	ICR Mice +/– kahweol or cafestol prior to CCl4 3 days	CCl4 + kahweol 50–100 or cafestol 50–100 dose-dependently ↓ transaminases and MDA rise vs CCl4 CCl4 + kahweol 50–100 or cafestol 50–100 dose-dependently ↑ glutathione content and activity vs CCl4 CCl4 + kahweol 50–100 or cafestol 50–100 dose-dependently ↓ necrosis and hepatic lesions vs CCl4 Kahweol and cafestol dose-dependently ↓ CYP2E1 activity, but ↔ its expression vs controls Kahweol and cafestol dose-dependently ↓ lipid peroxidation (MDA) and superoxide scavenging activity vs controls
Poyrazoglu, 2008[184]	Unfiltered (Turkish) coffee	Sprague- Dawley rats +/– CCl4 +/– Turkish coffee 7 days	CCl4 + Turkish coffee ↑ transaminase and MDA and ↓ serum albumin vs CCl4 CCl4 + Turkish coffee ↑ inflammation and necrosis vs CCl4, Turkish coffee alone ↑ steatosis, inflammation and necrosis vs control
Seo, 2017[185]	Kahweol	*In vivo* C57BL/6 mice +/– TAA +/– kahweol 8 weeks *In vitro* AML-12 cell line, LX2-cells or primary hepatocytes	*In vivo* TAA + kahweol ↓ fibrosis and expression of type I collagen, αSMA, CTGF and TGFβ mRNA vs TAA TAA + kahweol ↓ transaminase rise vs TAA TAA + kahweol ↓ nuclear translocation of Smad2 and 3 (=signalling pathway TGFβ) in nucleus vs TAA TAA + kahweol ↓ STAT3 phosphorylation vs TAA Kahweol ↓ MAPK pathways leading to ERK phosphorylation and C-Jun N terminal protein kinase vs TAA + kahweol *In vitro* Kahweol ↓ TGFβ stimulated type I collagen and CTGF expression Kahweol ↓ TGFβ stimulated Smad3 protein and STAT3 phosphorylation expression Kahweol ↓ TGFβ stimulated Erk and C-Jun N terminal protein expression
He, 2001[186]	CC Caffeine Different tea types	Wistar rats treated CC, Caffeine, Tea or Water 10 days prior to LPS & D-galactosamine	All beverages ↓ LPS-induced enhancement of transaminases Caffeine dose-dependently ↓ LPS-induced enhancement of transaminases

Table 2 (*continued*)

Author	Coffee compound	Animal Model	Main study findings
Ozercan, 2006[187]	Instant CC	Sprague Dawley rats +/– CCl4 +/– instant CC 7days	CCl4 + instant CC ↓ transaminase rise, necrosis and inflammation, but ↔ steatosis vs CCl4 CCl4 + instant CC ↓ serum MDA rise by CCl4, but ↔ MDA hepatic tissue vs CCl4 CCl4 + instant CC restored the lowered total antioxidant capacity vs CCl4
Shi, 2010[188]	Nescafe CC	Sprague Dawley +/– CCl4 +/– CC 8 weeks	CCl4 + CC 300 ↓ fibrosis scores and ↓ necrosis and infiltration of lymphocytes vs CCl4 CCl4 + CC 300 ↓ Collagen I and III, VEGF and TGFβ1 vs CCl4 CCl4 + CC 150–300 ↑ Bax mRNA expression vs CCl4 CCl4 + CC 150 ↓expression of αSMA, GRP 78, and GRP 94 vs CCl4
Shin, 2010[189]	Nestlé CC Nestlé DC Caffeine	*In vivo* Sprague Dawley rats +/– DMN +/– CC 4 weeks *In vitro* rat peritoneal macrophages from Sprague Dawley rats treated with LPS + DC or CC or caffeine	*In vivo* DMN + CC 100–300 dose-dependently ↑ body, liver and spleen weight vs DMN DMN + CC 100–300 ↓ transaminase rise and ↑ albumin decrease vs DMN DMN + CC 300 ↓ MDA vs DMN DMN + CC 100–300 dose-dependently ↓ hydroxyproline and αSMA expression, and ↑ tissue glutathione, SOD and catalase activity and SOD mRNA expression vs DMN DMN + CC 100–300 ↓ hepatocyte necrosis, atrophy, yellow pigment and inflammation vs DMN DMN + CC 100–300 ↓ mRNA expressions of PDGFβ, TGFβ, TNFα, IL1, inducible NOS vs DMN *In vitro* CC and DC ↓ nitrite production through macrophage LPS activation. Caffeine ↔
Abreu, 2011[190]	Coffee extract	Male littermates from pregnant rats given CC or control rats were treated +/– acetaminophen +/– CC	CC +/– acetaminophen ↓ TBARS (= lipid peroxidation) and ↑ glutathione vs controls CC +/– acetaminophen ↓ glutathione peroxidase activity and ↑ glutathione reductase vs controls CC +/– acetaminophen ↑ biotransformation phase II enzymes (GST, UGT1 and UGT2) vs controls
Moreno, 2011[191]	Instant CC Ground CC	Wistar rats +/– CCl4 +/– instant CC or ground CC 8 weeks	CCl4 + instant or ground CC ↓ ALP rise and ↓ TBARS, but ↔ ALT rise vs CCl4 CCl4 + ground CC ↑ glutathione vs CCl4 CCl4 + instant or ground CC partially ↓ hydroxyproline vs CCl4 CCl4 + instant < ground CC partially ↓ collagen, but ↔ necrosis vs CCl4 CCl4 + instant CC partially or ground CC completely ↓ TGFβ protein level, both ↓ TGFβ mRNA rise vs CCl4
Furtado, 2012[192]	CC DC Caffeine	Wistar rats +/– TAA +/– DC or caffeine or CC 8 weeks	TAA + DC or CC or caffeine ↓ ALT rise vs TAA TAA + DC or CC or caffeine ↓ liver injury score + collagen fibres fraction + TGFβ1 protein levels vs TAA TAA + DC or CC or caffeine ↑ reduced glutathione and ↓ oxidized glutathione vs TAA TAA + DC or CC ↓ proliferating cell nuclear antigen rise and ↓development of PNL vs TAA TAA + CC ↓ MMP-2 rise vs TAA, whereas TAA + DC ↑ MMP2 vs TAA

Table 2 (*continued*)

Author	Coffee compound	Animal Model	Main study findings
Arauz, 2013[193]	CC DC	Wistar rats +/– TAA +/– CC or DC 8 weeks	TAA + DC or CC ↓ transaminase and MDA rise vs TAA TAA + DC or CC partially ↑ glutathione peroxidase activity vs TAA TAA + DC or CC partially ↓ hydroxyproline, necrosis, TGFβ, αSMA and CTGF expression rise vs TAA TAA + DC or CC partially ↓ IL10, MMP2, 9, and 13 expression rise vs TAA
Arauz, 2017[194]	CC DC Caffeine	Wistar rats +/– BDL +/– CC or DC or caffeine	BDL + CC or DC ↔ ALT rise, but BDL + caffeine ↓ ALT rise vs BDL BDL + CC or DC or caffeine ↓ alkaline phosphatase and GGT vs BDL BDL + CC or DC or caffeine ↑ glutathione (peroxidase activity) and ↓ glutathione disulphide vs BDL BDL + CC or DC partially ↓ and caffeine completely ↓ MDA vs BDL BDL + CC or DC or caffeine ↓ hydroxyproline and collagenα1 protein expression + mRNA levels vs BDL BDL + CC or caffeine ↓ TGFβ mRNA + protein expression and CTGF, αSMA and IL-10 expression vs BDL
Sugiyama, 2001[195]	Caffeine	Mice injected anti-Fas antibody/TNFα or placebo + force-fed caffeine	Caffeine ↔ transaminase rise by anti-Fas antibody, but ↓ transaminase rise by TNFα Caffeine ↓ DNA fragmentation (i.e. apoptosis)
Chan, 2006[196]	Caffeine	*In vivo* Adenosine A2a/a3 receptor deficient mice and wildtype control + CCl4 or TAA for 9 weeks AND C57BL/6 mice + CCl4 or TAA for 7 weeks + adenosine receptor (ant) agonists *In vitro* LX-2 and HepG2 cells treated with methotrexate or ethanol to generate adenosine	*In vivo* TAA ↑ adenosine A2a receptor expression in wildtype mice Adenosine receptor deficient mice developed no fibrosis and mild transaminase rise upon CCl4 or TAA Caffeine mimicked the protective effect of Adenosine receptor deficient mice *In vitro* Adenosine A2a receptor agonists dose-dependently ↑ collagen production in rat HSC Adenosine A2a agonists ↓ activity and expression of MMP (western blotting) which lead to ↓ collagen degradation and ↑ collagen production by HSC
Klemmer, 2011[198]	Paraxanthine	Sprague Dawley rats underwent sham or BDL +/– paraxanthine 30 days	BDL + paraxanthine ↔ transaminase rise vs BDL BDL + paraxanthine ↓ bile acid toxicity and bridging fibrosis on histology and ↓ MDA vs BDL BDL + paraxanthine ↓ intrahepatic levels CTGF, total SMAD2 and 3 vs BDL, but ↔ serum CTGF level
Gordillo-Bastidas, 2013[199]	Caffeine	Wistar rats +/– TAA or BDL +/– caffeine 7 weeks	BDL + caffeine ↓ transaminase rise vs BDL BDL + caffeine & TAA + caffeine ↓ extracellular matrix, CTGF Collagen I & TGFβ1 vs BDL & TAA BDL + caffeine & TAA + caffeine ↓ fibrosis and necro-inflammation vs BDL & TAA BDL + caffeine & TAA + caffeine ↓ CD11b, IL1β, IL6 and TNFα expression rise vs BDL & TAA BDL + caffeine & TAA + caffeine ↑ SOD expression and catalase activity vs BDL & TAA BDL + caffeine & TAA + caffeine ↑ protein level of Nrf2 vs BDL & TAA BDL + caffeine & TAA + caffeine ↓ Snail-1 (pro-fibrogenic transcription factor) vs BDL & TAA

Table 2 (*continued*)

Author	Coffee compound	Animal Model	Main study findings
Shim, 2013[116]	Caffeine	*In vivo* Sprague Dawley rats +/– TAA +/– caffeine 8 weeks In vitro LX-2 cells	*In vivo* TAA + caffeine ↓ fibrosis and peri-portal inflammation histologically vs TAA TAA + caffeine ↓ αSMA and TGFβ level rise vs TAA *In vitro* Caffeine ↓ cell proliferation, wound healing and wound size and >50% ↑ apoptotic cells Caffeine ↓ F-actin expression and stellate cell adhesion (=involved cell migration & adhesion) Caffeine ↑ intracellular cAMP levels, modulating TGFβ/SMAD-induced expression of ECM Caffeine ↓ αSMA & procollagen I production, time and dose-dependently
Arauz, 2014[200]	Caffeine	Wistar rats +/– TAA +/– caffeine 8 weeks	TAA + caffeine ↓ transaminase and MDA rise vs TAA TAA + caffeine ↑ glutathione peroxidase vs TAA TAA + caffeine ↓ hydroxyproline, TGFβ protein level, αSMA expression and necrosis vs TAA TAA + caffeine ↓ IL10, MMPs, ↔ TIMP and ↓ TGFβ, αSMA and collagen α1 mRNA expression vs TAA
Hsu, 2015[197]	Caffeine	Sprague Dawley rats +/– BDL or TAA +/– caffeine prophylactically or therapeutically 4 weeks	BDL + therapeutic & prophylactic caffeine ↓ portal pressure vs BDL BDL + therapeutic caffeine & prophylactic caffeine ↓ portosystemic shunting and ↓ fibrosis vs BDL BDL + prophylactic caffeine ↓ intrahepatic angiogenesis, endothelial NOS, VEGF vs BDL BDL + prophylactic caffeine ↔ inducible NOS, COX1 and 2 and, p-ERK vs BDL BDL + prophylactic caffeine + adenosine A2a and A1a receptor agonists ↔ on portal pressure and fibrosis TAA + therapeutic caffeine & prophylactic caffeine ↓ portal pressure and ↓ fibrosis vs TAA
Wang, 2015[201]	Caffeine	Sprague-Dawley rats +/– ethanol +/– caffeine 8 or 12 weeks	Ethanol + caffeine ↓ transaminase and serum level fibrosis markers (hyaluronic acid, N-terminal procollagen type III, laminin, and type IV collagen) vs ethanol Ethanol + caffeine ↓ steatosis and inflammatory necrosis and ↓mRNA expression of procollagen I and III vs ethanol Ethanol + caffeine 20 ↓ collagen fiber and caffeine 10 and 20 ↓ expression of αSMA vs ethanol Ethanol + caffeine dose-dependently ↓ cAMP vs ethanol
Amer, 2017[202]	Caffeine	Albino rats +/– TAA +/– caffeine 8 weeks	TAA + caffeine ↓ transaminase and MDA rise vs TAA TAA + caffeine ↓ TNFα, IL-1β and IL-6 and TBARS vs TAA TAA + caffeine ↑ glutathione and glutathione peroxidase activity and SOD of cytoplasm vs TAA TAA + caffeine ↓ collagen deposition, MMP-9 and collagen type IV histologically vs TAA
Cachón, 2017[203]	Caffeine	Wistar rats +/– CCl4 +/– caffeine 10 weeks	CCl4 + caffeine 5–10 or N-acetyl cysteine ↓ relative liver weight vs CCl4 CCl4 + caffeine 5–10 or N-acetyl cysteine ↓ transaminase rise, (un) conjugated bilirubin vs CCl4 CCl4 + caffeine 5–10 or N-acetyl cysteine ↑ glutathione vs CCl4 CCl4 + caffeine 5–10 or N-acetyl cysteine ↓ fibrosis, but ↔ steatosis, inflammation and collagen level

Table 2 (*continued*)

Author	Coffee compound	Animal Model	Main study findings
Mohamed, 2017[204]	Caffeine	Wistar rats +/– CCl4 +/– caffeine 5 weeks	CCl4 + caffeine or N-acetyl cysteine ↓ fibrosis markers (i.e. TGFβ1 + hydroxyproline) vs CCl4 CCl4 + caffeine or N-acetyl cysteine ↓ inflammatory markers (TNFα + myeloperoxidase) vs CCl4 CCl4 + caffeine or N-acetyl cysteine ↓ relative liver weight vs CCl4 CCl4 + caffeine or N-acetyl cysteine transaminase, bilirubin, and MDA, and ↑ albumin vs CCl4 CCl4 + caffeine or N-acetyl cysteine ↑ glutathione + catalase activity vs CCl4 CCl4 + caffeine partly ↓ dilated congested blood vessel and activated Von Kuppfer cells
Eraky, 2018[205]	Caffeine	Sprague-Dawley rats +/– TAA +/– caffeine and/or silymarin 8 weeks	TAA + caffeine or silymarin or both ↑ albumin and ↓ transaminase and bilirubin rise vs TAA TAA + caffeine or silymarin but most in both ↓ inflammation and necrosis vs TAA TAA + caffeine or silymarin or both ↓ fibrosis% vs TAA TAA + caffeine or silymarin or both ↓ TGFβ1, CTGF and αSMA mRNA expressions vs TAA

Abbreviations ALT: alanine transferase, BDL: bile-duct ligation, CC: conventional coffee, CCl4: carbon tetrachloride, CCR: C-C chemokine receptor, CD: cluster differentiation, CGA: chlorogenic acid, COX: Cyclo-oxygenase, CTGF: connective tissue growth factor, CYP: cytochrome P, DC: decaffeinated coffee, DMN: dimethylnitrosamine, ECM: extracellular matrix, EGR: early growth response, ERK: extracellular signal-regulated protein kinase, GADD: growth arrest and DNA damage inducible gene, GGT: γ-glutamyl transferase, GST: glutathione S-transferase, IL: interleukin, KEAP: Kelch-like ECH-associated protein, LPS: lipopolysaccharide, MDA: malondialdehyde, MMP: matrix metalloproteinase, MyD: Myeloid differentiation primary response, NOS: nitric oxidase synthase, Nrf: Nuclear factor (erythroid-derived)-like, PDGF: platelet derived growth factor, ROS: reactive oxygen species, SMA: smooth muscle antigen, SOD: superoxidase dismutase, STAT: signal transducer activator of transcription, TAA: thioacetamide, TBARS: thiobarbituric acid reactive substances, TGF: tissue growth factor, TIMP: tissue inhibitor matrix metalloproteinase, TLR: toll-like receptor, TNF: tumour necrosis factor, UCT: uncoupling protein glucuronosyl transferase, VEGF: vascular endothelial growth factor.

IL-1β, IL-6, and TNFα. The authors hypothesized that the protective effect of CGA could be mediated via the inhibition of the toll-like receptor 4/ myeloid differentiation factor 88/nuclear factor κ of activated B-cells (TLR4-MyD88-NFκB) signalling pathway, as TLR4 activates pro-inflammatory producing Kupffer cells, HSCs, and liver endothelial cells.[177] NFκB additionally induces cyclo-oxygenase (COX)2 expression, which is involved in HCC development.[182]

Diterpenoids are fairly well studied in fibrosis.[183-185] Lee and colleagues first studied diterpenoids in CCl4-induced liver damage. Both kahweol and cafestol attenuated the CCl4-induced rise in transaminases and MDA. In addition, the diterpenoids-treated mice appeared protected from glutathione depletion, showed less necrosis and less hepatic lesions. The authors believed these effects were mediated via the reduction of CYP2E1 activity, an enzyme that induces liver injury upon CCl4 administration.[183] The elegant in vivo/in vitro study of Seo et al. examined kahweol administration in thioacetamide (TAA)-poisoned

mice.[185] They ascribed the transaminase and fibrosis lowering capacity of kahweol to the downregulation of TGFβ, the master pro-fibrogenic cytokine, and thereby downregulation of HSC activation and stimulating proteins involved in CTGF expression in vitro (i.e. Smad 2 and 3, signal transducer activator of transcription 3, ERK, and C-Jun N terminal protein).[185] Paradoxically there was one study that showed a deleterious effect of Turkish (unfiltered, diterpenoid-rich) coffee on fibrosis, inflammation, and lipid peroxidation even without exposure to an exogenous toxin, suggesting a potential toxic effect of this type of coffeep.[184] This study is in contrast to all other studies on whole CC or DC and fibrosis that did not find a direct toxic effect.[186-194]

Many studies found comparable effects of CC and DC on fibrosis in presence of various exogenous toxins.[189,192,193] This hepatoprotective effect included increased glutathione levels, reduced fibrosis on histology, decreased hydroxyproline levels (a proxy of total collagen content), reduced protein expression of αSMA, and lowered mRNA expression of TGFβ. In addition, these studies showed that both CC and DC reduced lipid peroxidation (as assessed by MDA), and inflammation (i.e. reduced IL 1 and 10, inducible nitric oxidase synthase, and TNFα). Only two studies found a more pronounced effect of CC than DC[192,194] on fibrosis, specifically they found attenuated matrix metalloproteinases 2 (MMP; involved in degradation of extracellular matrix), upon TAA administration in the CC-treated arm.[192] In addition, four studies on instant CC, ground CC or coffee extract, demonstrated similar results via similar pathways, i.e. I) increased glutathione, II) decreased collagen content assessed by hydroxyproline, histology, or collagen and αSMA expression, and III) decreased lipid peroxidation assessed by MDA or thiobarbituric acid reactive substances.[187,188,190,191] One of these studies suggested that apoptosis played a prominent anti-fibrogenic role, while pro-apoptotic Bax mRNA was upregulated, and anti-apoptotic Bcl-2 mRNA was downregulated after whole coffee administration in CCl4 poisoned rats.[188]

Sugiyama et al. first studied the effect of caffeine-only on TNFα-intoxicated mice. The authors found lowered transaminases and less DNA fragmentation upon caffeine treatment and hence concluded that caffeine had hepatoprotective characteristics, possibly mediated via induction apoptosis.[195] The in vivo/in vitro study of Chan and colleagues, nicely established a plausible mechanistic explanation as to how caffeine could prevent fibrosis.[196] The authors showed that adenosine A-deficient mice did not develop fibrosis upon TAA or CCl4 administration, and that caffeine mimicked these effects. Indeed, caffeine was found to act as non-selective adenosine A(2A) receptor antagonist, suppressing collagen production. Hsu et al. examined caffeine in bile duct ligated rats and found attenuated fibrosis, lowered portal pressure and less portosystemic shunting.[197] Caffeine here acted again as an antagonist of adenosine receptor A2a and A1a, as the presence of a competing adenosine agonist attenuated the results. Later studies all confirmed the anti-fibrotic properties of caffeine as assessed by histology or liver biochemistry.[116,197-205] Most studies explained this by the inhibition of TGFβ and the subsequent decreased αSMA and CTGF

expression upon caffeine treatment.[116,199,200,204,205] Also, caffeine decreased MDA (i.e. lipid peroxidation), increased superoxidase dismutase and glutathione concentration.[199,200,202-204] Gordillo-Bastidas and colleagues treated rats with either bile duct ligation or TAA, and discovered that caffeine decreased transcriptional factor Snail-1 (important in the activation of HSCs) and increased Nrf2.[199] Another elegant in vivo/in vitro study suggested that the anti-fibrogenic activity of caffeine was due to its apoptosis-inducing capacity[116] as caffeine decreased cell viability, reduced cell adhesion and proliferation, decreased the wound healing capacity, and increased the apoptotic cell count by over 50%. Lastly, Klemmer et al. examined the specific effect of paraxanthine, the most effective and least toxic metabolic methylxanthine of caffeine[206] in bile duct ligated rats and found lower transaminases and less bridging fibrosis.[198]

Hepatocellular Carcinoma

Stich and colleagues first described coffee as anti-mutagenic substance and ascribed this effect to the polyphenolic compounds and their ability to reduce the formation of mutagenic units.[207] Subsequently, Mori et al. assessed the effect of CGA on methylazoxymethanol-induced carcinogenesis in hamsters and found significantly less liver cell foci in CGA-treated hamsters (*Table 3*).[208] Decades later, the anti-mutagenic capacity of CGA was examined again in an in vivo/in vitro study of Yan et al.[209] In this study, intraperitoneal CGA administration reduced liver tumour volume and weight in HepG2 xenografts in vivo. The authors found a decreased phosphorylation of ERK1/2, possibly due to reduced mitogen activated protein kinase activation in vitro. In addition, hepatic MMP-2 was lowered, but as tissue inhibitors of MMP (TIMP) were not affected, MMP-2/TIMP ratio was decreased, leading to prevention of extracellular matrix degradation and tumour inhibition.

Diterpenoids are well-studied coffee compounds in carcinogenesis.[210-214] Schilter et al. studied the effect of diterpenoids in rats without administration of an exogenous toxin.[210] The authors found a dose-dependent increase of glutathione S-transferases (GST)-placental form. GSTs are glutathione catalyzing enzymes and induction of GST has been associated with reduced carcinogenesis.[215] The GST-inducing capacity of kahweol and cafestol, with[211] or without[212-214] presence of an exogenous toxin, was confirmed by later studies. In addition, Cavin et al. found a 50% decrease in DNA adduct formation after diterpenoid administration, potentially leading to fewer mutations in proto-oncogenes and tumour suppressor genes.[211] Lastly, mRNA expression of carcinogen-detoxifying phase II mechanisms (CYP450[211] and sulfotransferase 1A1)[214] was decreased upon diterpenoid treatment but not after unfiltered coffee administration. Moreover, CYP2B2 and CYP1A1 activities were increased upon both filtered and unfiltered CC in this study.[213]

Ten studies on whole coffee consumption in relation to hepatic carcinogenesis were conducted,[216-219] of which six specifically looked into tumour morphology.[220-225] Only Hasegawa

Table 3: Experimental evidence on coffee effects on carcinogenesis

Author	Coffee compound	Animal Model	Main study findings
Mori, 1986[208]	CGA	Syrian golden hamsters +/– methylazoxymethanol +/– CGA 24 weeks	Methylazoxymethanol + CGA ↓ adenocarcinoma in intestine vs methylazoxymethanol ↔ liver tumours Methylazoxymethanol + CGA ↓ liver cell foci vs methylazoxymethanol
Yan, 2017[209]	CGA	*In vivo* Nude mice with HepG2 xenograft + placebo or CGA 6 weeks *In vitro* hepG2 cells	*In vivo* CGA 30 and 60 ↓ tumour volume and tumour weight vs placebo CGA ↓ phosphorylation of ERK 1/2 vs placebo CGA ↓ MMP2 and 9, but ↔ TIMP2: hence, ↓ MMP-2/TIMP2 ratio vs placebo *In vitro* CGA 250uM ↔ cell viability, but ↓ CGA 500uM cell viability (50%)à inhibiting in vitro proliferation CGA ↓ MAPK activation of ERK1/2 CGA ↓ MMP2, but ↔ MMP-9 and TIMP2. Hence ↓ MMP-2/TIMP2 ratio
Schilter, 1996[210]	Kahweol Cafestol	Sprague-Dawley rats + kahweol and cafestol 90 days	Kahweol + cafestol ↔ GSTα and GSTμ activity, but doses 2300 and 6200 ↑ GST-P mRNA expression, protein and enzymatic level Results were reversible after 1 month withdrawal
Cavin, 1998[211]	Kahweol Cafestol	Sprague Dawley rats + aflatoxin B1 +/– placebo or kahweol and cafestol 90 days	Aflatoxin B1 + kahweol + cafestol dose-dependently ↓ aflatoxin B1 DNA-binding Aflatoxin B1 + kahweol + cafestol 2300 and 6200 ↓ DNA adduct formation (50%) vs aflatoxin B1 Aflatoxin B1 + kahweol + cafestol ↓ CYP450 (i.e. CYP2C11, CYP3A2), but ↔ CYP1A1 & CYP1A2 expression vs aflatoxin B1 Aflatoxin B1 + kahweol + cafestol ↑ GST subunit Yc2 vs aflatoxin B1
Huber, 2002[212]	Kahweol Cafestol	F344 rats + control or kahweol and cafestol 10 days	Kahweol + cafestol ↑ UGT 9-fold Kahweol + cafestol ↑ GST 3-fold (all subclasses, including GST-θ for the first time)
Huber, 2004[213]	Kahweol Cafestol	F344 rats + kahweol and cafestol 10 days	Kahweol + cafestol dose-dependently ↓ N-acetyltransferase activation in the liver, but reversible upon withdrawal Kahweol + cafestol dose-dependently ↑ GST-μ, GST-α, and GST-4VP in the liver, but reversible upon withdrawal
Huber, 2008[214]	Kahweol Cafestol Turkish coffee Paper-filtered CC	F344 rats + control or kahweol and cafestol or filtered CC or Turkish coffee 10 or 20 days	Kahweol + cafestol ↓ hepatic CYP450 metabolism/mRNA (CYP1A1/2 and CYP2B1/2), but ↔ CYP2E1 vs controls Kahweol + cafestol ↓ sulfotransferase 1A1, Turkish coffee ↔ sulfotransferase 1A1 vs controls
Higgins, 2008[216]	CC	*In vivo* C57BL/6 mice and DBA/20 mice, wild type (Nrf2+/+ and Nrf2-/-) + CC 5 days *In vitro* Mouse embryonic fibroblasts Nrf2 -/- and Nrf2+/+	*In vivo* Nrf2 -/- ↓ NQO1 protein and ↓ CYP1A2 mRNA vs Nrf2+/+ Nrf2+/+ & coffee 3% or 6% dose-dependently ↑ hepatic NQO1 protein vs Nrf2+/+ Nrf2 -/- & coffee 6% ↑ hepatic NQO1 protein vs Nrf2-/- Nrf2+/+ & coffee 3% or 6% ↑ GSTα1/2 and GSTα4 vs Nrf2+/+ Nrf2 -/- & coffee ↔ GST α1/2 or α4 vs Nrf2-/- Nrf2+/+ ↓ UGT1A6 expression vs Nrf2-/- Coffee ↔ effect UGT1A6 Nrf2 +/+ & coffee 6% ↑ CYP1A2 mRNA vs Nrf2+/+

Table 3 (*continued*)

Author	Coffee compound	Animal Model	Main study findings
			In vitro Kahweol + cafestol ↑ NQO1 induction on antioxidant response elements Nrf2+/+ & kahweol + cafestol ↓ acrolein toxicity Nrf2+/+ & CGA or Nrf2-/- ↔ acrolein toxicity
Morii, 2009[217]	Instant CC	ICR Mice +/– low vitamin diet +/– CC 2, 4, and 8 months	↔ hydrogen peroxide deleting capacity, SOD activities and MDA Low vitamin + CC ↓ 8-hydroxydeoxyguanosine activity but ↔ gene expression vs low vitamin + water Low vitamin + CC ↓ glutathione peroxidase expression vs low vitamin + water
Kalthoff, 2010[218]	Filtered CC Undiluted 12% CC Metal-filtered Paper-filtered CC DC Instant CC Boiled CC Cocoa Teas	*In vivo* humanized transgenic mice (human UGT1A-expression) +/– undiluted CC 3 days *In vitro* HepG2 cells	*In vivo* Undiluted CC ↑ UGT1A1 liver vs controls *In vitro* filtered, instant, boiled CC/DC ↑ UGT1A1, UGT1A7, UGT1A10 activity & protein expression vs controls (caffeine-independent) CCs and DC ↓ effect on UGT1A1 and UGT1A7 in genetic variants vs wild-type constructs UGT1A siRNA knockdown ↔ on proliferation Coffee compounds caffeine, paraxanthine, theobromine, theophylline, kahweol and cafestol ↔ UGT1A1, 1A10 or 1A7 CC and DC ↔ UGT1A1, UGT1A7 and UGT1A10 induction upon siRNA aryl-hydrocarbon receptor and Nrf2 knockdown CC and DC ↑ UGT1A genes by aryl-hydrocarbon receptor and Nrf2 signaling (transcriptional level) vs controls
Pietrocola, 2014[219]	Instant CC, Instant DC	C57BL/6 Mice and transgenic C57BL/6 mice expression fusion protein GFP-LC3 "long-term" design CC for 2 weeks "short-term" design CC or DC max 6h	CC and DC ↑ autophagic flux (lipidation of LC3) à ↓ overall abundance of the autophagic substrate sequestome-1 CC and DC ↑ phosphorylation cAMPK in short-term, but ↓ phosphorylation cAMPK long-term CC and DC ↓ phosphorylation of mTORC1 substrates both short and long-term
Hasegawa, 1995[220]	Instant CC Instant DC Brewed CCCaffeine Theophylline Theobromine	F3444 rats + DEN or saline injection +/– Instant CC or brewed CC or caffeine or theophylline or theobromine 6 weeks	Theophylline was the only component causing toxic effects DEN + instant CC or DC or Brewed CC ↔ liver foci frequency and area
Miura, 2004[221]	Instant CC	*In vivo* Donryu rats subcutaneously implanted with cells of AH109A in the back +/– instant CC 14 days *In vitro* AH109A cells or rat ascites hepatoma cell line + instant CC	*In vivo* Instant CC ↓ tumour growth speed and size vs controls Instant CC ↓ hepatoma weight vs controls and ↓ metastasis (0/9 vs 3/11) in controls Instant CC ↓ serum TBARS (indicating anti-oxidant capacity) vs controls *In vitro* Instant CC 0.3 mg/mL ↑ cells in S-phase à ↓ cells in the G1 phase and G2/M phase, and ↑ DNA fragmentation Instant CC 0.3 mg/mL ↓ intracellular peroxide level
Silva-Oliveira, 2010[222]	Roasted CC	Rats of which 50% was lactated from a coffee drinking mum treated +/– 2AAF and DEN and partial hepatectomy +/– CC	Toxins + CC ↔ hepatic tissue morphologically vs toxins + water Toxins + CC ↓ number of premalignant, persistent and remodelling lesions vs toxins + water

Table 3 (*continued*)

Author	Coffee compound	Animal Model	Main study findings
Ferk, 2014[223]	Metal-filtered CC Paper-filtered CC Metal-filtered DC	Sprague Dawley rats +/– aflatoxin B1 +/– metal-filtered CC or DC, or paper-filtered CC	Aflatoxin + metal/paper filtered CC dose-dependently ↓ DNA migration, aflatoxin + DC ↔ DNA migration vs aflatoxin Aflatoxin + caffeine pure dose-dependently ↓ DNA migration vs aflatoxin Aflatoxin + metal/paper filtered CC ↓82% or Aflatoxin + DC 57% ↓ number of liver foci vs aflatoxin Aflatoxin + metal/paper filtered CC or DC ↑ UGTs, but ↔ glutathione
Furtado, 2014[224]	Brewed CC Instant CC Caffeine	Wistar rats DEN + CCl4 +/– instant or brewed CC or caffeine 0.1% 23 weeks	Toxin + instant CC or caffeine ↓ number of neoplastic lesions per liver area vs toxin Toxin + instant CC or caffeine ↑ number of small preneoplastic lesions and ↓ number of large preneoplastic lesions vs toxin Toxin + instant/brewed CC or caffeine ↓ PCNA and ↑ GST-P vs toxin Toxin + brewed CC or caffeine ↓ liver collagen fibres, collagen I and III mRNA expression vs toxin Toxin + instant CC or caffeine ↑ Bax, but ↔ Bcl-2 and TGFβ protein levels vs toxins
Katayama, 2014[225]	Nestlé CC	Long-Evans Cinnamon rats +/– CC 27 weeks	CC ↓ glutamic pyruvic transaminase activity up to week 19, then ↔ vs controls ↔ copper and iron accumulation in liver and ↔ CTGF, TGFβ and Smad2 mRNA expression CC ↓ expression of inflammatory cytokines IL6 and TNFα and ↓ small GST-positive premalignant foci vs controls
Fujise, 2012[226]	Caffeine	Wistar rats +/– DEN +/– caffeine 14 weeks	DEN + caffeine ↓ HCC and number/size of lesions and ↓ PCNA positive cells vs DEN. All rats survived DEN + caffeine ↓ GST vs DEN
Hosaka, 2001[227]	Caffeine	ACI Male rats +/– 2AAF +/– caffeine12 weeks	2-AAF + caffeine 0.1%: 2 deaths, 2-AAF: 1 death 2-AAF + caffeine dose-dependently ↓ HCC-type, incident, and number of tumours vs 2-AAF

Abbreviations 2-AAF: 2-acetylaminofluorene, AMPK; adenosine monophosphate-activated protein kinase, CC: conventional coffee, CGA: chlorogenic acid, CTGF: connective tissue growth factor, CYP: cytochrome P, DC: decaffeinated coffee, DEN: diethylnitrosamine, ERK: extracellular signal-regulated protein kinase, GST: glutathione S-transferase, HCC: hepatocellular carcinoma, LC: lipidation of microtubule-associated protein light-chain, MDA: malondialdehyde, MAPK: mitogen activated protein kinase, MMP: matrix metalloproteinase, mTOR: mammalian target of rapamycin, NQO: NAD(P)H:quinone oxidoreductase 1, Nrf: Nuclear factor (erythroid-derived)-like, PCNA: proliferation cell nuclear antigen, SOD: superoxidase dismutase, TBARS: thiobarbituric acid reactive substances, TGF: tissue growth factor, TIMP: tissue inhibitor matrix metalloproteinase, TNF: tumour necrosis factor.

et al. found no difference in the frequency and location of diethylnitrosamine-induced tumours in coffee-treated rats.[220] All other studies found either reduced tumour frequency or size, upon coffee consumption in rats with endogenously or exogenously induced carcinogenesis.[221-225] Furtado et al. identified higher expression of pro-apoptotic Bax proteins in rats treated with instant coffee or caffeine, suggesting apoptosis-mediated protection against liver carcinogenesis.[224] This apoptosis-mediated hypothesis was supported by a preceding

in vivo/in vitro study on rats implanted with a hepatoma cell line treated with instant CC. In this study, instant CC had the ability to induce cell cycle arrest and lower DNA fragmentation, and therefore, apoptosis.[221] Another in vivo/in vitro study showed a coffee-mediated upregulation of genes involved in chemoprevention, i.e. nicotinamide adenine dinucleotide (phosphate) quinone oxidoreductase 1 and GSTα1-2 and α4, particularly in Nrf2-possessing mice.[216] In addition, CYP1A2 mRNA was upregulated upon high coffee administration in these mice. Nrf2-deficient mice exhibited lower UDP glucuronosyltransferase (UGT)-1A6 expression (proteins with indirect antioxidant and chemo-protective properties), and this was not affected by coffee treatment. These UGTs were studied in more detail by Kalthoff and colleagues, who found upregulation of these proteins regardless of coffee type in vitro.[218] The antiproliferative effect of coffee was attributed to this protein, as supported by UGT1A siRNA knockdown experiments. The authors hypothesized a synergistic effect of the combined components, as the compounds caffeine, paraxanthine, and diterpenoids alone had no effect on UGT upregulation. Most studies comparing CC with DC found similar anti-carcinogenic effects,[218-220] only Ferk et al. found a dose-dependent decrease of DNA migration in CC and caffeine, but not in DC. In addition, the number of liver foci decreased with 82% in CC-treated mice as compared to only 57% in DC-treated mice.[223] Two studies specifically examined the effect of caffeine on liver carcinogenesis and found a protective effect in terms of hepatocellular carcinoma incidence and number of lesions.[226,227] Of note, mortality rate was slightly higher in the caffeine-treated group as compared to the control group, suggesting a possible toxic effect of caffeine itself.[227]

Human experimental studies

To the best of our knowledge, only five experimental studies on coffee consumption and liver health have been conducted in humans so far. Boekschoten et al. conducted two trials in which healthy volunteers were given unfiltered coffee or coffee oils, high in cafestol. Both trials showed an increase in serum transaminases (with a large inter-individual variance) and a slight decrease in GGT and alkaline phosphataseoek.[228,229] Bichler et al. performed an experimental study with coffee (2/3 paper-filtered, 1/3 metal-filtered) on healthy volunteers. DNA-damage caused by reactive oxygen radical treatment in these individuals was found to be strongly reduced after coffee consumption but not after consumption of diterpenoids alone. Also, there was an upregulation of antioxidant enzymes, in particular superoxide dismutase.[230] Shaposhnikov and colleagues completed a placebo controlled intervention trial in 160 healthy volunteers administering water, three, or five cups of coffee per day during eight weeks. The authors did not find an effect of coffee on liver biochemistry (except for a small increase in GGT) nor on biomarker assays for oxidative stress or inflammation.[231] Finally, a recent randomized clinical trial was conducted among 44 patients with steatosis who were given green coffee bean extracts or placebo for eight

weeks. The coffee extracts significantly improved serum transaminases, lipid profile, insulin sensitivity and total antioxidant capacity as compared to placebo. However, steatosis was not affected by the coffee extract.[232]

Summary, conclusion and future perspectives

Over the past decades, both epidemiological and experimental evidence has accumulated on the hepatoprotective effect of coffee. In vivo studies using different experimental models to induce liver disease found beneficial effects of the various individual compounds of coffee as well as of whole coffee.

Several molecular pathways on the anti-steatotic, anti-fibrotic and anti-carcinogenic effects of coffee have been postulated. In *Figure 1* we graphically summarized the most important pathways. Briefly, the steatosis-lowering effect of coffee mainly resulted in decreased FAS

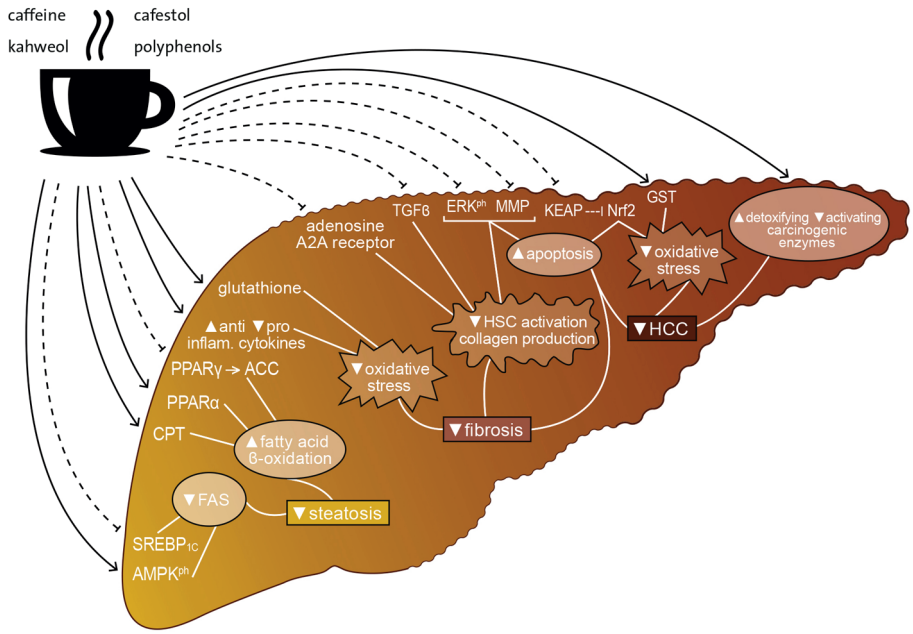

Figure 1: Graphical summary of potential mechanisms underlying the role of coffee in liver health

Simplified illustration of potential mechanisms of action underlying the role of coffee in liver health, specifically in steatosis, fibrosis and hepatocellular carcinoma.

Symbols ▲increasing; ▼decreasing; ➔stimulating; --I blocking.

Abbreviations ACC: acetyl-CoA carboxylase, AMPK: adenosine monophosphate-activated protein kinase, CPT: carnitine palmitoyltransferase, ERK: extracellular signal-regulated protein kinase, FAS: fatty acid synthesis, GST: glutathione S-transferase, HCC: hepatocellular carcinoma, HSC: hepatic stellate cell, KEAP: Kelch-like ECH-associated protein, MMP: matrix metalloproteinase, Nrf: Nuclear factor (erythroid-derived)-like, Ph: phosphorylated PPAR: peroxisome proliferator-activated receptor, SREBP: sterol element binding transcription factor, TGF: transcription growth factor

and increased fatty acid β-oxidation. Also, hepatic glutathione content was increased upon coffee administration, leading to reduced oxidative stress, and hence less steatosis and fibrosis. Coffee-induced inhibition of liver fibrosis mainly included the lowering of TGFβ and the antagonizing effect of caffeine specifically, on adenosine A2A receptors, inhibiting hepatic stellate activation. Both fibrosis and carcinogenesis were inhibited through upregulation of Nrf2, decreased ERK1/2 phosphorylation and increased apoptosis. In addition the balance between detoxifying and activating carcinogenic enzymes was altered by coffee consumption, resulting in reduced tumour growth. The mechanisms of action per coffee compound are depicted more comprehensively in *Table 4*.

Beyond the mechanisms described above, there is some evidence that coffee consumption may influence liver health via the gut microbiome, by modulation of gut microbiota[233-235] or by maintaining intestinal balance.[234]

That all said, it is important to realize that the observed hepatoprotective effects in experimental models cannot be directly translated to the human situation. Studies are largely heterogeneous in using many differences in doses, ways of administration, and coffee constitutes.[236] Similarly, there are the obvious differences between man and mice, and

Table 4: Proposed mechanisms of action per coffee compound

Compound	Mechanism of Action	Effect
Polyphenols	↑ glutathione	
	↑ fatty acid β oxidation	
	↓ C-C chemokine Receptor 2	anti-steatogenic
	↑ anti-inflammatory, ↓ pro-inflammatory cytokines	
	↓ mRNA expression PPARγ and fatty-acid binding protein	
	↑ mRNA expression of FGF21 and CPT1	
	↓ ROS formation	
	↓ lipid peroxidation	
	↓ collagen content	anti-fibrotic
	↓ KEAP -> ↑ Nrf2 -> ↑ phase II antioxidative enzymes	
	↓ TLR4-MyD88-NFkB -> ↓ pro-inflammatory cytokines	
	↓ MAPK activation -> ↓ ERK phosphorylation -> ↓ pro-inflammatory cytokines & ↑ cell viability	anti-fibrotic & anti-carcinogenic
	↓ MMP2/TIMP ratio	anti-carcinogenic
Diterpenoids	↑ glutathione	
	↓ TGFβ	anti-fibrotic
	↓ CYP2E1 activity	
	↑ GST	anti-fibrotic & anti-carcinogenic
	↓ carcinogen-activating enzymes	anti-carcinogenic
	↓ DNA adduct formation	
Whole coffee	↓ PPARγ -> ↓ ACC & ↓ fatty-acid binding protein	anti-steatogenic

Table 4 (*continued*)

Compound	Mechanism of Action	Effect
	↓ pro-inflammatory cytokines	
	↑ glutathione	
	↓ TGFβ	anti-fibrotic
	↓ collagen content	
	↓ MMP2	
	↓ anti-apoptotic Bcl-2, ↑ pro-apoptotic Bax	anti-fibrotic & anti-carcinogenic
	↑ Nrf2	anti-carcinogenic
	↑ phase II carcinogen-detoxifying enzymes	
Caffeine	↓ SREBP-1c	
	↓ ACC	
	↓ mRNA expression CPT2	anti-steatogenic
	↓ pro-inflammatory cytokines	
	↑ fatty acid β oxidation	
	↑ apoptosis	anti-steatogenic & anti-fibrotic & anti-carcinogenic
	↓ adenosine A2A receptor	
	↓ lipid peroxidation	
	↓ collagen content	
	↑ glutathione	anti-fibrotic
	↓ Snail -> ↓ TGFβ	
	↓ pro-inflammatory cytokines	
	↑ Nrf2	
	↑ SOD expression	
	↑ GST	anti-carcinogenic

Abbreviations ACC: acetyl-CoA carboxylase, CPT: carnitine palmitoyltransferase, CYP: cytochroom P, ERK: extracellular signal-regulated protein kinase, FGF: fibroblast growth factor, GST: glutathione S-transferase, KEAP: Kelch-like ECH-associated protein, MAPK: mitogen activated protein kinase, MMP: matrix metalloproteinase, Nrf: Nuclear factor (erythroid-derived)-like, PPAR: peroxisome proliferator-activated receptor, ROS: reactive oxygen species, SOD: superoxidase dismutase, SREBP: sterol element binding transcription factor, TGF: tissue growth factor, TIMP: tissue inhibitor matrix metalloproteinase; TLR4-MyD88-NFκB: toll-like receptor 4/ myeloid differentiation factor 88/nuclear factor κ of activated B-cells

between real life circumstances and laboratory conditions. Likewise, although different coffee compounds seem to all contribute to the observed effects on liver health, specific recommendations on using whole coffee or these compounds separately to treat human diseases cannot be made as of yet. Moving forward however, data from these experimental animal models can be used to design human phase I studies, examining bioavailability and dose-response relation of the various compounds, and eventually well-conducted clinical trials using standardized amounts and preparation methods of coffee. Ultimately this may then lead to clinical recommendations on the optimal preparation method of coffee, and on the optimal amount of coffee to benefit the liver.

Part III

DIET & NON-ALCOHOLIC FATTY LIVER DISEASE

MEDITERRANEAN FOO

Chapter 5

Association of dietary macronutrient composition and non-alcoholic fatty liver disease in an aging population: The Rotterdam Study

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Abstract

Introduction A healthy lifestyle is the first-line treatment in NAFLD, but specific dietary recommendations are lacking. Therefore, we aimed to determine whether dietary macronutrient composition is associated with NAFLD.

Methods Participants from the Rotterdam Study were assessed on 1) average intake of macronutrients (protein, carbohydrate, fat, fibre) using a Food Frequency Questionnaire, and 2) NAFLD presence using ultrasonography, in absence of excessive alcohol, steatogenic drugs, and viral hepatitis. Macronutrients were analysed using the nutrient density method and ranked (Q1-Q4). Logistic regression analyses were adjusted for sociodemographic, lifestyle, and metabolic covariates. Moreover analyses were adjusted for and stratified by BMI (25kg/m²). Also, substitution models were built.

Results In total, 3882 participants were included (age 70±9, 58% female). NAFLD was present in 1337 (34%) participants of whom 132 were lean and 1205 overweight. Total protein was associated with overweight NAFLD after adjustment for sociodemographic and lifestyle covariates ($OR_{Q4vs.Q1}$ 1.40; 95%CI 1.11–1.77). This association was driven by animal protein ($OR_{Q4vs.Q1}$ 1.54; 95%CI 1.20–1.98). After adjustment for metabolic covariates, only animal protein remained associated with overweight NAFLD ($OR_{Q4vs.Q1}$ 1.36; 95%CI 1.05–1.77). Mono and disaccharides were associated with lower overall NAFLD prevalence (OR_{Q4vsQ1} 0.66; 95%CI 0.52–0.83) but this effect diminished after adjustment for metabolic covariates and BMI. No consistent associations were observed for fat subtypes or fibre. There were no substitution effects.

Conclusion This large population-based study shows that high animal protein intake is associated with NAFLD in overweight, predominantly aged Caucasians, independently of well-known risk factors. Contrary to previous literature, our results do not support a harmful association of mono and disaccharides with NAFLD.

Introduction

Since the first case description by Ludwig et al. in 1980, the prevalence of non-alcoholic fatty liver disease (NAFLD) increased expeditiously paralleling the obesity epidemic.[13,237] NAFLD is characterised by fat deposition in the liver in absence of excessive alcohol consumption or established liver disease.[13] It is referred to as the hepatic manifestation of the metabolic syndrome, and has now become the most common liver disease affecting an estimated one-third of adults in the general population of developed countries.[6] In high-risk populations with type 2 diabetes and metabolic comorbidities, prevalence of NAFLD even reaches up to 70%.[238] Progression of NAFLD can lead to fibrosis, cirrhosis, hepatocellular carcinoma and liver failure with corresponding life-threatening complications. The end-stage disease often requires liver transplantation. Indeed NAFLD already constitutes the second most common indication for liver transplantation in the United States and is predicted to become the number one indication soon.[22] In addition to the above-mentioned, NAFLD also contributes to an increased risk for metabolic and cardiovascular morbidity and mortality.[239] Hence, NAFLD has emerged as a great global health threat and subsequently, prevention and treatment thereof are of strong public interest.

NAFLD is more common in people with an unhealthy lifestyle, i.e. with an unhealthy diet and physical inactivity.[13] Although there are several hundreds of promising pharmacological trials ongoing, there is still no registered drug for the treatment of NAFLD. Therefore, in daily practice, lifestyle modification remains the first-line treatment in NAFLD.[55] At present, weight loss of 5–7% or more is recommended, based on two prospective (randomised controlled) trials in overweight patients.[240,241] However, not all overweight individuals will develop NAFLD, and likewise, not all individuals with NAFLD are overweight.[242] This gives food for thought on whether dietary quality, rather than dietary quantity, is important in the pathogenesis and treatment of NAFLD. Current dietary recommendations include caloric restriction and adherence to the macronutrient composition of the Mediterranean diet.[55] However, evidence on the mono-unsaturated fatty acid (MUFA)–rich Mediterranean diet for NAFLD is limited by small study populations (N=12–90 subjects), suboptimal nutritional analyses, or use of surrogate primary endpoints (i.e. liver transaminases) rather than imaging diagnosis of NAFLD.[59] Moreover, health recommendations on fat and carbohydrate consumption have been widely debated.[243,244] Only a minority of studies examined the effect of all macronutrients combined, and those who did, showed conflicting results.[57,245-247] In addition, these studies too are hampered by small sample size (N=56–349 subjects) and/or by suboptimal methodology (e.g. not correcting for energy intake, BMI, overall dietary quality, or other potential confounders).

So far, no study has examined macronutrient composition in relation to NAFLD on a large scale using comprehensive nutritional analyses methods including energy density and substitution models, taking into account potentially important sociodemographic, lifestyle,

and metabolic risk factors. We therefore conducted a large population-based study in elderly Caucasians, who completed a validated 389-item food frequency questionnaire (FFQ) and underwent hepatic ultrasound, to determine whether macronutrient intake is associated with NAFLD independently of total energy intake and a large number of potentially confounding traits.

Methods

Study population

The Rotterdam Study is a large ongoing population-based cohort of predominantly elder participants residing in a suburb of Rotterdam, the Netherlands. The design and rationale of this population-based study have been described in detail previously.[248] In short, the study commenced in 1989 and comprises three different cohorts (RS I, RS II, and RS III). All residents aged 55 (RS I, RS II) or 45 (RS III) and above were invited to participate. Participation rate of these cohorts were 78%, 67% and 65%, respectively. Liver imaging is part of the core protocol since 2009. Hence, all participants who underwent abdominal US between January 2009 and June 2014 were included. The Rotterdam Study has been approved by the institutional review board (Medical Ethics Committee) of the Erasmus MC University Medical Centre Rotterdam and by the review board of The Netherlands Ministry of Health, Welfare, and Sports. Written informed consent was obtained from the participants.

Dietary Data

Participants were asked to complete an externally validated semi-quantitative 389-item FFQ developed for Dutch adults during their visit at the research centre.[98,249] This questionnaire included detailed questions on food item consumption over the last month and addressed frequency, portion size, type of food item, and preparation methods. Servings were estimated in grams per day using standardised household measures,[250] and macronutrient intake was extracted from the questionnaires using the Dutch Food Composition Table (NEVO v2011) that includes information on nutrient content per gram or serving per product. Incomplete or unreliable FFQs, defined as caloric intake of less than 500 or more than 7500 kilocalories (kcal), were excluded. To correct for potential measurement error and to examine the relative contribution of a macronutrient to the diet, we adjusted for energy intake using the nutrient density method.[251] For example, 1 gram of protein equals 4 kcal, hence to calculate the energy percent of protein (E%) = (total protein intake(g)*4/ total kcal intake)*100. Similarly, the E% of carbohydrates (4kcal/g), fats (9kcal/g), fibre

(2kcal/g) and alcohol (6kcal/g) were determined. Subsequently, all E% were ranked into quartiles (Q1=lowest quartile).
Additionally, to account for confounding by overall dietary quality, the Dutch Healthy Diet Index (DHDI) was derived from the FFQ and added to the multivariable analyses.[100] A higher DHDI indicates stricter adherence to the Dutch dietary guidelines. DHDI is an adherence score designed specifically for the Netherlands, but correlates highly with the perhaps more familiar (Alternate)- Healthy Eating Index, the (A)HEI ($r \geq 0.60$).[252] For the purpose of this study the DHDI was modified in multivariable models to avoid multicollinearity (e.g. macronutrient analyses of fat is adjusted for DHDI minus the trans fatty acid and saturated fat components of the DHDI).

Liver imaging

Steatosis was assessed using abdominal ultrasound, which was carried out by a certified and experienced technician on Hitachi HI VISION 900. All participants were unaware of the presence of steatosis before completing the FFQs. Ultrasound images were stored digitally and re-evaluated by a single hepatologist with over 10 years of experience (RK). The ultrasound technician and hepatologist were blinded for the FFQ data. Diagnosis of steatosis was determined dichotomously according to Hamaguchi et al.,[104] as presence or absence of hyperechogenic liver parenchyma. Participants with possible secondary causes for steatosis were excluded from this study, i.e. 1) excessive alcohol consumption (>30g/day for men and >20g/day for women) as assessed by the FFQ; 2) use of steatogenic drugs, i.e. amiodarone, systemic corticosteroids, methotrexate, or tamoxifen, extracted from linked pharmacy data; and 3) viral hepatitis, based on hepatitis B surface antigen and anti-hepatitis C virus serology, as measured by an automatic immunoassay (Roche Diagnostic GmbH). Of note, for participants from RS-III, there was a median time-gap of 5.5 years between completing the FFQ (before introduction of liver ultrasound) and the performance of ultrasound. Because dietary data are known to be stable over time, RS-III was included in the total study population.[109]

Biochemistry and additional covariates

All blood samples were collected after overnight fasting just before abdominal US. Blood lipids, platelet count, glucose, alanine aminotransferase (ALT), aspartate aminotransferase, gamma-glutamyl transferase (GGT), and total bilirubin were measured using automatic enzyme procedures (Roche Diagnostic GmbH, Mannheim, DE). Insulin was determined using an automatic immunoassay (Roche Diagnostic GmbH).
Data concerning demographics, physical activity, smoking, educational level, and comorbid conditions were obtained during an extensive home interview by trained interviewers. An-

thropometric measurements were carried out by well-trained research assistants measuring height (m), weight (kg) and waist circumference (WC in cm). Blood pressure measurements (mmHg) were obtained at a single visit using two subsequent measurements in upright position after a minimum of 5 minutes rest. BMI was calculated as weight/height² (kg/m²) and considered lean if <25 kg/m² and overweight if ≥25 kg/m².

Insulin resistance was assessed using the homeostasis model assessment of insulin resistance (HOMA-IR), as fasting glucose (mmol/dl) multiplied by fasting insulin (mU/L) divided by 22.5.[106] The metabolic syndrome was diagnosed when at least three of the following traits were present: 1) abdominal obesity, defined as WC ≥102 cm in men and ≥88 cm in women; 2) serum triglycerides ≥150 mg/dl (1.7 mmol/L), or drug treatment for elevated triglycerides; 3) serum high-density lipoprotein cholesterol (HDL-C) ≤40 mg/dl (1.0 mmol/L) in men and ≤50 mg/dl (1.3 mmol/L) in women, or drug treatment for low HDL-C; 4) blood pressure ≥130/85 mmHg or drug treatment for elevated blood pressure; and 5) fasting plasma glucose (FPG) ≥100 mg/dl (5.6 mmol/L) or drug treatment for elevated blood glucose.[28]

Statistical Analyses

After excluding participants with either missing or unreliable FFQs and participants with more than 30% missing study variables, the remaining missing values (range of 0.02–10.79% within covariates) were imputed using multiple imputation (fully conditioned specification) to reduce bias due to missing data.[253] Ten imputed datasets were created using the R-package mice and the analyses were performed in each dataset before the results were pooled by Rubin's rules, to take into account uncertainty with the prediction of missing data.[254] The imputation process is described in more detail in the *Supplementary Methods*.

Descriptive statistics were used to describe population characteristics. Continuous data were presented as mean ± standard deviation (SD) or median with 25th and 75th percentile (P25-P75) according to the distribution of the variable. Categorical data were presented as percentage. Chi-square test, Student's T test, or Wilcoxon Rank Sum test were used to evaluate differences in categorical, normally distributed, and not-normally distributed data, between subjects with and without NAFLD. In order to give more insight in the composition of the different macronutrients, we created 49 relevant food groups out of the 389 food items and performed a Spearman correlation to identify the 3 topmost correlations between macronutrients and food groups.

All macronutrients were analysed continuously (in E%) using standardised values (increase per 1SD) as well as in quartiles using Q1 as reference. We used three separate multivariable logistic regression models to assess the associations of macronutrients with NAFLD. The first 'socio-demographic' model (model 1) includes adjustment for age, gender, education level (low/moderate/high), and study cohort. The second 'lifestyle confounding' model

(model 2) is additionally adjusted for smoking status (never vs. past/current), alcohol in E%, energy intake (kcal), physical activity (metabolic equivalent of task (MET) hours/week), and DHDI. Finally, model 3, the 'metabolic' model, was additionally adjusted for presence of diabetes, metabolic syndrome, and total cholesterol (mg/dL). Moreover, analyses with carbohydrates were adjusted for fibre intake and vice versa, and all subtypes of one macronutrient were adjusted for each other. Results were presented as odds ratio (OR) with 95% confidence interval (95%CI).

BMI is an important covariate, it could act as potential mediator (in the pathway between the exposure [diet] and the outcome [NAFLD]), as a confounder (affecting both exposure and outcome, causing a false association), as a collider (a covariate that is not in the pathway but is influenced by both the exposure and the outcome, it may create a non-existing association between the exposure and outcome), or as effect modifier (indicating different associations in subgroups of patients). Also, participants with NAFLD and a normal BMI (lean NAFLD) could have a different pathophysiological pathway from overweight NAFLD, e.g. through genetic predisposition or body composition. In addition, measurement error, eating habits, and hence, macronutrient associations with NAFLD could have differed between lean and overweight individuals. Therefore, we used the following approaches to account for BMI: First, we evaluated the linearity of BMI in model 3 in relation to steatosis using cubic splines and found a non-linear effect using log-likelihood ratio testing. The figure of the spline showed that a log-shaped form could improve the fit of the model, we therefore created a model 4, adding log-transformed BMI as covariate to the metabolic model to evaluate changes in effect estimates. We believe the effect of BMI can be studied best in this model 4, because here we already adjusted for all other potential confounding factors. Second, we tested for interaction between BMI and each macronutrient (e.g. BMI x total protein). And third, all analyses were stratified for lean and overweight participants at a cut-point of 25 kg/m², while this is a widely used cut-off as established by the WHO and because there was no clear cut-off point visible in the cubic splines.

Also, to evaluate whether the observed associations were due to higher intake of that specific macronutrient rather than lower intake of another macronutrient, we performed substitution analyses in the metabolic model.[255] For example, the substitution model of replacing total protein for total fat included the following dietary covariates, total protein, total carbohydrates, total fibre, and alcohol, but not total fat, in addition to the above-mentioned metabolic and environmental covariates. The obtained estimate from the regression coefficient for protein from this model reflect the theoretical effect of replacing all fat intake completely with protein intake (in E%). Additional sensitivity analyses were performed comparing the analyses of imputed data to that of the complete case, and assessed differences between the group with and without missing cases in order to assess the robustness of our data. In addition, we excluded the third cohort from the final analyses as they filled in their FFQ 5.5 years prior to liver imaging.

To correct for the inflated type I error that arises due to multiple testing we applied the method proposed by Sidák,[256] adapted as described in Galwey et al.,[257] using the effective number of tests instead of the actual number of tests. This adaptation is necessary to take into account that dietary exposures inter-correlate (i.e. the intake of individual macronutrients are not fully independent from each other), and hence, the corresponding tests are not independent from each other. The resulting corrected significance level for all macronutrient analyses was $P<0.021$. All analyses were performed using SPSS 21.0 (SPSS Inc., Chicago, IL, USA) and R version 3.4.1.

Patient involvement

Participants were not involved in setting the research question or the outcome measures, nor were they involved in developing plans for recruitment, design, or implementation of the study. Participant were not asked for advise on the interpretation of results. All participants were regularly updated on study outcomes via a home-sent newsletter and the study website.

Results

Study Characteristics

The flowchart of the study population is depicted in *Figure 1*. 5967 participants were eligible for this study. First, we excluded participants due to missing, incomplete, or unreliable FFQs (n=1173; 19.7%). These participants were younger (mean age 68.5 vs. 69.6 years old; $P<0.01$), less often of Caucasian origin (95.0% vs. 98.0%; $P<0.01$), and had a slightly higher BMI (27.5 vs. 26.9 kg/m²; $P<0.01$) than the included study group, whereas gender and frequency of steatosis were similar (55.8% vs. 57.5% female; $P=0.27$ and 37.2% vs. 35.5% steatosis; $P=0.27$). Second, we excluded participants with more than 30% of missing study variables (0.8%), and participants with secondary causes for steatosis (18.3%). Hence, the total study population included 3882 participants. Population characteristics are presented in *Table 1* and original and imputed data in *Supplementary Table 1*. In short, mean age was 69.7 ± 8.8, 58.3% were female, the majority of participants were of Caucasian origin (97.6%), and median BMI was 26.9 (24.5 – 29.7) kg/m². NAFLD prevalence was 34.4% (n=1337). Participants with NAFLD had lower education level, were more often current or former smokers, performed less physical activity, had higher BMI, more comorbidities, and more deviant mean or median laboratory values albeit within the normal range.

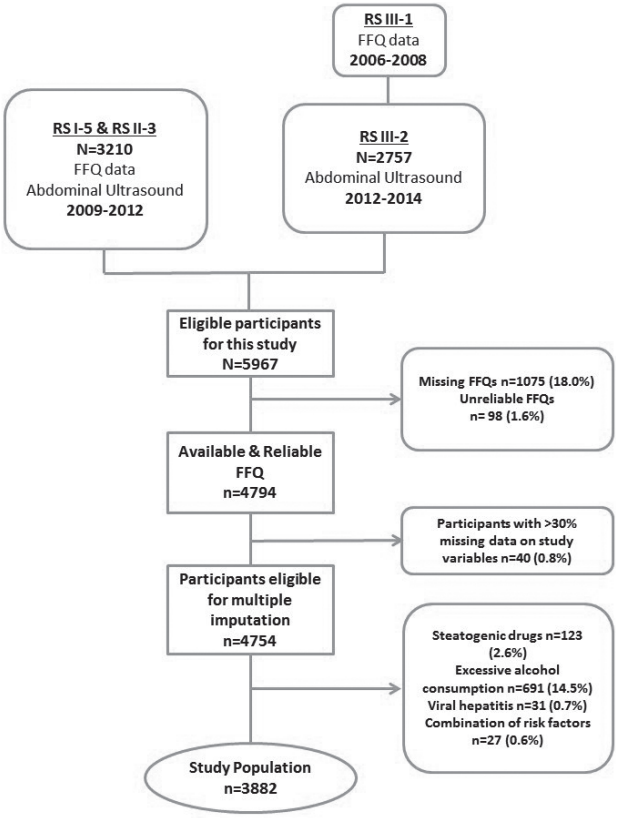

Figure 1: Flowchart of the study
Legend: RS is Rotterdam Study, I-III is number of cohort, 1–5 is number of times cohort visited. FFQ is Food Frequency Questionnaire.

Dietary Data

Dietary characteristics are presented as relative consumption (E%) in *Table 1*. Animal protein correlated most with red meat (r=0.35), refined meat (r=0.25), and fish (r=0.24); and vegetable protein with whole grain, rice, bread and pasta (r=0.50), cruciferous vegetables (r=0.27), and vegetarian food products (r=0.24). Mono and disaccharides correlated most with fruit (r=0.63), sweets (r=0.30), and fruit juice (r=0.24); and polysaccharides with whole grain, rice, bread and pasta (r=0.39), refined grain, rice, bread and pasta (r=0.29), and potatoes (r=0.27). Lastly, saturated fat correlated most with full fat cheese/ cream (r=0.50), full/ non-fluid fat (r=0.42), and desserts (0.27); MUFAs with sauce (r=0.29), fried snacks (r=0.29), and oil fats (r=0.29); poly-unsaturated fatty acids (PUFA) with diet/ fluid fats (r=0.35), full/ non-fluid fats (r=0.32), and peanuts (r=0.25); and trans fatty acid with full fat cheese/cream (r=0.39), full/ non-fluid fat (r=0.38), and desserts (r=0.32). NAFLD

Table 1: Characteristics of the study population

	Total Population N=3882	No NAFLD n=2545 (65.6%)	NAFLD n=1337 (34.4%)	*P*-value*
Demographics				
Age (years)	69.7 ± 8.8	69.6 ± 9.2	70.0 ± 8.2	0.164
Female (%)	58.3	59.1	56.8	0.184
Caucasian (%)	97.6	97.4	98.0	0.458
Education Level (%)				
Low	48.4	45.0	54.9	<0.001
Intermediate	30.2	31.4	28.1	
High	21.3	23.6	17.0	
Smoking status (%)				
Never	36.1	38.3	31.8	<0.001
Past or Current	63.9	61.7	68.2	
Alcohol (units/d)	0.45 (0.05 – 1.20)	0.45 (0.06 – 1.21)	0.43 (0.05 – 1.19)	0.422
Physical Activity†	40.6 (15.7 – 77.6)	43.8 (17.2 – 81.3)	34.6 (13.5 – 70.0)	<0.001
Physical examination				
BMI (kg/m²)	26.9 (24.5 – 29.7)	25.8 (23.7 – 28.0)	29.3 (27.0 – 32.3)	<0.001
Lean	30.2	40.9	9.9	<0.001
Overweight	69.7	59.1	90.1	
Waist Circumference (cm)				
Men	98.2 ± 10.6	95.0 ± 9.1	104.1 ± 10.5	<0.001
Women	89.1 ± 12.2	85.0 ± 10.5	97.4 ± 11.3	<0.001
Biochemistry				
AST (U/L)	24 (21 – 28)	24 (21 – 28)	25 (21 – 29)	<0.001
ALT (U/L)	18 (15 – 24)	17 (14 – 22)	21 (16 – 29)	<0.001
GGT (U/L)	23 (17 – 34)	21 (15 – 30)	28 (20 – 39)	<0.001
Platelets (*10^9/L)	262 (223 – 305)	260 (222 – 303)	266 (225 – 310)	0.031
HOMA-IR	2.6 (1.7 – 4.1)	2.1 (1.5 – 3.1)	4.1 (2.7 – 6.1)	<0.001
Total Cholesterol (mmol/L)	5.4 ± 1.1	5.5 ± 1.1	5.4 ± 1.1	0.002
HDL-C (mmol/L)	1.5 ± 0.4	1.5 ± 0.4	1.3 ± 0.4	<0.001
Triglycerides (mmol/L)	1.3 (1.0 – 1.7)	1.2 (0.9 – 1.5)	1.6 (1.2 – 2.1)	<0.001
Comorbidities				
Metabolic Syndrome	51.9	40.8	73.0	<0.001
-WC>88cm (♀) or >120cm (♂)	43.2	29.1	69.9	<0.001
- Triglycerides >150mg/dL	46.0	39.0	59.4	<0.001
- HDL-C <40mg/dL (♂) or 50mg/dL (♀)	44.7	38.3	56.7	<0.001
- Blood pressure ≥130/85mmHg	84.3	80.5	91.5	<0.001
- FPG>100mg/dL	41.5	31.1	61.2	<0.001
Diabetes Mellitus (%)	13.1	7.5	23.7	<0.001
Hypertension (%)	74.0	68.9	83.7	<0.001

Table 1 (*continued*)

	Total Population N=3882	No NAFLD n=2545 (65.6%)	NAFLD n=1337 (34.4%)	*P*-value*
Dietary Data (E%)				
Total Kilocalories/ day	2031 (1620 – 2515)	2052 (1642 – 2537)	1996 (1579 – 2456)	0.003
Total Protein	15.6 (14.0 – 17.4)	15.4 (13.8 – 17.2)	16.0 (14.3 – 17.8)	<0.001
Animal protein	9.2 (7.5 – 11.2)	9.0 (7.2 – 10.9)	9.5 (7.9 – 11.6)	<0.001
Vegetable protein	6.3 (5.5 – 7.1)	6.3 (5.5 – 7.1)	6.2 (5.4 – 7.1)	0.076
Total Fat	31.9 (27.9 – 36.1)	31.7 (27.8 – 35.9)	32.4 (28.2 – 36.5)	0.039
Saturated fat	11.5 (9.7 – 13.3)	11.4 (9.7 – 13.2)	11.6 (9.9 – 13.5)	0.018
MUFA	10.7 (9.2 – 12.4)	10.7 (9.1 – 12.3)	10.8 (9.3 – 12.6)	0.055
PUFA	6.4 (5.3 – 7.6)	6.4 (5.4 – 7.6)	6.4 (5.3 – 7.6)	0.276
Trans fatty acid	0.51 (0.41 – 0.62)	0.50 (0.41 – 0.61)	0.53 (0.42 – 0.64)	0.002
Total Carbohydrate	46.2 (41.9 – 50.7)	46.6 (42.2 – 51.0)	45.5 (41.5 – 50.3)	<0.001
Mono & disaccharide	22.8 (18.2 – 27.7)	23.2 (18.7 – 28.0)	21.9 (17.5 – 26.9)	<0.001
Polysaccharide	22.7 (19.8 – 26.0)	22.7 (19.7 – 26.0)	22.7 (19.9 – 25.9)	0.683
Fibre	2.6 (2.2 – 3.0)	2.6 (2.2 – 3.1)	2.6 (2.1 – 3.0)	0.013
Total Alcohol	1.4 (0.2 – 3.4)	1.4 (0.2 – 3.4)	1.3 (0.1 – 3.5)	0.939

Pooled data based on 10 imputations represent % for categorical variables and for continuous variables mean ± SD or median (P25-P75).*P-value is based on T-test, Wilcoxon rank sum test, Chi-square test or Fisher's exact test and is a comparison between the no NAFLD and NAFLD columns. †Physical activity in metabolic equivalent task hours/week. Abbreviations ALT: alanine aminotransferase; AST: aspartate aminotransferase; BMI: body mass index; FPG: fasting plasma glucose; GGT: gamma-glutamyltransferase; HDL-C: high-density lipoprotein cholesterol; HOMA-IR: homeostasis model assessment of insulin resistance; MUFA: mono-unsaturated fatty acid; PUFA: poly-unsaturated fatty acid

participants reported lower median caloric consumption than participants without NAFLD (1996 vs. 2052 kcal, *Table 1*). The same was observed for BMI (overweight individuals reported 2006 kcal vs 2089 kcal in lean individuals; *P*<0.01). NAFLD participants reported higher median total protein, animal protein, total fat, saturated fat, and trans fatty acid intake (all in E%, *Table 1*). Moreover, they reported lower total carbohydrate and mono and disaccharide consumption, and marginally lower fibre consumption (*Table 1*). Absolute consumption of macronutrients in grams and energy percentage per quartile are given in *Supplementary Table 2*.

Protein consumption and NAFLD

Both total and animal protein were associated with higher odds for NAFLD in the first 3 models (*Table 2*). Vegetable protein was not associated with NAFLD in any of the models. After adjustment for log-transformed BMI none of the associations remained. However, effect modification by BMI was suggested: interaction with BMI was *P*=0.10 for total protein, *P*=0.04 for animal protein, and *P*=0.19 for vegetable protein. Indeed,

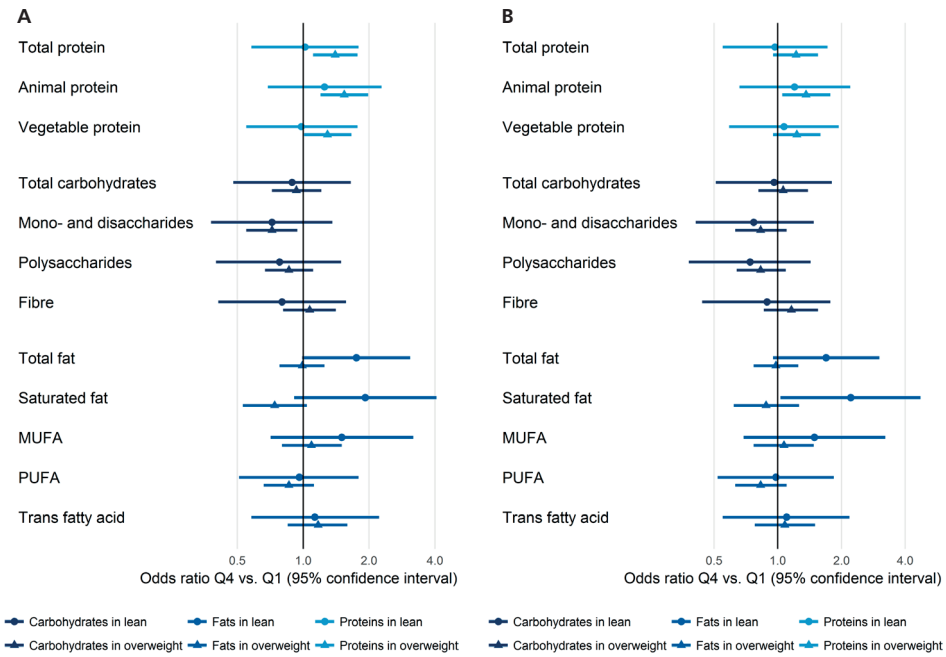

Figure 2: Stratified multivariable analyses for the association of macronutrients and its subtypes with NAFLD (OR with 95%CI)

Results of multivariable logistic regression of the association of the highest quartile (Q4) versus the lowest quartile (Q1) of consumption of a particular (subtype) of macronutrient with NAFLD as dependent outcome. A) Odds ratios are adjusted for age, gender, education level, study cohort, past or current smoking, alcohol in E%, physical activity and DHDI. B) Odds ratios are adjusted for age, gender, education level, past or current smoking, alcohol in E%, physical activity, DHDI, diabetes mellitus, cholesterol and metabolic syndrome. Y-axis: all macronutrients and the P-value for trend over quartiles. X-axis: multivariable OR (95%CI) of Q4 vs. Q1 on a semi-log scale, circle bullets reflect lean participants and triangle bullets reflect overweight participants.

stratified analyses by BMI revealed that both total protein and animal protein intake were associated with overweight NAFLD in model 1 and 2 (*Figure 2A)*. Vegetable protein was associated with overweight NAFLD as well, but only in model 2. In model 3, the association with overweight NAFLD attenuated but remained significant for animal protein ($OR_{Q4vs.Q1}$ 1.36, 95%CI 1.05–1.77; $P_{for\ trend}$=0.09) while not for total protein ($OR_{Q4vs.Q1}$ 1.22, 95%CI 0.95–1.55; $P_{for\ trend}$=0.09), as shown in *Figure 2B*. Detailed results of the stratified models are displayed in *Supplementary Table 3*.

Carbohydrate consumption and NAFLD

Total carbohydrate intake and mono and disaccharide intake were inversely related with NAFLD prevalence in both model 1 and 2, but the associations attenuated in the third and fourth models (*Table 2*). In contrast, polysaccharide consumption was not associated in the first two models, but after adjustment for metabolic traits in model 3, there was an

Table 2: Stepwise logistic regression models between macronutrients as independent variables and NAFLD as dependent variable using quartile 1 as reference in the total study population

	Q2	Q3	Q4	*P* for trend	Continuous (per SD increase)
Model 1 (Sociodemographic)					
Total protein	**1.24 (1.02 – 1.51)**	**1.49 (1.23 – 1.81)‡**	**1.71 (1.41 – 2.07)‡**	**<0.001‡**	**1.22 (1.14 – 1.30)‡**
Animal protein	**1.45 (1.19 – 1.77)‡**	**1.41 (1.16 – 1.72)‡**	**1.90 (1.55 – 2.33)‡**	**<0.001‡**	**1.24 (1.15 – 1.33)‡**
Vegetable protein	1.06 (0.88 – 1.28)	0.98 (0.81 – 1.20)	1.12 (0.92 – 1.38)	0.411	1.05 (0.97 – 1.13)
Total carbohydrate	0.94 (0.78 – 1.14)	**0.76 (0.62 – 0.92)‡**	**0.74 (0.60 – 0.91)‡**	**0.001‡**	**0.87 (0.81 – 0.93)‡**
Mono-disaccharide	**0.73 (0.61 – 0.88)‡**	**0.67 (0.55 – 0.82)‡**	**0.60 (0.49 – 0.75)‡**	**<0.001‡**	**0.85 (0.78 – 0.92)‡**
Polysaccharide	0.99 (0.82 – 1.20)	0.94 (0.77 – 1.15)	0.83 (0.67 – 1.03)	0.088	0.96 (0.89 – 1.04)
Fibre	0.84 (0.69 – 1.02)	0.93 (0.76 – 1.13)	0.93 (0.75 – 1.15)	0.745	1.01 (0.94 – 1.09)
Total fat	0.96 (0.79 – 1.16)	1.13 (0.94 – 1.37)	1.17 (0.97 – 1.41)	0.**037**	1.05 (0.99 – 1.13)
Saturated fat	0.94 (0.77 – 1.15)	0.98 (0.78 – 1.22)	0.94 (0.71 – 1.24)	0.759	0.91 (0.80 – 1.04)
MUFA	1.07 (0.87 – 1.31)	1.07 (0.85 – 1.33)	1.25 (0.95 – 1.64)	0.141	**1.15 (1.03 – 1.29)‡**
PUFA	0.98 (0.81 – 1.19)	0.89 (0.73 – 1.08)	**0.79 (0.63 – 1.00)**	**0.033**	**0.89 (0.81 – 0.98)‡**
Trans fatty acid	0.95 (0.78 – 1.17)	1.16 (0.93 – 1.44)	1.25 (0.96 – 1.63)	**0.044**	1.11 (0.99 – 1.25)
Model 2 (Lifestyle Confounding)					
Total protein	**1.23 (1.01 – 1.50)**	**1.46 (1.20 – 1.78)‡**	**1.61 (1.31 – 1.97)‡**	**<0.001‡**	**1.20 (1.11 – 1.29)‡**
Animal protein	**1.44 (1.18 – 1.76)‡**	**1.38 (1.12 – 1.69)‡**	**1.80 (1.45 – 2.23)‡**	**<0.001‡**	**1.21 (1.12 – 1.31)‡**
Vegetable protein	1.10 (0.91 – 1.33)	1.03 (0.84 – 1.26)	1.18 (0.95 – 1.47)	0.218	1.07 (0.99 – 1.16)
Total carbohydrate	**0.97 (0.83 – 0.97)‡**	0.82 (0.67 – 1.01)	0.82 (0.66 – 1.03)	**0.037**	**0.90 (0.83 – 0.97)‡**
Mono-disaccharide	**0.76 (0.63 – 0.93)‡**	**0.73 (0.59 – 0.89)‡**	**0.66 (0.52 – 0.83)‡**	**0.001‡**	**0.89 (0.81 – 0.97)‡**
Polysaccharide	0.99 (0.82 – 1.20)	0.93 (0.76 – 1.14)	0.81 (0.65 – 1.01)	0.057	0.95 (0.88 – 1.03)
Fibre	0.89 (0.72 – 1.08)	1.00 (0.81 – 1.25)	1.03 (0.81 – 1.32)	0.532	1.06 (0.97 – 1.16)
Total fat	0.94 (0.78 – 1.14)	1.09 (0.90 – 1.32)	1.07 (0.87 – 1.32)	0.293	1.03 (0.95 – 1.11)
Saturated fat	0.91 (0.74 – 1.12)	0.91 (0.73 – 1.15)	0.83 (0.62 – 1.11)	0.266	**0.86 (0.75 – 0.99)**
MUFA	1.07 (0.87 – 1.32)	1.06 (0.84 – 1.33)	1.24 (0.94 – 1.63)	0.176	**1.17 (1.05 – 1.32)‡**
PUFA	1.00 (0.82 – 1.21)	0.89 (0.73 – 1.09)	**0.79 (0.63 – 1.00)**	**0.032**	**0.89 (0.81 – 0.98)‡**
Trans fatty acid	0.95 (0.78 – 1.17)	1.15 (0.93 – 1.44)	1.22 (0.93 – 1.60)	0.069	1.11 (0.99 – 1.24)
Model 3 (Metabolic)					
Total protein	1.19 (0.97 – 1.47)	**1.33 (1.08 – 1.64)‡**	**1.34 (1.08 – 1.67)‡**	**0.004‡**	**1.13 (1.04 – 1.22)‡**
Animal protein	**1.40 (1.14 – 1.73)‡**	1.23 (0.99 – 1.52)	**1.53 (1.22 – 1.92)‡**	**0.002‡**	**1.14 (1.05 – 1.24)‡**
Vegetable protein	1.13 (0.92 – 1.39)	1.03 (0.83 – 1.28)	1.19 (0.95 – 1.49)	0.262	1.07 (0.98 – 1.16)
Total carbohydrate	1.07 (0.87 – 1.31)	0.92 (0.74 – 1.14)	0.95 (0.75 – 1.20)	0.432	0.94 (0.86 – 1.02)
Mono-disaccharide	**0.80 (0.65 – 0.99)**	0.82 (0.66 – 1.02)	**0.78 (0.61 – 0.99)**	0.067	0.94 (0.86 – 1.03)
Polysaccharide	0.91 (0.74 – 1.12)	0.95 (0.77 – 1.17)	**0.78 (0.62 – 0.99)**	0.076	0.95 (0.87 – 1.03)
Fibre	0.96 (0.77 – 1.18)	1.13 (0.89 – 1.42)	1.14 (0.88 – 1.49)	0.166	1.09 (0.99 – 1.20)
Total fat	1.01 (0.82 – 1.24)	1.15 (0.94 – 1.42)	1.08 (0.87 – 1.34)	0.310	1.02 (0.94 – 1.10)
Saturated fat	0.97 (0.78 – 1.20)	1.07 (0.84 – 1.37)	1.04 (0.76 – 1.42)	0.628	0.97 (0.84 – 1.12)
MUFA	1.15 (0.93 – 1.44)	1.09 (0.85 – 1.38)	1.20 (0.90 – 1.60)	0.349	1.10 (0.97 – 1.24)

Table 2 (*continued*)

	Q2	Q3	Q4	*P* for trend	Continuous (per SD increase)
PUFA	1.06 (0.86 – 1.30)	0.89 (0.71 – 1.10)	0.80 (0.63 – 1.02)	**0.033**	**0.89 (0.81 – 0.99)**
Trans fatty acid	0.95 (0.77 – 1.18)	1.15 (0.91 – 1.45)	1.12 (0.84 – 1.49)	0.232	1.05 (0.93 – 1.18)
Model 4 (Metabolic + log-transformed BMI)					
Total protein	1.13 (0.91 – 1.41)	1.13 (0.91 – 1.42)	0.99 (0.79 – 1.25)	0.933	1.01 (0.93 – 1.09)
Animal protein	**1.31 (1.05 – 1.64)‡**	1.01 (0.80 – 1.27)	1.15 (0.90 – 1.48)	0.725	1.01 (0.92 – 1.10)
Vegetable protein	1.07 (0.86 – 1.33)	0.98 (0.78 – 1.23)	1.12 (0.88 – 1.43)	0.543	1.04 (0.95 – 1.14)
Total carbohydrate	1.15 (0.93 – 1.44)	1.05 (0.83 – 1.33)	1.17 (0.91 – 1.51)	0.357	1.02 (0.93 – 1.11)
Mono-disaccharide	0.87 (0.70 – 1.08)	1.00 (0.79 – 1.27)	0.94 (0.72 – 1.23)	0.940	1.02 (0.92 – 1.13)
Polysaccharide	0.99 (0.80 – 1.23)	1.04 (0.83 – 1.31)	0.85 (0.66 – 1.10)	0.339	1.00 (0.92 – 1.10)
Fibre	0.93 (0.74 – 1.16)	1.09 (0.85 – 1.40)	1.08 (0.81 – 1.42)	0.372	1.06 (0.95 – 1.17)
Total fat	1.06 (0.85 – 1.32)	1.18 (0.95 – 1.47)	1.05 (0.84 – 1.33)	0.470	1.00 (0.92 – 1.09)
Saturated fat	1.02 (0.81 – 1.29)	1.07 (0.82 – 1.39)	1.10 (0.79 – 1.53)	0.537	1.01 (0.86 – 1.18)
MUFA	1.20 (0.95 – 1.51)	1.05 (0.81 – 1.36)	1.14 (0.84 – 1.56)	0.672	1.03 (0.90 – 1.17)
PUFA	1.13 (0.91 – 1.40)	0.99 (0.78 – 1.24)	0.91 (0.70 – 1.18)	0.343	0.93 (0.84 – 1.04)
Trans fatty acid	0.90 (0.72 – 1.14)	1.08 (0.84 – 1.39)	1.04 (0.76 – 1.41)	0.508	1.03 (0.91 – 1.18)

Values are odds ratios with 95% confidence intervals taking quartile 1 as reference. **P*-trend is calculated across quartiles. Bold values indicate $P<0.05$. ‡Indicates significant values using $P < 0.021$ as determined by Sidák. Model 1 (socio-demographic) is adjusted for age, gender, education level and study cohort. Model 2 (lifestyle confounding) is in addition previous model adjusted for past or current smoking, alcohol in E%, physical activity, energy intake and DHDI. Model 3 (metabolic) is in addition to the previous model adjusted for cholesterol, metabolic syndrome and diabetes mellitus. Model 4 (metabolic + log-transformed BMI) is in addition to the previous model adjusted for log-transformed BMI. Abbreviations BMI: body mass index; MUFA: mono-unsaturated fatty acid; PUFA: poly-unsaturated fatty acid; Q: Quartile.

inverse association with NAFLD (*Table 2*). Finally, after correction for log-transformed BMI, none of the associations remained significant. Also, no association between dietary fibre and NAFLD was found. The associated did not differ by BMI (*P* for interaction was *P*=0.08 for total carbohydrate, *P*=0.45 for mono-and disaccharides, *P*=0.16 for polysaccharides, and *P*=0.55 for fibre intake). Comprehensive results of the stratified models are shown in *Supplementary Table 3*. As depicted in *Figure 2A*, only mono and disaccharides were significantly associated with lower odds for NAFLD in overweight individuals. Yet, direction and magnitude of estimates in lean participants were comparable to those of overweight participants (OR 0.72 for both, model 2). After adjustment for metabolic covariates in model 3, the association for mono and disaccharides in overweight dissipated ($OR_{Q4vs.Q1}$ 0.83, 95%CI 0.63–1.10; $P_{for\ trend}$=0.28, *Figure 2B* and *Supplementary Table 3*).

Fat consumption and NAFLD

PUFA consumption was associated with lower odds for NAFLD in the first 3 models, but results diminished after adjustment for log-transformed BMI (*Table 2)*. No effect modification by BMI was observed (*P* for interaction was *P*=0.16 for total fat, *P*=0.20 for saturated fat, *P*=0.21 for MUFAs, *P*=0.54 for PUFAs, and *P*=0.96 for trans fatty acids). *Figure 2A & Figure 2B* show the stratified models 2 and 3, in which PUFA intake was no longer associated with NAFLD (*Supplementary Table 3)*. Saturated fat became associated with lean NAFLD after metabolic adjustment ($OR_{Q4vs.Q1}$ 2.21, 95%CI 1.03–4.72; $P_{for\ trend}$=0.03, *Figure 2B* and *Supplementary Table 3)*.

Substitution Analyses

We did not observe consistent substitution effects when one (sub)type of macronutrient was substituted for another (sub)type of macronutrient (*Supplementary Table 4*).

Sensitivity analyses

First, 3259 participants had complete data on all variables, and 623 had missing data on at least one covariate. Estimates derived from the complete case analyses were more pronounced than the imputed analyses, with all results pointing in the same direction. For example, in the imputed analyses (*Supplementary Table 3)* animal protein had an $OR_{Q4vs.Q1}$ of 1.36 (95%CI 1.05–1.77; $P_{for\ trend}$=0.09) for overweight NAFLD compared to an $OR_{Q4vs.Q1}$ of 1.52 (95%CI 1.14–2.03; $P_{for\ trend}$=0.03) in the complete case analyses *(Supplementary Table 5).* We therefore compared the group with complete cases to the group with missing data. In the latter group (total n=623), physical activity was the variable most often missing before imputation (n=358), followed by smoking status (n=203), and education level (n=50). The participants with missing variables were older (71.1 vs. 69.5), and were less often female (47% vs. 60%). Moreover, they had higher prevalence of diabetes (17.3% vs. 12.4%), and NAFLD (38.4% vs. 33.7%) and had a higher BMI (27.4 vs. 26.8kg/m²). More detailed information is shown in *Supplementary Table 6*.

Second, we excluded the third cohort from the final analysis in order to avoid possible bias induced by a time lag of 5.5 years (median) between completion of the FFQs and liver imaging. The direction of the results did not change, but significance attenuated as can be seen in *Supplementary Table 7*. However, the results should be interpreted in light of decreased power and the difference in cohort characteristics, such as age (mean age RSI and II was 75.3 vs. 62.0 years in RS III). Third, additional adjustment for coffee as potential confounding covariate did not change the association between macronutrients and NAFLD (data not shown).

Discussion

This is the first large population-based cohort study to examine macronutrient intake using an extensive and externally validated semi-quantitative FFQ in relation to ultrasound-confirmed NAFLD. The results of this cross-sectional analysis, with FFQs preceding ultrasound, imply that specific macronutrients are associated with NAFLD independent of energy intake. Specifically, high animal protein intake was associated with higher prevalence of NAFLD in overweight participants. In addition, we found a trend towards lower prevalence of NAFLD in those with high consumption of mono and disaccharides. However, this association did not hold true after adjustment for log-transformed BMI. We did not observe consistent substitution effects of macronutrient replacement, emphasizing the need of a diverse diet. Recent dietary review papers on NAFLD have advocated implementation of the Mediterranean diet, which is rich in MUFAs, fruits, legumes, and nuts; and low in saturated fat, carbohydrates, and red meat.[258] Although we analysed diet based on macronutrient composition and not on predefined dietary patterns, we found that intake of animal protein was significantly associated with overweight NAFLD independent of socio-demographic, lifestyle, and metabolic traits. Furthermore, we found that the association between animal protein and NAFLD is mainly present in the highest quartile and does not appear to be dose-responsive.

Our findings are in line with previous studies, which showed that NAFLD patients consumed significantly more meat than controls[259] even after adjustment for confounders and energy intake.[57] Moreover, another recent Dutch population study found similar results, showing higher intake of protein from animal sources in individuals with a fatty liver, identified by the Fatty Liver Index (a non-invasive algorithm) rather than liver imaging.[247] In this study however, BMI was not taken into account as a covariate, and macronutrients were not adjusted for dietary quality (e.g. DHDI). Interestingly, a large epidemiological study showed that high red meat intake was associated with all-cause mortality, and in particular with mortality from liver diseases (hazard ratio 2.30, highest vs. lowest quintile).[260] In addition, a study from Israel found that high meat consumption, specifically high red and processed meat consumption were associated with NAFLD and insulin resistance, independent of saturated fat intake and BMI.[261] Yet, two other studies did either not find a difference in protein consumption between patients and controls, or found that controls consumed slightly more protein than patients.[245,246] However, both studies used absolute consumption in grams instead of energy adjusted intake and did not distinguish between animal or vegetable protein. Another recent study suggested a beneficial effect of protein. This was an intervention study in 37 diabetics with mild steatosis (<30% lipid content on MRI), in whom intrahepatic lipid content reduced upon a strict high vegetable or animal protein diet for 6 weeks.[262] Since this study differs from ours in various ways (i.e. our study included N=3882 individuals with a low prevalence of diabetes (13%) in an observational

rather than an interventional study design with an outcome defined by >30% steatosis as set by the detection limits of ultrasonography), direct comparison is difficult.

Contrary to common belief, we did not find a 'harmful' association between carbohydrates and NAFLD. In contrast, participants with high mono and disaccharide intake initially showed lower odds for NAFLD, but this association attenuated after BMI adjustment. Although the general assumption is that fructose intake harms the liver, evidence for this assumption is indeterminate.[263] In most studies, it is difficult to separate the contribution of fructose-containing sugars from that of other dietary factors, such as origin of food item, energy intake, and overall dietary quality.[244] This lack of clarity is supported by experimental studies showing that isocaloric carbohydrate intake was not associated with steatosis, but rather with amount of calories.[264,265] Most studies to date have shown detrimental effects on NAFLD, but focussed only on fructose intake from soft drinks.[266,267] In fact, in this predominantly elderly population median soft drink consumption was less than 1 glass per day (i.e. 44.5% consumers that have a median consumption of 0.36 glasses/day (0.14–0.91)). Indeed, the main food group contributing to mono and disaccharides in this population was actually fruit, and soda was not amongst the top 3 correlated contributors. This may partly explain why we did not observe a negative association.

Our results also do not suggest a "beneficial" role for MUFAs. This is in line with previous studies on macronutrient associations with NAFLD, which either did not find an association with MUFAs or found higher MUFA consumption in the NAFLD-group than in controls.[57,245-247] In contrast, a randomised controlled trial from Italy showed a reduction in liver fat following a MUFA enriched-diet in patients with diabetes.[268] However, this study was small (9 participants/arm) and showed a significant but small decrease in liver fat percentage (2.2% in the MUFA-arm). In addition, MUFA-intake in this study was much higher (~25%) than in our study (10.7%).[268] Dietary fat consumption, in particular saturated fat, remains a widely debated topic[269] and evidence on associations of (subtypes of) fat with incident metabolic disease is heterogeneous and suffer from residual confounding.[270] Our substitution analyses did imply a favourable trend when substituting PUFAs for animal and total protein intake in overweight participants. However, this association was not significant.

There are several possible mechanistic explanations as to how high animal protein intake could be associated with overweight NAFLD. Although we adjusted for overall dietary quality, the association might be explained by other dietary components. One hypothesis is, that constituents, such as nitrate, nitrite, haem iron and their by-products, in both unprocessed and processed meat could act as mediators between dietary intake and cardiovascular and metabolic homeostasis.[260] Haem iron is associated with increased oxidative stress and insulin resistance.[271] Nitrate and nitrite have been associated with endothelial dysfunction and insulin resistance.[272] Moreover, a large prospective cohort study found

that nitrate, nitrite, and haem iron from red meat intake were all associated with higher risk of chronic liver disease.[273]

Another possible mechanism through which animal protein could be associated with NAFLD is low-grade metabolic acidosis induced by a high diet-dependent acid load. The Western diet, characterized by high intake of acidic food items (e.g. animal protein) and low intake of alkali, potassium-rich food items (e.g. vegetables/fruits) increases daily acid load.[274] Recently, diet-dependent acid load has been associated with a higher risk of NAFLD.[259,275] The authors of these studies hypothesized that high dietary acid load might suppress growth hormone secretion and subsequent insulin-like growth factor-1 response, which both have been associated with NAFLD. In addition, some experimental studies have showed that high dietary acid load reduces extracellular pH, and insulin sensitivity and decreases beta cell response.[276] This could lead not only to diabetes, but also to NAFLD, as insulin resistance is the key dysfunction in this disease.[13]

The main strength of this study is the use of a large unselected study population and a robust statistical analysis with correction for a great number of sociodemographic, lifestyle, and metabolic traits, as assessed by well-validated tools. Furthermore, we used widely accepted nutritional epidemiologic methods and performed sensitivity analyses, emphasizing the robustness of our results. Also, we corrected for multiple comparisons using Sidák-corrected alpha-levels, taking into account that dietary exposures inter-correlate. Finally, abdominal ultrasound is a widely used and reliable imaging technique that yields high sensitivity and specificity for moderate and severe steatosis.[50]

Nonetheless, there are some limitations that need to be addressed. First, due to the cross-sectional design of this study, it is not possible to draw conclusions on causality. Although reverse causality is unlikely (participants were not aware of having NAFLD when filling in the FFQs), residual confounding may still remain. In particular, participants with diabetes (13%) might have adapted their eating habits due to this comorbidity. Nonetheless, we corrected for this potential confounder in the analyses of the third, metabolic model. Second, part of our study population completed the FFQ 5.5 years prior to abdominal ultrasound (i.e. RS III). Because dietary data is known to be stable over time,[109] we assumed that dietary habits in this elderly population would be rather constant. This was indeed recently shown in a paper from The Rotterdam Study.[277] Nevertheless, study cohort was added as covariate in all regression models and sensitivity analysis was performed excluding the third cohort from all main analyses. The results of this sensitivity were largely similar albeit no longer statistically significant due to smaller sample size. Third, as with any self-administered questionnaire, data are subject to potential reporter and recall bias. This is reflected in the probable caloric under-reporting in overweight participants.[278] However, the 389-item FFQ used in this study has been extensively validated in previous studies.[98,249] In addition, unreliable FFQs were excluded. Moreover, we adjusted for total energy intake and thereby accounted for extraneous variation in energy intake and potential measurement error.[251]

Fourth, in an attempt to avoid bias due to missing data, we performed multiple imputations on our data. Contrary to our expectation, the complete case analyses showed more pronounced associations with NAFLD. Differences between the complete and imputed cases were marginal, but the imputed group had more frequently metabolic disorders. We therefore hypothesize that this somewhat more unhealthy group could have affected the association through a phenomenon in which a relative contribution of a macronutrient to an "already higher risk group" is less pronounced.[279] Either way, this could have more likely led to underestimation (rather than overestimation) of the effect that we observed. Finally, in terms of generalisability to younger, non-Caucasian cohorts, results have to be interpreted in the context of a different range of consumption of the various macronutrients by this predominant elderly population.

In conclusion, we found an independent association of high animal protein consumption and NAFLD in an overweight, predominantly aged Caucasian population. The results of this large study add to the current evidence on the importance of dietary composition in NAFLD. In particular, it shifts focus from the carbohydrate and fat debate towards the third, previously underexplored macronutrient, protein. The cause-effect relation and mechanistic pathways of this association remain unanswered for which more studies are needed. Ultimately, we need to understand more about the dietary components that put individuals at risk for NAFLD, before we can make any firm dietary recommendations for the prevention and treatment of NAFLD.

Supplementary Files

Supplementary Methods: details on the multiple imputation process

	Multiple imputation
Software used	R version 3.4.1
Imputation method and key settings	Fully conditional specification (package mice version 2.25)*;* maximum iterations: 50
No. of imputed data sets created	10
Analyses variables	total cholesterol; triglycerides; high density lipoprotein cholesterol; body mass index; glucose; weight; physical activity; smoking status; systolic blood pressure; diastolic blood pressure; ethnicity; education level; alcohol consumption; fat intake; waist circumference; kilocalorie intake; Dutch healthy diet index; age; anti-diabetic drugs; lipid-lowering drugs; antihypertensive drugs; fibre intake; study cohort, gender, steatosis, protein intake, carbohydrate intake;
Auxiliary variables	aspartate transaminase; alanine transaminase; homeostasis model assessment of insulin resistance; hip circumference; heart rate; spleen size; calcium intake; creatinine; glomerular filtration rate; vitamin E intake; potassium intake; magnesium intake; phosphorus intake; gamma-glutamyl transferase
Treatment of not normally distributed continuous variables	Predictive mean matching
Treatment of normally distributed variables	Linear regression
Treatment of binary/categorical variables	(Proportional odds) logistic regression
Population	For the imputation we used reliable and completed FFQs. In addition participants had to have less than 30% missing on study variables. Imputed population (n=4.754).

Supplementary Table 1: Imputation Characteristics

	Original Data n=3882	Imputed data*
Demographics		
Age (years)	**69.7 ± 8.8**	no missing data
Female (%)	**58.3**	no missing data
Caucasian (%)	**97.7**	97.6
Education Level (%)		
Low	**48.4**	48.4
Intermediate	**30.3**	30.2
High	**21.3**	21.3
Smoking status (%)		
Never / Past or Current	**36.7 / 63.3**	36.1 / 63.9
Alcohol (units/d)	**0.45 (0.05 – 1.20)**	no missing data
Physical Activity†	**41.3 (15.8 – 78.6)**	40.6 (15.7 – 77.6)
Caloric Intake (kcal/day)	**2031 (1620 – 2515)**	no missing data
Physical examination		
BMI (kg/m^2)	**26.9 (24.5 – 29.7)**	26.9 (24.5 – 29.7)

Supplementary Table 1 (*continued*)

	Original Data n=3882	Imputed data*
Lean	**30.2**	30.2
Overweight	**46.7**	46.7
Obese	**23.0**	23.0
WC (cm)		
Men	**98.2 ± 10.6**	98.2 ± 10.6
Women	**89.1 ± 12.2**	no missing data
Biochemistry		
AST (U/L)	**24 (21 – 28)**	24 (21 – 28)
ALT (U/L)	**18 (15 – 24)**	18 (15 – 24)
GGT (U/L)	**23 (17 – 34)**	23 (17 – 34)
Platelets (*10^9/L)	**262 (223 – 305)**	262 (223 – 305)
HOMA-IR	**2.6 (1.7 – 4.1)**	2.6 (1.7 – 4.1)
Total Cholesterol (mmol/L)	**5.4 ± 1.1**	5.4 ± 1.1
HDL-C (mmol/L)	**1.5 ± 0.4**	1.5 ± 0.4
Triglycerides (mmol/L)	**1.3 (1.0 – 1.7)**	1.3 (1.0 – 1.7)
Comorbidities		
Metabolic Syndrome (%)	**51.9**	51.9
- WC>88cm (♀) or >120cm (♂)	**43.2**	43.2
- Triglycerides >150mg/dL	**46.2**	46.0
- HDL-C <40mg/dL (♂) or 50mg/dL (♀)	**44.8**	44.7
- Blood pressure ≥130/85mmHg	**84.3**	84.3
- FPG>100mg/dL	**41.5**	41.5
Diabetes Mellitus (%)	**13.2**	13.1
Hypertension (%)	**74.0**	74.0
NAFLD (%)	**34.4**	no missing data

*Pooled data based on 10 imputations represent % for categorical variables and for continuous variables mean ± SD or median (P25-P75).†Physical activity in metabolic equivalent task hours/week.

Supplementary Table 2: Absolute macronutrient consumption in grams and energy percent per quartile

	Quartile 1	Quartile 2	Quartile 3	Quartile 4
Total Protein				
in grams per Q	74.0 ± 24.6	81.5 ± 25.0	85.0 ± 26.2	86.3 ± 31.0
in energy % per Q	12.4 ± 1.5	14.8 ± 0.47	16.4 ± 0.49	19.5 ± 2.2
Animal protein				
in grams per Q	35.2 ± 14.3	46.3 ± 14.5	51.4 ± 15.7	61.1 ± 23.6
in energy % per Q	5.9 ± 1.3	8.3 ± 0.48	10.1 ± 0.58	13.6 ± 2.6
Vegetable protein				
in grams per Q	26.0 ± 9.9	31.2 ± 10.2	34.3 ± 10.7	41.7 ± 15.8
in energy % per Q	4.7 ± 0.68	5.9 ± 0.23	6.7 ± 0.24	8.1 ± 0.93

Supplementary Table 2 (*continued*)

	Quartile 1	Quartile 2	Quartile 3	Quartile 4
Total Fat				
in grams per Q	54.2 ± 20.1	67.8 ± 21.8	80.4 ± 25.6	108.1 ± 57.8
in energy % per Q	24.4 ± 3.0	30.0 ± 1.2	33.9 ± 1.2	41.3 ± 6.7
Saturated fat				
in grams per Q	19.0 ± 6.9	24.5 ± 8.2	29.0 ± 9.6	38.9 ± 19.4
in energy % per Q	8.3 ± 1.1	10.6 ± 0.48	12.3 ± 0.55	15.6 ± 2.6
MUFA fat				
in grams per Q	17.3 ± 6.7	22.5 ± 7.6	27.0 ± 8.0	39.9 ± 24.8
in energy % per Q	7.8 ± 1.1	10.0 ± 0.43	11.5 ± 0.48	14.9 ± 3.5
PUFA fat				
in grams per Q	10.0 ± 4.0	13.5 ± 4.6	16.6 ± 5.3	24.2 ± 14.2
in energy % per Q	4.5 ± 0.65	5.9 ± 0.31	7.0 ± 0.33	9.3 ± 2.3
Trans fatty acid				
in grams per Q	0.74 ± 0.31	1.07 ± 0.36	1.32 ± 0.44	1.92 ± 0.92
in energy % per Q	0.33 ± 0.07	0.46 ± 0.03	0.56 ± 0.03	0.76 ± 0.15
Total Carbohydrate				
in grams per Q	194.3 ± 75.9	233.1 ± 75.9	255.1 ± 79.0	289.8 ± 103.4
in energy % per Q	36.9 ± 5.2	44.1 ± 1.2	48.4 ± 1.3	54.9 ± 3.5
Mono- & disaccharides				
in grams per Q	75.1 ± 34.7	107.9 ± 37.0	134.0 ± 42.6	174.3 ± 64.0
in energy % per Q	14.3 ± 3.2	20.6 ± 1.3	25.1 ± 1.4	32.7 ± 4.4
Polysaccharides				
in grams per Q	91.3 ± 36.4	115.0 ± 37.9	126.6 ± 40.6	148.2 ± 53.6
in energy % per Q	16.8 ± 2.8	21.3 ± 0.85	24.2 ± 0.93	29.4 ± 3.3
Fibre				
in grams per Q	19.5 ± 7.5	25.4 ± 8.7	29.5 ± 9.5	36.0 ± 13.1
in energy % per Q	1.8 ± 0.30	2.4 ± 0.13	2.8 ± 0.14	3.6 ± 0.48

Mean energy intake in grams or energy% (±SD) per quartile.

Supplementary Table 3: Stratified stepwise logistic regression models with macronutrient as independent variables and NAFLD as dependent variable using quartile 1 as reference

	Lean (BMI < 25 kg/m²) n=1174				
	Q2	Q3	Q4	*P* for trend	Continuous (per SD increase)
Model 1 (Sociodemographic)					
Total protein	1.10 (0.69 – 1.77)	0.91 (0.54 – 1.53)	1.08 (0.64 – 1.84)	0.965	0.99 (0.82 – 1.20)
Animal protein	**1.75 (1.09 – 2.81)‡**	0.94 (0.52 – 1.70)	1.33 (0.76 – 2.35)	0.718	1.00 (0.82 – 1.22)
Vegetable protein	1.00 (0.60 – 1.68)	0.85 (0.50 – 1.45)	0.78 (0.45 – 1.36)	0.304	0.92 (0.75 – 1.12)
Total carbohydrate	1.05 (0.64 – 1.74)	0.72 (0.42 – 1.23)	0.66 (0.38 – 1.16)	0.075	0.84 (0.69 – 1.02)
Mono-disaccharide	0.73 (0.44 – 1.21)	0.64 (0.38 – 1.09)	**0.55 (0.31 – 0.98)**	**0.038**	0.82 (0.66 – 1.01)
Polysaccharide	1.13 (0.66 – 1.93)	1.19 (0.69 – 2.04)	0.70 (0.37 – 1.32)	0.354	0.95 (0.76 – 1.17)
Fibre	0.69 (0.41 – 1.17)	0.65 (0.38 – 1.09)	0.68 (0.38 – 1.22)	0.173	0.94 (0.75 – 1.17)
Total fat	0.97 (0.55 – 1.71)	1.42 (0.83 – 2.43)	**1.70 (1.01 – 2.87)**	**0.017‡**	1.16 (0.98 – 1.37)
Saturated fat	1.03 (0.57 – 1.84)	1.22 (0.66 – 2.27)	1.83 (0.89 – 3.77)	0.089	1.16 (0.84 – 1.60)
MUFA	1.29 (0.72 – 2.29)	1.29 (0.68 – 2.43)	1.62 (0.77 – 3.39)	0.246	1.08 (0.82 – 1.41)
PUFA	0.92 (0.54 – 1.57)	0.79 (0.45 – 1.38)	0.85 (0.46 – 1.55)	0.503	0.90 (0.71 – 1.16)
Trans fatty acid	0.77 (0.44 – 1.35)	0.76 (0.42 – 1.37)	1.17 (0.60 – 2.28)	0.716	1.02 (0.75 – 1.39)
Model 2 (Lifestyle Confounding)					
Total protein	1.08 (0.67 – 1.73)	0.89 (0.52 – 1.50)	1.02 (0.58 – 1.79)	0.869	0.98 (0.81 – 1.20)
Animal protein	**1.69 (1.05 – 2.72)**	0.93 (0.51 – 1.69)	1.25 (0.69 – 2.28)	0.862	0.98 (0.79 – 1.21)
Vegetable protein	1.13 (0.66 – 1.91)	1.03 (0.59 – 1.80)	0.98 (0.55 – 1.77)	0.866	1.00 (0.81 – 1.23)
Total carbohydrate	1.19 (0.71 – 2.00)	0.88 (0.49 – 1.55)	0.89 (0.48 – 1.65)	0.503	0.93 (0.74 – 1.15)
Mono-disaccharide	0.83 (0.49 – 1.40)	0.76 (0.43 – 1.32)	0.72 (0.38 – 1.36)	0.297	0.91 (0.71 – 1.15)
Polysaccharide	1.16 (0.68 – 1.98)	1.26 (0.73 – 2.18)	0.78 (0.40 – 1.49)	0.591	0.99 (0.79 – 1.23)
Fibre	0.73 (0.43 – 1.25)	0.70 (0.39 – 1.25)	0.80 (0.41 – 1.57)	0.493	1.04 (0.80 – 1.35)
Total fat	1.01 (0.57 – 1.78)	1.46 (0.84 – 2.54)	1.75 (0.99 – 3.08)	**0.024**	1.15 (0.95 – 1.38)
Saturated fat	1.01 (0.56 – 1.83)	1.21 (0.64 – 2.28)	1.92 (0.91 – 4.06)	0.080	1.18 (0.84 – 1.65)
MUFA	1.23 (0.69 – 2.20)	1.25 (0.66 – 2.36)	1.50 (0.71 – 3.18)	0.334	1.03 (0.78 – 1.36)
PUFA	0.95 (0.56 – 1.64)	0.85 (0.48 – 1.49)	0.96 (0.51 – 1.79)	0.803	0.95 (0.74 – 1.22)
Trans fatty acid	0.75 (0.42 – 1.32)	0.73 (0.40 – 1.33)	1.13 (0.58 – 2.22)	0.782	1.02 (0.75 – 1.39)
Model 3 (Metabolic)					
Total protein	1.08 (0.66 – 1.75)	0.87 (0.51 – 1.49)	0.97 (0.55 – 1.72)	0.734	0.99 (0.81 – 1.20)
Animal protein	1.57 (0.97 – 2.55)	0.90 (0.49 – 1.65)	1.20 (0.66 – 2.20)	0.960	0.98 (0.79 – 1.22)
Vegetable protein	1.22 (0.71 – 2.09)	1.10 (0.62 – 1.94)	1.07 (0.59 – 1.94)	0.940	1.04 (0.84 – 1.28)
Total carbohydrate	1.21 (0.71 – 2.07)	0.96 (0.54 – 1.73)	0.96 (0.51 – 1.80)	0.721	0.95 (0.77 – 1.19)
Mono-disaccharide	0.85 (0.50 – 1.45)	0.81 (0.46 – 1.44)	0.77 (0.41 – 1.48)	0.436	0.94 (0.74 – 1.20)
Polysaccharide	1.06 (0.61 – 1.83)	1.25 (0.72 – 2.18)	0.74 (0.38 – 1.43)	0.560	0.99 (0.79 – 1.24)
Fibre	0.79 (0.45 – 1.36)	0.81 (0.45 – 1.45)	0.89 (0.44 – 1.77)	0.743	1.08 (0.83 – 1.40)
Total fat	1.01 (0.56 – 1.80)	1.41 (0.80 – 2.48)	1.69 (0.95 – 3.02)	**0.038**	1.12 (0.92 – 1.35)
Saturated fat	0.95 (0.52 – 1.72)	1.28 (0.67 – 2.44)	**2.21 (1.03 – 4.72)**	**0.030**	1.26 (0.89 – 1.78)
MUFA	1.20 (0.66 – 2.17)	1.24 (0.65 – 2.38)	1.49 (0.69 – 3.22)	0.342	0.98 (0.73 – 1.30)

Supplementary Table 3 (*continued*)

	Lean (BMI < 25 kg/m²) n=1174				
	Q2	Q3	Q4	*P* for trend	Continuous (per SD increase)
PUFA	1.04 (0.60 – 1.80)	0.88 (0.49 – 1.56)	0.98 (0.52 – 1.84)	0.804	0.96 (0.74 – 1.24)
Trans fatty acid	0.74 (0.41 – 1.31)	0.71 (0.39 – 1.31)	1.10 (0.55 – 2.18)	0.862	0.96 (0.70 – 1.32)
Model 1 (Sociodemographic)					
Total protein	1.17 (0.93 – 1.47)	**1.36 (1.09 – 1.69)‡**	**1.49 (1.19 – 1.85)‡**	**<0.001‡**	**1.16 (1.08 – 1.26)‡**
Animal protein	**1.30 (1.03 – 1.64)**	1.16 (0.93 – 1.45)	**1.62 (1.28 – 2.05)‡**	**<0.001‡**	**1.18 (1.08 – 1.28)‡**
Vegetable protein	1.08 (0.87 – 1.34)	0.98 (0.78 – 1.22)	1.25 (0.99 – 1.58)	0.144	1.08 (0.99 – 1.17)
Total carbohydrate	0.93 (0.75 – 1.15)	**0.78 (0.63 – 0.98)**	0.86 (0.68 – 1.09)	0.095	**0.91 (0.84 – 0.99)**
Mono-disaccharide	**0.73 (0.59 – 0.90)‡**	**0.71 (0.57 – 0.89)‡**	**0.67 (0.52 – 0.85)‡**	**0.001‡**	**0.89 (0.81 – 0.97)‡**
Polysaccharide	1.01 (0.81 – 1.25)	0.97 (0.78 – 1.22)	0.90 (0.71 – 1.15)	0.386	1.00 (0.91 – 1.09)
Fibre	0.85 (0.68 – 1.06)	1.00 (0.80 – 1.26)	0.93 (0.73 – 1.18)	0.910	1.01 (0.92 – 1.10)
Total fat	0.98 (0.79 – 1.22)	1.09 (0.88 – 1.36)	1.10 (0.89 – 1.37)	0.249	1.03 (0.96 – 1.12)
Saturated fat	0.96 (0.76 – 1.21)	0.95 (0.73 – 1.24)	0.86 (0.62 – 1.19)	0.406	0.91 (0.78 – 1.06)
MUFA	1.02 (0.81 – 1.30)	0.96 (0.74 – 1.24)	1.10 (0.80 – 1.50)	0.727	1.09 (0.96 – 1.25)
PUFA	1.02 (0.82 – 1.27)	0.97 (0.77 – 1.22)	0.89 (0.68 – 1.15)	0.351	0.94 (0.84 – 1.04)
Trans fatty acid	0.93 (0.74 – 1.17)	1.16 (0.90 – 1.48)	1.21 (0.89 – 1.65)	0.101	1.12 (0.98 – 1.27)
Model 2 (Lifestyle Confounding)					
Total protein	1.16 (0.92 – 1.46)	**1.32 (1.05 – 1.66)‡**	**1.40 (1.11 – 1.77)‡**	**0.003‡**	**1.14 (1.05 – 1.24)‡**
Animal protein	**1.29 (1.02 – 1.63)**	1.13 (0.90 – 1.43)	**1.54 (1.20 – 1.98)‡**	**0.004‡**	**1.14 (1.05 – 1.24)‡**
Vegetable protein	1.11 (0.89 – 1.38)	1.01 (0.81 – 1.28)	**1.29 (1.01 – 1.66)**	0.094	1.10 (1.00 – 1.20)
Total carbohydrate	0.95 (0.76 – 1.18)	0.84 (0.67 – 1.07)	0.93 (0.72 – 1.21)	0.435	0.94 (0.86 – 1.03)
Mono-disaccharide	**0.76 (0.61 – 0.95)**	**0.76 (0.60 – 0.96)**	**0.72 (0.55 – 0.94)‡**	**0.022**	0.93 (0.84 – 1.02)
Polysaccharide	1.00 (0.80 – 1.25)	0.95 (0.76 – 1.20)	0.86 (0.67 – 1.11)	0.233	0.98 (0.89 – 1.07)
Fibre	0.91 (0.72 – 1.15)	1.10 (0.85 – 1.42)	1.07 (0.81 – 1.41)	0.377	1.06 (0.96 – 1.18)
Total fat	0.96 (0.77 – 1.20)	1.04 (0.83 – 1.30)	0.99 (0.78 – 1.25)	0.896	1.00 (0.92 – 1.09)
Saturated fat	0.93 (0.73 – 1.17)	0.87 (0.67 – 1.14)	0.74 (0.53 – 1.04)	0.094	**0.84 (0.72 – 0.99)**
MUFA	1.04 (0.82 – 1.32)	0.96 (0.74 – 1.24)	1.09 (0.80 – 1.50)	0.775	1.12 (0.98 – 1.28)
PUFA	1.02 (0.82 – 1.27)	0.97 (0.77 – 1.22)	0.86 (0.66 – 1.12)	0.262	0.93 (0.83 – 1.04)
Trans fatty acid	0.92 (0.73 – 1.16)	1.15 (0.89 – 1.48)	1.17 (0.85 – 1.59)	0.161	1.12 (0.98 – 1.27)
Model 3 (Metabolic)					
Total protein	1.14 (0.90 – 1.45)	1.25 (0.99 – 1.59)	1.22 (0.95 – 1.55)	0.091	1.09 (1.00 – 1.18)
Animal protein	**1.31 (1.03 – 1.67)**	1.08 (0.84 – 1.67)	**1.36 (1.05 – 1.77)‡**	0.092	1.09 (1.00 – 1.20)
Vegetable protein	1.11 (0.88 – 1.40)	0.99 (0.78 – 1.26)	1.23 (0.95 – 1.59)	0.252	1.07 (0.97 – 1.18)
Total carbohydrate	1.05 (0.84 – 1.32)	0.93 (0.73 – 1.19)	1.06 (0.81 – 1.39)	0.906	0.97 (0.88 – 1.07)
Mono-disaccharide	0.80 (0.63 – 1.01)	0.85 (0.66 – 1.09)	0.83 (0.63 – 1.10)	0.283	0.98 (0.88 – 1.09)
Polysaccharide	0.93 (0.74 – 1.17)	0.96 (0.75 – 1.22)	0.83 (0.64 – 1.09)	0.246	0.97 (0.88 – 1.07)
Fibre	0.96 (0.76 – 1.23)	1.17 (0.90 – 1.52)	1.16 (0.86 – 1.55)	0.181	1.08 (0.97 – 1.20)
Total fat	1.01 (0.80 – 1.27)	1.10 (0.87 – 1.39)	0.98 (0.77 – 1.25)	0.915	0.99 (0.91 – 1.09)
Saturated fat	1.00 (0.78 – 1.28)	0.99 (0.75 – 1.31)	0.88 (0.62 – 1.26)	0.547	0.93 (0.78 – 1.09)
MUFA	1.13 (0.88 – 1.45)	1.00 (0.76 – 1.31)	1.07 (0.77 – 1.48)	0.958	1.08 (0.94 – 1.24)

Supplementary Table 3 (*continued*)

	Lean (BMI < 25 kg/m²) n=1174				
	Q2	**Q3**	**Q4**	***P* for trend**	**Continuous (per SD increase)**
PUFA	1.04 (0.83 – 1.31)	0.93 (0.73 – 1.18)	0.83 (0.63 – 1.10)	0.142	0.92 (0.82 – 1.03)
Trans fatty acid	0.94 (0.74 – 1.20)	1.16 (0.89 – 1.51)	1.08 (0.78 – 1.50)	0.332	1.07 (0.94 – 1.23)

Bold values indicate P<0.05. ‡ Indicates significant values using P < 0.021. Model 1 (socio-demographic) is adjusted for age, gender, education level and study cohort. Model 2 (lifestyle confounding) is in addition previous model adjusted for past or current smoking, alcohol in E%, physical activity, energy intake and DHDI. Model 3 (metabolic) is in addition to the previous model adjusted for cholesterol, metabolic syndrome and diabetes mellitus.

Supplementary Table 4: Substitution analyses of macronutrients in the metabolic model

Polysaccharide intake						
Mono-and disaccharides	1.00 (0.98 – 1.02)	0.938	1.01 (0.96 – 1.05)	0.800	1.00 (0.98 – 1.02)	0.824
Total protein	1.00 (0.97 – 1.03)	0.974	1.01 (0.94 – 1.09)	0.780	0.97 (0.94 – 1.00)	0.084
Animal protein	1.00 (0.96 – 1.03)	0.867	1.02 (0.94 – 1.11)	0.635	0.97 (0.94 – 1.01)	0.135
Vegetable protein	1.00 (0.88 – 1.13)	0.995	1.09 (0.82 – 1.45)	0.554	0.98 (0.86 – 1.12)	0.820
Total fat	1.00 (0.98 – 1.02)	0.744	0.99 (0.95 – 1.04)	0.730	0.99 (0.97 – 1.01)	0.600
Saturated fat	0.99 (0.94 – 1.04)	0.658	0.92 (0.82 – 1.04)	0.194	1.02 (0.96 – 1.08)	0.506
MUFA	0.98 (0.95 – 1.03)	0.458	1.02 (0.93 – 1.12)	0.708	0.96 (0.92 – 1.00)	0.067
PUFA	1.03 (0.98 – 1.08)	0.298	1.04 (0.92 – 1.18)	0.493	1.01 (0.96 – 1.07)	0.603
Fibre	0.97 (0.85 – 1.10)	0.619	0.92 (0.67 – 1.27)	0.618	0.93 (0.82 – 1.06)	0.297
Total fat intake						
Total carbohydrates	1.00 (0.99 – 1.02)	0.669	1.02 (0.99 – 1.05)	0.223	1.00 (0.99 – 1.02)	0.712
Mono- di saccharides	1.00 (0.99 – 1.02)	0.696	1.02 (0.99 – 1.05)	0.187	1.00 (0.99 – 1.02)	0.799
Polysaccharides	1.00 (0.98 – 1.02)	0.701	1.01 (0.96 – 1.06)	0.719	1.01 (0.99 – 1.03)	0.604
Total protein	1.00 (0.97 – 1.03)	0.957	1.02 (0.95 – 1.09)	0.657	0.97 (0.94 – 1.01)	0.114
Animal protein	1.00 (0.97 – 1.03)	0.961	1.02 (0.95 – 1.09)	0.652	0.97 (0.94 – 1.01)	0.123
Vegetable protein	1.01 (0.92 – 1.11)	0.820	1.04 (0.83 – 1.30)	0.730	1.00 (0.90 – 1.11)	0.995
Fibre	0.99 (0.88 – 1.11)	0.883	1.02 (0.75 – 1.37)	0.915	0.95 (0.84 – 1.07)	0.370
Saturated fat intake						
MUFA	0.99 (0.91 – 1.07)	0.794	1.10 (0.92 – 1.32)	0.289	0.92 (0.85 – 1.01)	0.066
PUFA	1.04 (0.97 – 1.11)	0.277	1.12 (0.96 – 1.31)	0.150	0.99 (0.92 – 1.06)	0.773
Total carbohydrates	1.01 (0.95 – 1.06)	0.792	1.09 (0.97 – 1.24)	0.150	0.96 (0.91 – 1.02)	0.218
Mono- di saccharides	1.01 (0.95 – 1.07)	0.780	1.11 (0.98 – 1.27)	0.105	0.96 (0.90 – 1.02)	0.179
Polysaccharides	1.01 (0.95 – 1.06)	0.803	1.09 (0.96 – 1.23)	0.188	0.97 (0.91 – 1.02)	0.230
Protein	1.00 (0.94 – 1.08)	0.907	1.11 (0.94 – 1.31)	0.209	**0.93 (0.86 – 1.00)**	**0.041**
Animal protein	1.01 (0.94 – 1.08)	0.839	1.11 (0.95 – 1.31)	0.197	**0.93 (0.86 – 1.00)**	**0.039**
Vegetable protein	0.97 (0.87 – 1.07)	0.522	1.06 (0.82 – 1.38)	0.650	0.91 (0.81 – 1.02)	0.093
Fibre	0.92 (0.81 – 1.05)	0.226	1.03 (0.74 – 1.43)	0.876	**0.83 (0.72 – 0.95)**[‡]	**0.006**[‡]
MUFA intake						
Saturated fat	1.00 (0.93 – 1.08)	0.975	0.92 (0.77 – 1.10)	0.357	1.05 (0.96 – 1.14)	0.291
PUFA	1.04 (0.96 – 1.13)	0.342	1.02 (0.84 – 1.23)	0.834	1.05 (0.96 – 1.15)	0.315
Total carbohydrates	1.01 (0.97 – 1.06)	0.502	1.00 (0.91 – 1.09)	0.929	1.03 (0.99 – 1.08)	0.166
Mono- di saccharides	1.01 (0.97 – 1.06)	0.498	1.00 (0.91 – 1.10)	0.941	1.03 (0.98 – 1.08)	0.206
Polysaccharides	1.01 (0.97 – 1.06)	0.553	0.98 (0.89 – 1.08)	0.700	1.04 (0.99 – 1.08)	0.149
Total protein	1.01 (0.97 – 1.06)	0.640	1.01 (0.91 – 1.12)	0.875	0.99 (0.94 – 1.05)	0.834
Animal protein	1.01 (0.97 – 1.06)	0.613	1.01 (0.91 – 1.12)	0.863	0.99 (0.94 – 1.05)	0.819
Vegetable protein	0.97 (0.88 – 1.07)	0.554	0.96 (0.76 – 1.22)	0.747	0.97 (0.87 – 1.08)	0.620
Fibre	0.93 (0.82 – 1.05)	0.258	0.93 (0.67 – 1.30)	0.686	0.89 (0.78 – 1.01)	0.072
PUFA intake						
Saturated fat	0.96 (0.90 – 1.02)	0.181	0.90 (0.78 – 1.05)	0.183	0.98 (0.92 – 1.06)	0.651
MUFA	0.95 (0.87 – 1.03)	0.209	0.99 (0.82 – 1.19)	0.903	0.92 (0.84 – 1.01)	0.065

Supplementary Table 4 (*continued*)

Polysaccharide intake						
Total carbohydrates	0.97 (0.92 – 1.02)	0.183	0.98 (0.87 – 1.11)	0.745	0.96 (0.91 – 1.02)	0.172
Mono- di saccharides	0.97 (0.92 – 1.02)	0.182	0.97 (0.86 – 1.10)	0.677	0.96 (0.91 – 1.02)	0.191
Polysaccharides	0.96 (0.91 – 1.02)	0.204	0.95 (0.83 – 1.09)	0.479	0.97 (0.91 – 1.03)	0.290
Total protein	0.96 (0.91 – 1.02)	0.199	0.99 (0.86 – 1.14)	0.921	**0.93 (0.88 – 0.99)‡**	**0.014‡**
Animal protein	0.96 (0.91 – 1.02)	0.166	0.98 (0.85 – 1.14)	0.821	**0.93 (0.88 – 0.99)‡**	**0.019‡**
Vegetable protein	0.92 (0.82 – 1.03)	0.151	0.94 (0.70 – 1.26)	0.667	0.91 (0.80 – 1.02)	0.117
Fibre	0.89 (0.77 – 1.02)	0.083	0.92 (0.65 – 1.30)	0.652	**0.83 (0.71 – 0.95)‡**	**0.009‡**

Bold values indicate P<0.05. ‡Indicates significant values using P < 0.021. Substitution model was performed adjusted for age, gender, education level, study cohort, past or current smoking, alcohol in E%, physical activity, energy intake, DHDI, cholesterol, metabolic syndrome, diabetes mellitus and for log-transformed BMI in the overall group.

Supplementary Table 5: Complete case analyses

	Overall n=3259				
	Q2	**Q3**	**Q4**	***P* for trend**	**Continuous (per SD increase)**
Model 4 (Metabolic + log-transformed BMI)					
Total protein	1.11 (0.87 – 1.42)	1.16 (0.91 – 1.48)	1.04 (0.80 – 1.34)	0.741	1.02 (0.93 – 1.12)
Animal protein	1.30 (1.01 – 1.66)	0.99 (0.77 – 1.27)	1.23 (0.94 – 1.61)	0.480	1.02 (0.92 – 1.13)
Vegetable protein	0.95 (0.75 – 1.21)	0.81 (0.63 – 1.04)	0.99 (0.76 – 1.30)	0.633	1.02 (0.93 – 1.13)
Total carbohydrate	1.22 (0.96 – 1.56)	1.10 (0.85 – 1.43)	1.24 (0.94 – 1.64)	0.241	1.04 (0.94 – 1.15)
Mono-disaccharide	0.83 (0.65 – 1.06)	0.96 (0.74 – 1.25)	0.97 (0.72 – 1.30)	0.891	1.04 (0.93 – 1.16)
Polysaccharide	0.97 (0.76 – 1.23)	1.04 (0.82 – 1.34)	0.83 (0.63 – 1.09)	0.320	1.02 (0.92 – 1.12)
Fibre	0.88 (0.68 – 1.13)	1.03 (0.78 – 1.35)	1.00 (0.73 – 1.36)	0.697	1.01 (0.90 – 1.14)
Total fat	1.17 (0.92 – 1.48)	1.15 (0.90 – 1.46)	1.03 (0.79 – 1.33)	0.840	0.98 (0.89 – 1.08)
Saturated fat	1.02 (0.79 – 1.32)	1.07 (0.80 – 1.43)	1.02 (0.71 – 1.48)	0.837	0.98 (0.83 – 1.17)
MUFA	**1.32 (1.02 – 1.70)**	1.15 (0.86 – 1.53)	1.29 (0.92 – 1.83)	0.326	1.05 (0.90 – 1.22)
PUFA	1.06 (0.84 – 1.35)	0.90 (0.70 – 1.16)	0.92 (0.69 – 1.23)	0.342	0.92 (0.81 – 1.03)
Trans fatty acid	0.98 (0.76 – 1.26)	1.07 (0.82 – 1.41)	1.02 (0.72 – 1.43)	0.745	1.01 (0.88 – 1.17)
	Lean (BMI < 25 kg/m²) n=1011				
	Q2	**Q3**	**Q4**	***P* for trend**	**Continuous (per SD increase)**
Model 3 (Metabolic)					
Total protein	1.02 (0.60 – 1.74)	0.97 (0.55 – 1.72)	0.85 (0.45 – 1.61)	0.636	0.98 (0.79 – 1.22)
Animal protein	1.53 (0.90 – 2.59)	0.83 (0.42 – 1.62)	1.13 (0.58 – 2.22)	0.839	0.98 (0.78 – 1.24)
Vegetable protein	1.16 (0.65 – 2.09)	0.89 (0.47 – 1.68)	0.99 (0.52 – 1.90)	0.770	1.03 (0.81 – 1.30)
Total carbohydrate	1.15 (0.64 – 2.08)	0.95 (0.50 – 1.82)	1.01 (0.50 – 2.02)	0.877	0.99 (0.77 – 1.26)
Mono-disaccharide	0.89 (0.49 – 1.61)	0.84 (0.44 – 1.60)	0.88 (0.43 – 1.81)	0.719	0.97 (0.74 – 1.27)
Polysaccharide	1.28 (0.69 – 2.35)	1.49 (0.81 – 2.76)	0.78 (0.36 – 1.65)	0.735	1.00 (0.78 – 1.29)
Fibre	0.67 (0.36 – 1.22)	0.70 (0.36 – 1.34)	0.81 (0.38 – 1.72)	0.614	1.04 (0.78 – 1.39)
Total fat	1.11 (0.59 – 2.09)	1.31 (0.70 – 2.44)	1.77 (0.94 – 3.33)	0.064	1.10 (0.89 – 1.37)
	Lean (BMI < 25 kg/m²) n=1011				
	Q2	**Q3**	**Q4**	***P* for trend**	**Continuous (per SD increase)**
Saturated fat	0.91 (0.47 – 1.75)	1.13 (0.55 – 2.32)	**2.67 (1.16 – 6.19)**	**0.022**	1.34 (0.91 – 1.97)
MUFA	1.17 (0.61 – 2.23)	1.12 (0.55 – 2.30)	1.52 (0.65 – 3.52)	0.388	0.92 (0.67 – 1.26)
PUFA	1.00 (0.55 – 1.83)	0.95 (0.51 – 1.79)	1.12 (0.57 – 2.23)	0.788	1.02 (0.77 – 1.36)
Trans fatty acid	0.77 (0.41 – 1.44)	0.64 (0.32 – 1.26)	1.10 (0.51 – 2.36)	0.976	0.91 (0.63 – 1.31)
	Overweight (BMI ≥ 25 kg/m²) n=2248				
	Q2	**Q3**	**Q4**	***P* for trend**	**Continuous (per SD increase)**
Model 3 (Metabolic)					
Total protein	1.15 (0.89 – 1.50)	1.26 (0.97 – 1.64)	**1.34 (1.02 – 1.76)**	**0.028**	**1.12 (1.02 – 1.23)**
Animal protein	1.29 (0.99 – 1.68)	1.05 (0.80 – 1.37)	**1.52 (1.14 – 2.03)‡**	**0.027**	**1.13 (1.02 – 1.25)**
Vegetable protein	0.98 (0.76 – 1.26)	0.83 (0.64 – 1.08)	1.13 (0.85 – 1.50)	0.735	1.06 (0.95 – 1.17)

Supplementary Table 5 (*continued*)

	Overall n=3259				
	Q2	**Q3**	**Q4**	***P* for trend**	**Continuous (per SD increase)**
Total carbohydrate	1.13 (0.88 – 1.46)	0.99 (0.76 – 1.29)	1.11 (0.83 – 1.48)	0.701	0.99 (0.89 – 1.11)
Mono-disaccharide	0.75 (0.58 – 0.97)	0.78 (0.59 – 1.02)	0.84 (0.62 – 1.14)	0.324	1.00 (0.89 – 1.12)
Polysaccharide	0.89 (0.69 – 1.14)	0.95 (0.73 – 1.23)	0.80 (0.60 – 1.07)	0.222	0.98 (0.88 – 1.09)
Fibre	0.95 (0.73 – 1.25)	1.13 (0.84 – 1.51)	1.10 (0.80 – 1.52)	0.354	1.04 (0.92 – 1.17)
Total fat	1.10 (0.86 – 1.42)	1.09 (0.85 – 1.41)	0.94 (0.72 – 1.23)	0.713	0.97 (0.88 – 1.07)
Saturated fat	1.01 (0.78 – 1.33)	1.01 (0.74 – 1.37)	0.78 (0.53 – 1.16)	0.292	0.90 (0.75 – 1.08)
MUFA	1.26 (0.96 – 1.66)	1.12 (0.83 – 1.51)	1.21 (0.84 – 1.75)	0.511	1.12 (0.95 – 1.32)
PUFA	0.98 (0.76 – 1.26)	0.83 (0.63 – 1.08)	0.82 (0.60 – 1.12)	0.120	0.88 (0.78 – 1.01)
Trans fatty acid	1.02 (0.79 – 1.34)	1.16 (0.87 – 1.55)	1.02 (0.71 – 1.46)	0.678	1.05 (0.90 – 1.23)

Bold values indicate P<0.05. ‡Indicates significant values using P < 0.021. Model 3 (metabolic) is in addition to the previous model adjusted for cholesterol, metabolic syndrome and diabetes mellitus. Model 4 (metabolic + log-transformed BMI) is in addition to the previous model adjusted for log-transformed BMI.

Supplementary Table 6: Characteristics of imputed and complete cases

	Complete cases (n=3259)	Imputed cases (n=623)	*P-value
Age (years)	69.5 ± 8.6	71.1 ± 9.7	<0.001
Female (%)	60.4	47.0	<0.001
Caucasian (%)	97.8	97.0	0.256
RS cohort I / II / III (%)	26.6 / 30.5 / 42.9	32.4 / 30.2 / 37.4	0.006
Education Level (%)			
Low	48.2	49.4	0.287
Intermediate	30.1	31.8	
High	21.7	18.8	
Smoking status (%)			
Never / Past or Current	37.2 / 62.8	33.6 / 66.4	0.151
Alcohol (units/d)	0.45 (0.06 – 1.19)	0.40 (0.03 – 1.21)	0.439
Physical Activity†	41.5 (15.8 – 79.0)	36.3 (18.0 – 73.9)	0.645
Caloric Intake (kcal/day)	2040 (1636 – 2514)	1976 (1541 – 2525)	0.079
BMI (kg/m²)	26.8 (24.4 – 29.5)	27.4 (24.8 – 30.6)	<0.001
Lean	31.0	26.2	0.015
Overweight	69.0	73.8	
ALT (U/L)	18 (15 – 24)	18 (15 – 24)	0.607
HOMA-IR	2.6 (1.7 – 4.0)	2.7 (1.8 – 4.3)	0.099
Total Cholesterol (mmol/L)	5.4 ± 1.1	5.3 ± 1.1	0.016
Metabolic Syndrome (%)	51.3	55.0	0.102
Hypertension (%)	73.6	76.1	0.192
Diabetes Mellitus (%)	12.4	17.3	0.001
NAFLD (%)	33.7	38.4	0.025

Data on imputed cases is original data, not imputed on the present variables. Data represent % for categorical variables and for continuous variables mean ± SD or median (P25 – P75).**P*-value is based on T-test, Wilcoxon rank sum test, Chi-square test or Fisher's exact test. †Physical activity in metabolic equivalent task hours/week.

Supplementary Table 7: Sensitivity analyses using RS-cohort I and II in which FFQs were completed at the same time as all other measurements, amongst them abdominal ultrasound

	RS I and II only n=2252				
	Q2	**Q3**	**Q4**	***P* for trend**	**Continuous (per SD increase)**
Model 4 (Metabolic + log-transformed BMI)					
Total protein	1.08 (0.80 – 1.45)	1.02 (0.76 – 1.37)	0.92 (0.68 – 1.25)	0.537	0.97 (0.88 – 1.07)
Animal protein	1.27 (0.93 – 1.72)	0.99 (0.73 – 1.34)	1.11 (0.80 – 1.53)	0.949	0.97 (0.87 – 1.08)
Vegetable protein	0.91 (0.69 – 1.20)	0.79 (0.59 – 1.06)	1.08 (0.78 – 1.49)	0.951	1.01 (0.89 – 1.13)
Total carbohydrate	**1.35 (1.01 – 1.80)**	1.20 (0.88 – 1.64)	1.18 (0.84 – 1.65)	0.493	1.02 (0.91 – 1.15)
Mono-disaccharide	0.91 (0.68 – 1.23)	1.02 (0.74 – 1.40)	0.91 (0.65 – 1.30)	0.786	1.03 (0.91 – 1.17)
Polysaccharide	0.92 (0.70 – 1.22)	0.90 (0.67 – 1.21)	0.88 (0.63 – 1.24)	0.454	0.99 (0.88 – 1.12)
Fibre	0.94 (0.69 – 1.27)	1.13 (0.81 – 1.56)	1.03 (0.71 – 1.50)	0.624	1.06 (0.92 – 1.21)
Total fat	1.19 (0.89 – 1.59)	**1.42 (1.06 – 1.90)‡**	1.16 (0.86 – 1.57)	0.180	1.03 (0.93 – 1.15)
Saturated fat	1.10 (0.80 – 1.51)	1.24 (0.88 – 1.76)	1.16 (0.75 – 1.79)	0.398	1.04 (0.85 – 1.27)
MUFA	1.34 (0.99 – 1.82)	1.16 (0.82 – 1.64)	1.01 (0.67 – 1.53)	0.850	0.96 (0.81 – 1.13)
PUFA	1.28 (0.97 – 1.70)	1.04 (0.77 – 1.41)	0.99 (0.70 – 1.39)	0.728	1.00 (0.87 – 1.14)
Trans fatty acid	0.97 (0.71 – 1.32)	1.20 (0.87 – 1.67)	1.26 (0.84 – 1.90)	0.158	1.08 (0.91 – 1.28)
	Lean (BMI < 25 kg/m²) in RSI and II n=664				
	Q2	**Q3**	**Q4**	***P* for trend**	**Continuous (per SD increase)**
Model 3 (Metabolic)					
Total protein	1.51 (0.81 – 2.85)	0.84 (0.40 – 1.79)	1.07 (0.50 – 2.32)	0.817	0.95 (0.74 – 1.23)
Animal protein	1.64 (0.85 – 3.17)	0.86 (0.37 – 1.97)	1.20 (0.54 – 2.64)	0.998	0.95 (0.72 – 1.25)
Vegetable protein	0.89 (0.45 – 1.77)	0.77 (0.37 – 1.61)	1.16 (0.53 – 2.52)	0.897	1.02 (0.77 – 1.36)
	Lean (BMI < 25 kg/m²) in RSI and II n=664				
	Q2	**Q3**	**Q4**	***P* for trend**	**Continuous (per SD increase)**
Total carbohydrate	1.00 (0.47 – 2.13)	1.44 (0.67 – 3.10)	1.04 (0.45 – 2.43)	0.729	1.00 (0.75 – 1.35)
Mono-disaccharide	0.68 (0.32 – 1.46)	0.82 (0.37 – 1.81)	0.82 (0.35 – 1.94)	0.794	1.02 (0.74 – 1.40)
Polysaccharide	0.83 (0.41 – 1.65)	0.90 (0.43 – 1.90)	0.69 (0.29 – 1.64)	0.482	0.98 (0.72 – 1.34)
Fibre	0.83 (0.40 – 1.74)	0.99 (0.44 – 2.24)	1.12 (0.43 – 2.91)	0.730	1.16 (0.80 – 1.66)
Total fat	1.15 (0.55 – 2.39)	1.44 (0.70 – 2.98)	1.33 (0.61 – 2.87)	0.386	1.07 (0.84 – 1.37)
Saturated fat	0.87 (0.39 – 1.92)	1.08 (0.45 – 2.60)	2.19 (0.80 – 6.01)	0.119	1.44 (0.91 – 2.26)
MUFA	1.50 (0.72 – 3.14)	1.04 (0.45 – 2.44)	1.05 (0.37 – 3.00)	0.850	0.83 (0.56 – 1.23)
PUFA	0.93 (0.45 – 1.90)	1.03 (0.49 – 2.16)	0.94 (0.40 – 2.23)	0.964	1.03 (0.73 – 1.45)
Trans fatty acid	0.85 (0.40 – 1.83)	0.66 (0.28 – 1.54)	1.07 (0.41 – 2.77)	0.947	0.91 (0.61 – 1.36)

Supplementary Table 7 *(contined)*

	Overweight (BMI ≥ 25 kg/m²) in RSI and II n=1588				
	Q2	**Q3**	**Q4**	***P* for trend**	**Continuous (per SD increase)**
Model 3 (Metabolic)					
Total protein	1.03 (0.75 – 1.41)	1.12 (0.82 – 1.53)	1.16 (0.85 – 1.59)	0.282	1.07 (0.96 – 1.18)
Animal protein	1.29 (0.93 – 1.78)	1.07 (0.78 – 1.47)	1.33 (0.95 – 1.85)	0.229	1.07 (0.96 – 1.20)
Vegetable protein	0.99 (0.74 – 1.31)	0.85 (0.63 – 1.16)	1.17 (0.84 – 1.64)	0.675	1.03 (0.91 – 1.16)
Total carbohydrate	1.30 (0.96 – 1.75)	0.96 (0.69 – 1.32)	1.04 (0.73 – 1.47)	0.747	0.97 (0.86 – 1.09)
Mono-disaccharide	0.93 (0.68 – 1.27)	0.88 (0.64 – 1.23)	0.81 (0.57 – 1.16)	0.251	0.98 (0.86 – 1.11)
Polysaccharide	0.89 (0.67 – 1.19)	0.84 (0.62 – 1.15)	0.88 (0.62 – 1.25)	0.388	0.96 (0.85 – 1.08)
Fibre	1.01 (0.73 – 1.38)	1.19 (0.85 – 1.68)	1.08 (0.74 – 1.59)	0.517	1.08 (0.94 – 1.24)
Total fat	1.05 (0.78 – 1.41)	1.33 (0.98 – 1.79)	1.08 (0.79 – 1.47)	0.345	1.02 (0.91 – 1.14)
Saturated fat	1.08 (0.78 – 1.49)	1.20 (0.84 – 1.71)	0.94 (0.60 – 1.47)	0.996	0.93 (0.75 – 1.15)
MUFA	1.23 (0.89 – 1.69)	1.20 (0.84 – 1.71)	1.04 (0.68 – 1.60)	0.885	1.07 (0.90 – 1.27)
PUFA	1.22 (0.91 – 1.64)	0.93 (0.68 – 1.28)	0.85 (0.60 – 1.21)	0.221	0.93 (0.81 – 1.08)
Trans fatty acid	0.97 (0.71 – 1.33)	1.26 (0.90 – 1.77)	1.29 (0.84 – 1.96)	0.124	1.11 (0.93 – 1.33)

Bold values indicate P<0.05. ‡Indicates significant values using P < 0.021. Model 3 (metabolic) is in addition to the previous model adjusted for cholesterol, metabolic syndrome and diabetes mellitus. Model 4 (metabolic + log-transformed BMI) is in addition to the previous model adjusted for log-transformed BMI.

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Chapter 6

Diet-dependent acid load – the missing link between an animal protein-rich diet and non-alcoholic fatty liver disease?

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Abstract

Introduction Our group recently showed that animal protein was independently associated with non-alcoholic fatty liver disease (NAFLD). We hypothesize that this may be explained by a high diet-dependent acid load (DAL).

Methods This cross-sectional study is embedded in a prospective population-based cohort. We estimated DAL-proxies via food-frequency questionnaires using potential renal acid load (PRAL; using dietary protein, phosphorus, potassium, calcium, and magnesium intake), net endogenous acid production (NEAP; using protein and potassium intake), and animal-protein-to-potassium-ratio (A:P). We defined NAFLD using ultrasound after excluding secondary steatogenic causes. We used logistic regression models –adjusted for socio-demographic, lifestyle, and metabolic traits– on categorized (Q1-Q4) and continuous DAL-proxies (allowing for non-linearity) and NAFLD.

Results We included 3882 participants of which 1337 had NAFLD. All DAL-proxies were higher, meaning more acidic, in individuals with NAFLD (PRAL: –2.9 vs –5.5mEq/day; NEAP: 37.0 vs 35.1mEq/day, and AP:13.3 vs 12.4; all $P<0.001$). The highest quartile of DAL-proxies was associated with NAFLD independent of socio-demographic and lifestyle confounders, but significance dissipated after correction for metabolic confounders and multiple testing. However, the *P*-value for non-linearity was significant in all DAL-proxies ($P<0.001$). Natural cubic splines performed better with than without DAL-proxies in the fully adjusted model (all $P\leq0.038$). The highest probability of NAFLD was found for an acidic diet.

Conclusions This study showed an independent non-linear association between an acidic diet and NAFLD. Further studies with acid-base biomarkers are needed, but our findings might provide a mechanistic explanation for the harmful association between an animal protein-rich diet and NAFLD.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease worldwide with an estimated prevalence of 25% in the adult population.[280] Its occurrence closely parallels the obesity epidemic. Obesity and insulin resistance are therefore reckoned as novel risk factors for liver disease in absence of the traditional risk factors, i.e. alcohol misuse or viral hepatitis.[281] NAFLD can progress to more severe liver disease with hepatic fibrosis or cirrhosis potentially leading to need for transplantation or even liver-related death.[22] In addition, NAFLD is a major risk factor for incidence cardiovascular disease,[30] and indeed the most common cause of death in NAFLD is related to cardiovascular events.[282] Given the above, it is of great public health interest to unravel NAFLD pathophysiology in order to improve understanding and treatment thereof.

Adhering to a healthy lifestyle, in terms of implementing a well-balanced diet and effectuating adequate physical activity, is the cornerstone of treatment of NAFLD across the entire spectrum of the disease.[283,284] Our contemporary Western diet, on the other hand, is related with an increased risk of NAFLD development.[285] This Western diet typically consists of a high intake of animal-based food products and sugar-containing beverages, and by low intake of fruit, vegetables and whole grains.[286] Our group recently showed that animal protein was the only (subtype of) macronutrient that was independently associated with higher prevalence of NAFLD in an elderly population-based cohort.[287] Interestingly, others have found that red meat intake was associated with increased overall mortality, and in particular with liver-related mortality.[260] However, the underlying mechanisms that contribute to this association remain elusive.

It has been previously postulated that a Western diet may cause low-grade metabolic acidosis, which may subsequently lead to metabolic disturbances such as type 2 diabetes[288] and cardiovascular diseases.[289] The rationale for this hypothesis is that this diet is rich in food items that supply acid precursors (i.e. non-carbonic acids such as sulfuric acid from meat and fish) and low in food items that supply base precursors (i.e. alkali salts from organic acids such as citrate and bicarbonate from vegetables and fruits), leading to a disturbance in acid-base balance.[290,291] Two preceding studies have suggested an association between diet-dependent acid load (DAL) and NAFLD, independent of body mass index (BMI).[259,275] Amongst them, Krupp et al. showed that potential renal acid load (PRAL, a proxy of DAL), was associated with alanine aminotransferase (ALT) and steatosis, as defined by a surrogate diagnostic algorithm, in a small study of healthy adolescents.[275] Moreover, Chan and colleagues demonstrated that net endogenous acid production (NEAP, another proxy of DAL) but not PRAL, was associated with MRI-diagnosed NAFLD in a Chinese population (n=793).[259] In addition, two recent Japanese studies have showed an association between low urine pH (indicator of metabolic acidosis) and incident NAFLD.[292,293]

To date, however, there has been no large-scale study on the association of diet-dependent acid load and NAFLD in Western adults. We therefore aimed to evaluate if DAL (as assessed by PRAL, NEAP, and animal protein-to-potassium ratio [A:P]), was independently associated with ultrasound-defined NAFLD in an elderly Western population.

Subjects and Methods

Study Population

This study is embedded in The Rotterdam Study, a prospective cohort study, that was initiated in the mid-1980s in order to study our aging population in more detail.[248] The design and rationale behind The Rotterdam Study have been described in more detail previously[248,287] and is also described in the *Supplementary Methods I*.[294] In short, all participants of The Rotterdam Study reside in Ommoord, a suburb of Rotterdam, the Netherlands, and all participants were aged 45 or 55 years or above at time of first enrolment. The Rotterdam Study consists of three different cohorts (RS I, RS II, and RS III) that each visited the research centre multiple times. From 2009 onwards the Hepatology department joined this research initiative by expanding the extensive physical work-up with liver imaging (this comprises cohort RS I-5, RS II-3, and RS III-2). The Rotterdam Study has been approved by the institutional review board (Medical Ethics Committee) of the Erasmus MC University Medical Centre Rotterdam and by the review board of The Netherlands Ministry of Health, Welfare, and Sports. Written informed consent was obtained from all participants.

Dietary data and diet-dependent acid load

We requested all participants to complete an externally validated, semi-quantitative, 389-item food frequency questionnaire (FFQ) that was developed for Dutch adults.[98,249] Habitual dietary intake was assessed by means of detailed questions on food item consumption over the last month that addressed not only type of food, but also quantity, portion size, and preparation methods. Servings were estimated in grams or in milligrams per day using standardised household measures.[250] We extracted macronutrient intake from the questionnaires using the Dutch Food Composition Table (NEVO v2011) that contains information on nutrient content per gram or serving per product. DAL was calculated using three previously defined algorithms, which we will refer to as DAL-proxies from this point forward. The DAL-proxies included: 1) potential renal acid load (PRAL),[295] 2) net endogenous acid production (NEAP),[291] and 3) animal protein-to-potassium ratio (A:P).[296]

Remer and Manz developed PRAL to proxy the renal net acid excretion using nutrient intake data.[295] PRAL was validated against urine pH in 24 hour urine samples from 63 healthy volunteers.

PRAL (mEq/day)[295] = *0.4888 x protein [g/day] +*
0.0366 x phosphorus [mg/day] -
0.0205 x potassium [mg/day] -
0.0125 x calcium [mg/day] –
0.0263 x magnesium [mg/day]

Shortly after, Frassetto et al. developed a simplified algorithm (NEAP) using dietary protein as acid precursor and potassium only, as base precursor from organic anions.[291] This algorithm was validated in 141 healthy men and women consuming 20 different diets, and NEAP accounted for over 70% of the variation in renal net acid excretion.

NEAP (mEq/day)[291] = *(54.5 x protein [g/day] / potassium [mEq/day]) – 10.2*

Finally, Zwart and colleagues suggested the use of the ratio between animal protein intake and potassium intake instead of total dietary protein, as animal protein is considered to be the main contributor to diet-dependent acid load.[296,297]

A:P[296] = *animal protein [g/day] / potassium [g/day]*

We assessed adherence to the Dutch Dietary Guidelines 2015, using a predefined index that describes a general advice to follow a balanced and healthy dietary pattern,[298] in order to assess dietary quality (DQ). Briefly, this guideline comprises specified recommendations on I) vegetables (≥200g/day), II) fruit (≥200g/day), III) whole-grain products (≥90g/day), IV) legumes (≥135g/week), V) unsalted nuts (≥15g/day), VI) fish (≥100g/week), VII) dairy (≥350g/day), VIII) tea (≥150mL/day), IX) whole grains ≥50% of total grains, X) unsaturated fats and oils ≥50% of total fats, XII) red and processed meat <300g/week, XIII) sugar-containing beverages (≤150mL/day), XIV) alcohol (≤10 g/day), and XV) salt (≤6 g/day). Incomplete FFQs were excluded as well as FFQs with unreliable energy intake (i.e. <500 calories/day or ≥7500 calories/day).

Assessment of steatosis

Abdominal ultrasound was performed by a certified and experienced technician on a Hitachi HI VISION 900 (PvW). Ultrasound images were stored digitally so re-evaluation by an experienced hepatologist (RdK) was possible. All FFQs were filled in prior to liver

imaging, so participants were unaware of the ultrasound results when completing the FFQ. Diagnosis of steatosis was determined dichotomously,[104] measuring the hyper-echogenicity of liver parenchyma. In order to define NAFLD, we first excluded participants that used steatogenic drugs (i.e. systemic corticosteroids, methotrexate, tamoxifen or amiodarone). This was identified through linkage with pharmacy data. Second, participants with viral hepatitis were excluded, based on hepatitis B surface antigen and anti-hepatitis C virus serology which was assessed using an automatic immunoassay (Roche Diagnostic GmbH). And third, we excluded participants with excessive alcohol consumption (>30g/day for men and >20 g/day for women, based on the FFQ).

Other Covariates

We obtained data on demographics, physical activity, and education level by means of an extensive home interview by qualified interviewers. Briefly, physical activity was assessed using the LASA Physical Activity Questionnaire and expressed in metabolic equivalent of task (MET)hours/week.[299] Blood pressure was measured at a single visit using two successive measurements in a sitting position, and blood samples were collected after overnight fasting. Automatic enzyme procedures were used to measure blood lipids, platelet count, glucose, ALT, aspartate aminotransferase, and gamma-glutamyltransferase (Roche Diagnostic GmbH, Mannheim, DE). An automatic immunoassay was used to determine insulin (Roche Diagnostic GmbH). Estimated glomerular filtration rate (GFR in [ml/min/1.73 m^2]) was based on calibrated creatinine levels using the CKD-EPI Creatinine Equation (2009). Creatinine levels were calibrated by aligning mean values of serum creatinine from our cohort with those of the Third National Health and Nutrition Examination Survey (NHANES III) in different age and sex specific categories.[300] Anthropometrics were measured by well-trained research assistants. The presence of hypertension was diagnosed if either systolic (≥140mmHg) or diastolic (≥90mmHg) blood pressure was increased or the participant was on anti-hypertensive medication. Diabetes was defined as fasting glucose above 7.0 mmol/L (≥126mg/dL) or drug treatment for elevated blood glucose. Metabolic syndrome was diagnosed if three out of five of the following traits were present: 1) high waist circumference (≥102 cm for men and ≥88 cm for women), 2) high blood pressure (≥130/85 mmHg), 3) HDL below 1.0 mmol/L (≤40 mg/dL) in men and below 1.3 mmol/L (≤50 mg/dL) in women or the use of lipid-lowering drugs, 4) triglycerides above 1.7 mmol/L (≥150 mg/dL) in both sexes or the use of lipid-lowering drugs, and finally 5) fasting glucose above 5.6mmol/L (≥100 mg/dL).[28] Insulin resistance was calculated using the homeostasis assessment model of insulin resistance (HOMA-IR): (fasting glucose [mmol/L] x fasting insulin [mU/lL]) / 22.5.[106]

Statistical Analyses

We excluded all participants with missing or unreliable FFQs, and participants with more than 30% missing study variables. Variables were imputed using multiple imputation under the fully conditioned specification to reduced bias due to missing data.[301] A more detailed description on the imputation process can be found in the *Supplementary Methods II*.[294] Population characteristics of both the original and imputed data were described using the mean (standard deviation, SD), median (25th and 75th percentile, P25-P75), or percentage. We carried out analyses of variance (ANOVA) to compare means for different strata (NAFLD vs no NAFLD and quartiles of DAL) and Kruskal-Wallis tests to compare medians for different strata. Chi-square tests were used to compare categorical variables across strata. We calculated Spearman rank correlation coefficients in order to give more insight in the correlations of DAL with dietary macronutrients, dietary micronutrients, and DQ. We used logistic regression models to assess the association between DAL-proxy categories and NAFLD for comparability with other dietary-acid load studies that used categorical analyses[275] and to facilitate clinical interpretation. Then we assessed linearity, fitting models with splines to allow for non-linearity using natural cubic splines. Thereafter, we tested the need for the non-linear terms and optimal degrees of freedom by comparing the spline model against linear models with the Akaike Information Criterion. And lastly, we tested the relevance of DAL as predictor for NAFLD by comparing the spline model to a model without DAL using a likelihood ratio test.

We adjusted all analyses for potential confounders using 4 models. In model 1, we adjusted for potential socio-demographic confounders, i.e. age, sex, education level (low/moderate/high), study cohort (RSI/RSII/RSIII), and for energy intake (kilocalories). In model 2, we adjusted for lifestyle confounding factors such as alcohol use (in units, one units is 10grams), physical activity (MET equivalent hours/week), and smoking (current/past or never). In model 3 (the main model) we adjusted for metabolic variables, i.e. HDL-cholesterol (in mmol/L), triglycerides (in mmol/L), presence of metabolic syndrome, estimated GFR (in ml/min/1.73m^2), presence of diabetes mellitus and BMI (in kg/m^2). And finally, in model 4 we tested the potential confounding effect of DQ on the association between DAL and NAFLD. The selection of covariates was based on previous literature.[6] Results were expressed as predicted probability or odds ratio (OR) per quartile, both with the accompanying 95% confidence interval (CI). In addition, we tested all models for multicollinearity (VIF>5.0). Furthermore, in order to test the robustness of our results we performed several sensitivity analyses. First, to account for potential measurement error in dietary intake and to remove extraneous variation arising from total energy intake, all DAL-proxies were adjusted for energy intake using the residual method: that part of the DAL-proxies that was not explained by total energy intake.[302] Second, in order to test the robustness of our results, we performed several stratified analyses. We stratified by sex, as gender-differences

in DAL have been previously suggested.[275,288] We stratified by cohort, as participants from various cohorts differ in terms of age (mean age RSIII: 62, RS II: 72, and RS I: 79 years old) and because there was a median time-gap of 5.5 years between completing the FFQ and performance of ultrasound in cohort three (RS-III, see *Supplementary Methods I*).[294] But as dietary data are known to be stable over time,[109] RS-III was included in the main analysis. We also stratified by GFR, using 60 ml/min/1.73m² as cut-off to distinguish between a normal and impaired renal function (as the kidneys play a crucial role in maintaining acid-base balance in the body).[303] We stratified by age (using 65 as cut-off), as DAL-associations have been previously observed particularly in young individuals.[304] And finally, we stratified by BMI at a cut-point of 25kg/m², as participants with a normal BMI (lean) could have a different pathophysiological pathway compared to overweight participants.[288,305] Third, we replaced presence of diabetes with the continuous proxy for insulin resistance, HOMA-IR, in model 3, in order to test the hypothesis that the mechanistic explanation behind DAL associations are mediated by insulin resistance.[276,306] And lastly, we alternately excluded the Q1 or the Q4 from the continuous spline analyses in order to assess whether it was the alkaline component or the acidic component (respectively), or both, that drives the association between DAL-proxies and NAFLD.

To correct for the inflated type I error that arises due to multiple testing we applied the method proposed by Sidák,[256] adapted as described in Galwey et al.,[257] using the effective number of tests (n=1.8) instead of the actual number of tests (n=3). This adaptation is necessary to take into account that dietary exposures inter-correlate instead of being independent from each other. The resulting corrected significance level for all DAL-proxy analyses was $P<0.028$. All analyses were performed using R version 3.5.1.

Results

Participant Characteristics

In total, 5967 participants were eligible for this study. We excluded unreliable FFQs (n=98; 1.6%) and missing FFQs (n=1075; 18%). These participants were significantly younger (68.5 vs 69.6 years; $P<0.01$), less often of European descent (95% vs 98%; $P<0.01$), and had a higher BMI (27.5 vs 26.9 kg/m²; $P<0.01$), but there was no difference in steatosis prevalence (37.2% vs 35.5%; $P=0.27$) and sex (55.8% vs 57.5%; $P=0.27$). Subsequently, we excluded 40 participants (0.8%) that had >30% of missing data on study variables. Lastly, we excluded 872 participants (18.3%) with potential secondary causes for steatosis (n=123 steatogenic drug use, n=691 alcohol misuse, n=31 viral hepatitis, n=27 combination of the aforementioned factors; *Supplementary Methods I*). Hence, the total study population consisted of 3882 participants of which 1337 individuals had NAFLD (34.4%).

The median DAL-proxies in this population were as follows: PRAL –4.7 mEq/day (–15.4; 4.4), NEAP 35.7 mEq/day (29.6–42.3), and A:P 12.7 (10.2–15.4). Population characteristics on both original and imputed data are shown in *Supplementary Table 1*. In short, mean age was 69.7 years (8.8), median BMI was 26.9 (24.5–29.7), 58.3% was female, and the majority was of European descent (97.6%). In *Supplementary Table 2* population characteristics are shown according to NAFLD stratum. Median DAL-proxies were significantly higher in participants with NAFLD than in participants without NAFLD, i.e. for PRAL –2.9 vs. –5.5 mEq/day (P=4.97e^{-6}), for NEAP 37.0 vs. 35.1 mEq/day (P=2.74e^{-6}), and for A:P 13.3 vs. 12.4 (P=1.70e^{-10}).

Characteristics of diet-dependent acid load

Population characteristics per PRAL-quartile are given in *Table 1*. The median PRAL of Q4 was 11.1 (7.4; 17.6) mEq/day. In addition, characteristics per NEAP and A:P quartile are depicted in *Supplementary Table 3 A–B*. The median NEAP in the Q4 of NEAP was 47.5 (44.6–53.0) mEq/day, and the median A:P in the Q4 of A:P was 17.8 (16.4–20.1). Similar characteristics for Q4 in all DAL-proxies were found, being lower proportion of females,

Table 1: Characteristics per quartile PRAL

	PRAL				
	Quartile 1	**Quartile 2**	**Quartile 3**	**Quartile 4**	***P*-value**
DAL					
PRAL	–24.4 (–33.7 ; –19.1)	–9.7 (–12.5 ; –7.2)	–0.33 (–2.3 ; 1.9)	11.1 (7.4 ; 17.6)	n/a
NEAP	25.6 (21.9 – 28.4)	32.7 (30.9 – 34.3)	38.7 (37.0 – 40.7)	47.4 (44.1 – 52.9)	<0.001
A:P	9.2 (7.6 – 10.5)	11.7 (10.4 – 13.2)	13.9 (12.4 – 15.4)	17.2 (15.0 – 19.9)	<0.001
Demographics					
Age	70.2 (8.8)	69.8 (8.6)	69.8 (8.8)	69.1 (9.2)	0.057
Female (%)	67.6	62.5	54.9	48.2	<0.001
Caucasian (%)	97.6	97.9	97.8	97.2	0.804
Education Level (%)					
Low	48.4	49.7	47.9	47.7	0.167
Intermediate	27.7	29.4	31.6	32.5	
High	23.9	20.9	20.5	19.8	
Smoking status (%)					
Never	39.5	37.7	34.5	32.6	0.009
Current / Former	60.5	62.3	65.5	67.4	
Alcohol (units/d)	0.45 (0.04 – 1.16)	0.49 (0.08 – 1.22)	0.45 (0.07 – 1.22)	0.43 (0.03 – 1.17)	0.203
Physical Activity (METh/wk)	46.4 (18.5 – 84.5)	43.0 (17.0 – 78.8)	37.0 (14.3 – 74.5)	34.6 (13.5 – 73.2)	<0.001
Energy intake (kcal/d)	2175 (1823 – 2667)	1929 (1557 – 2363)	1891 (1488 – 2360)	2105 (1700 – 2668)	<0.001

Table 1 (*continued*)

	PRAL				
	Quartile 1	**Quartile 2**	**Quartile 3**	**Quartile 4**	***P*-value**
Physical examination					
BMI (kg/m²)	26.5 (24.3 – 29.4)	26.8 (24.5 – 29.4)	27.0 (24.6 – 29.9)	27.2 (24.7 – 30.0)	0.010
Biochemistry					
AST (U/L)	24 (21 – 28)	24 (21 – 28)	24 (21 – 28)	25 (21 – 29)	0.125
ALT (U/L)	18 (14 – 24)	18 (14 – 23)	18 (14 – 24)	20 (15 – 25)	<0.001
GGT (U/L)	21 (16 – 30)	22 (16 – 32)	23 (17 – 34)	25 (18 – 37)	<0.001
Platelets (*10^9/L)	269 (232 – 309)	263 (224 – 305)	259 (220 – 303)	257 (218 – 303)	0.002
HOMA-IR	2.4 (1.7 – 3.7)	2.5 (1.7 – 3.9)	2.6 (1.7 – 4.2)	2.9 (1.8 – 4.6)	<0.001
Total Cholesterol (mmol/L)	5.6 (1.1)	5.5 (1.1)	5.4 (1.1)	5.3 (1.1)	<0.001
HDL-C (mmol/L)	1.5 (0.4)	1.5 (0.4)	1.5 (0.4)	1.4 (0.4)	<0.001
Triglycerides (mmol/L)	1.3 (1.0 – 1.7)	1.3 (1.0 – 1.7)	1.3 (1.0 – 1.7)	1.3 (1.0 – 1.8)	0.482
GFR	76 (66 – 86)	76 (66 – 86)	75 (66 – 85)	76 (65 – 86)	0.796
Comorbidities					
Metabolic Syndrome	48.4	52.6	54.2	57.1	0.003
- Waist circumference	40.2	43.2	43.8	45.6	0.112
- Triglycerides	41.6	45.4	48.4	48.5	0.007
- HDL-Cholesterol	40.1	44.7	46.5	47.2	0.007
- Blood pressure	84.6	85.0	83.7	83.7	0.794
- Fasting Glucose	41.8	45.1	46.6	53.5	<0.001
Diabetes Mellitus (%)	11.1	11.6	12.5	17.3	<0.001
Hypertension (%)	73.8	75.7	72.1	74.3	0.363
NAFLD	30.9	31.8	35.2	39.9	<0.001

Data is expressed as mean (SD), median (P25-P75) or percentage. *P-value is based on ANOVA, Kruskall-Wallis test or Chi-square test.

Abbreviations ALT: alanine aminotransferase; A:P: animal protein to potassium ratio; AST: aspartate aminotransferase; BMI: body mass index; DAL: dietary acid load; GFR: glomerular filtration rate; GGT: gamma-glutamyltransferase; HDL-C: high-density lipoprotein cholesterol; HOMA-IR: homeostasis model assessment of insulin resistance; NAFLD: non-alcoholic fatty liver disease; NEAP: net endogenous acid production; PRAL; potential renal acid load.

lower physical activity, more current or former smokers, higher BMI and higher gamma glutamyltransferase. In addition, there were more comorbidities, in particular higher prevalence of NAFLD and diabetes (*Table 1* and *Supplementary Table 3 A - B)*.

PRAL, NEAP and A:P correlations with other dietary parameters are depicted in *Table 2*. All DAL-proxies correlated inversely with DQ, meaning that the higher the DAL-proxy the lower dietary quality (r_s=–0.29 for PRAL, r_s=–0.29 for NEAP, and r_s=–0.32 for A:P). In particular fruit intake had a strong inverse correlation with DAL (r_s=–0.51 for PRAL, r_s=–0.50 for NEAP, and r_s=–0.37 for A:P). Also, mono and disaccharides and fiber intake were inversely correlated (*Table 2*). As expected, all DAL-proxies correlated positively with animal protein (r_s=0.26 for PRAL, r_s=0.32 for NEAP, and r_s=0.51 for A:P).

Table 2: Correlations between DAL-proxies and diet

	PRAL	NEAP	A:P
	r_s	r_s	r_s
Total protein (g)	0.17	0.23	0.21
Animal protein	0.26	0.32	0.51
Vegetable protein	–0.08	–0.03	–0.31
Total carbohydrates (g)	–0.27	–0.23	–0.32
Mono-and disaccharides	–0.45	–0.42	–0.35
Polysaccharides	0.02	0.06	–0.20
Fiber	–0.40	–0.36	–0.48
Total fat (g)	0.20	0.25	0.12
Saturated fat	0.24	0.27	0.21
Mono-unsaturated fatty acids	0.20	0.25	0.12
Poly-unsaturated fatty acids	0.11	0.16	–0.04
Trans fatty acids	0.23	0.24	0.18
Minerals (mg)			
Vitamin E	–0.03	0.03	–0.09
Magnesium	–0.23	–0.18	–0.26
Potassium	–0.44	–0.39	–0.30
Phosphorus	0.05	0.08	0.08
Calcium	–0.05	–0.03	0.06
DQ score (points)	–0.29	–0.29	–0.32
↑ Vegetables	–0.27	–0.23	–0.24
↑ Fruit	–0.51	–0.50	–0.37
↑ Whole grain products	0.09	0.10	–0.12
↑ Legumes	–0.03	–0.01	–0.08
↑ Nuts	0.03	0.04	–0.09
↑ Dairy	–0.02	–0.04	0.09
↑ Fish	0.04	0.08	0.14
↑ Tea	–0.05	–0.05	–0.05
↑ Whole/Refined grains	–0.04	–0.06	–0.13
↑ Unsaturated fats/oils	–0.06	–0.05	–0.06
↓ Red and processed meat	–0.17	–0.22	–0.32
↓ Sugar containing drinks	0.07	0.06	0.06
↓ Alcohol	–0.02	–0.01	–0.02
↓ Salt	–0.22	–0.25	–0.13

By Spearman's rank correlation. DQ score can theoretically vary from 0–14 points. DQ subtypes are dichotomous.
Legend Spearman correlation:

negative			positive		
moderate –0.59 to –0.40	**weak** –0.39 to –0.20	**very weak** –0.19 to –0	**very weak** 0 to 0.19	**weak** 0.20 to 0.39	**moderate** 0.40 to 0.59

Abbreviations A:P: animal protein to potassium ratio; DAL: dietary acid load; NEAP: net endogenous acid production; PRAL; potential renal acid load.

Categorized diet-dependent acid load and NAFLD

The highest quartile (Q4) of PRAL was associated with higher prevalence of NAFLD (using Q1 as reference), in all models (*Table 3*). However, after correction for multiple testing, the association between PRAL and NAFLD was no longer significant in model 3 (OR_{Q4vsQ1} 1.26, 95%CI 1.01–1.58; *P* = 0.041). A similar association was seen for NEAP, in which the Q4 of NEAP was only significantly associated with NAFLD in model 1 and 2 (model 3: OR_{Q4vsQ1} 1.24, 95%CI 0.99–1.56; *P*=0.058). Lastly, the Q4 of A:P had a more pronounced

Table 3: Logistic regression analyses of DAL-proxies with NAFLD as dependent variable

	Total population (n=3882)		
	Q2	Q3	Q4
PRAL n per quartile	**(n=970)**	**(n=971)**	**(n=970)**
NEAP n per quartile	**(n=970)**	**(n=971)**	**(n=970)**
A:P n per quartile	**(n=970)**	**(n=971)**	**(n=970)**
Model 1 (sociodemographic)			
PRAL	0.99 (0.81 – 1.20)	1.14 (0.94 – 1.38)	**1.42 (1.17 – 1.72)†**
NEAP	1.02 (0.84 – 1.24)	1.24 (1.02 – 1.50)	**1.40 (1.15 – 1.69)†**
A:P	1.14 (0.94 – 1.39)	**1.36 (1.12 – 1.65)†**	**1.63 (1.35 – 1.97)†**
Model 2 (lifestyle)			
PRAL	0.98 (0.81 – 1.19)	1.12 (0.92 – 1.36)	**1.38 (1.14 – 1.67)†**
NEAP	1.03 (0.85 – 1.25)	**1.22 (1.01 – 1.48)**	**1.36 (1.12 – 1.65)†**
A:P	1.14 (0.94 – 1.39)	**1.33 (1.09 – 1.61)†**	**1.58 (1.31 – 1.92)†**
Model 3 (metabolic)			
PRAL	0.97 (0.77 – 1.21)	1.09 (0.87 – 1.36)	**1.26 (1.01 – 1.58)**
NEAP	0.97 (0.78 – 1.22)	1.18 (0.94 – 1.47)	1.24 (0.99 – 1.56)
A:P	0.96 (0.77 – 1.21)	1.09 (0.87 – 1.36)	1.22 (0.97 – 1.52)
Model 4 (metabolic + DQ)			
PRAL	0.97 (0.77 – 1.21)	1.09 (0.87 – 1.37)	**1.27 (1.01 – 1.60)**
NEAP	0.98 (0.78 – 1.22)	1.18 (0.94 – 1.47)	1.25 (0.99 – 1.57)
A:P	0.96 (0.77 – 1.21)	1.09 (0.87 – 1.37)	1.22 (0.97 – 1.54)

Values are odds ratios with 95% confidence intervals taking quartile 1 as reference. Bold values indicate P<0.05.
† Indicates significant values using P < 0.028 as determined by Sidák.
Model 1 (socio-demographic) is adjusted for age, gender, education level, energy intake and study cohort.
Model 2 (lifestyle) is in addition previous model adjusted for past or current smoking, units of alcohol, and physical activity.
Model 3 (metabolic) is in addition to the previous model adjusted for HDL-cholesterol, triglycerides, metabolic syndrome, GFR, diabetes mellitus and BMI.
Model 4 (metabolic + DQ) is in addition to the previous model adjusted for DQ.
Abbreviations A:P: animal protein-potassium ratio; DQ: Dietary Quality; NEAP: net endogenous acid production; PRAL: potential renal acid load; Q: Quartile.
Abbreviations A:P: animal protein to potassium ratio; BMI: body mass index; DAL: dietary acid load; DQ: dietary quality; GFR: glomerular filtration rate; HDL: high density lipoprotein; NAFLD: non-alcoholic fatty liver disease; NEAP: net endogenous acid production; PRAL; potential renal acid load.

association with NAFLD than PRAL and NEAP in the first two models, but this association was confounded by metabolic factors –in particular by the metabolic syndrome, BMI, and diabetes mellitus– in model 3 (OR_{Q4vsQ1} 1.22, 95%CI 0.97–1.52; *P*=0.089).

Continuous diet-dependent acid load and NAFLD

Logistic regression with natural cubic splines for DAL showed a clear non-linear effect of DAL (*P* for non-linearity for PRAL: $6.5{\cdot}10^{-4}$, NEAP: $3.7{\cdot}10^{-4}$, and A:P: $2.7{\cdot}10^{-5}$). The predicted probability of NAFLD in model 3 (metabolic) was lowest within the alkaline PRAL range –45 to –4 mEq/day, with a minimum predicted probability of NAFLD that was 29%. Whereas the predicted probability of NAFLD increased to 36% for acidic PRAL-values 9 to 11 mEq/day (equals Q4; *Figure 1A).* A similar shape was seen for NEAP and A:P (*Figure 1B – C*). For NEAP Q1 and Q2 NAFLD probability was low (<33%), but increased for towards 37% (*Figure 1B)*. For Q1 and Q2 of A:P probability of NAFLD was low (again <33%), and the highest predicted probability of NAFLD (36%) was seen at an A:P of 17 in (again equals Q4; *Figure 1C)*. The shape of the splines for models 1 and 2 were very similar to that of model 3, but generally with higher predicted probabilities of NAFLD and more pronounced differences between high and low DAL (*Supplementary Figure 1 A – B*). All models performed better with DAL in the model than without DAL, this even remained significant for NEAP and A:P after multiple comparison correction (log-likelihood ratio test: *P* = 0.038 for PRAL; *P* = 0.015 for NEAP; *P* = 0.012 for A:P).

Diet-dependent acid load and potential confounding by dietary quality

We assessed whether DAL was confounded by DQ in a separate model 4. Categorical analyses of DAL-proxies with NAFLD adjusted for DQ are depicted in *Table 3; model 4.* Although the results were not statistically significant, the associations hardly attenuated after adjustment of DQ. Hence, DQ could not fully explain the association between DAL and NAFLD. The same effect of DQ was seen on the splines (*Supplementary Figure 1C*: PRAL *P*=0.043, NEAP *P*=0.017; and A:P *P*=0.014, comparing the models with and without DAL-proxies).

Sensitivity analyses

To test the robustness of our findings we carried out multiple sensitivity analyses. First, we used the residual method to account for extraneous variation in DAL arising from total energy intake. The associations with PRAL attenuated slightly, but the results for NEAP and A:P were similar to the main analysis (*Supplementary Table 4*). Second, we stratified

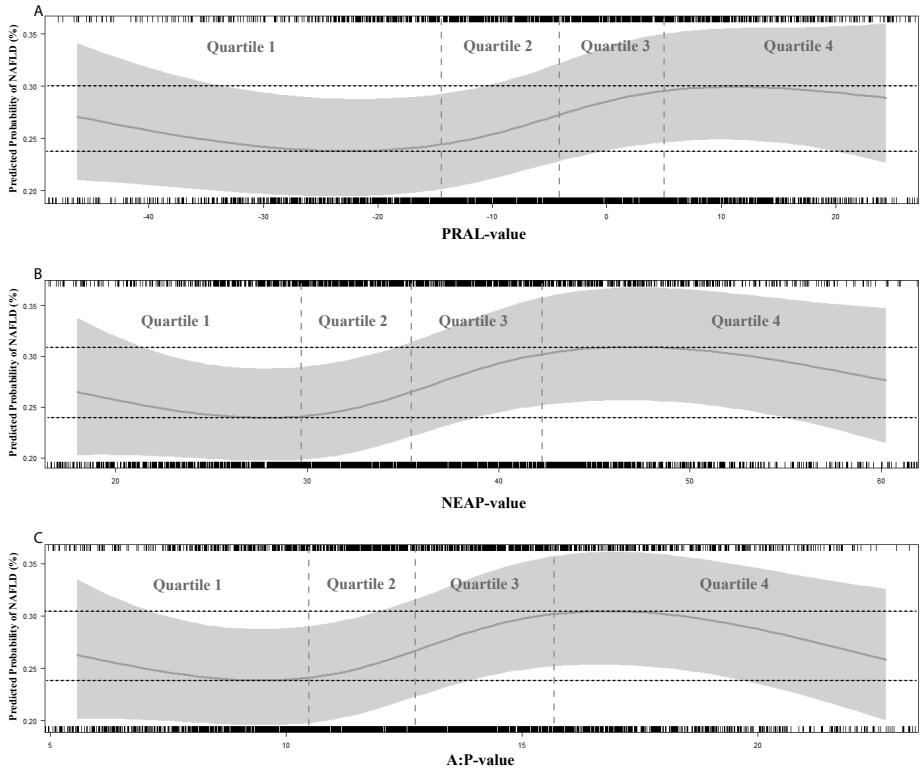


Figure 1: Natural cubic splines for the association of A: PRAL, B: NEAP, and C: A:P with the predicted probability of NAFLD in (metabolic) model 3

A: Model with PRAL vs. model without PRAL (P=0.038). B: Model with NEAP vs. model without NEAP (P=0.015). C: Model with A:P vs. model without A:P (P=0.012).

Y-axis represents predicted probability of NAFLD in (metabolic) model 3: NAFLD ~ DAL-proxy + age + gender + education level + energy intake + study cohort + smoking + units of alcohol + physical activity + HDL-cholesterol + triglycerides + metabolic syndrome + GFR + diabetes mellitus + BMI.

X-axis represent values of PRAL, NEAP or A:P. The grey-colored bar represents the 95% confidence interval. The upper and lower 2.5th centile were excluded from the graphs.

Abbreviations A:P: animal protein to potassium ratio; BMI: body mass index; DAL: dietary acid load; DQ: dietary quality; GFR: glomerular filtration rate; NAFLD: non-alcoholic fatty liver disease; NEAP: net endogenous acid production; PRAL; potential renal acid load.

by several predefined covariates (*Supplementary Tables 5–9*). All stratified results largely resembled the original analyses (*Figure 2*). Interestingly, PRAL was nominally significant associated with higher NAFLD prevalence in participants with an impaired kidney function after full adjustment in the metabolic model (OR_{Q4vsQ1} 1.81 95%CI 1.01–3.24, P = 0.047, *Supplementary Table 7*). Third, we have additionally adjusted model 3 for insulin resistance (instead of diabetes presence, using HOMA-IR) and found that the association dissipated, indicating the mediating role of insulin resistance in this association (OR_{Q4vsQ1} for PRAL: 1.19, 95%CI 0.95–1.50; for NEAP 1.15 95%CI 0.88–1.38; for A:P 1.12 95%CI

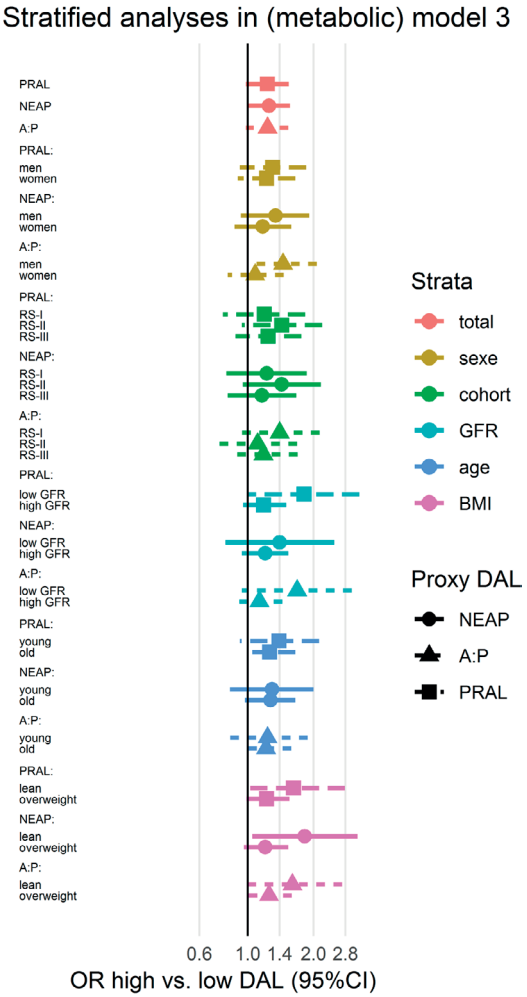


Figure 2: Associations of DAL-proxies with NAFLD stratified by various covariates in the metabolic model

Values are odds ratios of the fourth quartile with 95% confidence intervals taking the first quartile as reference. All strata are analysed within modified (metabolic) model 3: NAFLD ~ DAL-proxy + (age) + (gender) + education level + energy intake + (study cohort) + smoking + units of alcohol + physical activity + HDL-cholesterol + triglycerides + metabolic syndrome + (GFR) + diabetes mellitus + (BMI).

This figure represents data that can also be found in more detail in Supplementary Table 4 – 9.

Abbreviations A:P: animal protein to potassium ratio; BMI: body mass index; DAL: dietary acid load; GFR: glomerular filtration rate; NAFLD: non-alcoholic fatty liver disease; NEAP: net endogenous acid production; PRAL; potential renal acid load.

0.89–1.41). And fourth, in order to assess whether it is the acidic component or the alkaline component that drives the association between DAL-proxies and NAFLD, we alternately excluded the alkaline component (Q1) and the acidic component (Q4) from the analyses. All models with DAL-proxies performed better than models without DAL-proxies only when

the alkaline component was excluded, but not when the acidic component was excluded (log-likelihood ratio test excluding Q1: *P*=0.032 for PRAL; *P*=0.015 for NEAP; *P*=0.010 for A:P; log-likelihood ratio test excluding Q4: *P*=0.310 for PRAL; *P*=0.115 for NEAP; *P*=0.253 for A:P) This may indicate that it is the acidic component drives the association between DAL-proxies and NAFLD.

Discussion

In this largest population-based cohort study to date, we found that diet-dependent acid load, as assessed by net-endogenous acid production and animal protein-to-potassium ratio, was independently associated with NAFLD. This association was not linear; but the highest probability of NAFLD (36–37%) was found for an acidic diet and the minimum predicted probability of NAFLD (29%) for an alkaline diet. Indeed, the association was driven mainly by the acidic component of the diet. Models with NEAP and A:P performed significantly better in predicting NAFLD than without these indices of DAL, importantly, even after correction for numerous confounders such as BMI and overall dietary quality. Moreover, the association between DAL-proxies and NAFLD was tested for in different subgroups of the population which showed overall consistent results.

Despite all ongoing pharmacological studies, lifestyle intervention remains the only available treatment for NAFLD today.[307] Recently, several studies found detrimental associations between high animal protein intake[247,287] and NAFLD. Amongst them, a recent study from Zelber-Sagi and colleagues found that high red and processed meat consumption was associated with NAFLD and insulin resistance.[261] This is in line with another recent study from Etemadi et al. that showed that a diet rich in red meat was associated with higher incidence of liver-related mortality.[260] Food items from the Western dietary pattern, such as red meat, generally contribute to a higher diet-dependent acid load.[303] Indeed, animal protein had the highest positive correlation with all DAL-proxies in our study as well. Interestingly, the highest inverse correlation with DAL-proxies was found for mono-and disaccharides. This is in line with our previous study in which we found an inverse association of mono-and disaccharides with NAFLD, though this was not independent from metabolic confounders. We also know that fruit was by far the most contributing group to this macronutrient. And fruits have indeed a alkalinizing potential.[303]

In this study we show that in particular an acidic diet was associated with higher NAFLD prevalence, and we therefore hypothesize that diet-dependent acid load may (partially) explain the link between an animal protein-rich diet and NAFLD. Interestingly, our findings are in line with two previous studies on DAL and NAFLD.[259,275] A study in German adolescents found that PRAL was associated with ALT, fatty liver index and hepatic steatosis index in girls but not in boys.[275] However, the study population was small (n=145), the study only

included adolescents and young adults (mean age 20 years old), and steatosis algorithms were assessed only continuously (but prevalence was most probably low as median FLI was ± 10). Another elegant study analysed PRAL and NEAP in association with NAFLD (assessed by MRI-PDFF), in 793 Chinese adults.[259] The authors found an association between NEAP and NAFLD in multivariable analysis. However, linearity of PRAL and NEAP was not assessed in these two studies,[259,275] which makes direct comparison (along with major sociodemographic differences) to our Western adult population difficult. Of note, mean diet-dependent acid load in the latter study was rather high (PRAL 24 mEq/day and NEAP 77 mEq/day)[259] in comparison to our study and that of others which was relatively more alkaline (i.e. the Rotterdam Study: PRAL –4.7mEq/day and NEAP 36 mEq/day; Nurses' Health Study: PRAL –3.1 mEq/day and NEAP: 44 mEq/day; and Health Professionals' Follow-up Study: PRAL 5.7 mEq/day and NEAP 48 mEq/day).[288]

Diet-dependent acid load has also been implicated in other health outcomes that are related to NAFLD, such as type 2 diabetes and hypertension.[303] An interesting Swedish study examined whether PRAL was associated with overall mortality in over 80.000 individuals followed for 13 years.[308] Similar to our study, the authors found a non-linear association between PRAL and all-cause mortality but rather a U-shaped spline. Thus, both dietary acid and alkali excess were associated with increased mortality risk. We did not observe a clear U-shape in our splines, but the predicted probability of NAFLD was indeed also higher in the distinct alkaline diets. To date, there is no study that showed that a diet high in alkaline load is detrimental for health, however, one could speculate that a one-sided, unbalanced diet might be unhealthy anyway – possibly also via other mechanisms than diet-dependent acid load alone. Rightfully so, the authors of this Swedish study argued whether the low impact (HR 1.06) of PRAL on mortality risk is important in terms of public health. Indeed, there are studies that found no association between diet-dependent acid load and health.[309,310] Differences in sociodemographic factors such as age, gender, ethnicity, and dietary habits could have contributed to these contrasting results. We have therefore performed several predefined subgroup analyses. Most subgroup analyses were not statistically significant, but should be interpreted in light of lower statistical power, and generally confirmed the main results. Yet, the highest quartile of PRAL was nominally significant in participants with an impaired renal function (estimated GFR < 60 ml/min/1.73m²) and, albeit not significant, the same trend was seen for both NEAP and A:P. The results were not significant after multiple testing correction, but again, they should be interpreted in light of lower power (n=597). This finding is not surprising as the kidney plays a crucial role in maintaining acid-base balance.[311] Likewise, a long-term high diet-dependent acid load could also contribute to the development of chronic kidney disease by increasing endothelin-1, angiotensin-II, and aldosterone production to meet the demand for hydrogen excretion[312].

Mechanistically, it is thought that consumed proteins, in particular sulphur-containing amino-acids (e.g. methionine and cysteine) found in animal proteins, form sulphate after

oxidation.[303] This is a non-volatile acid that can be neutralized by bicarbonate but forms a hydrogen-bond as end product. In contrast, plant protein often contains glutamate which can be metabolized without this formation. Fruit on the other hand, is potassium-rich and often accompanied by citrate and malate, which consume hydrogen-bonds to become neutral and hence are alkalinizing.[313] This low-grade or subclinical metabolic acidosis has been associated with various metabolic alterations.[314] Amongst others, a change in glucose homeostasis has been found, being a lower insulin response to high glucose in the presence of a low pH.[276] Also, long-term subclinical metabolic acidosis may increase adrenal cortisol production (modulated by the hypothalamic-pituitary-adrenal axis) and subsequently may lead to visceral obesity and insulin resistance.[306,315] In our study, adjustment for insulin resistance, as assessed by HOMA-IR, weakened the association between DAL and NAFLD, confirming our hypothesis that the association between DAL and NAFLD is mediated via insulin resistance.[276,306] Based on experimental studies, it has been proposed that low-grade metabolic acidosis influenced the growth hormone/ insulin growth factor 1 system, leading to hepatocellular growth hormone resistance and subsequent hepatic lipid accumulation.[316,317] Opponents of the diet-dependent acid load hypothesis argue that DAL-proxies are merely a different way of scoring adherence to a healthy diet. Although we cannot exclude this possibility, separate correction for adherence to dietary quality did not fully explain the association between DAL and NAFLD. Indeed, food items can effectuate health changes without affecting acid-base balance. For example, fruits and vegetables also contain dietary fiber that is beneficial for glycaemic control.[318] Also, it has been suggested that the metabolization of sulphur-containing amino-acids (i.e. methionine and cysteine found in animal protein) in the liver can directly cause liver injury.[319] Lastly, an experimental rat study found that a high protein diet upregulated mRNA expression of genes encoding proteins involved in amino acid uptake and enhanced lipid synthesis.[320] In this study, hepatic mRNA and protein levels of heat shock protein 90, a marker of liver injury, were markedly increased in these rats fed a high-protein diet. Of note, high protein intake did not result in elevated hepatic lipid concentrations in these rats.

Our study has several strengths, including a large sample size, availability of a great number of well-defined covariates, the (predefined) subgroup analyses to confirm the robustness of our findings, the evaluation of linearity and subsequent use of natural cubic splines, and the correction for multiple testing. Nonetheless, there are several considerations that need to be addressed. First, this study comprises a large predominantly elderly and Caucasian population, with a relatively alkaline diet. Despite the reassuring similar results in several subgroups of participants, caution on generalizability should be exercised. Second, due to the cross-sectional design of this study it is not possible to draw any conclusions on causality. Third, although we have corrected for a large number of different traits, we cannot exclude the possibility of residual confounding. Fourth, as with any self-administered questionnaire, the FFQ is susceptible for recall and reporter bias. Nonetheless, we have

tried to limit this bias by excluding potential unreliable FFQs and by performing a sensitivity analyses with DAL-proxies corrected by the residual method to account for extraneous variation in energy intake. Fifth, one of the three included cohorts (RS III) completed the FFQ 5.5 years prior to liver imaging. We assumed that dietary data was stable over time,[98] which was indeed recently shown in another paper from The Rotterdam Study.[321] Nonetheless, we used study cohort as covariables in all regression models, moreover, we performed sensitivity analysis per study cohort. All the separate cohorts showed results that were comparable to the main analysis. Sixth, we used ultrasonography to assess steatosis, which has a good sensitivity to detect moderate steatosis but is poor in detecting mild steatosis and in grading steatosis. Moreover, the golden standard to assess NAFLD –and its advanced subtype non-alcoholic steatohepatitis– is a liver biopsy. However, it is unethical to perform this invasive procedure on a large scale in presumed healthy individuals. Finally, we had no dispose of acid-base biomarkers to estimate actual metabolic acidosis. However, NEAP and PRAL have been previously validated in healthy individuals and accounted for 71% of the variation in renal net acid excretion.[291,295] And, although A:P has not been validated as of yet, results of A:P were very comparable to NEAP and PRAL.

In conclusion, this study shows that an acid-base (un)balanced diet is associated with NAFLD and that differences in sociodemographics, lifestyle, metabolic factors, and dietary quality did not fully explain the observed associations. Though the results should be interpreted in context of a relatively alkaline diet, our findings might explain the previously observed association of an animal protein-rich diet with NAFLD. Future research initiatives should use acid-base biomarkers, such as urinary ammonium,[322] to study low-grade metabolic acidosis more objectively. Nevertheless, dietary recommendations that are in agreement with an acid-base balanced diet, i.e. a diet rich in fruit and vegetables and poor in animal protein, are generally considered beneficial for health. Therefore, adherence to such a diet low in animal protein while awaiting the results of future studies seem justifiable.

Supplementary Methods: details on the multiple imputation process

Variables (range missing values per variable: 0.02 to 10.79%) were imputed using multiple imputation under the fully conditioned specification to reduced bias due to missing data.[301] Thirty imputed datasets were created using the R package mice,[323] and analysed separately. Results from these analyses were pooled using Rubin's rules in order to take into account the added uncertainty due to the missing data.[254]

Supplementary Methods: details on the multiple imputation process

	Multiple imputation
Software used	R version 3.5.0
Imputation method and key settings	Fully conditional specification (package mice version 3.1.0)*;* maximum iterations: 20
No. of imputed data sets created	30
Analyses variables	total cholesterol; triglycerides; calcium intake; high density lipoprotein cholesterol; body mass index; glucose; weight; physical activity; smoking status; systolic blood pressure; diastolic blood pressure; ethnicity; education level; alcohol consumption; fat intake; waist circumference; kilocalorie intake; Dietary Quality; age; anti-diabetic drugs; lipid-lowering drugs; antihypertensive drugs; fibre intake; study cohort; gender; steatosis; protein intake; carbohydrate intake; vitamin E intake; potassium intake; magnesium intake; phosphorus intake; glomerular filtration rate
Auxiliary variables	aspartate transaminase; alanine transaminase; homeostasis model assessment of insulin resistance; hip circumference; heart rate; spleen size; creatinine; gamma-glutamyltransferase
Treatment of not normally distributed continuous variables	Predictive mean matching
Treatment of normally distributed variables	Linear regression
Treatment of binary/categorical variables	(Proportional odds) logistic regression
Population	For the imputation we used reliable and completed FFQs. In addition participants had to have less than 30% missing on study variables. Imputed population (n=4.754).

Supplementary Table 1: Imputation Characteristics

	Original Data n=3882	After imputation
Demographics		
Age (years)	**69.7 (8.8)**	no missing data
Female (%)	**58.3**	no missing data
Caucasian (%)	**97.7**	97.6
Education Level (%)		
Low	**48.4**	48.4
Intermediate	**30.3**	30.3
High	**21.3**	21.3
Smoking status (%)		
Never / Past or Current	**36.7 / 63.3**	36.1 / 63.9
Alcohol (units/d)	**0.45 (0.05 – 1.19)**	no missing data
Physical Activity (METh/wk)	**41.3 (15.8 – 78.5)**	40.5 (15.8 – 77.7)
Caloric Intake (kcal/day)	**2031 (1621 – 2514)**	no missing data
DQ	**7 (6 – 8)**	no missing data
PRAL	**–4.7 (–15.4; 4.4)**	no missing data
NEAP	**35.7 (29.6 – 42.3)**	no missing data
A:P	**12.7 (10.2 – 15.4)**	no missing data
Physical examination		
BMI (kg/m²)	**26.9 (24.5 – 29.7)**	26.9 (24.5 – 29.7)
Lean (%)	**30.2**	30.2
Overweight (%)	**69.8**	69.8
WC (cm)		
Men	**98.2 (10.6)**	98.2 (10.6)
Women	**89.2 (12.2)**	no missing data
Biochemistry		
AST (U/L)	**24 (21 – 28)**	24 (21 – 28)
ALT (U/L)	**18 (15 – 24)**	18 (15 – 24)
GGT (U/L)	**23 (17 – 33)**	23 (17 – 34)
Platelets (*10^9/L)	**262 (223 – 305)**	262 (223 – 305)
HOMA-IR	**2.6 (1.7 – 4.1)**	2.6 (1.7 – 4.1)
Total Cholesterol (mmol/L)	**5.4 (1.1)**	5.4 (1.1)
HDL-C (mmol/L)	**1.5 (0.4)**	1.5 (0.4)
Triglycerides (mmol/L)	**1.3 (1.0 – 1.7)**	1.3 (1.0 – 1.7)
GFR	**76.1 (65.7 – 85.8)**	76.1 (65.6 – 85.8)
Comorbidities		
Metabolic Syndrome (%)	**53.2**	53.2
- WC>88cm (♀) or >120cm (♂)	**43.2**	43.2
- Triglycerides >150mg/dL	**46.2**	46.0
- HDL-C <40mg/dL (♂) or 50mg/dL (♀)	**44.8**	44.7

Supplementary Table 1 (*continued*)

	Original Data n=3882	After imputation
- Blood pressure ≥130/85mmHg	**84.3**	84.3
- FG>100mg/dL	**46.8**	46.8
Diabetes Mellitus (%)	**13.2**	13.1
Hypertension (%)	**74.0**	74.0
NAFLD (%)	**34.4**	no missing data

Data is expressed as mean (SD), median (P25-P75) or percentage.
Abbreviations ALT: alanine aminotransferase; A:P: animal protein to potassium ratio; AST: aspartate aminotransferase; BMI: body mass index; DAL: dietary acid load; GFR: glomerular filtration rate; GGT: gamma-glutamyltransferase; HDL-C: high-density lipoprotein cholesterol; HOMA-IR: homeostasis model assessment of insulin resistance; NAFLD: non-alcoholic fatty liver disease; NEAP: net endogenous acid production; PRAL; potential renal acid load.

Supplementary Table 2: Characteristics of the study population

	No NAFLD n=2545 (65.6%)	NAFLD n=1337 (34.4%)	P-value*
Demographics			
Age (years)	69.6 (9.2)	70.0 (8.2)	0.180
Female (%)	59.1	56.8	0.195
Caucasian (%)	97.4	98.1	0.237
Education Level (%)			
Low	45.0	54.9	<0.001
Intermediate	31.4	28.2	
High	23.6	16.9	
Smoking status (%)			
Never	38.2	32.0	<0.001
Past or Current	61.8	68.0	
Alcohol (units/d)	0.45 (0.06 – 1.21)	0.43 (0.05 – 1.19)	0.422
Physical Activity (METh/wk)	43.7 (17.5 – 81.6)	34.6 (13.5 – 70.3)	<0.001
Energy intake	2052 (1642 – 2537)	1996 (1579 – 2456)	0.003
PRAL (mEq/d)	−5.5 (−16.1; 3.4)	−2.9 (−13.7; 6.1)	<0.001
NEAP (mEq/d)	35.1 (29.2 – 41.6)	37.0 (30.2 – 43.6)	<0.001
A:P (mEq/d)	12.4 (10.0 – 15.0)	13.3 (10.6 – 16.0)	<0.001
Physical examination			
BMI (kg/m^2)	25.8 (23.7 – 28.1)	29.3 (27.0 – 32.3)	<0.001
lean (%)	40.9	9.9	<0.001
overweight (%)	59.1	90.1	
Waist Circumference (cm)	89.1 (11.1)	100.3 (11.4)	<0.001
Biochemistry			
AST (U/L)	24 (21 – 28)	25 (21 – 29)	<0.001
ALT (U/L)	17 (14 – 22)	21 (16 – 29)	<0.001
GGT (U/L)	21 (15 – 30)	28 (20 – 39)	<0.001

Supplementary Table 2 (*continued*)

	No NAFLD n=2545 (65.6%)	NAFLD n=1337 (34.4%)	P-value*
Platelets (*109/L)	260 (222 – 303)	266 (225 – 310)	0.053
HOMA-IR	2.1 (1.5 – 3.1)	4.1 (2.7 – 6.1)	<0.001
Total Cholesterol (mmol/L)	5.5 (1.1)	5.4 (1.1)	0.002
HDL-C (mmol/L)	1.5 (0.4)	1.3 (0.4)	<0.001
Triglycerides (mmol/L)	1.2 (0.9 – 1.5)	1.6 (1.2 – 2.1)	<0.001
GFR	76 (65 – 85)	76 (66 – 86)	0.272
<60/>60 (%)	15.7 / 84.3	14.9 / 85.1	0.544
Comorbidities			
Metabolic Syndrome	41.7	75.0	<0.001
- Waist Circumference	29.1	69.9	<0.001
- Triglycerides	38.9	59.4	<0.001
- HDL-Cholesterol	38.3	56.7	<0.001
- Blood pressure	80.5	91.4	<0.001
- Fasting Glucose	36.3	66.6	<0.001
Diabetes Mellitus (%)	7.5	23.7	<0.001
Hypertension (%)	68.9	83.7	<0.001

Pooled data based on 30 imputations represent % for categorical variables and for continuous variables mean (SD) or median (P25-P75).*P-value is based on ANOVA, Kruskall-Wallis test or Chi-square test and is the comparison between the no NAFLD and NAFLD columns.

Abbreviations ALT: alanine aminotransferase; A:P: animal protein to potassium ratio; AST: aspartate aminotransferase; BMI: body mass index; DAL: dietary acid load; GFR: glomerular filtration rate; GGT: gamma-glutamyltransferase; HDL-C: high-density lipoprotein cholesterol; HOMA-IR: homeostasis model assessment of insulin resistance; NAFLD: non-alcoholic fatty liver disease; NEAP: net endogenous acid production; PRAL; potential renal acid load

Supplementary Table 3A: Characteristics per quartile NEAP

	NEAP				
	Quartile 1	Quartile 2	Quartile 3	Quartile 4	P-trend*
DAL					
PRAL	–24.1 (–33.6 ; –17.7)	–9.8 (–13.2 ; –6.8)	–0.33 (–2.5 ; 2.2)	10.9 (6.8 ; 17.6)	<0.001
NEAP	25.3 (21.9 – 27.8)	32.7 (31.1 – 34.1)	38.7 (37.2 – 40.4)	47.5 (44.6 – 53.0)	n/a
A:P	9.0 (7.5 – 10.4)	11.7 (10.5 – 13.1)	13.9 (12.5 – 15.3)	17.4 (15.3 – 20.0)	<0.001
Demographics					
Age	71.0 (8.7)	69.6 (8.5)	68.8 (8.8)	69.5 (9.2)	<0.001
Female (%)	68.5	60.8	54.0	49.9	<0.001
Caucasian (%)	97.8	98.2	97.9	96.7	0.160
Education Level (%)					
Low	49.9	49.1	45.2	49.6	0.022
Intermediate	28.2	27.8	33.7	31.5	
High	21.9	23.1	21.1	18.9	

Supplementary Table 3A (*continued*)

	NEAP				
	Quartile 1	**Quartile 2**	**Quartile 3**	**Quartile 4**	**P-trend***
Smoking status (%)					0.002
Never	40.8	36.4	34.7	32.5	
Current / Former	59.2	63.6	65.3	67.5	
Alcohol (units/d)	0.36 (0.03 – 1.08)	0.55 (0.09 – 1.25)	0.52 (0.09 – 1.25)	0.39 (0.03 – 1.16)	<0.001
Physical Activity (METh/wk)	43.2 (17.3 – 78.9)	47.1 (18.0 – 84.0)	38.1 (15.0 – 76.0)	33.3 (13.5 – 70.6)	<0.001
Energy intake (Kcal)	2019 (1613 – 2457)	2037 (1665 – 2491)	2066 (1626 – 2532)	2015 (1588 – 2551)	0.525
Physical examination					
BMI (kg/m^2)	26.6 (24.3 – 29.4)	26.6 (24.4 – 29.5)	27.0 (24.6 – 29.7)	27.3 (24.7 – 30.0)	0.012
Biochemistry					
AST (U/L)	24 (21 – 28)	24 (21 – 28)	24 (21 – 28)	25 (21 – 29)	0.357
ALT (U/L)	18 (14 – 23)	18 (14 – 24)	19 (15 – 24)	19 (15 – 25)	<0.001
GGT (U/L)	21 (16 – 30)	22 (17 – 32)	23 (17 – 34)	25 (18 – 37)	<0.001
Platelets (*109/L)	267 (232 – 312)	265 (220 – 303)	259 (220 – 300)	259 (220 – 306)	0.002
HOMA-IR	2.4 (1.7 – 3.6)	2.6 (1.7 – 4.0)	2.6 (1.8 – 4.1)	2.9 (1.9 – 4.7)	<0.001
Total Cholesterol (mmol/L)	5.6 (1.1)	5.5 (1.1)	5.4 (1.1)	5.3 (1.1)	<0.001
HDL-C (mmol/L)	1.5 (0.4)	1.5 (0.4)	1.5 (0.4)	1.4 (0.4)	<0.001
Triglycerides (mmol/L)	1.3 (1.0 – 1.7)	1.2 (1.0 – 1.7)	1.3 (1.0 – 1.7)	1.3 (1.0 – 1.8)	0.708
GFR	76 (65 – 85)	77 (66 – 86)	76 (66 – 86)	76 (65 – 86)	0.898
Comorbidities					
Metabolic Syndrome	49.9	51.1	54.9	56.6	0.009
- Waist Circumference	40.6	42.6	43.8	45.8	0.131
- Triglycerides	43.1	44.5	48.3	48.1	0.045
- HDL-Cholesterol	41.9	43.2	46.0	47.5	0.050
- Blood pressure	86.2	83.8	82.5	84.6	0.150
- Fasting Glucose	40.4	46.6	46.6	53.5	<0.001
Diabetes Mellitus (%)	11.2	12.5	11.7	17.1	<0.001
Hypertension (%)	75.9	72.9	71.8	75.3	0.131
NAFLD	31.0	31.5	35.8	39.4	<0.001

Data is expressed as mean (SD), median (P25-P75) or percentage. *P-value is based on ANOVA, Kruskall-Wallis test or Chi-square test.

Abbreviations ALT: alanine aminotransferase; A:P: animal protein to potassium ratio; AST: aspartate aminotransferase; BMI: body mass index; DAL: dietary acid load; GFR: glomerular filtration rate; GGT: gamma-glutamyltransferase; HDL-C: high-density lipoprotein cholesterol; HOMA-IR: homeostasis model assessment of insulin resistance; NAFLD: non-alcoholic fatty liver disease; NEAP: net endogenous acid production; PRAL; potential renal acid load.

Supplementary Table 3B: Characteristics per quartile AP-ratio

	A:P				
	Quartile 1	**Quartile 2**	**Quartile 3**	**Quartile 4**	***P*-trend***
DAL					
PRAL	–21.4 (–32.4 ; –13.0)	–10.4 (–16.4 ; –3.9)	–1.7 (–6.8 ; 3.1)	9.2 (3.5 ; 16.4)	<0.001
NEAP	26.1 (21.9 – 30.2)	32.4 (29.5 – 36.0)	37.7 (34.6 – 41.1)	46.2 (42.2 – 52.1)	<0.001
A:P	8.5 (7.2 – 9.5)	11.4 (10.8 – 12.0)	13.9 (13.3 – 14.6)	17.8 (16.4 – 20.1)	n/a
Demographics					
Age	69.9 (8.9)	69.2 (8.6)	69.4 (9.0)	70.4 (8.9)	0.009
Female (%)	63.5	57.3	57.1	55.3	0.001
Caucasian (%)	96.6	98.3	97.6	97.9	0.116
Education Level (%)					
Low	49.0	45.2	45.6	53.9	<0.001
Intermediate	28.1	31.7	31.2	30.2	
High	22.9	23.1	23.2	15.9	
Smoking status (%)					
Never	40.1	38.2	35.7	30.4	<0.001
Current / Former	59.9	61.8	64.3	69.6	
Alcohol (units/d)	0.38 (0.03 – 1.11)	0.52 (0.09 – 1.22)	0.45 (0.07 – 1.24)	0.45 (0.04 – 1.18)	0.002
Physical Activity (METh/wk)	45.0 (18.0 – 82.8)	44.4 (18.0 – 82.2)	36.8 (14.5 – 74.5)	32.2 (13.5 – 73.2)	<0.001
Energy intake (kcal)	2099 (1702 – 2598)	2063 (1686 – 2505)	2022 (1611 – 2526)	1940 (1504 – 2405)	<0.001
Physical Examination					
BMI (kg/m^2)	26.0 (23.9 – 28.9)	26.9 (24.5 – 29.4)	27.0 (24.8 – 29.8)	27.5 (24.9 – 30.5)	<0.001
Biochemistry					
AST (U/L)	24 (21 – 28)	24 (21 – 28)	24 (21 – 29)	24 (21 – 28)	0.605
ALT (U/L)	18 (14 – 23)	18 (14 – 23)	19 (15 – 24)	19 (15 – 25)	0.004
GGT (U/L)	21 (16 – 31)	22 (16 – 31)	24 (17 – 35)	25 (18 – 36)	<0.001
Platelets (*10^9/L)	268 (228 – 308)	261 (225 – 304)	259 (219 – 304)	261 (221 – 305)	0.054
HOMA-IR	2.3 (1.6 – 3.5)	2.5 (1.8 – 3.8)	2.7 (1.8 – 4.2)	2.9 (1.8 – 4.7)	<0.001
Total Cholesterol (mmol/L)	5.5 (1.1)	5.5 (1.1)	5.4 (1.1)	5.3 (1.1)	<0.001
HDL-C (mmol/L)	1.5 (0.4)	1.5 (0.4)	1.5 (0.4)	1.4 (0.4)	0.012
Triglycerides (mmol/L)	1.2 (1.0 – 1.7)	1.3 (1.0 – 1.7)	1.3 (1.0 – 1.8)	1.3 (1.0 – 1.8)	0.053
GFR	77 (66 – 86)	77 (66 – 85)	75 (66 – 86)	75 (64 – 85)	0.241
Comorbidities					
Metabolic Syndrome	45.3	52.0	55.6	59.7	<0.001
- Waist Circumference	35.0	41.4	45.7	50.5	<0.001
- Triglycerides	40.9	45.3	48.1	49.7	<0.001
- HDL-Cholesterol	39.5	44.7	46.5	48.0	0.001
- Blood pressure	83.4	83.0	84.9	85.9	0.276
- Fasting Glucose	37.7	46.4	49.7	53.3	<0.001
Diabetes Mellitus (%)	10.1	12.1	13.6	16.6	<0.001
Hypertension (%)	71.4	71.5	76.0	77.0	0.005
NAFLD	28.9	31.8	35.7	41.3	<0.001

Data is expressed as mean (SD), median (P25-P75) or percentage. *P-value is based on ANOVA, Kruskall-Wallis test or Chi-square test.
Abbreviations ALT: alanine aminotransferase; A:P: animal protein to potassium ratio; AST: aspartate aminotransferase; BMI: body mass index; DAL: dietary acid load; GFR: glomerular filtration rate; GGT: gamma-glutamyltransferase; HDL-C: high-density lipoprotein cholesterol; HOMA-IR: homeostasis model assessment of insulin resistance; NAFLD: non-alcoholic fatty liver disease; NEAP: net endogenous acid production; PRAL; potential renal acid load.

Supplementary Table 4: Logistic regression analyses of DAL-residuals with outcome variable NAFLD

	Total population (n=3882)		
	Q2	Q3	Q4
PRAL n per quartile	**(n=970)**	**(n=971)**	**(n=970)**
NEAP n per quartile	**(n=970)**	**(n=971)**	**(n=970)**
A:P n per quartile	**(n=970)**	**(n=971)**	**(n=970)**
Model 1 (sociodemographic)			
PRAL	0.98 (0.81 – 1.20)	1.16 (0.96 – 1.41)	**1.37 (1.13 – 1.66)†**
NEAP	1.03 (0.85 – 1.25)	**1.25 (1.04 – 1.52)†**	**1.40 (1.16 – 1.69)†**
A:P	1.16 (0.96 – 1.41)	**1.37 (1.13 – 1.66)†**	**1.61 (1.33 – 1.95)†**
Model 2 (lifestyle)			
PRAL	0.98 (0.81 – 1.19)	1.14 (0.94 – 1.39)	**1.33 (1.10 – 1.62)†**
NEAP	1.03 (0.85 – 1.25)	**1.24 (1.02 – 1.50)**	**1.36 (1.12 – 1.65)†**
A:P	1.16 (0.95 – 1.41)	**1.33 (1.10 – 1.62)†**	**1.56 (1.29 – 1.89)†**
Model 3 (metabolic)			
PRAL	0.97 (0.78 – 1.22)	1.10 (0.88 – 1.38)	1.23 (0.98 – 1.54)
NEAP	0.98 (0.78 – 1.22)	1.19 (0.96 – 1.49)	1.25 (1.00 – 1.56)
A:P	1.01 (0.81 – 1.27)	1.13 (0.90 – 1.41)	1.23 (0.98 – 1.53)
Model 4 (metabolic + DQ)			
PRAL	0.97 (0.77 – 1.22)	1.10 (0.88 – 1.38)	1.23 (0.97 – 1.55)
NEAP	0.98 (0.78 – 1.23)	1.20 (0.96 – 1.50)	1.25 (1.00 – 1.58)
A:P	1.01 (0.81 – 1.27)	1.13 (0.90 – 1.41)	1.23 (0.97 – 1.55)

Values are odds ratios with 95% confidence intervals taking quartile 1 as reference. **Bold** values indicate P<0.05. † Indicates significant values using P < 0.028 as determined by Sidák.
Model 1 (socio-demographic) is adjusted for age, gender, education level, energy intake and study cohort **Model 2** (lifestyle) is in addition previous model adjusted for past or current smoking, units of alcohol, and physical activity **Model 3** (metabolic) is in addition to the previous model adjusted for HDL-cholesterol, triglycerides, metabolic syndrome, GFR, diabetes mellitus and log-transformed BMI **Model 4** (metabolic + DQ) is in addition to the previous model adjusted for DQ.
Abbreviations A:P: animal protein to potassium ratio; BMI: body mass index; DAL: dietary acid load; DQ: dietary quality; GFR: glomerular filtration rate; NAFLD: non-alcoholic fatty liver disease; NEAP: net endogenous acid production; PRAL; potential renal acid load.

Supplementary Table 5: Logistic regression analyses of DAL associations with outcome variable NAFLD stratified by sex

	Men (n=1619)		
	Q2	**Q3**	**Q4**
PRAL n per quartile	**(n=364)**	**(n=438)**	**(n=502)**
NEAP n per quartile	**(n=380)**	**(n=447)**	**(n=486)**
A:P n per quartile	**(n=414)**	**(n=417)**	**(n=434)**
Model 2 (lifestyle)			
PRAL	0.95 (0.68 – 1.33)	1.20 (0.87 – 1.64)	**1.42 (1.05 – 1.93)†**
NEAP	1.12 (0.80 – 1.55)	1.34 (0.98 – 1.85)	**1.49 (1.09 – 2.04)†**
A:P	1.35 (0.98 – 1.85)	**1.59 (1.16 – 2.18)†**	**1.87 (1.37 – 2.55)†**
Model 3 (metabolic)			
PRAL	0.90 (0.61 – 1.33)	1.14 (0.79 – 1.63)	1.30 (0.92 – 1.85)
NEAP	0.97 (0.66 – 1.42)	1.35 (0.94 – 1.94)	1.34 (0.93 – 1.91)
A:P	1.06 (0.74 – 1.52)	1.31 (0.92 – 1.87)	1.45 (1.02 – 2.07)
	Women (n=2263)		
	Q2	**Q3**	**Q4**
PRAL n per quartile	**(n=606)**	**(n=533)**	**(n=468)**
NEAP n per quartile	**(n=590)**	**(n=524)**	**(n=484)**
A:P n per quartile	**(n=556)**	**(n=554)**	**(n=536)**
Model 2 (lifestyle)			
PRAL	1.02 (0.80 – 1.30)	1.05 (0.81 – 1.34)	**1.34 (1.04 – 1.72)†**
NEAP	1.00 (0.79 – 1.28)	1.15 (0.90 – 1.47)	1.28 (1.00 – 1.65)
A:P	1.05 (0.82 – 1.36)	1.20 (0.93 – 1.54)	**1.42 (1.11 – 1.82)†**
Model 3 (metabolic)			
PRAL	0.99 (0.75 – 1.32)	1.04 (0.78 – 1.40)	1.22 (0.90 – 1.65)
NEAP	0.98 (0.74 – 1.30)	1.08 (0.81 – 1.45)	1.17 (0.87 – 1.58)
A:P	0.91 (0.68 – 1.22)	0.94 (0.70 – 1.26)	1.08 (0.81 – 1.46)

Values are odds ratios with 95% confidence intervals taking quartile 1 as reference. P-trend is calculated across the quartiles. Bold values indicate P<0.05. † Indicates significant values using P < 0.028 as determined by Sidák. Model 1 (socio-demographic) is adjusted for age, education level, energy intake and study cohort Model 2 (lifestyle) is in addition previous model adjusted for past or current smoking, units of alcohol, and physical activity Model 3 (metabolic) is in addition to the previous model adjusted for HDL-cholesterol, triglycerides, metabolic syndrome, GFR, diabetes mellitus and BMI Model 4 (metabolic + DQ) is in addition to the previous model adjust for DQ.

Abbreviations A:P: animal protein to potassium ratio; BMI: body mass index; DAL: dietary acid load; DQ: dietary quality; GFR: glomerular filtration rate; NAFLD: non-alcoholic fatty liver disease; NEAP: net endogenous acid production; PRAL; potential renal acid load.

Supplementary Table 6: Logistic regression analyses of DAL associations with outcome variable NAFLD stratified by study cohort

	RS-cohort I (n=1069)		
	Q2	**Q3**	**Q4**
PRAL n per quartile	**(n=259)**	**(n=259)**	**(n=263)**
NEAP n per quartile	**(n=259)**	**(n=234)**	**(n=263)**
A:P n per quartile	**(n=239)**	**(n=262)**	**(n=283)**
Model 2 (lifestyle)			
PRAL	0.93 (0.64 – 1.34)	0.92 (0.63 – 1.34)	1.07 (0.74 – 1.54)
NEAP	1.10 (0.77 – 1.57)	1.08 (0.74 – 1.55)	1.10 (0.77 – 1.57)
A:P	1.21 (0.83 – 1.76)	1.11 (0.77 – 1.60)	**1.45 (1.02 – 2.07)**
Model 3 (metabolic)			
PRAL	1.03 (0.67 – 1.58)	1.00 (0.64 – 1.55)	1.19 (0.77 – 1.83)
NEAP	1.13 (0.74 – 1.72)	1.14 (0.73 – 1.76)	1.22 (0.80 – 1.86)
A:P	1.22 (0.78 – 1.90)	1.05 (0.68 – 1.62)	1.40 (0.92 – 2.13)
	RS-cohort II (n=1183)		
	Q2	**Q3**	**Q4**
PRAL n per quartile	**(n=303)**	**(n=315)**	**(n=270)**
NEAP n per quartile	**(n=284)**	**(n=295)**	**(n=289)**
A:P n per quartile	**(n=297)**	**(n=281)**	**(n=316)**
Model 2 (lifestyle)			
PRAL	1.00 (0.70 – 1.42)	1.32 (0.93 – 1.87)	**1.73 (1.21 – 2.46)†**
NEAP	0.99 (0.69 – 1.40)	1.31 (0.93 – 1.85)	**1.65 (1.17 – 2.32)†**
A:P	1.10 (0.77 – 1.57)	**1.61 (1.13 – 2.29)†**	**1.70 (1.20 – 2.40)†**
Model 3 (metabolic)			
PRAL	0.75 (0.49 – 1.15)	1.20 (0.80 – 1.82)	1.43 (0.94 – 2.19)
NEAP	0.76 (0.50 – 1.16)	1.14 (0.76 – 1.70)	1.43 (0.95 – 2.16)
A:P	0.77 (0.51 – 1.18)	1.22 (0.80 – 1.86)	1.11 (0.73 – 1.68)
	RS-cohort III (n=1630)		
	Q2	**Q3**	**Q4**
PRAL n per quartile	**(n=408)**	**(n=397)**	**(n=437)**
NEAP n per quartile	**(n=427)**	**(n=442)**	**(n=418)**
A:P n per quartile	**(n=434)**	**(n=428)**	**(n=371)**
Model 2 (lifestyle)			
PRAL	1.05 (0.77 – 1.44)	1.10 (0.80 – 1.51)	**1.44 (1.07 – 1.95)†**
NEAP	1.00 (0.73 – 1.38)	1.24 (0.90 – 1.69)	**1.40 (1.02 – 1.92)**
A:P	1.12 (0.83 – 1.52)	1.30 (0.96 – 1.77)	**1.61 (1.18 – 2.19)†**
Model 3 (metabolic)			
PRAL	1.12 (0.79 – 1.60)	1.03 (0.73 – 1.47)	1.24 (0.88 – 1.76)
NEAP	1.01 (0.71 – 1.45)	1.19 (0.83 – 1.69)	1.16 (0.81 – 1.67)
A:P	0.94 (0.66 – 1.33)	1.03 (0.73 – 1.45)	1.18 (0.83 – 1.69)

Values are odds ratios with 95% confidence intervals taking quartile 1 as reference. Bold values indicate P<0.05.
† Indicates significant values using P < 0.028 as determined by Sidák.
Model 1 (socio-demographic) is adjusted for age, gender, education level and energy intake Model 2 (lifestyle) is in addition previous model adjusted for past or current smoking, units of alcohol, and physical activity Model 3

(metabolic) is in addition to the previous model adjusted for HDL-cholesterol, triglycerides, metabolic syndrome, GFR, diabetes mellitus and BMI Model 4 (metabolic + DQ) is in addition to the previous model adjust for DQ.
Abbreviations A:P: animal protein to potassium ratio; BMI: body mass index; DAL: dietary acid load; DQ: dietary quality; GFR: glomerular filtration rate; NAFLD: non-alcoholic fatty liver disease; NEAP: net endogenous acid production; PRAL; potential renal acid load.

Supplementary Table 7: Logistic regression analyses of DAL associations with outcome variable NAFLD stratified by GFR (cut-off 60)

	GFR < 60 (n=597)		
	Q2	Q3	Q4
PRAL n per quartile	(n=147)	(n=143)	(n=162)
NEAP n per quartile	(n=142)	(n=149)	(n=156)
A:P n per quartile	(n=144)	(n=144)	(n=174)
Model 2 (lifestyle)			
PRAL	1.00 (0.59 – 1.69)	0.98 (0.58 – 1.68)	1.49 (0.90 – 2.47)
NEAP	0.89 (0.53 – 1.51)	1.09 (0.66 – 1.82)	1.23 (0.75 – 2.02)
A:P	1.08 (0.63 – 1.86)	1.33 (0.78 – 2.26)	**1.66 (1.00 – 2.74)**
Model 3 (metabolic)			
PRAL	1.05 (0.57 – 1.91)	1.01 (0.55 – 1.87)	**1.81 (1.01 – 3.24)**
NEAP	0.86 (0.47 – 1.56)	1.25 (0.70 – 2.23)	1.40 (0.79 – 2.49)
A:P	1.11 (0.60 – 2.05)	1.39 (0.75 – 2.55)	1.68 (0.94 – 2.99)
	GFR > 60 (n=3285)		
	Q2	Q3	Q4
PRAL n per quartile	(n=823)	(n=828)	(n=808)
NEAP n per quartile	(n=828)	(n=822)	(n=814)
A:P n per quartile	(n=826)	(n=827)	(n=796)
Model 2 (lifestyle)			
PRAL	0.99 (0.80 – 1.22)	1.14 (0.92 – 1.41)	**1.37 (1.11 – 1.69)†**
NEAP	1.05 (0.85 – 1.30)	**1.25 (1.02 – 1.55)**	**1.38 (1.12 – 1.70)†**
A:P	1.16 (0.94 – 1.43)	**1.34 (1.08 – 1.65)†**	**1.56 (1.27 – 1.93)†**
Model 3 (metabolic)			
PRAL	0.96 (0.75 – 1.23)	1.09 (0.86 – 1.39)	1.18 (0.92 – 1.50)
NEAP	0.99 (0.78 – 1.27)	1.16 (0.91 – 1.48)	1.20 (0.94 – 1.53)
A:P	0.93 (0.73 – 1.18)	1.04 (0.82 – 1.33)	1.13 (0.88 – 1.44)

Values are odds ratios with 95% confidence intervals taking quartile 1 as reference. P-trend is calculated across the quartiles. Bold values indicate P<0.05. † Indicates significant values using P < 0.028 as determined by Sidák. Model 1 (socio-demographic) is adjusted for age, gender, education level, energy intake and study cohort Model 2 (lifestyle) is in addition previous model adjusted for past or current smoking, units of alcohol, and physical activity Model 3 (metabolic) is in addition to the previous model adjusted for HDL-cholesterol, triglycerides, metabolic syndrome, diabetes mellitus and BMI Model 4 (metabolic + DQ) is in addition to the previous model adjust for DQ.
Abbreviations A:P: animal protein to potassium ratio; BMI: body mass index; DAL: dietary acid load; DQ: dietary quality; GFR: glomerular filtration rate; NAFLD: non-alcoholic fatty liver disease; NEAP: net endogenous acid production; PRAL; potential renal acid load.

Supplementary Table 8: Logistic regression analyses of DAL associations with outcome variable NAFLD stratified by age (cut-off 65 years)

	Age <65 years old (n=1160)		
	Q2	**Q3**	**Q4**
PRAL n per quartile	**(n=281)**	**(n=276)**	**(n=333)**
NEAP n per quartile	**(n=289)**	**(n=318)**	**(n=319)**
A:P n per quartile	**(n=301)**	**(n=311)**	**(n=257)**
Model 2 (lifestyle)			
PRAL	1.14 (0.78 – 1.68)	1.32 (0.90 – 1.94)	**1.58 (1.10 – 2.28)†**
NEAP	1.06 (0.71 – 1.57)	1.33 (0.91 – 1.96)	**1.47 (1.00 – 2.16)**
A:P	1.14 (0.78 – 1.64)	1.33 (0.93 – 1.92)	**1.63 (1.13 – 2.37)†**
Model 3 (metabolic)			
PRAL	1.28 (0.83 – 1.98)	1.29 (0.83 – 1.98)	1.39 (0.92 – 2.12)
NEAP	1.12 (0.71 – 1.76)	1.36 (0.88 – 2.09)	1.29 (0.83 – 2.00)
A:P	0.99 (0.65 – 1.50)	1.100 (0.73 – 1.66)	1.23 (0.80 – 1.88)
	Age ≥ 65 years old (n=2722)		
	Q2	**Q3**	**Q4**
PRAL n per quartile	**(n=689)**	**(n=695)**	**(n=637)**
NEAP n per quartile	**(n=681)**	**(n=653)**	**(n=651)**
A:P n per quartile	**(n=669)**	**(n=660)**	**(n=713)**
Model 2 (lifestyle)			
PRAL	0.94 (0.75 – 1.18)	1.05 (0.83 – 1.32)	**1.34 (1.07 – 1.69)†**
NEAP	1.02 (0.81 – 1.28)	1.19 (0.95 – 1.49)	**1.34 (1.07 – 1.68)†**
A:P	1.14 (0.90 – 1.44)	**1.33 (1.06 – 1.68)†**	**1.56 (1.24 – 1.95)†**
Model 3 (metabolic)			
PRAL	0.87 (0.66 – 1.13)	1.01 (0.77 – 1.32)	1.26 (0.96 – 1.65)
NEAP	0.92 (0.71 – 1.20)	1.10 (0.84 – 1.43)	1.27 (0.97 – 1.65)
A:P	0.94 (0.72 – 1.23)	1.10 (0.84 – 1.44)	1.21 (0.93 – 1.58)

Values are odds ratios with 95% confidence intervals taking quartile 1 as reference. *P*-trend is calculated across the quartiles. **Bold** values indicate P<0.05. † Indicates significant values using P < 0.028 as determined by Sidák. **Model 1** (socio-demographic) is adjusted for gender, education level, energy intake and study cohort **Model 2** (lifestyle) is in addition previous model adjusted for past or current smoking, units of alcohol, and physical activity **Model 3** (metabolic) is in addition to the previous model adjusted for HDL-cholesterol, triglycerides, metabolic syndrome, GFR, diabetes mellitus and BMI **Model 4** (metabolic + DQ) is in addition to the previous model adjust for DQ.

Abbreviations A:P: animal protein to potassium ratio; BMI: body mass index; DAL: dietary acid load; DQ: dietary quality; GFR: glomerular filtration rate; NAFLD: non-alcoholic fatty liver disease; NEAP: net endogenous acid production; PRAL; potential renal acid load.

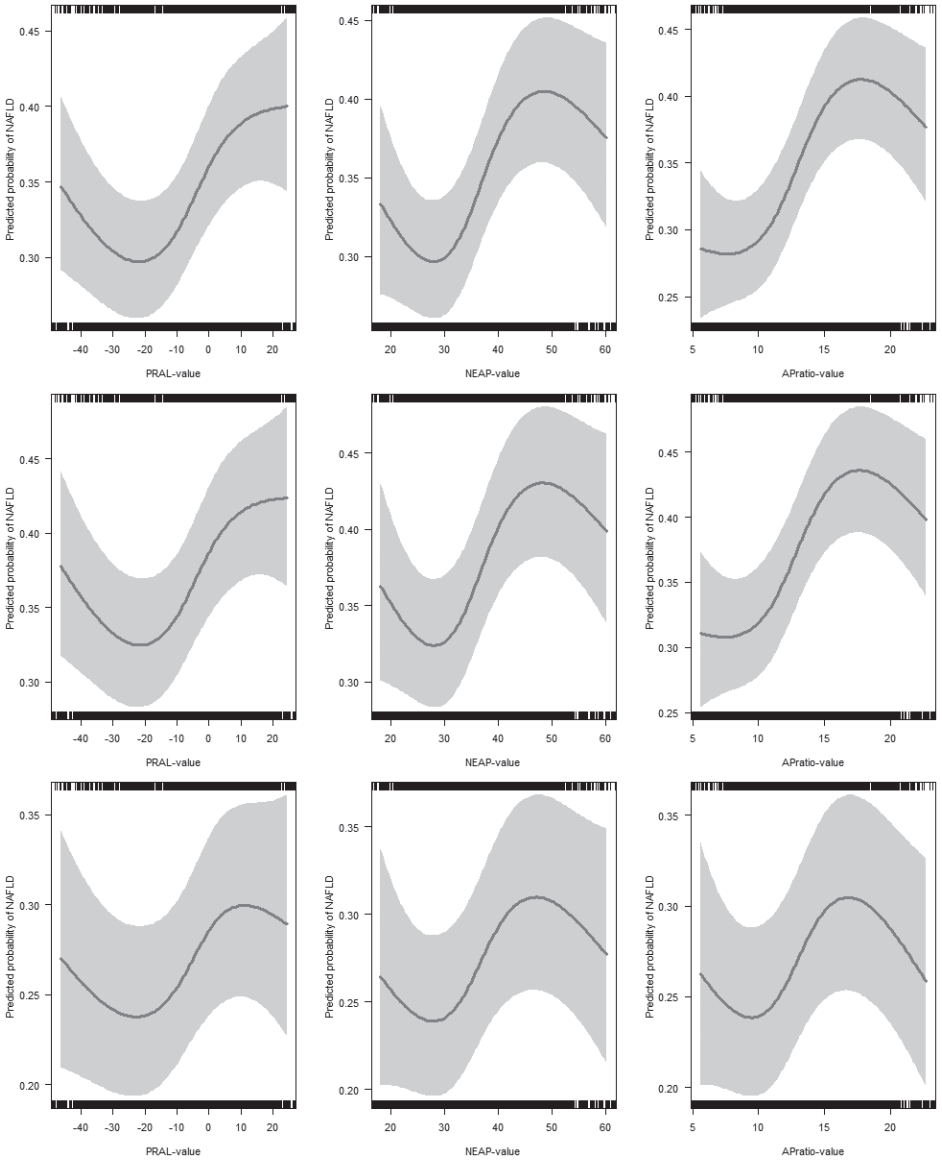
Supplementary Table 9: Logistic regression analyses of DAL associations with outcome variable NAFLD stratified by BMI (cut-off 25)

	BMI < 25 kg/m² (n=1174)		
	Q2	**Q3**	**Q4**
PRAL n per quartile	**(n=296)**	**(n=285)**	**(n=267)**
NEAP n per quartile	**(n=308)**	**(n=273)**	**(n=277)**
A:P n per quartile	**(n=289)**	**(n=259)**	**(n=251)**
Model 2 (lifestyle)			
PRAL	1.20 (0.71 – 2.05)	1.11 (0.64 – 1.92)	1.53 (0.91 – 2.58)
NEAP	1.41 (0.82 – 2.41)	1.30 (0.74 – 2.29)	1.70 (0.99 – 2.90)
A:P	1.32 (0.79 – 2.19)	1.18 (0.69 – 2.02)	1.63 (0.98 – 2.70)
Model 3 (metabolic)			
PRAL	1.15 (0.67 – 1.99)	1.00 (0.57 – 1.78)	1.62 (0.95 – 2.78)
NEAP	1.35 (0.78 – 2.36)	1.18 (0.66 – 2.12)	**1.82 (1.05 – 3.18)**
A:P	1.19 (0.70 – 2.03)	1.08 (0.62 – 1.89)	1.60 (0.95 – 2.70)
	BMI ≥ 25 kg/m² (n=2708)		
	Q2	**Q3**	**Q4**
PRAL n per quartile	**(n=674)**	**(n=686)**	**(n=703)**
NEAP n per quartile	**(n=662)**	**(n=698)**	**(n=693)**
A:P n per quartile	**(n=681)**	**(n=712)**	**(n=719)**
Model 2 (lifestyle)			
PRAL	0.91 (0.73 – 1.14)	1.10 (0.88 – 1.38)	**1.32 (1.06 – 1.65)†**
NEAP	0.95 (0.76 – 1.19)	1.16 (0.93 – 1.45)	**1.31 (1.05 – 1.64)†**
A:P	0.94 (0.75 – 1.18)	1.13 (0.90 – 1.41)	**1.34 (1.07 – 1.68)†**
Model 3 (metabolic)			
PRAL	0.88 (0.69 – 1.13)	1.11 (0.87 – 1.41)	1.22 (0.96 – 1.55)
NEAP	0.92 (0.72 – 1.17)	1.21 (0.96 – 1.53)	1.20 (0.96 – 1.53)
A:P	0.92 (0.72 – 1.17)	1.10 (0.86 – 1.39)	1.25 (0.98 – 1.59)

Values are odds ratios with 95% confidence intervals taking quartile 1 as reference. *P*-trend is calculated across the quartiles.

Model 1 (socio-demographic) is adjusted for age, gender, education level, energy intake and study cohort **Model 2** (lifestyle) is in addition previous model adjusted for past or current smoking, units of alcohol, and physical activity **Model 3** (metabolic) is in addition to the previous model adjusted for HDL-cholesterol, triglycerides metabolic syndrome, GFR and diabetes mellitus **Model 4** (metabolic + DQ) is in addition to the previous model adjust for DQ.

Abbreviations A:P: animal protein to potassium ratio; BMI: body mass index; DAL: dietary acid load; DQ: dietary quality; GFR: glomerular filtration rate; NAFLD: non-alcoholic fatty liver disease; NEAP: net endogenous acid production; PRAL; potential renal acid load.



Supplementary Figure 1: Natural cubic splines for the association of DAL-proxies with predicted probability of NAFLD in model 1, 2, and 4

Model 1 (socio-demographic) is adjusted for age, gender, education level, energy intake and study cohort

Model 2 (lifestyle) is in addition previous model adjusted for past or current smoking, units of alcohol, and physical activity

Model 4 (metabolic + DQ) is in addition to model 2 adjusted for BMI, HDL-cholesterol, triglycerides, metabolic syndrome, GFR, diabetes mellitus, and DQ.

Abbreviations A:P: animal protein to potassium ratio; BMI: body mass index; DAL: dietary acid load; DQ: dietary quality; GFR: glomerular filtration rate; NAFLD: non-alcoholic fatty liver disease; NEAP: net endogenous acid production; PRAL; potential renal acid load.

Comparison model with DAL-proxy vs model without DAL-proxy

A. (Sociodemographic) model 1 (PRAL: P=7.4e^{-5}, NEAP: P=1.2e^{-5}, and A:P: P=1.9e^{-8}).
B. (Lifestyle) model 2 (PRAL: P=2.3e^{-4}, NEAP: P=4.0e^{-5}, and A:P: P=1.3e^{-7})
C. (Metabolic + DQ) model 4 (PRAL: P=0.043, NEAP: P=0.017, and A:P: P=0.014)

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Chapter 7

Adherence to a plant-based, high-fibre dietary pattern is related to regression of non-alcoholic fatty liver disease in an elderly population

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Abstract

Dietary lifestyle intervention is key in treating non-alcoholic fatty liver disease (NAFLD). We aimed to examine the longitudinal relation between well-established dietary patterns as well as population-specific dietary patterns and NAFLD. Participants from two subsequent visits of the Rotterdam Study were included. All underwent serial abdominal ultrasonography (median follow-up: 4.4 years) and filled in a food frequency questionnaire. Secondary causes of steatosis were excluded. Dietary data from 389 items were collapsed into 28 food groups and a-posteriori dietary patterns were identified using factor analysis. Additionally, we scored three a-priori dietary patterns (Mediterranean Diet Score, Dutch Dietary Guidelines and WHO-score). Logistic mixed regression models were used to examine the relation between dietary patterns and NAFLD. Analyses were adjusted for demographic, lifestyle and metabolic factors. We included 963 participants of whom 343 had NAFLD. Follow-up data was available in 737 participants. Incident NAFLD was 5% and regressed NAFLD was 30%. We identified five a-posteriori dietary patterns (cumulative explained variation [R^2]=20%). The patterns were characterised as: *vegetable & fish*, *red meat & alcohol*, *traditional*, *salty snacks & sauces* and *fats & sweets*. Adherence to the *traditional* pattern (i.e. high intake of vegetable oils/stanols, margarines/butters, potatoes, whole grains and sweets/desserts) was associated with regression of NAFLD per SD increase in Z-score (0.40,95%CI 0.15–1.00). Adherence to the three a-priori patterns all showed regression of NAFLD, but only the WHO-score showed a distinct association (0.73,95%CI 0.53–1.00). Hence, in this large elderly population, adherence to a plant-based, high-fibre and low-fat diet was related to regression of NAFLD.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease worldwide and characterized by fat accumulation in the liver without the presence of the well-known risk factors for liver disease such as alcohol misuse and viral hepatitis.[281] The increase of NAFLD parallels the worldwide rise of non-communicable metabolic diseases, such as obesity and type 2 diabetes mellitus.[324,325] In fact, it is predicted that NAFLD prevalence will continue to increase, which will subsequently lead to even more advanced liver disease and liver-related mortality in the coming years.[326] But apart from the liver-related sequelae, NAFLD is also regarded as the hepatic manifestation of the metabolic syndrome.[327] It is not only strongly associated with metabolic health, but it also actively contributes to the risk of cardiovascular disease incidence.[182,328]

Pathogenesis of NAFLD is multifactorial – there are many molecular pathways that contribute to the development of NAFLD – and it is likely that NAFLD pathogenesis differs between individuals.[281] A common denominator in the development of NAFLD, however, is diet. Furthermore, as there is no registered drug for the treatment of NAFLD, the mainstay of treatment is implementing a healthy diet and stimulating physical activity.[55] It has been repeatedly shown that weight loss of 5% or more of the total body weight is beneficial for liver health,[240,241,329] meanwhile the Mediterranean diet has been shown to reduce liver fat independent of weight loss.[59,330] To date, the Mediterranean dietary pattern is indeed regarded as the diet of choice for NAFLD.[59] And although there is a paramount of studies on the association between separate food items or groups with NAFLD, evidence on diet as a whole almost exclusively originates from either small or cross-sectional studies.[59,286,331,332] Studying pre-hypothesized individual food items or nutrients can be of value, nonetheless, this approach has some important drawbacks.[333] For instance, one does not eat isolated nutrients but instead a complex mix of foods which can interact with each other, and moreover, foods are highly inter-correlated with each other. In addition, the effect of one nutrient can be small. Therefore, studying their separate effects is challenging in a real-life setting. Conceptually, the study of dietary quality –by means of dietary patterns– represents more information on dietary habits, food and nutrient consumption.[334] Recently, *Ma et al.* showed for the first time, that high dietary quality, as assessed by the a-priori hypothesized Mediterranean Diet Score (MDS) and the American Heart Eating Index, was associated with reduced odds of steatosis development in a large longitudinal cohort study.[335]

Another way to study dietary patterns is the use of factor analysis to derive population-specific patterns, so-called a-posteriori dietary patterns. To date, a few small cross-sectional studies appraised the association between a-posteriori dietary patterns and steatosis. All identified an unhealthy dietary pattern that was associated with steatosis.[286,336,337].

The combination of both a-priori and a-posteriori patterns might reveal a better understanding of the relation between dietary quality and NAFLD. The objective of this study

was, therefore, to examine the relation between dietary patterns and NAFLD prospectively in an elderly predominantly Caucasian population. Specifically, we studied the following a-priori dietary patterns: 1) the Mediterranean Diet Score, 2) the Dutch Dietary Guidelines, and 3) the World Health Organization recommendations. In addition, we identified five population-specific a-posteriori patterns.

Methods

Study Cohort

This study is embedded in the large ongoing population-based cohort study entitled the Rotterdam Study (RS). The RS commenced in 1989 and was designed to study elderly diseases as a response to the increasing proportion of elderly people in the population. A detailed description of the design and rationale has been described previously.[248] For the purpose of this study, we used two subsequent visits from the second cohort (RSII-3 and RSII-4). All individuals in this cohort were included based on their ZIP-code (being the suburb Ommoord in Rotterdam) and their age (at first inclusion 55 years or older).
We excluded participants that did not undergo ultrasound and participants with missing or unreliable food frequency questionnaires (FFQ), i.e. caloric consumption below 500 or above 7500 kilocalories (kcal) per day. We excluded unreliable transient elastography (TE), i.e. an interquartile range (IQR) / median liver stiffness measurement (LSM) > 0.30 in measurements with a median of ≥ 7.1 kilopascals (kPa) or above[338] and failure of TE (i.e. if no LSM was measured after at least ten attempts). Lastly, we excluded participants with well-known risk factors for steatosis, such as the presence of viral hepatitis (as measured by HbsAg and anti-HCV), the presence of alcohol misuse (measured using FFQs and defined as ≥ 2 units of alcohol per day in women and ≥ 3 units in men), or the use of pharmacy-registered drugs that are known to cause steatosis (i.e. tamoxifen, methotrexate, systemic corticosteroids, and amiodarone).
The RS has been approved by the institutional review board (Medical Ethics Committee) of the Erasmus MC University Medical Centre Rotterdam and by the review board of The Netherlands Ministry of Health, Welfare and Sports. Written informed consent was obtained from all participants.

Food Frequency Questionnaires

Participants were asked to fill in a semi-quantitative 389-item FFQ, specifically developed for Dutch adults, during both research visits (RSII-3 and RSII-4).[98,249] This FFQ includes detailed questions on consumption over the last month and deals with type of food item,

portion size, preparation method and frequency of consumption.[250] Questions within the FFQ are for example: *"Did you eat eggs last month? If yes, how were they prepared (boiled or baked)? How often did you eat eggs per month (once or 2–3 times) or per week (once, 2–3, 4–5, 6–7 times)? How many eggs did you eat on an average day then?"*. The 389 food items were grouped into 28 empirical food groups (*supplementary table 1*), based on previous publications,[336,337,339] and adapted based on the food item and group quantities (i.e. merged similar groups with a very low median intake).

A-priori dietary patterns

We chose to study the Mediterranean Diet Score (MDS), the Dutch Dietary Guidelines (DDG), and the World Health Organization (WHO) recommendations as a-priori dietary patterns.

The MDS, first described by Trichopoulou et al.[340], originally has 9 components, of which 7 components are regarded beneficial (i.e. vegetables, legumes, fruits, nuts, whole grains, fish and mono-unsaturated fatty acids (MUFA)–to–saturated fatty acids ratio) and 2 components are regarded hazardous (i.e. red meat intake and excessive alcohol use). For the purpose of this study, we adapted the MDS by excluding the alcohol component from our calculation, as the MDS cut-off for hazardous alcohol use is 50 grams per day, which is a very high cut-off in the context of a hepatic health outcome.[341] Moreover, alcohol consumption was included in the multivariable model as potential confounder. Hence, our adapted MDS has 8 components. All components were given a score of 0 (unhealthy) or 1 (healthy) based on sex specific median cut-offs, and summed up.

The DDG is a predefined index that was developed in 2015 and describes a general advice to follow a balanced and healthy dietary pattern.[298] The DDG is scored on the following points, consumption of I) vegetables (≥200g/day), II) fruit (≥200g/day), III) whole-grain products (≥90g/day), IV) legumes (≥135g/week), V) unsalted nuts (≥15g/day), VI) fish (≥100g/week), VII) dairy (≥350g/day), VIII) tea (≥150mL/day), IX) whole grains ≥50% of total grains, X) unsaturated fats and oils ≥50% of total fats, XII) red and processed meat <300g/week, XIII) sugar-containing beverages (≤150mL/day), XIV) alcohol (≤10 g/day), and XV) salt (≤6 g/day).

We calculated the WHO-score based on the recent revised guidelines of the WHO (October 2018).[342] This score is composed of 6 components which are scored as 0 if unhealthy and 1 if healthy. The components are scored as healthy if they satisfied the following criteria: I) vegetables and fruit intake of ≥400g per day, II) sugar intake (added and sugar sweetened beverages) of <10 grams per day, III) energy percentage from fat intake <30%, IV) energy percentage from saturated fat intake <10%, V) energy percentage from trans fatty acid intake is <1%, and VI) salt intake of <5 grams per day.

A-posteriori dietary patterns

A priori dietary patterns signify patterns described/identified in previous studies or specific habits of certain populations. They therefore do not necessarily 'fit' every population. For example, the Mediterranean Diet is natural for the Greek population in which this diet has been developed, whereas other populations such as the Dutch or Asian have different eating habits. We believe it is therefore of interest to also use a-posteriori dietary patterns. These are population-specific dietary patterns were derived using factor analysis on the 28 food groups at baseline with Varimax rotation and minimum residual estimation, using the function "fa" from the R package *psych*.[343] We included 5 dietary patterns based on the bend in the scree plot (*supplementary fig. 1*). The factor loadings for each food group reflect the relationship between the food group and the respective factor (i.e. dietary pattern). Subsequently we calculated adherence scores, separately for both visits, by multiplying the factors (determined for the food groups at baseline) with the observed values of the food groups at baseline (RSII-3) and follow-up (RSII-4), respectively. Each score at baseline was scaled to have a mean zero and a standard deviation (SD) of one. The same scaling parameters were used for the corresponding score at follow-up, to optimize comparability.

Hepatic imaging

For the purpose of this study, all participants underwent an abdominal ultrasound (Hitachi HI VISION 900) and TE (FibroScan®, EchoSens, Paris, France). Both examinations were performed at the same visit by an experienced nurse technician. The diagnosis of steatosis was dichotomized as yes or no, because of the poor sensitivity for the grading of steatosis but the good performance for diagnosing moderate/severe steatosis.[50] Steatosis was defined as hyperechogenic liver parenchyma as compared to the kidney parenchyma.[104] The practical performance of the transient elastography has been described in detail previously.[78] In short, both M and XL probe were available for the liver stiffness measurements (LSM) and used dependent on the subcutaneous fat layer as instructed by the manufacturer. Reliability criteria are described in the paragraph above ("Study Cohort"). Additionally, participants with an intra-cardiac device were excluded from the analyses. LSM were given as kilopascals (kPa). We used the previously proposed cut-off value of 8 kPa to proxy the presence of fibrosis in participants with steatosis, from this point forward referred to as non-alcoholic steatofibrosis (NASF).[54] As the main focus of this present study is NAFLD, participants with an LSM of 8kPa or higher without steatosis were excluded.

Other covariates

All blood samples were drawn after overnight fasting. Automatic enzyme procedures (Roche Diagnostics GmbH, Mannheim, DE) were used to determine lipid profile, glucose, alanine aminotransferase, aspartate aminotransferase and gamma-glutamyltransferase. Insulin and viral hepatitis B or C were determined using an automatic immunoassay (Roche Diagnostics GmbH, Mannheim, DE). Detailed information on drug use was obtained via automated pharmacy linkage (with which 98% of the participants were registered). A three hour home interview was carried out by trained research nurses and included questions on physical activity, smoking behaviour, education level, medical history, and demographics. In the research centre, anthropometrics were measured (i.e. weight, height, and waist and hip circumference), as well as blood pressure (median value after two measures in an upright position). The metabolic syndrome was defined using the harmonizing consensus criteria from Alberti et al. and contained 5 components on abdominal obesity, lipid profile, blood pressure, and fasting plasma glucose.[28] The comorbidities diabetes mellitus and hypertension were established on the basis of drug use for the respective comorbidity or findings at physical examination, as described in detail previously.[344]

Statistical analyses

Participant's characteristics at baseline and follow-up are summarized using the median and first and third quartile, median and range (for dietary variables), or percentages.

To examine the association between the dietary patterns, micronutrient and macronutrient composition, we calculated and tested Spearman correlation coefficients between the raw values of (subtypes of) macronutrients as well as adjusted for total energy intake and the components of the a-posteriori dietary patterns, and the adherence scores at baseline. Differences between energy-adjusted and energy & dietary pattern-adjusted correlation coefficients may be explained by overlapping characteristics of the different a-posteriori patterns, which could outweigh each other's effects.

In addition, as a supplementary analysis, we assessed the cross-sectional association between the different energy-adjusted food groups and NAFLD at baseline using univariable logistic regression. The energy-adjustment was carried out using the residual method.[345]

To investigate the association of diet with NAFLD over time in the presence of missing values in the covariates we used Bayesian logistic mixed models, as implemented in the R package JointAI.[346] In this approach missing values in covariates are imputed simultaneously with the estimation of the regression coefficients of interest, and the added uncertainty in the coefficients due to the missing values is automatically taken into account.[347,348] This imputation was done using the covariates of our most extensive set (i.e. Model 2, given below). The choice of a mixed model allowed us to include data from all patients that

fulfilled the above mentioned inclusion criteria, even when no follow-up measurement was available. A random intercept was included in the mixed model to take into account correlation between repeated measurements within the same subject. Separate models were fitted for each of the a-priori dietary patterns and the five a-posteriori patterns. As the five a posteriori dietary patterns explain approximately 20% of the variation in dietary intake in the population, they were analysed together in one model.

Two sets of covariates were created. The first set ("Model 1") contains baseline age (in years), physical activity (in metabolic equivalent task hour/week) and education level (low/intermediate/high), and in addition, sex, energy intake (in kilocalories per day), alcohol consumption (in units per day), and follow-up time (in years). The second set ("Model 2") additionally contains covariates that reflect potential confounding, colliding, or mediating factors, i.e. baseline type 2 diabetes mellitus, baseline hypertension, and BMI. To allow the effect of diet to change over time and to allow effect modification by BMI interaction terms between the respective dietary pattern variable(s) and follow-up time (in Model 1 and 2) and BMI (only in Model 2) were included.

To obtain results for Model 1, ten sets of imputed values were extracted from each of the analyses of Model 2, then Model 1 was fitted on each dataset. Output from the ten repeated analyses per model was combined to calculate overall results. Since none of the interaction terms mentioned above had relevant contribution to any of the models, and the presence of interaction terms in a model complicates the interpretation of the regression coefficients substantially, we re-fitted Model 1 and Model 2 without the interaction terms (using imputed values from the original models) and present only the results of these simplified models.

We also investigated the role of BMI as a mediating factor between diet and NAFLD: we performed additional analyses with BMI (continuous) as outcome measure. For this, Bayesian linear mixed models were used and incomplete covariates were again simultaneously imputed. The models contained the confounders from Model 2, an interaction term between the dietary patterns and follow-up time, and a random intercept.

We also examined adherence to dietary patterns in relation to NAFLD severity. Due to the low number of NASF patients, we were not able to perform mixed effects logistic regression models on this outcome. In order to gain insight into the association between dietary patterns and NASF, we therefore plotted the (a-posteriori and a-priori) dietary pattern adherence scores across participants with NASF, participants with 'simple' steatosis, and participants without steatosis.

We used non-informative priors for our Bayesian analyses. Results from the Bayesian analyses are presented as posterior means and 95% credible intervals (CI). All analyses were performed using R version 3.5.2 and the packages JointAI (version 0.5.1) and psych (version 1.8.12). More detailed information of the statistical analyses can be found in the *supplementary methods.*

Results

Participant Characteristics

The flowchart of the study is illustrated in *figure 1*. After exclusion, 963 (60.1%) participants were eligible for this study at baseline. Prevalence of men (n=424/963 vs n=280/639)

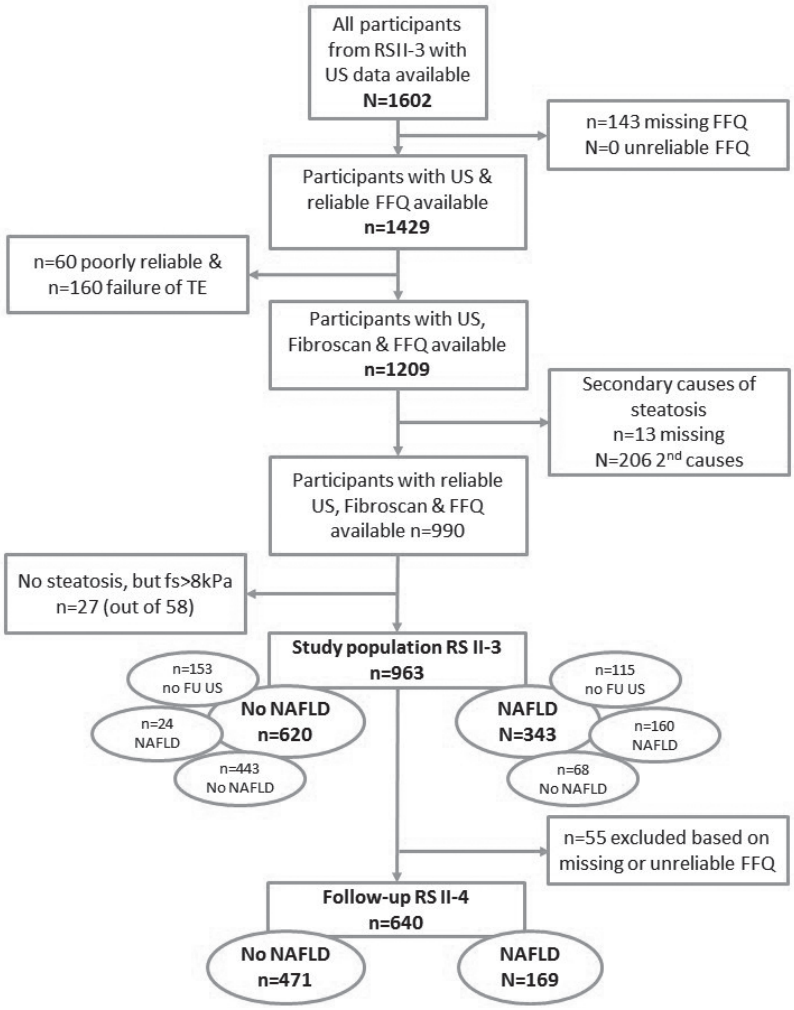


Figure 1: Flowchart of the study

The dotted encircled ovals depict the diagnosis at follow up RS II-4 for the 'no NAFLD' and 'NAFLD' group in RS II-3. The numbers of 'No NAFLD' and 'NAFLD' in RS II-4 depicts these encircled numbers minus the exclusion of 55 unreliable or missing FFQ at follow-up. Please find supplementary table 2 for more information.

Abbreviations: FFQ: Food Frequency Questionnaire, FU: follow-up, KPa: Kilopascals, LSM: Liver Stiffness Measurements, NAFLD: Non-Alcoholic Fatty Liver Disease, RS: Rotterdam Study, TE: Transient Elastography, US: ultrasound

Table 1: Participant Characteristics

	Baseline data n=963	Follow-up data n=640
Demographics		
Age (years)	71.0 [68.6, 73.4]	75.1 [72.7 – 77.4]
Female (%)	56.0	54.7
Caucasian (%)*	97.7	-
Education Level (%)†		
Low / Intermediate / High	48.2 / 31.7 / 20.1	-
Smoking status (%)‡		
Never / Past or Current	37.7 / 62.3	-
Alcohol (units/d)	0.49 [0.08, 1.22]	0.42 [0.04, 1.21]
Physical Activity (metEqh/wk)§	44.7 [18.0, 84.6]	-
Physical examination		
BMI (kg/m^2)¶	26.9 [24.5, 29.4]	26.5 [24.3, 29.2]
Waist Circumference (cm)	93.4 [84.8, 101.2]	94.3 [85.4, 101.5]
Biochemistry		
AST (U/L)	25.00 [21.00, 28.00]	-
ALT (U/L)	17.00 [14.00, 22.00]	-
GGT (U/L)¶	23.00 [17.00, 32.00]	-
Platelets (*10^9/L) ¶	254 [218, 301]	
HOMA-IR	2.68 [1.73, 4.14]	-
Total Cholesterol (mmol/L)	5.40 [4.70, 6.20]	-
HDL-C (mmol/L)	1.44 [1.20, 1.72]	-
Triglycerides (mmol/L)	1.29 [0.98, 1.75]	-
Comorbidities		
Metabolic Syndrome (%)	55.6	-
Diabetes Mellitus (%)	15.2	-
Hypertension (%)	82.8	-
Liver Imaging		
NAFLD (%)	35.6	26.4
Liver Stiffness (kPa)	4.90 [4.00, 6.10]	5.10 [4.20, 6.10]
NASF (%)	3.2	1.6
Diet		
Energy intake (kcal/day)	1932 (1529, 2354)	1964 (1573, 2424)
MDS (range 0–10)	5.00 (4.00, 6.00)	5.00 (4.00, 6.00)
DDG (range 0–13)	7.00 (6.00, 8.00)	7.00 (6.00, 8.00)
WHO score (range 0–5)	2.00 (2.00, 4.00)	2.00 (2.00, 3.00)
Vegetable & Fish Pattern (SD)	–0.19 (–0.70, 0.56)	–0.21 (–0.80, 0.51)
Red Meat & Alcohol Pattern (SD)	0.06 (–0.53, 0.68)	0.03 (–0.59, 0.57)
Traditional Pattern (SD)	–0.05 (–0.68, 0.60)	–0.21 (–0.86, 0.56)

Table 1 (*continued*)

	Baseline data n=963	Follow-up data n=640
Salty Snacks & Sauces Pattern (SD)	–0.13 (–0.68, 0.57)	–0.14 (–0.67, 0.53)
High Fat Dairy & Refined Grains Pattern (SD)	0.04 (–0.54, 0.52)	0.04 (–0.51, 0.54)

Data represents original non-imputed data as median [P25-P75], percentage, or median (range) for dietary data. Baseline data was complete except for *ethnicity data: missing in n=179 cases, †education level: missing in n=26 cases, ‡smoking status: missing in n=55 cases, §physical Activity: missing in n=68 cases, ¶covariables with <0.5% missing values. Follow-up data was complete.
Abbreviations: ALT: alanine aminotransferase, AST: aspartate aminotransferase, BMI: body mass index, DDG: Dutch dietary guidelines, GGT: gamma glutamyltransferase, HDL-C: high density lipoprotein cholesterol, HOMA-IR: homeostasis model assessment of insulin resistance, MDS: Mediterranean diet score, NAFLD: non-alcoholic fatty liver disease, kcal: kilocalories, metEqh: metabolic equivalent hours, SD: standard deviation, WHO: world health organization.

and steatosis (n=343/963 vs n=252/639) were similar in the included and excluded group (*P*=0.93 and *P*=0.12 respectively). BMI was slightly lower in the included group (mean 27.2 ± 3.8 kg/m²) than in the excluded (mean 28.0 ± 4.4 kg/m²) group (*P*<0.01) as was the mean age (72.0 ± 4.8 in the included group and 72.7 ± 5.7 in the excluded group; *P*<0.01). Of these, 343 participants had NAFLD (35.6%), of which 31 (9%) had coincident elevated LSM, i.e. NASF (*table 1*).

Follow-up data of 737 participants (76.5%) was available, measured at a median time of 4.4 years [4.3 – 4.5] after the baseline measurement. Of those lost to follow-up, no participant was registered as deceased. Most participants had a similar ultrasound diagnosis at baseline and follow-up (*figure 1*). However, in the group without steatosis, 24 participants progressed to steatosis (5.1%), and in the group with steatosis, 68 participants regressed to no steatosis (29.8%). In *supplementary table 2* a detailed overview of follow-up by liver status is given.

Participant characteristics at baseline and follow-up are given in *table 1*. In short, at baseline, median age was 71 [69 – 73] years, 56% were female, median BMI was 26.9 [24.5 – 29.4] kg/m², and median LSM was 4.9 [4.0 – 6.1] kPa. At follow-up, median age was 75 [73 – 77] years, 55% was female, median BMI was 26.5 [24.3 – 29.2] kg/m², and median LSM was 5.1 [4.2 – 6.1] kPa.

Adherence scores to all dietary patterns at baseline and follow-up are given in *table 1*. In general, adherence to healthy eating patterns was similar and low. Median energy intake, as well as adherence to dietary guidelines was comparable at baseline and follow-up. In this population, absolute intake was quite low, the most consumed food groups were coffee (406 grams or 1.6 cups/day), fruit (301 grams/day), and vegetables (211 grams/day) (*table 2).* The energy-adjusted associations between food groups and NAFLD at baseline are given in *supplementary figure 2*.

Table 2: Median absolute intake and factor loadings for food groups per a-posteriori dietary pattern

		Food group factor loadings				
	Median [P25, P75] intake in grams	Vegetable & Fish Pattern	Red Meat & Alcohol Pattern	Traditional Pattern	Salty Snacks & Sauces Pattern	High Fat Dairy & Refined Grains Pattern
Explained variation		4.8%	4.5%	4.3%	3.3%	2.5%
Fruit	301 [132, 529]	**0.292**	**–0.225**	0.144		
Fruit juice	21 [0, 107]		–0.105			**0.257**
Nuts	0.7 [0.0, 4.5]	0.110	–0.116		**0.265**	
Vegetable oils & Stanols	27 [14, 43]		0.182	**0.543**		
Margarine or butter	11 [6, 19]		0.109	**0.391**		0.185
Tomatoes	18 [5, 35]	**0.586**	–0.111		0.143	
Vegetables	211 [131, 325]	**0.584**		0.168		
Potatoes	68 [35, 102]		0.112	**0.314**		
Legumes	9 [0, 30]			0.142	**0.236**	
Whole grains	105 [68, 143]			**0.396**		–0.196
Refined grains	30 [14, 56]	0.169	0.167			**0.346**
Egg products	13 [7, 21]	0.110	**0.229**			
Red meat	41 [23, 61]	0.196	**0.599**	**0.223**		0.174
Refined or organ meat	24 [13, 38]	0.177	**0.546**	**0.214**		0.167
Poultry	9 [4, 15]	**0.308**	0.185			
Fish	22 [10, 38]	**0.358**			0.141	
Low fat dairy products	193 [96, 319]	0.147		0.183		–0.270
High fat dairy products	20 [7, 46]			0.114		**0.401**
Salty snacks	22 [10, 40]		**0.246**		**0.622**	
Sauces	2.7 [0.6, 6.3]	**0.267**	0.118		**0.480**	0.158
Sweet snacks or desserts	79 [48, 121]			**0.439**	0.181	**0.246**
Sugary drinks	0 [0, 43]		0.164		0.137	0.178
Diet soda or water	13 [0, 150]	0.191		–0.149	0.123	
Tea	174 [54, 406]	0.138	**–0.308**	0.179		
Coffee	406 [174, 406]		**0.212**			
Wine	21 [0, 83]	0.186				
Beer or spirits	0 [0, 27]	–0.105	**0.248**			
Soy products	0 [0, 0]		–0.157		0.129	

A-posteriori dietary patterns

The a-posteriori patterns obtained by factor analysis are presented in *table 2* and *figure 2*. We identified five dietary patterns which together explained 19.5% of the variation in food intake. Specifically, the patterns explained respectively 4.8%, 4.5%, 4.3%, 3.3% and 2.5% of the variation in food intake. The first pattern was characterised by high intake of vegetables, poultry, fish, and fruit. This pattern was therefore named *vegetable & fish*. The second pattern was characterised by high intake of red, refined or organ meat, salty

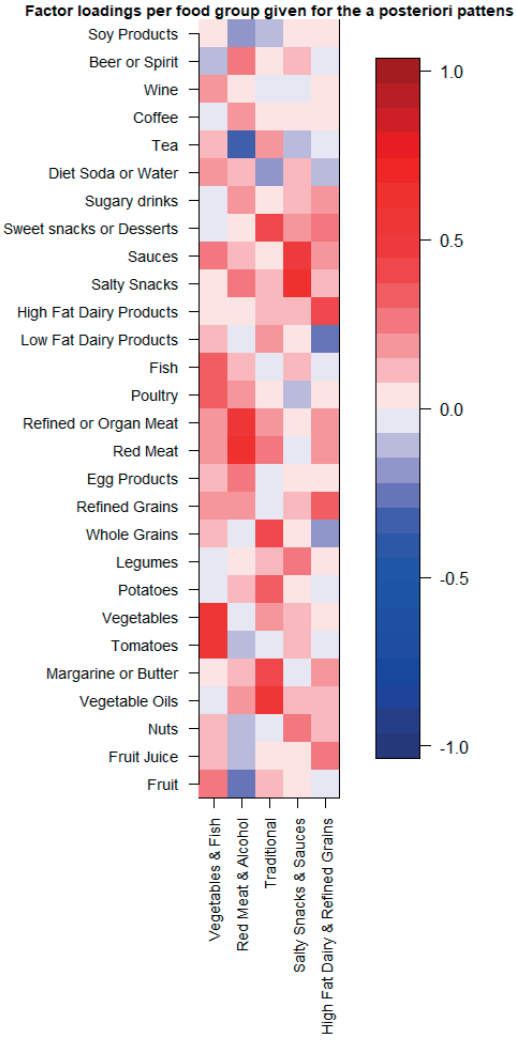
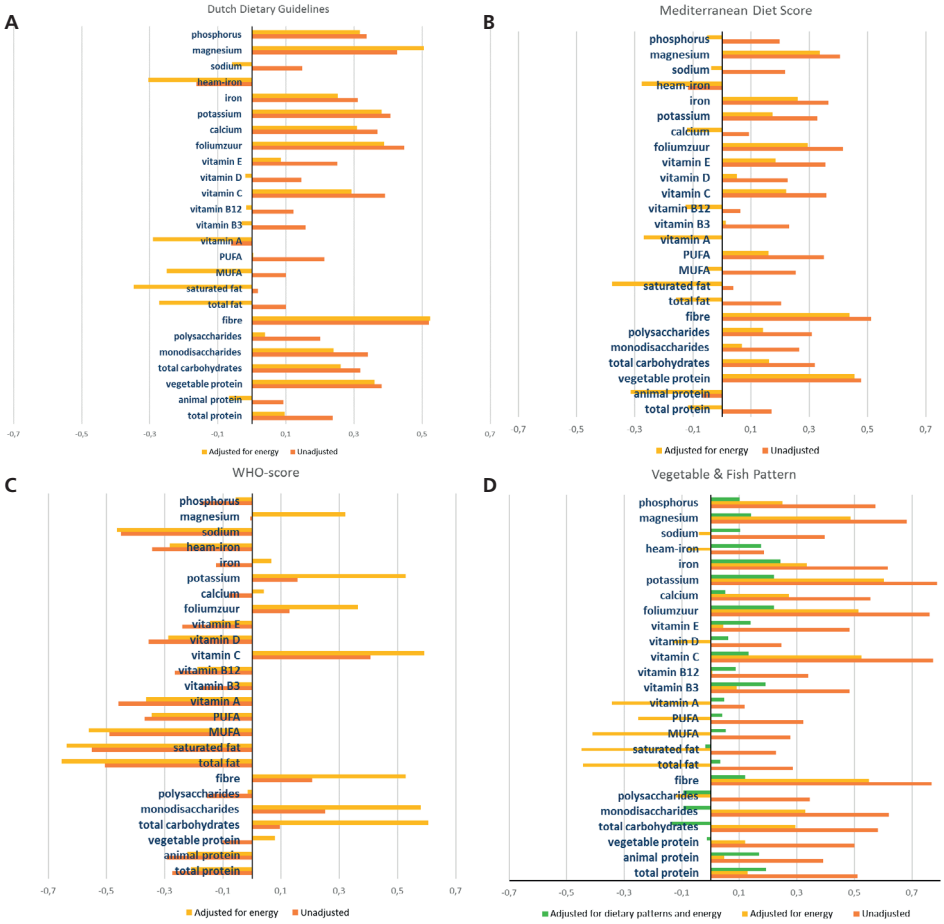


Figure 2: Heatmap of factor loadings from a-posteriori dietary patterns
Factor loadings of a-posteriori patterns per food group visualized using a heatmap. Red reflects a positive load, blue a negative load.

snacks, and beer or spirits, and low intake of fruit and tea. We therefore called this pattern *red meat & alcohol*. The third pattern was defined by high intake of vegetable oils & stanols and margarines or butters, potatoes, whole grains, and sweet snacks or desserts. This pattern was therefore called *traditional*. The fourth pattern was represented by high intake of savoury food groups such as nuts, legumes, salty snacks, and sauces. We thus named this pattern *salty snacks & sauces*. The last pattern was defined by high intake of fruit juice, refined grains, high-fat dairy products, and sweet snacks or desserts. This pattern was therefore called *high-fat dairy & refined grains.*

Dietary pattern characteristics

In order to give more insight in the overall macronutrient and micronutrient composition of the dietary patterns, we performed Spearman rank correlation analyses at baseline (*figure 3a-h*).



The bars in orange reflect the unadjusted correlation coefficients and those in yellow the energy-adjusted coefficients. If the energy-adjusted correlation is lower than the unadjusted one, this indicates that, although the relative intake may be high, the absolute intake of the nutrient for that pattern is low. Which is, for example, the case in fat consumption for the DDG and the MDS (*figure 3a and b*). All a-priori dietary patterns had a high energy-adjusted correlation with fibre and vegetable protein intake, and a negative correlation with overall fat intake (*figure 3a-c*). In addition, the DDG and the WHO-score had a relatively high correlation for mono-and disaccharide intake.

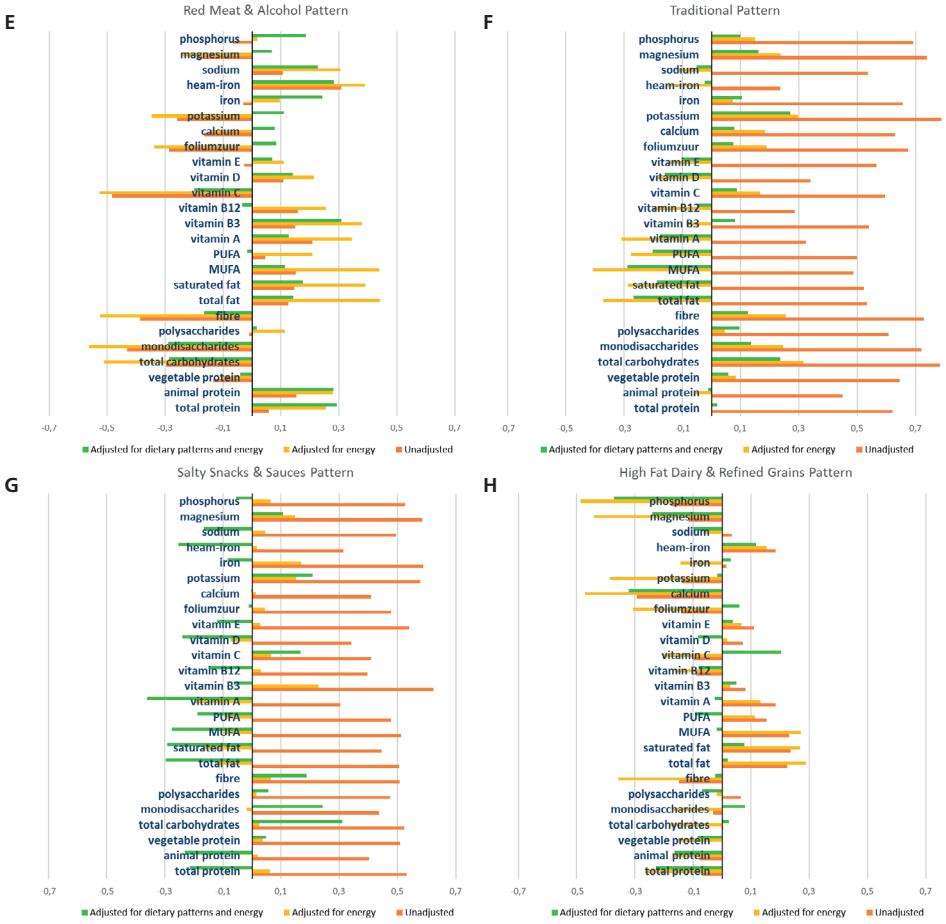


Figure 3: Correlation coefficients of adherence scores with standardized unadjusted, energy adjusted and energy & dietary pattern adjusted macronutrients & micronutrients

These figures reflect the Spearman correlation coefficients of various micronutrients and macronutrients per dietary pattern. The orange bars reflect the unadjusted correlation coefficients, the yellow bars the correlation adjusted for energy intake using the residual method, and the third green bar (in the a posteriori dietary patterns) reflects the correlation adjusted for other a-posteriori dietary patterns and energy intake.

Abbreviations: MUFA: mono-unsaturated fatty acid, PUFA: poly-unsaturated fatty acid.

In *figure 3d-h* a third, green bar reflects the energy & dietary pattern-adjusted correlation. We added these results here since all patterns are analysed together in one regression model.

A-posteriori patterns that resemble the healthy a-priori dietary patterns most are the *traditional* and *salty snacks & sauces* patterns, which are also low in animal protein, fats, sodium and haem-iron, and high in vegetable protein, fibre, mono-and disaccharides and potassium (*figure 3f and 3g*). Whereas the *red meat & alcohol* and *high-fat dairy & refined grains* patterns were low in fibre and potassium and high in fats and haem iron (*figure 3e and 3h*).

Relation between Dietary Patterns and NAFLD

A summary of the multivariable logistic mixed models between the a-priori dietary patterns and risk of NAFLD are given in *table 3*. Results indicate regression of NAFLD in all patterns, but only with an unambiguous credible interval for the WHO-score after full adjustment for sociodemographic, lifestyle and metabolic confounders (*table 3*; OR 0.73, 95%CI 0.35–1.00).

Table 3: Summary of parameters of interest from logistic mixed models for risk of NAFLD with adherence to a-priori dietary patterns

	risk of NAFLD	
	Model 1 OR (95%CI)	Model 2 OR (95%CI)
A-priori dietary patterns		
MDS	0.86 [0.70, 1.07]	0.86 [0.70, 1.07]
DDG	0.88 [0.71, 1.08]	0.89 [0.71, 1.12]
WHO-score	**0.74 [0.55, 0.99]**	**0.73 [0.53, 1.00]**

This table reflects six different mixed logistic regression models. Please find **supplementary table 3** for results on all coefficients from these models. Numbers in bold reflect a tail-probability of <0.05.
Model 1 is adjusted for: sex, age, baseline education level, baseline physical activity, energy intake, alcohol intake, and follow-up time.
Model 2 is additionally adjusted for BMI, baseline type 2 diabetes mellitus and baseline hypertension.
Abbreviations: MDS: Mediterranean Diet Score, CI: credible interval, DDG: Dutch Dietary Guidelines, NAFLD: non-alcoholic fatty liver disease, WHO: World Health Organization.

The multivariable associations between a-posteriori dietary patterns and risk of NAFLD is shown in *table 4*. Adherence to the *traditional* dietary pattern was associated with regression of NAFLD after full adjustment (*table 4*; OR 0.40, 95%CI 0.15–1.00) and adherence to the *salty snacks & sauces* pattern showed a similar result (OR 0.43, 95% CI 0.17–1.04). Factors that were independently associated incidence of NAFLD were incremental energy intake, BMI, baseline diabetes and baseline hypertension. Factors that were associated with regression of NAFLD were female gender, follow-up time, and high education level (*table 4 and supplementary table 3a-c*).

Table 4: Logistic mixed model for risk of NAFLD with adherence to a-posteriori dietary patterns

	risk of NAFLD	
	Model 1 mean (95%CI)	Model 2 mean (95%CI)
follow-up time (years)	**0.74 [0.66, 0.84]**	**0.70 [0.60, 0.81]**
age (years)*	0.93 [0.82, 1.04]	**0.88 [0.77, 1.00]**
sex (female)	**0.17 [0.04, 0.57]**	0.21 [0.05, 0.78]
energy intake (kcals)	1.00 [1.00, 1.00]	1.00 [1.00, 1.00]
Education level*		
low	ref	ref
intermediate	**0.24 [0.06, 0.84]**	0.35 [0.09, 1.33]
high	**0.06 [0.01, 0.30]**	**0.12 [0.02, 0.58]**
physical activity* (metEqhs)	**0.99 [0.97, 1.00]**	1.00 [0.99, 1.01]
alcohol intake (units)	0.85 [0.52, 1.40]	1.06 [0.61, 1.85]
BMI (kg/m^2)	-	**3.01 [2.28, 4.19]**
diabetes mellitus*	-	41.5 [8.49, 273]
hypertension*	-	7.93 [1.60, 50.8]
A-posteriori dietary patterns		
Vegetable & Fish Pattern	1.88 [0.90, 4.05]	1.31 [0.57, 3.01]
Red Meat & Alcohol Pattern	1.40 [0.70, 2.80]	0.79 [0.36, 1.69]
Traditional Pattern	**0.33 [0.14, 0.77]**	**0.40 [0.15, 1.00]**
Salty Snacks & Sauces Pattern	0.63 [0.28, 1.35]	0.43 [0.17, 1.04]
High Fat Dairy & Refined Grains Pattern	0.82 [0.55, 1.22]	1.23 [0.79, 1.96]

This table shows results (OR and 95% CIs) of two multivariable logistic mixed models. *Depicts baseline variables. Numbers in bold reflect a tail-probability of <0.05.
Abbreviations: BMI: body mass index, CI: credible interval, NAFLD: non-alcoholic fatty liver disease, kcal: kilocalories, metEqh: metabolic equivalent hours.

Additional Analyses for BMI

In *supplementary table 4* we show the association between adherence to a-posteriori and a-priori dietary patterns with BMI over time. Taking interactions into account, BMI over time depended on the adherence of DDG and WHO-scores (i.e. low scores: BMI decreased, high scores: BMI increased). For MDS the interaction effect was so small that irrespective of the dietary score BMI generally increased over time. At baseline, none of the dietary patterns was associated with BMI, except for a-priori pattern WHO which was associated with a lower BMI (*supplementary table 4d*). In the model using a-posteriori dietary patterns the effect of time on BMI depended on the score of the *sweets & fats* pattern. For low scores in this pattern the model estimated that BMI increased over time, whereas for high scores a slight decrease of BMI was found (*supplementary table 4a*). On the other hand, there was a significant positive interaction between follow-up time and DDG and between

follow-up time and WHO, which means the that BMI increased over time when adhering to these patterns and decreases for participants that had low scores on these patterns (*supplementary table 4c and d*).

Visualization of Dietary Patterns for NAFLD severity

Adherence to the different dietary patterns are visualized for NAFLD severity in *supplementary figure 3*. There was no particular difference in adherence between participants without steatosis, participants with simple steatosis and participants with NASF visible.

Discussion

The first step in treating NAFLD is lifestyle modification and an important pillar in this treatment is diet. Although there is a paramount of studies on nutrition and NAFLD, there is no study yet that assesses the longitudinal association between well-known dietary quality scores, population-specific dietary pattern scores and NAFLD adjusted for important covariates such as BMI and metabolic confounders. We, therefore, examined both a-priori and a-posteriori dietary patterns in relation to NAFLD using comprehensive multivariable logistic mixed regression models in a large elderly white population-based cohort. After a follow-up period of almost 4.5 years, only 5% of the participants had incident NAFLD, whereas as much as 30% had regression of NAFLD. Amongst the three pre-hypothesized (a-priori) dietary quality scores and the five a-posteriori population-specific dietary patterns, two dietary pattern scores were associated with a lower risk of NAFLD, i.e. the WHO-score and the *Traditional* dietary pattern score. The latter was characterized by high intake vegetable oils & stanols, margarines or butters, potatoes, whole grains, and sweet snacks or desserts.

The three a-priori dietary patterns that we studied in this paper –the Mediterranean diet, the Dutch dietary guidelines, and the world health organization diet score– largely overlapped in macro & micronutrient composition. All were highly correlated with fibre, vegetable protein, carbohydrate intake and potassium, and all were inversely correlated with animal protein, fat, sodium and haem iron intake. The only exception was the MDS, which was highly correlated with fat by its characteristic unsaturated fatty acid intake. Of the three patterns MDS is the one best-studied in relation to liver health, and accumulating evidence indeed suggests that this diet is beneficial for metabolic and liver health.[58,59] Even though the evidence for MDS as therapeutic strategy for NAFLD predominantly originates form observational studies, the EASL consensus guidelines for NAFLD advocates the implementation of this diet as therapeutic strategy.[55] Our results are consistent with a beneficial effect of adherence to the MDS as well as a slight negative effect on the risk of NAFLD.

To date, only one other longitudinal study in dietary quality has been performed.[335] This study from Ma et al. showed that adherence to the MDS was significantly associated with reduced risk of steatosis within the Framingham Heart Study. Important strengths of this study are the large sample size and the use of computed tomography. A limitation, on the other hand, was the fact that regression models were not adjusted for metabolic covariates such as diabetes and hypertension – of which we know and show in this paper – are strongly related to both NAFLD and diet.

To our knowledge, only few studies have examined the association between diet and NAFLD using factor analyses.[286,336,337] One of them was the cross-sectional study of Koch et al. that used 38 food groups and found an a-posteriori pattern that was high in soups, beer, wine, juice, poultry and eggs and was associated with steatosis as assessed by MRI.[336] Another cross-sectional study identified four different a-posteriori patterns (i.e. a *fast-food*, *prudent*, *high protein*, and *unsaturated fatty acid pattern*) using 15 food groups, and found that only the *fast-food* pattern was associated with ultrasound-diagnosed NAFLD.[337] Lastly, there has been one longitudinal study in adolescents (n=995) which identified two a-posteriori patterns, a *healthy* and a *western/unhealthy* pattern. This study found that adherence to the *western* dietary pattern at 14 years of age was associated with higher odds of NAFLD at 17 years. We did not find a detrimental association between dietary patterns and NAFLD, again, possibly because of the low number of incident NAFLD cases over time. But another important difference between our study and the others is that we adjusted for BMI, whereas they had either not taken BMI into account,[336,337] or found that their results dissipated after correction for BMI.[286] The only a-posteriori pattern in our study for which we found a clear association was the *traditional* pattern. Interestingly, this diet resembled the macronutrient & micronutrient composition of the three a-priori healthy diet scores, i.e. having a high correlation with fibre intake, mono-and disaccharide, vegetable protein, and potassium intake and an inverse correlation with fat, haem iron, sodium, and animal protein intake.

The rationale to use a-posteriori dietary patterns is that they reflect the actual population-based dietary eating habits and may therefore reveal new insights in the relation between diet and disease in a particular population.[333] Together the five a-posteriori dietary patterns explained almost 20% of the variation in food intake, but their independent explanatory ability was less than five percent. In addition, the five identified patterns were quite heterogeneous, which complicates unambiguous interpretation. Indeed appearing paradoxical results were obtained when comparing factor loadings and macro-& micro-nutrients correlation coefficients. For example, the factor loadings for the food group red meat is above 0.2 in the *traditional* pattern, whereas the macronutrient animal protein is negatively correlated to this pattern. There are two possible explanations for this paradox; 1) absolute intake of the food group red meat is low (median 41 grams/day), which is supported by the difference in unadjusted and energy-adjusted correlation coefficients;

and 2) the food group red meat is even more prominent in the *red meat & alcohol* pattern, as both patterns were adjusted for one another in the mixed logistic regression models, red meat is therefore not a unique feature of the *traditional* pattern. In other words, another combination of food groups may be more distinctive for the traditional pattern than red meat. The first explanation also reflects why adjusting for energy intake is important in this elderly population in which absolute intake is generally low. Indeed, semi-quantitative FFQs cannot measure exact energy intake and it is therefore important to look at the relative food intake. However, a diet high in energy-dense foods may not reflect the effect of the actual foods themselves, but rather an association with the high energy intake.[345] That is why we chose to show both energy-adjusted and unadjusted results.

An important characteristic of the healthy dietary patterns was fibre. Recently, a systematic umbrella review and meta-analyses, showed that high intakes of dietary fibre – from 25 grams per day onwards – could prevent non-communicable diseases in the general population.[349] In addition, another population-based study showed that adherence to a plant-based diet promoted an increase in fibre-degrading bacteria in the gut. In that study, high adherence to the MDS (defined as the highest population-specific tertile in the study) was related to higher levels of short-chain fatty acid production in the faeces.[350] Indeed, butyrate production, an important short-chain fatty acid, is considered to be anti-obesogenic and has been proposed to decrease gut permeability and affect insulin sensitivity.[351] Additionally, in a recent study of our group we show that (in a larger subset of the population) animal protein was the only macronutrient that was associated with higher prevalence of NAFLD.[344] This finding is in line with other recent studies that demonstrate a detrimental effect of (particularly red) meat on liver health, insulin sensitivity, and overall mortality.[260,352] In a spin-off study we examined whether diet-dependent acid load could explain the association between an animal protein-rich diet and NAFLD.[353] In this study, we found that an acidic diet was associated with high animal protein intake and low fruit intake as well as with NAFLD. An alkaline diet is generally accompanied by high potassium intake, which is an alkalinizing micronutrient.[295] The healthy dietary patterns in this study all had a positive correlation with potassium.

Although a major strength of our current study is that diet is taken into account as a whole, we cannot exclude the possibility that the associations found between dietary patterns and NAFLD are driven by particular food groups instead of by the entire dietary pattern.[333] The pattern *salty snacks & sauces*, for example, are characterised by "unhealthy" food groups that have a high sodium load, such as salty snacks and sauces. However, this pattern is also characterised by food groups that can exert specific beneficial effects on metabolic health, such as nuts and legumes.[354] Indeed, we have previously shown that specific food items, such as coffee and tea, are associated with NAFLD independent of dietary quality.[130] On the other hand, specific constituents within the diet can also be detrimental, such as haem iron from meat, which is associated to oxidative stress and insulin resistance.[355] Noteworthy

is that both beneficial dietary patterns, the WHO-score and the *traditional* dietary pattern, had a negative correlation with vitamin E, an anti-oxidant that has been proposed as treatment of NAFLD.[55] But whereas vitamin E has been proposed to exert anti-oxidant beneficial effect on the liver[63], vitamin E has also repeatedly been associated with detrimental health outcomes such as haemorrhagic stroke[356] and mortality.[357]

The large sample size and prospective design of our study enabled us to include important sociodemographic, lifestyle and metabolic confounders in the analysis. Nonetheless, there are several limitations that need mentioning. This cohort comprises an elderly, almost exclusively Caucasian population, which might hamper generalization towards other populations. Also, although none of the participants was deceased at time of follow-up, we cannot exclude the possibility that follow-up measurements were missing *not at random* during follow-up. In addition, although we have a large study cohort of almost a thousand participants, only a small number of incident NAFLD and regressed NAFLD was found. This might have reduced our power to detect relevant effects. In line with this, the number of NASF cases was also quite small which hindered us from performing multivariable analyses on this phenotype. However, the number of NASF cases that we found in our study is in line with the prevalence of elevated LSM in a general NAFLD population.[358] Also, the presence of steatosis could have influenced LSM, we therefore used a conservative cut-off proposed for patients with severe steatosis. This could have led to underestimation of individuals with significant fibrosis. But even with this conservative cut-off no evident difference in adherence to dietary patterns was found.[54] In addition, ultrasound and transient elastography as diagnostic tool for steatosis is not the gold standard, ultrasound is a subjective measure with a sensitivity of 79.7% and specificity of 86.2%. Diagnosis of steatosis could be subject to time of measure and operator.[359] Liver biopsy is the gold standard. However, it is ethically debatable and practically infeasible to perform an invasive procedure such as the liver biopsy in large cohorts. However, all our ultrasound measurements were performed by a single operator, which reduces the risk of bias. Furthermore, transient elastography over time is not validated in patients with NAFLD as of yet, but in hepatitis B longitudinal assessment of fibrosis using transient elastography is being used.[360] Moreover, the use of food frequency questionnaires could have led to recall bias as the questions concern dietary behaviour over the last month. In particular non-differential measurement error could have occurred (i.e. over or underreporting, as reflected in total energy intake). We have, however, dealt with the potential of this type of bias by adjusting for energy intake using the residual method and by adjusting for energy intake in the multivariable analysis to account for the extraneous variation in energy intake. In addition, previous studies have confirmed that FFQs provide sufficient information on diet to study overall dietary quality.[361] Also, BMI in elderly is a suboptimal measure for adiposity, we therefore cannot exclude the possibility that a decrease in BMI is caused by a loss in muscle mass rather than fat mass, which is generally an expression of poor

nutritional and physical health.[362] Indeed, if adherence to a presumed healthy diet is low, this could also be explained by low overall food intake rather than imply that overall dietary quality is poor. In this case, low adherence to healthy diets could lead to weight loss. As the absolute consumption is low, snacking might not be detrimental at all in this frail elderly group. In line with this, as our results comprise an elderly white population, a word of caution on generalisability is therefore warranted.

In summary, in this large prospective elderly epidemiological study we found that adherence to the World Health Organization Diet and to a *Traditional* diet - both characterized as plant-based, high-fibre and low-fat- was related to regression of NAFLD over time. This finding is in line with increasing evidence that (red) meat is negatively associated with NAFLD and other metabolic comorbidities.[260,352] However, we are the first longitudinal study to examine full dietary patterns with adjustment for BMI and energy intake in relation to NAFLD. While adding important new information, we believe that at the current moment, without external validation of our results and with the unknown generalizability outside our elderly population, it is too premature to firmly adapt guidelines or clinical management accordingly. Future (randomized) interventional studies on for example a meat-based vs no meat diet, ideally in younger populations, are needed. Also, there is still a gap in knowledge on the potential underlying mechanisms, such as dietary acid load or metagenomic alterations in the blood that effectuate the steatogenic effect of diet on the liver. On the other hand, to the best of our knowledge a diet that is rich in fibre and vegetables and low in red meat hasn't been described to be detrimental for health either. And with the alarming rise in NAFLD incidence in children[363], clear and effective recommendations on the treatment of NAFLD are desperately needed. We therefore think that adherence to such a diet while awaiting the results of future studies may be beneficial.

Supplementary Files

Supplementary Methods:

For our main analyses we used the R package JointAI[346] which performs simultaneous analysis and imputation using the Bayesian framework. We opted for the Bayesian method as opposed to "standard" mixed models since the latter suffered from numerical problems that prevented us from obtaining any results. Moreover, the Bayesian approach has been shown to be superior for handling incomplete covariates in the presence of longitudinal data and non-linear associations (such as interaction terms), compared to standard implementations of multiple imputation (the current gold-standard for handling missing covariate values).[348,364,365]

In the method used here, in addition to the analysis model of interest (the logistic mixed model for NAFLD), models are specified for each of the longitudinal covariates (energy intake, alcohol intake, BMI (1 value missing at follow-up) and the dietary pattern variables) and each of the incomplete baseline covariates (socioeconomic status (26 missing values) and physical activity (68 missing values). These additional models allow us to obtain imputed values for missing covariate values. The choice of models used for the covariates depends on the type of each of the variables. Specifically, we used

a cumulative logit model for socioeconomic status (ordered factor with three categories),
a gamma regression model with a log-link for physical activity (right-skewed continuous),
a gamma mixed regression model with a log-link for alcohol intake (right-skewed continuous, measured at baseline and follow-up), and
linear mixed models for energy intake, BMI and each of the dietary pattern variables.

The analysis model of interest and covariate models are estimated jointly so that the imputed values of incomplete covariates are used directly in the main analysis model.

To use all available information for the imputation, and since the different dietary pattern variables contain overlapping but not identical information, we included all dietary pattern variables in the models for covariates. For example, during the analysis of the association of NAFLD with the DDG pattern, the other dietary pattern variables (WHO, MDS and the five a-posteriori patterns) were not included in the logistic mixed model for NAFLD, but all eight variables were included in the models for the covariates.

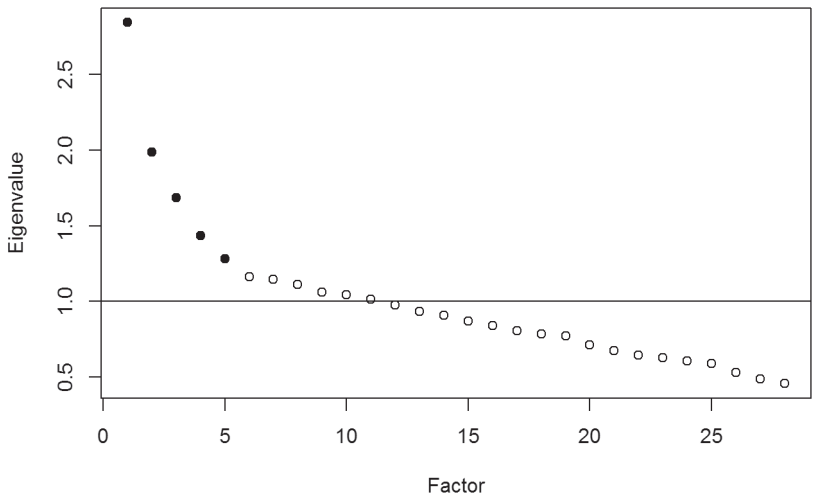
For the same reason (to utilize all information available) the most extensive model ("Model 2"), including both interactions (dietary pattern with time, and dietary pattern with BMI) was used to extract imputed values which were then used in the estimation of the smaller models (models without interactions and/or models with the smaller set of confounders).

In the Bayesian framework, the estimation of regression coefficients is typically performed using the Markov Chain Monte Carlo (MCMC) method. In simple terms, this is an iterative procedure in which a chain of values is created for each regression coefficient and each

missing value. The values in such a chain represent samples of the distribution of the respective coefficient or missing value. Results of a Bayesian regression model are obtained by summarizing this sample using the mean and 2.5% and 97.5% quantiles, which form the credible interval.

To obtain imputed values, random draws from the chains for missing values were performed. The values from each draw were then filled into the original incomplete data, thereby creating multiple completed versions of the data.

Each completed dataset was analysed to obtain results for "Model 1" as well as the models without interaction terms. To obtain overall results from these models, pooled over the multiple versions of the completed data, the chains resulting from each of the analyses were merged and the summary (mean and quantiles) calculated from the merged sample.



Supplementary Figure 1: Scree plot of the factor analysis

The y-axis reflects the Eigenvalue and the x-axis the derived factors. The filled dots reflect the factors that we included in this manuscript as a-posteriori patterns.

Supplementary Table 1: food items in food groups

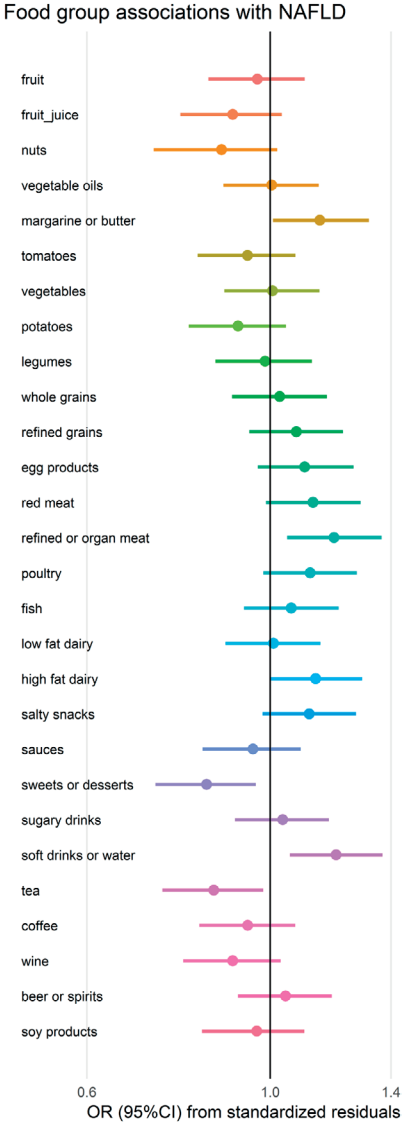
Food group	Included food items
Fruit	fruit miscellaneous, apples, bananas, pears, oranges, strawberries, grapes
Fruit juice	orange juice, other fruit juices
Nuts	(mixed) nuts, flax seed
Vegetable Oils & Stanols	diet/low-fat/fluid margarine or frying-fat, gravy, low-fat butter, full-fat/low-fat stanoil margarine, vinegar-oil, olive oil, mono or polyunsaturated fatty acid rich oil
Margarines or Butters	full-fat butter/margarine/frying-fat, lard, gravy n.o.s., spreads n.o.s.
Tomatoes	tomato sauce, ketchup, tomatoes
Vegetables	spinach, cauliflower, broccoli, cabbage, carrots, vegetable soup, wok vegetables, onions, beans, lettuce, cooked vegetables, vegetable juice, raw vegetables, salads
Potatoes	potatoes (without fat), oven fries, mash potato
Legumes	legumes, legume soup
Whole Grain Products	muesli, fibre-rich breakfast cereals/ knackerbrod, rye bread, brown rice/rolls/bread, whole-grain bread/ rolls, whole-grain dough, whole grain rusk, multigrain rolls/bread
Refined Grain Products	biscuits, cornflakes, white rolls/bread/dough/rice, toasts
Eggs	eggs (baker or boiled)
Red Meat	ham, minced meat, pork chop, beef, meatball, satay
Refined or Organ Meat	bacon, gammon, meat n.o.s., rolled fillet, miscellaneous meat, smoked sausage, cold cuts, cooked liver, liver products, organ meat n.o.s., liver sausage
Poultry	chicken
Fish	mussels, European flounder, salmon, trout, herring, fish n.o.s.
Low-fat Dairy	fat-free/low-fat yoghurt or quark, low-fat cheese (20–30%), fat-free/low-fat (pasteurized) milk, buttermilk
Full-fat Dairy	full-fat yoghurt, full-fat cheese (40+), cheese n.o.s., whipped cream, cheese fondue, cream, full-fat (pasteurized) milk, coffee cream, creamer
Salty Snacks	peanutbutter, peanuts, peanut sauce, pizza, fries, snackbar products, spring rolls, crisps, salty salad snack
Sauce	halvanaise, mayonaise, warm sauces, sauce/dressing n.o.s.
Sweet extras or Desserts	chocolates (dark/milk/white), sugar (additives), candybar, sweets, mousse, custard, pie, icecream, rusk, croissants, currant bun, *ontbijtkoek*, pancake, sweet biscuits, cake
Sugary Drinks	Ice pop, soda, breakfast drinks, fat-free/full-fat/low-fat chocolate milk
Diet Soda or Water	diet soda, mineral water
Tea	herbal, black and green tea
Coffee	coffee
Wine	red wine, white wine
Beer or Spirit	beer, low-alcohol beer, strong liquors, *advocaat*
Soy Products	bean curd, meat substitutes, soy dessert, soy milk

Food items in *italic* reflect typical Dutch food items of which there is no sufficient translation
Abbreviations: n.o.s.: not otherwise specified

Supplementary Table 2: Follow-up data according to liver status

	no NAFLD*	NAFLD without elevated LSM†	NAFLD with elevated LSM‡	lost to follow-up
no NAFLD	443	23	1	153
NAFLD without elevated LSM	62	144	4	102
NAFLD with elevated LSM	6	6	6	13

*40 additional participants without reliable FFQ available. †14 additional participants without reliable FFQ available. ‡1 additional participants without reliable FFQ available.
Abbreviations: FFQ: food-frequency questionnaire, LSM: liver stiffness measurement, NAFLD: non-alcoholic fatty liver disease.



Supplementary Figure 2: Food group univariate associations with NAFLD adjusted for energy intake
This figure reflects the association between the different 28 food groups adjusted for energy intake using the residual method with NAFLD. The data is represented as odds ratio with 95% confidence interval. This figure represents cross-sectional data at baseline.

Supplementary Table 3a: Complete logistic mixed model for risk of NAFLD with adherence to the MD

	risk of NAFLD Full model mean (95%CI)
follow-up time (years)	**0.73 [0.64, 0.83]**
age (years)*	0.91 [0.81, 1.01]
sex (female)	**0.31 [0.09, 0.95]**
energy intake (kcals)	**1.00 [1.00, 1.00]**
Education level*	
low	ref
intermediate	0.41 [0.11, 1.36]
high	**0.17 [0.04, 0.72]**
physical activity* (metEqhs)	1.00 [1.00, 1.00]
alcohol intake (units)	1.08 [0.66, 1.80]
BMI (kg/m²)	**2.66 [2.11, 3.61]**
diabetes mellitus*	**29.4 [7.01, 161]**
hypertension*	**6.28 [1.49, 30.8]**
A-priori dietary patterns	
MDS	0.84 [0.66, 1.05]

Supplementary Table 3b: Complete logistic mixed model for risk of NAFLD with adherence to the DDG

	risk of NAFLD Full model mean (95%CI)
follow-up time (years)	**0.74 [0.64, 0.83]**
age (years)*	0.91 [0.81, 1.01]
sex (female)	**0.30 [0.09, 0.92]**
energy intake (kcals)	1.00 [1.00, 1.00]
Education level*	
low	ref
intermediate	0.38 [0.10, 1.27]
high	**0.16 [0.03, 0.65]**
physical activity* (metEqhs)	1.00 [0.99, 1.01]
alcohol intake (units)	1.02 [0.61, 1.73]
BMI (kg/m²)	**2.69 [2.12, 3.70]**
diabetes mellitus*	**29.6 [6.78, 178]**
hypertension*	**6.47 [1.49, 34.5]**
A-priori dietary pattern	
DDG	0.89 [0.71, 1.12]

Supplementary Table 3c: Complete logistic mixed model for risk of NAFLD with adherence to the WHO

	risk of NAFLD Full model mean (95%CI)
follow-up time (per year)	**0.73 [0.64, 0.82]**
age (per year)	0.91 [0.81, 1.01]
sex (female)	0.33 [0.10, 1.04]
energy intake (per kcal)	1.00 [1.00, 1.00]
Education level*	
low	ref
intermediate	0.38 [0.11, 1.29]
high	**0.16 [0.03, 0.69]**
physical activity* (per metEqh)	1.00 [0.99, 1.01]
alcohol intake (per unit)	1.13 [0.68, 1.89]
BMI (per kg/m^2)	**2.69 [2.13, 3.55]**
diabetes mellitus*	**29.7 [7.05, 161]**
hypertension*	**6.79 [1.56, 36.1]**
A-priori dietary pattern	
WHO-score	**0.73 [0.53, 1.00]**

These tables reflect the mixed logistic regression models for the three a-priori patterns (A: MDS, B: DDG, C: WHO). *Depicts baseline variables. Numbers in bold reflect a tail-probability of <0.05.
Abbreviations: BMI: body mass index, CI: credible interval, DDG: Dutch dietary guidelines, kcal: kilocalories, MDS: Mediterranean Diet Score, metEqh: metabolic equivalent hours, NAFLD: non-alcoholic fatty liver disease, WHO: world health organization.

Supplementary table 4a: Additional linear mixed regression between a-posteriori dietary patterns and BMI

	BMI as outcome Full model mean (95%CI)
follow-up time (years)	0.01 [–0.01, 0.04]
age (years)*	–0.04 [–0.09, 0.01]
sex (female)	0.13 [–0.37, 0.65]
energy intake (kcals)	0.000 [0.000, 0.001]
Education level*	
low	ref
intermediate	–0.17 [–0.71, 0.39]
high	**–0.85 [–1.54, –0.21]**
physical activity* (metEqhs)	**–0.008 [–0.013, –0.003]**
alcohol intake (units)	0.04 [–0.10, 0.17]
diabetes mellitus*	**2.05 [1.38, 2.67]**
hypertension*	**1.43 [0.83, 2.03]**

Supplementary table 4a (*continued*)

	BMI as outcome Full model mean (95%CI)
A-posteriori dietary patterns	
Vegetable & Fish Pattern	0.002 [–0.22, 0.24]
Red Meat & Alcohol Pattern	0.15 [–0.07, 0.36]
Traditional Pattern	–0.09 [–0.31, 0.14]
Salty Snacks & Sauces Pattern	0.07 [–0.16, 0.29]
High Fat Dairy & Refined Grains Pattern	–0.09 [–0.22, 0.03]
Interaction terms	
FU-time × Vegetable & Fish Pattern	–0.004 [–0.06, 0.05]
FU-time × Red Meat & Alcohol Pattern	–0.02 [–0.07, 0.05]
FU-time × Traditional Pattern	–0.02 [–0.06, 0.01]
FU-time × Salty Snacks & Sauces Pattern	0.01 [–0.04, 0.05]
FU-time × High Fat Dairy & Refined Grains Pattern	**–0.04 [–0.07, –0.01]**

Supplementary table 4b: Additional linear mixed regression between MDS and BMI

	BMI as outcome Full model mean (95%CI)
follow-up time (years)	0.001 [–0.08, 0.08]
age (years)*	–0.05 [–0.10, 0.001]
sex (female)	0.06 [–0.44, 0.57]
energy intake (kcals)	0.000 [0.000, 0.000]
Education level*	
low	ref
intermediate	–0.18 [–0.73, 0.39]
high	**–0.87 [–1.52, –0.21]**
physical activity* (metEqhs)	**–0.008 [–0.013, –0.003]**
alcohol intake (units)	0.04 [–0.09, 0.18]
diabetes mellitus*	**2.08 [1.43, 2.77]**
hypertension*	**1.46 [0.82, 2.10]**
A-priori dietary patterns	
MDS	–0.005 [–0.08, 0.06]
Interaction terms	
FU-time × MDS	0.003 [–0.01, 0.018]

Supplementary table 4c: Additional linear mixed regression between DDG and BMI

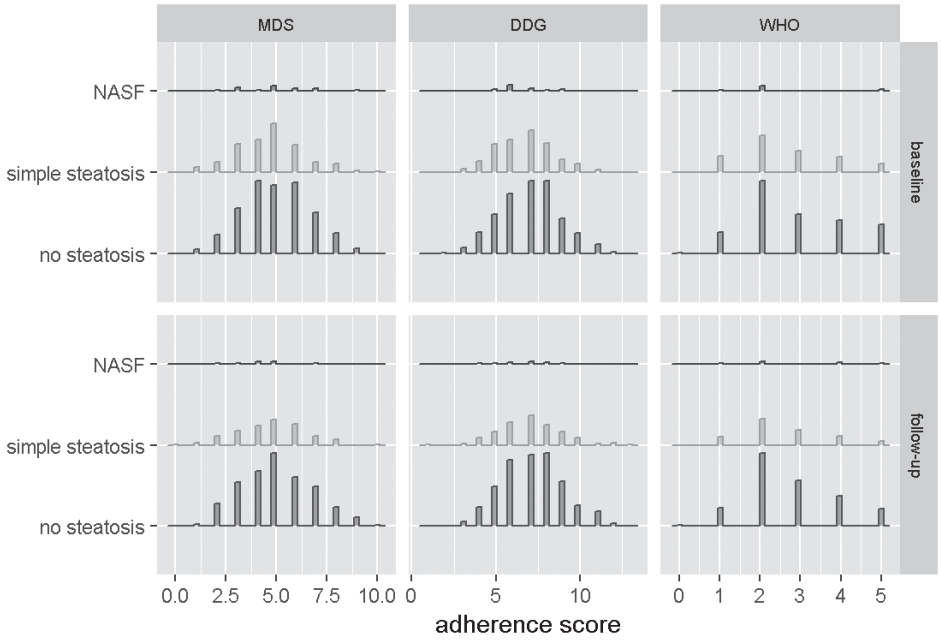
	BMI as outcome
	Full model mean (95%CI)
follow-up time (years)	–0.104 [–0.21, 0.002]
age (years)*	–0.05 [–0.10, 0.003]
sex (female)	0.06 [–0.46, 0.58]
energy intake (kcals)	0.000 [0.000, 0.000]
Education level*	
low	ref
intermediate	–0.18 [–0.75, 0.40]
high	**–0.89 [–1.55, –0.24]**
physical activity* (metEqhs)	**–0.008 [–0.01, –0.003]**
alcohol intake (units)	0.05 [–0.09, 0.18]
diabetes mellitus*	**2.06 [1.43, 2.71]**
hypertension*	**1.47 [0.83, 2.09]**
A-priori dietary patterns	
DDG	–0.02 [–0.09, 0.04]
Interaction terms	
FU-time × DDG	**0.016 [0.002, 0.031]**

Supplementary table 4d: Additional linear mixed regression between WHO and BMI

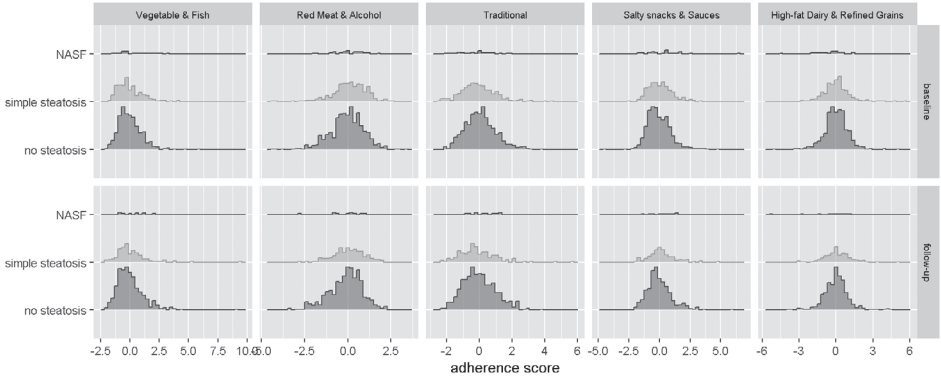
	risk of NAFLD
	Full model mean (95%CI)
follow-up time (years)	–0.09 [–0.17, 0.003]
age (years)*	–0.05 [–0.10, 0.003]
sex (female)	0.07 [–0.44, 0.58]
energy intake (kcals)	0.000 [0.000, 0.000]
Education level*	
low	ref
intermediate	–0.18 [–0.75, 0.36]
high	**–0.87 [–1.52, –0.21]**
physical activity* (metEqhs)	**–0.01 [–0.01, –0.003]**
alcohol intake (units)	0.05 [–0.09, 0.18]
diabetes mellitus*	**2.07 [1.40, 2.72]**
hypertension*	**1.47 [0.87, 2.08]**
A-priori dietary patterns	
WHO	–0.089 [–0.17, 0.002]
Interaction terms	
FU-time × WHO	**0.03 [0.01, 0.05]**

These tables show the results (mean estimate and 95% CIs) of multivariable linear mixed models f a posteriori dietary patterns (A) and a priori dietary patterns (B-D). *Depicts baseline variables. Numbers in bold reflect a tail-probability of <0.05.

Abbreviations: BMI: body mass index, CI: credible interval, DDG: Dutch Dietary Guidelines, MDS: Mediterranean Diet Score, NAFLD: non-alcoholic fatty liver disease, kcal: kilocalories, metEqh: metabolic equivalent hours, WHO: World Health Organization.



Supplementary Figure 3a: Visualization of adherence scores to the a-priori dietary patterns across participants without NAFLD, with simple steatosis, and NASF



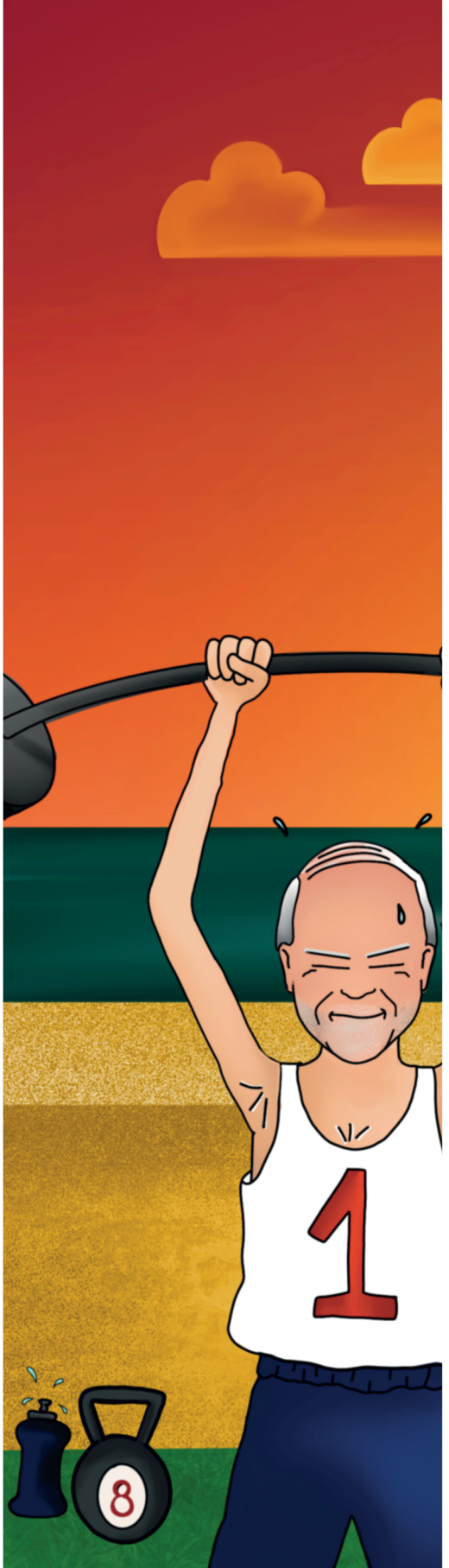
Supplementary Figure 3b: Visualization of adherence scores to the a-posteriori dietary patterns across participants without NAFLD, with simple steatosis, and NASF

These figures represent the absolute number of individuals per adherence score (on the x-axis) against NAFLD severity on the y-axis (i.e. no steatosis, simple steatosis: steatosis without elevated liver stiffness, and non-alcoholic steatofibrosis: steatosis with elevated liver stiffness) illustrated per dietary pattern (A: a priori dietary patterns, B: a posteriori dietary patterns).

Abbreviations: DDG: Dutch dietary guideline, MDS: Mediterranean diet score, NASF: non-alcohol steatofibrosis, WHO: world health organization

Part IV

BODY COMPOSITION & NON-ALCOHOLIC FATTY LIVER DISEASE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Chapter 8

Non-alcoholic fatty liver disease in The Rotterdam Study: about muscle mass, sarcopenia, fat mass and fat distribution

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is the most prevalent liver disease worldwide. Obesity is a major risk factor for NAFLD and recently, low skeletal muscle mass emerged as additional risk factor for NAFLD. However, the different contributions of BMI to the risk of NAFLD are not yet well-known. We therefore studied body composition and muscle function with NAFLD in an elderly population-based study. Participants of European descent underwent dual X-ray absorptiometry and hepatic ultrasonography. NAFLD was defined as liver steatosis in absence of secondary causes for steatosis. Skeletal muscle index (SMI) was defined as appendicular lean mass/height^2 and (pre)sarcopenia was defined using the EWGSOP-consensus guidelines. All analyses were stratified by sex and BMI (cut-point: 25kg/m^2) and adjusted for age, weight, height, HOMA-IR, triglycerides, and android-fat-to-gynoid-fat ratio (AGR). We included 4609 participants of whom 1623 had NAFLD (n=161 normal-weight and n=1462 overweight). Pre-sarcopenia and sarcopenia prevalence was low (5.9% and 4.5% respectively) and both were not associated with NAFLD. SMI was associated with less NAFLD in normal-weight women (OR 0.48, 95%CI 0.29–0.80). A similar association for SMI and NAFLD was seen in normal-weight men, but significance dissipated after adjustment for AGR (OR 0.63, 95%CI 0.39–1.02). Generally, fat mass was a better predictor for NAFLD than lean mass. In particular, android fat mass was associated with all NAFLD subgroups (OR from 1.77 in overweight men to 8.34 in normal-weight women, P_{max}=0.001), whereas substitution of gynoid fat mass for other body components had a significant protective association with NAFLD in every subgroup, but normal-weight men. Likewise, AGR was the best performing predictor for NAFLD prevalence (OR from 1.97 in normal-weight men to 4.81 in normal-weight women, P_{max}<0.001). In conclusion, both high fat mass and low SMI were associated with normal-weight NAFLD. However, fat distribution (as assessed by AGR) could best predict NAFLD prevalence.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most prevalent chronic liver disease today and it parallels the epidemic of obesity and diabetes mellitus.[6,13] NAFLD constitutes a major public health threat as it leads not only to an increased risk of liver-specific,[22] but also cardiovascular morbidity and mortality.

Obesity is strongly related to NAFLD.[13] That said, certainly not all obese individuals have NAFLD and not every NAFLD patient is obese. In fact, about 1 in 6 of the NAFLD patients have a normal body mass index (BMI).[242] Therefore, the accuracy of BMI as the all-encompassing measure of adiposity is debated.[366] Presence of excess (visceral) fat mass is a well-established risk factor for NAFLD, independent of BMI.[367,368] Recently, emerging evidence suggested that low skeletal muscle mass (or pre-sarcopenia) also contributes to the risk of NAFLD.[369-371] Indeed, as skeletal muscle mass is the primary tissue responsible for insulin-mediated glucose disposal, skeletal muscle plays an important role in glucose homeostasis and insulin resistance, which are key in the pathogenesis of NAFLD.[372]

However, evidence on the association between skeletal muscle mass and NAFLD predominantly originates from young Asian populations with a high BMI.[12,370] This gives room for thought whether it is a true shortage of muscle mass, or a relative excess of fat mass, or both that is associated with NAFLD. Interestingly, a recent population-based study found that the two body components, lean body mass and fat mass, both explained the relation between BMI and mortality.[373] To the best of our knowledge, there are no studies that have examined the role of both components, independent of each other, in relation to NAFLD. But considering the above, there are arguments to think that both contributions of BMI, i.e. (lean) muscle mass and fat mass, are risk factors for NAFLD.

We therefore studied the independent association of the different components of the body with NAFLD, stratified by sex and BMI, in a large elderly European population. We were particularly interested in the association between skeletal muscle mass with (sarcopenia) or without (pre-sarcopenia) loss of muscle function, and NAFLD. In addition, we assessed which body composition parameter could best predict NAFLD prevalence.

Participants and methods

Study Population

This cross-sectional analysis included participants from The Rotterdam Study, a large ongoing population-based cohort of participants aged 45 years or older in the Netherlands. The design and rationale of this population-based study have been described in detail previously.[248] In short, the study commenced in 1989 and comprises three cohorts. Hepatic

imaging has been part of the physical work-up since 2009. There are no specific eligibility criteria for The Rotterdam Study, except for the minimum age and residential area (ZIP-codes). As body composition differs amongst ethnicities[12] and The Rotterdam Study is predominantly of European background, the low number of non-European participants were excluded to examine a homogenous population. Ethnicity was determined using genome-wide genotypes into HapMap CEU release 22 (build 36). The genotype data was pruned in order to end up with variants in linkage equilibrium and the ancestry component for each individual was estimated on the basis of the maximum likelihood using the ADMIXTURE software.[248] The Rotterdam Study is approved by the institutional review board of the Erasmus MC University Medical Centre Rotterdam and by the review board of The Netherlands Ministry of Health, Welfare and Sports. Written informed consent was obtained from all participants.

Dual X-ray Absorptiometry Scanning

Body components were assessed using dual X-ray absorptiometry (DXA)-scans with iDXA total body fan-beam densitometer (GE Lunar Corp, Madison, WI, USA). All scans were analysed using enCORE software, which divides scan-results into total lean mass, total fat mass and bone/organ mass. Total lean mass is the sum of trunk lean mass and appendicular lean mass (ALM; the sum of lean tissue from the arms and legs), and total body fat mass is the sum of android fat mass (localized around the waist), gynoid fat mass (localized around the breasts, hips and thighs), and fat mass not otherwise specified. The latter includes mainly trunk fat mass and a small proportion of appendicular fat mass, but as this was not further specified, we refer to this remaining component as trunk fat mass. The relative proportion (or fraction) of each component to the body was expressed as percentage of total body weight. For instance, ALM-fraction was calculated as ALM/total body weight*100.
Skeletal muscle mass was estimated by the skeletal muscle index (SMI) using ALM divided by squared body height (kg/m²) in order to adjust for variation in skeletal size. Low SMI was defined as ≤7.25 kg/m² in men and ≤5.67 kg/m² in women, based on cut-off values suggested by the European Working Group on Sarcopenia in Older People (EWGSOP).[374]
Fat distribution was assessed using the standardized android-fat-to-gynoid-fat ratio (AGR).

Pre-sarcopenia and Sarcopenia

Grip strength (proxy for overall muscle strength) was examined using a hydraulic hand dynamometer (Fabrication Enterprises Inc., USA). The maximum grip strength was defined as the maximum value (in kg) out of three serial attempts using the non-dominant hand. EWGSOP cut-off values for low grip strength were ≤29kg for BMI≤24kg/m², ≤30kg for BMI 24.1–28kg/m², or ≤32kg for BMI>28kg/m² in men; and ≤17kg for BMI≤23kg/m²,

≤17.3kg for BMI 23.1–26kg/m², ≤18kg for BMI 26.1–29kg/m², or ≤21kg for BMI>29kg/m² in women.[374]

Gait speed (measure of physical performance)[375] was examined using the GAITRite walkway (CIR Systems, Inc., Sparta, New Jersey), a 5.79 meters long electronical walkway. Again, EWGSOP cut-offs were applied for the definition of low gait speed, i.e. for men:<0.65m/s if height≤173cm, or <0.76m/s if height>173cm; and for women:<0.65m/s if height≤159cm, or <0.76m/s if height>159cm.[374]

Pre-sarcopenia and Sarcopenia were defined according to the EWGSOP consensus guideline.[374,376] Pre-sarcopenia was defined as presence of low SMI, and sarcopenia as low SMI plus either low muscle strength or low physical performance.

Hepatic Imaging

Hepatic steatosis was assessed using abdominal ultrasound (US), which was carried out by a certified and experienced technician (Hitachi HI VISION 900). Images were stored digitally. Diagnosis of steatosis was determined dichotomously as presence of a hyperechogenic liver parenchyma.[104] Participants with secondary causes for steatosis were excluded from this study, i.e. those with 1) excessive alcohol consumption (men>30g/day and women>20g/day); 2) use of steatogenic drugs, i.e. amiodarone, systemic corticosteroids, methotrexate, or tamoxifen (extracted from automated pharmacy linkage); and 3) viral hepatitis, based on hepatitis B surface antigen and anti-hepatitis C virus, measured by an automatic immunoassay (Roche Diagnostic GmbH). The remainder participants with steatosis were considered to have NAFLD.

NAFLD severity was assessed using transient elastography (FibroScan®, EchoSens, Paris, France). Practical implementation of this examination has been described in detail previously.[78] Liver stiffness measurements (LSM, in kilopascals [kPa]) were available for a subset of the study population (from January 2011 onwards). A single operator obtained 10 serial measurements using either the M or XL-probe dependent on the thickness of the subcutaneous fat layer. We excluded participants with 1) unreliable measurements (i.e., interquartile range/median LSM>0.3 and LSM≥7.1kPa)[101], 2) failure of assessment, or 3) presence of an intracardial device. For this study, non-alcoholic steatohepatitis (NASH), or advanced NAFLD, was defined as presence of steatosis and LSM≥8.0 kPa, a proxy for fibrosis.[54]

Biochemistry and additional covariates

Fasting blood lipids, platelet count, glucose, alanine aminotransferase (ALT), aspartate aminotransferase, and gamma-glutamyl transferase were measured using automatic enzyme procedures (Roche Diagnostic GmbH, Mannheim, DE). Insulin was determined using an automatic immunoassay (Roche Diagnostic GmbH).

Data on demographics, physical activity, smoking behaviour, educational level, and comorbid conditions were obtained during an extensive home interview. Daily energy intake in kilocalories and alcohol intake in grams was assessed using a 389-item semi-quantitative food frequency questionnaire.[98] We excluded unreliable energy consumption of <500 or >7500 kcal/day. Blood pressure measurements were obtained using two subsequent measurements in upright position. BMI was calculated as weight/height² (kg/m²) and dichotomized into normal-weight:<25kg/m² and overweight:≥25kg/m².

Insulin resistance was determined using the homeostasis model assessment of insulin resistance (HOMA-IR = fasting glucose (mmol/dl) times fasting insulin (mU/L) divided by 22.5).[106] The metabolic syndrome was diagnosed when three out of five metabolic traits were present: 1) abdominal obesity, i.e. waist circumference>102cm in men and >88cm in women; 2) serum triglycerides ≥130mg/dl or drug treatment for elevated triglycerides; 3) serum high-density lipoprotein cholesterol (HDL-C) <40mg/dl in men and <50mg/dl in women or drug treatment for low HDL-C; 4) Blood pressure ≥130/85mmHg or drug treatment for elevated blood pressure; 5) fasting plasma glucose ≥100mg/dl or drug treatment for elevated blood glucose.[28] Hypertension was diagnosed if either systolic (≥140mmHg) or diastolic (≥90mmHg) blood pressure was increased and/or if the participant was on anti-hypertensive medication. Diabetes was diagnosed as fasting glucose above 7.0mmol/L and/or drug treatment for elevated blood glucose.

Statistical analyses

To reduce bias due to missing data, missing values were imputed using multiple imputation (fully conditioned specification). Details on this imputation process can be found in the *Supplementary Methods*. Continuous data were presented as mean ± standard deviation (SD) or median with 25th or 75th percentile (P25-P75). Categorical data were presented as percentage. Chi-square test, one-way analyses of variance, or Kruskall-Wallis test were used to assess differences by strata.

Associations between body composition and NAFLD were examined using logistic regression models stratified by BMI and sex, because of the known sex-differences in body composition.[377] We examined the association between 1) the different body components, 2) SMI, and 3) (pre)sarcopenia with NAFLD. We evaluated weight, height, weight and height, BMI, and body fat fraction as adjustment for body composition using the Akaike Information Criterion (AIC).[378] After evaluation, models adjusted for weight and height performed best. Moreover, without weight as factor in the model, all body components were associated with higher prevalence of NAFLD. If body fat fraction was put in the model together with ALM (or SMI), the beta for ALM could reflect two scenarios. First, the beta could reflect an increase in ALM and subsequent increase in total body weight. In this case, total body fat would increase too (as body fat fraction is set and total weight increases).

Second, the beta could reflect an increase of ALM at a set weight, while body fat fraction remains the same (weight is set) and bone and organ mass hardly varies. Thus an increase of ALM would then be at the expense of trunk lean mass. The latter scenario reflects the substitution of one component of the body at the expense of another. We performed such a substitution analyses[255,379], which is often used in nutritional epidemiology to assess the relative replacement of one nutrient by another for a given caloric intake. A similar formula can be applied in order to assess the relative replacement of one body component with another for a given body weight. For example:

NAFLD ~ total lean mass(%) + bone/organ mass(%) + total body weight.

In this example, only total fat mass (%) is not included in this formula and hence, the beta for total lean mass reflects the increase of total lean mass at the expense of total body fat mass (in % body weight).

In addition to weight and height, all analyses were adjusted for age and study cohorts as well. Potential confounding of a nested set of sociodemographic, lifestyle, and metabolic predictor variables (based on the literature[6]) was tested, taking potential overfitting into account.

Furthermore, in order to assess which parameter had the best performance for NAFLD (i.e. explained more variation of the outcome and thus resulted in a better model fit), we compared SMI, ALM-fraction, AGR, body fat fraction, (pre)sarcopenia, grip, and speed using the AIC.

To test the robustness of our conclusions we performed three sensitivity analyses. First, we explored ALM-fraction as alternative proxy for skeletal muscle mass to facilitate comparison with previous reports.[370,371] Second, as gait speed measurements were performed on a separate day at the research facility, this measurement was missing in 32.6% of individuals. In the main analysis, we assumed that if one of both proxies for physical functioning was normal, there would be no sarcopenia. In the sensitivity analysis, we used imputed grip strength and gait speed data to re-classify sarcopenia. Third, we analysed the association of SMI with NASH in order to assess whether SMI was also associated with NAFLD severity.

We checked all analyses for potential multicollinearity using the variance inflation factor. In addition we corrected for the inflated type I error that arises due to multiple testing. We applied the adapted method proposed by Sidák,[256,257] using the effective number of tests instead of the actual number of tests (n=7). This adaptation took into account that the different body components are strongly interrelated and, hence, are not independent from each other. The corrected significance level was $P<0.010$. All analyses were performed using R version 3.5.1 (R core team, Vienna, Austria).

Results

Study population

The flowchart of the study is illustrated in *Figure 1*. In total, 5967 participants underwent abdominal US. We excluded 253 participants (4.2%) because of unreliable food questionnaires, missing data on DXA-scans or outlier values. Second, 887 participants (15.5%) were excluded while having secondary causes for steatosis. Lastly, 218 participants were excluded because of non-European background. Hence, the total number of eligible study participants was 4609. Mean age was 69.3±9.2 years, 57.0% was female and mean BMI was 27.5±4.2 kg/m² (range 15.0–47.2). Both original and imputed data of the total population are depicted in *Supplementary Table 1*.

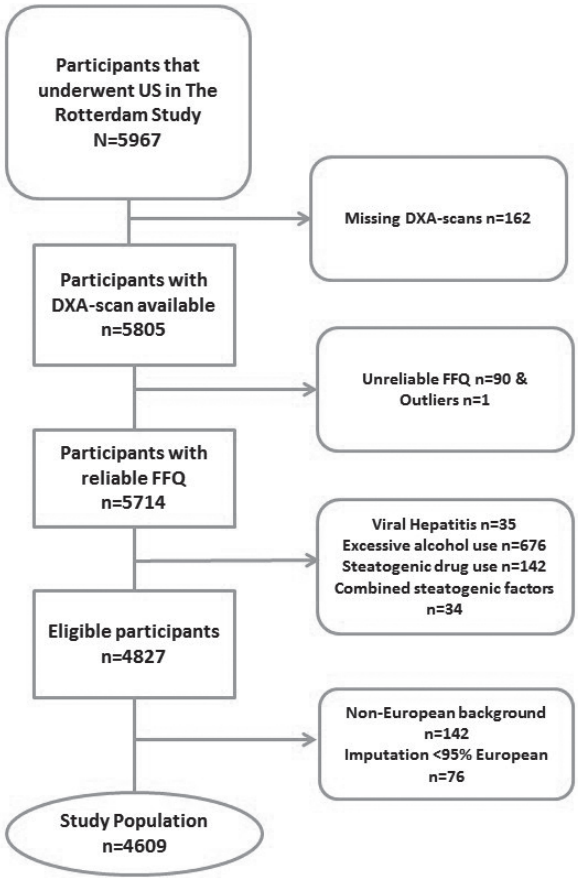


Figure 1: Flowchart of the study
Abbreviations: DXA: dual X-ray absorptiometry, FFQ: food frequency questionnaire, US: ultrasound

NAFLD characteristics

The overall prevalence of NAFLD in this study population was 35.2% (n=1623). Of those, 161 individuals were normal-weight (10%) and 1462 were overweight (90%). Data on demographics, biochemistry and comorbidities are given, stratified by BMI, in *Supplementary Table 2*. Differences between NAFLD strata were most pronounced in the overweight group. However, ALT, gamma-glutamyl transferase, insulin resistance and triglycerides were significantly different in both NAFLD subgroups.

Body composition and NAFLD

Table 1 depicts the data on body composition stratified by sex and BMI. Fat mass parameters were higher in every NAFLD subgroup, whereas lean mass parameters were lower mostly in the normal-weight NAFLD subgroups. Interestingly, ALM-fraction was the only parameter

Table 1: Study Characteristics of participants for different NAFLD strata

	Normal weight n=1339			Overweight n=3270		
			Men N=1980			
	No NAFLD n=432	NAFLD n=67	*P*-value*	No NAFLD n=822	NAFLD n=659	*P*-value*
Height (m)	177.0 (7.1)	176.8 (7.8)	0.796	176 (7.1)	176 (6.9)	0.703
Weight (kg)	72.9 (7.0)	74.8 (7.6)	0.041	86.8 (10.1)	93.2 (12.9)	<0.001
Total fat mass (kg)	19.1 [16.5, 21.8]	21.0 [18.6, 24.1]	<0.001	26.6 [23.3, 30.5]	30.9 [26.6, 36.3]	<0.001
Body fat fraction (%)	26.0 (4.6)	28.6 (4.3)	<0.001	31.4 (4.6)	34.3 (4.6)	<0.001
Android fat mass (kg)	1.70 [1.28, 2.08]	2.01 [1.75, 2.42]	<0.001	2.73 [2.21, 3.24]	3.40 [2.82, 4.08]	<0.001
Trunk/Appendicular fat mass (kg)	14.9 [12.8, 16.8]	16.2 [14.6, 18.5]	<0.001	20.4 [18.0, 23.3]	23.6 [20.5, 27.7]	<0.001
Gynoid fat mass (kg)	2.56 [2.19, 2.95]	2.71 [2.35, 3.08]	0.028	3.38 [2.94, 3.98]	3.86 [3.22, 4.65]	<0.001
AGR	0.66 [0.54, 0.76]	0.76 [0.68, 0.84]	0.028	0.79 [0.69, 0.89]	0.87 [0.79, 0.97]	<0.001
Total lean mass (kg)	51.0 [47.4, 54.8]	49.9 [47.0, 54.5]	0.371	55.7 [51.8, 59.9]	57.0 [52.9, 61.2]	<0.001
Trunk lean mass (kg)	27.6 [26.0, 29.6]	26.9 [25.5, 28.9]	0.209	29.5 [27.6, 31.6]	30.1 [28.0, 32.4]	0.001
Appendicular lean mass (kg)	23.3 [21.1, 25.4]	23.1 [20.8, 25.2]	0.998	26.0 [24.0, 28.7]	26.9 [24.7, 29.4]	<0.001
SMI (kg/m²)	7.43 (0.74)	7.41 (0.69)	0.769	8.45 (0.80)	8.70 (0.91)	<0.001
ALM-fraction (%)	31.9 [30.3, 33.9]	31.2 [29.1, 33.2]	0.015	30.5 [28.7, 32.3]	29.4 [27.7, 31.0]	<0.001
Normal SMI	58.8	58.2		94.3	94.7	
Presarcopenia	24.5	20.9	0.630	3.0	2.0	0.337
Sarcopenia	16.7	20.9		2.7	3.3	
Gait speed (m/s)	1.26 [1.11, 1.40]	1.27 [1.13, 1.38]	0.727	1.24 [1.09, 1.35]	1.23 [1.09, 1.34]	0.990
Hand grip strength (kg)	35.4 (8.5)	33.0 (9.2)	0.036	36.8 (8.9)	36.7 (9.1)	0.841

Table 1 (*continued*)

	Women N=2629					
	No NAFLD n=746	**NAFLD n=94**	***P*-value***	**No NAFLD n=986**	**NAFLD n=803**	***P*-value***
Height (m)	164.0 (6.6)	163.4 (6.4)	0.420	162.5 (6.7)	162.7 (6.4)	0.435
Weight (kg)	61.0 (6.6)	63.1 (5.6)	0.003	75.8 (10.5)	82.6 (11.7)	<0.001
Total fat mass (kg)	20.9 [17.7, 23.4]	23.5 [21.1, 25.0]	<0.001	29.9 [26.5, 34.5]	34.8 [30.3, 40.8]	<0.001
Body fat fraction (%)	34.1 (4.9)	37.4 (3.6)	<0.001	41.8 (4.4)	44.3 (4.3)	<0.001
Android fat mass (kg)	1.48 [1.12, 1.81]	1.95 [1.70, 2.29]	<0.001	2.47 [2.06, 2.99]	3.20 [2.67, 3.92]	<0.001
Trunk/Appendicular fat mass (kg)	16.1 [13.7, 17.9]	17.9 [16.4, 19.0]	<0.001	22.7 [20.2, 26.4]	26.5 [23.1, 30.9]	<0.001
Gynoid fat mass (kg)	3.33 [2.81, 3.83]	3.46 [2.97, 3.86]	0.080	4.67 [3.99, 5.48]	5.16 [4.35, 6.13]	<0.001
AGR	0.43 [0.35, 0.51]	0.56 [0.50, 0.65]	0.080	0.53 [0.46, 0.61]	0.62 [0.55, 0.71]	<0.001
Total lean mass (kg)	37.3 [34.7, 40.1]	37.1 [34.4, 38.7]	0.118	40.3 [37.6, 43.7]	42.3 [39.3, 45.8]	<0.001
Trunk lean mass (kg)	20.8 [19.4, 22.3]	20.5 [19.2, 21.8]	0.135	21.9 [20.6, 23.7]	22.9 [21.3, 24.8]	<0.001
Appendicular lean mass (kg)	16.5 [15.0, 18.1]	16.2 [15.2, 17.3]	0.109	18.3 [16.8, 20.2]	19.3 [17.6, 21.3]	<0.001
SMI (kg/m²)	6.14 (0.60)	6.03 (0.45)	0.088	7.01 (0.71)	7.36 (0.84)	<0.001
ALM-fraction (%)	27.7 [25.8, 29.4]	26.2 [24.7, 27.5]	<0.001	25.2 [23.7, 26.6]	24.2 [22.9, 25.5]	<0.001
Normal SMI	79.9	76.6		98.8	98.8	
Pre-sarcopenia	12.2	14.9	0.727	0.5	0.7	0.691
Sarcopenia	7.9	8.5		0.7	0.5	
Gait speed (m/s)	1.24 [1.11, 1.36]	1.21 [1.08, 1.31]	0.994	1.17 [1.03, 1.28]	1.15 [1.02, 1.27]	0.570
Hand grip strength (kg)	21.8 (6.0)	20.9 (5.1)	0.176	21.77 (5.75)	21.61 (5.66)	0.553

Pooled data based on 74 imputations is presented as mean (SD), median [P25-P75] or percentage. Data is presented stratified by sex and BMI. *P-value is calculated using analyses of variance, Kruskall-Wallis, or Chi-squared test.

Abbreviations: AGR: android-fat-to-gynoid-fat ratio; ALM: appendicular lean mass; BMI: body mass index; NAFLD: non-alcoholic fatty liver disease; SMI; skeletal muscle index.

that was lower in *every* NAFLD subgroup. Also, overall prevalence of pre-sarcopenia and sarcopenia was low (5.9% and 4.5% respectively) and not associated with NAFLD (*Table 1*). We observed no clear effect modification, but the association between body components and NAFLD was most pronounced in normal-weight women (*Table 2*). Again, fat mass was generally associated with higher NAFLD and lean mass with lower NAFLD prevalence. In both sexes, android fat mass remained associated with NAFLD. In addition, ALM remained independently associated with normal-weight women (OR 0.75, 95%CI 0.62–0.91, *P*=0.003; *Table 2*).

Supplementary Table 3 shows the substitution analyses. Substituting ALM for fat mass was associated with lower prevalence of NAFLD, except when replaced for gynoid fat mass. Indeed, replacing components by gynoid fat mass was associated with lower NAFLD prevalence, whereas the opposite was true for android fat mass.

Table 2: Association of different parts of body with NAFLD

	Men			
	Normal-weight n=499	***P*-Value**	**Overweight n=1481**	***P*-Value**
		Model 1		
Total lean mass	**0.87 (0.80 – 0.96)**	**0.003**	**0.93 (0.90 – 0.96)**	**<0.001**
Appendicular lean mass	**0.80 (0.69 – 0.93)**	**0.003**	**0.91 (0.86 – 0.96)**	**0.001**
Trunk lean mass	0.86 (0.74 – 1.00)	0.045	**0.90 (0.85 – 0.95)**	**<0.001**
Total body fat	**1.16 (1.06 – 1.27)**	**0.001**	**1.08 (1.05 – 1.12)**	**<0.001**
Trunk fat mass	**1.22 (1.08 – 1.37)**	**0.001**	**1.11 (1.06 – 1.17)**	**<0.001**
Gynoid fat mass	1.46 (0.81 – 2.63)	0.203	0.97 (0.80 – 1.17)	0.720
Android fat mass	**3.49 (1.95 – 6.22)**	**<0.001**	**2.49 (2.00 – 3.11)**	**<0.001**
		Model 2		
Total lean mass	0.90 (0.82 – 0.99)	0.026	0.96 (0.93 – 1.00)	0.038
Appendicular lean mass	0.84 (0.72 – 0.97)	0.020	0.95 (0.89 – 1.01)	0.105
Trunk lean mass	0.90 (0.77 – 1.06)	0.199	0.94 (0.89 – 1.00)	0.065
Total body fat	1.13 (1.03 – 1.24)	0.011	1.04 (1.01 – 1.08)	0.022
Trunk fat mass	1.17 (1.04 – 1.33)	0.012	1.06 (1.01 – 1.11)	0.030
Gynoid fat mass	1.32 (0.71 – 2.42)	0.377	0.96 (0.78 – 1.18)	0.685
Android fat mass	**2.94 (1.59 – 5.45)**	**0.001**	**1.77 (1.40 – 2.25)**	**<0.001**
		Model 3		
Total lean mass	0.94 (0.85 – 1.04)	0.202	0.97 (0.94 – 1.01)	0.147
Appendicular lean mass	0.87 (0.74 – 1.01)	0.071	0.97 (0.91 – 1.03)	0.296
Trunk lean mass	0.99 (0.83 – 1.17)	0.867	0.96 (0.90 – 1.02)	0.169
Total body fat	1.09 (0.99 – 1.20)	0.094	1.03 (1.00 – 1.07)	0.094
	Women			
	Normal-weight n=840	***P*-Value**	**Overweight n=1789**	***P*-Value**
		Model 1		
Total lean mass	**0.79 (0.71 – 0.87)**	**<0.001**	0.97 (0.93 – 1.01)	0.098
Appendicular lean mass	**0.68 (0.57 – 0.81)**	**<0.001**	0.95 (0.88 – 1.02)	0.146
Trunk lean mass	**0.76 (0.64 – 0.87)**	**0.001**	0.97 (0.91 – 1.02)	0.227
Total body fat	**1.22 (1.10 – 1.35)**	**<0.001**	1.04 (1.00 – 1.08)	0.034
Trunk fat mass	**1.30 (1.14 – 1.49)**	**<0.001**	1.06 (1.01 – 1.12)	0.020
Gynoid fat mass	0.73 (0.47 – 1.12)	0.150	**0.63 (0.54 – 0.73)**	**<0.001**
Android fat mass	**12.5 (6.28 – 24.9)**	**<0.001**	**3.40 (2.71 – 4.27)**	**<0.001**
		Model 2		
Total lean mass	**0.80 (0.71 – 0.89)**	**<0.001**	0.96 (0.92 – 1.00)	0.074
Appendicular lean mass	**0.69 (0.57 – 0.83)**	**<0.001**	0.98 (0.90 – 1.06)	0.545
Trunk lean mass	**0.76 (0.64 – 0.90)**	**0.001**	0.93 (0.88 – 1.00)	0.035
Total body fat	**1.20 (1.08 – 1.33)**	**0.001**	1.05 (1.01 – 1.10)	0.020

Table 2 (*continued*)

	Women			
	Normal-weight n=840	***P*-Value**	**Overweight n=1789**	***P*-Value**
Trunk fat mass	**1.26 (1.09 – 1.44)**	**0.001**	1.07 (1.01 – 1.13)	0.021
Gynoid fat mass	1.00 (0.62 – 1.61)	0.996	0.85 (0.73 – 1.00)	0.050
Android fat mass	**8.34 (3.95 – 17.6)**	**<0.001**	**2.16 (1.69 – 2.76)**	**<0.001**
	Model 3			
Total lean mass	**0.84 (0.75 – 0.94)**	**0.002**	0.97 (0.93 – 1.01)	0.170
Appendicular lean mass	**0.75 (0.62 – 0.91)**	**0.003**	1.01 (0.94 – 1.10)	0.772
Trunk lean mass	0.82 (0.69 – 0.98)	0.029	0.93 (0.87 – 0.99)	0.025
Total body fat	1.16 (1.03 – 1.29)	0.011	1.06 (1.01 – 1.10)	0.010

Results are given as OR (95%CI) for NAFLD as outcome stratified by sex and BMI. Results in bold reflect significant findings with a P-value <0.010. Model 1: adjusted for age, study cohorts, weight, and height. Model 2: in addition: HOMA-IR, and triglycerides. Model 3: in addition AGR. Additional adjustments for confounding by education level, physical activity, alcohol intake, energy intake, ALT, and smoking resulted in negligible changes in odds ratio (<5%).
Abbreviations: AGR: android-fat-to-gynoid-fat ratio; BMI: body mass index; CI: confidence interval; HOMA-IR: homeostasis model assessment of insulin resistance; NAFLD: non-alcoholic fatty liver disease; OR: odds ratio.

Comparing the performance of the different body composition parameters for NAFLD, fat mass parameters explained more variation of the outcome (resulted in a better model fit) than lean mass parameters (as indicated by a lower AIC). AGR was the predictor with the overall best model fit (*Supplementary Table 4*). Interestingly, SMI and ALM-fraction performed equally with regard to the model fit in the analysis for NAFLD.

SMI & NAFLD

Table 3 shows the association between our exposure variable of interest, SMI, and NAFLD. SMI was associated with less NAFLD prevalence in normal-weight men, but significance dissipated after correction for multiple testing and adjustment for AGR. In normal-weight women, however, SMI remained associated with less NAFLD after full adjustment (OR 0.48, 95%CI 0.29–0.80, *P*=0.005. *Table 3*). Using ALM-fraction (instead of SMI) provided similar conclusions, underscoring the robustness of our results (*Supplementary Table 5*). No independent association was found between SMI and overweight NAFLD. However, SMI was relatively high in this population. The prevalence of low SMI (i.e. ≤7.25 kg/m² or ≤5.67 kg/m²) was only 5% in overweight individuals against 28% in the normal-weight. Prevalence of (pre)sarcopenic obesity (i.e. BMI≥30kg/m²) was even lower, in fact, only five out of the 1091 *obese* individuals had a low SMI. Interestingly, four of them had NAFLD.
The analysis of pre-sarcopenia and sarcopenia is given in *Supplementary Table 6*. In model 1, sarcopenia was associated with NAFLD in men and pre-sarcopenia with NAFLD in

Table 3: SMI & NAFLD

	Men			
	Normal-weight (n=499)	***P*-value**	**Overweight (n=1481)**	***P*-value**
Model 1				
SMI	**0.50 (0.32 – 0.79)**	**0.003**	**0.75 (0.63 – 0.90)**	**0.002**
Model 2				
SMI	0.57 (0.36 – 0.91)	0.018	0.88 (0.72 – 1.06)	0.169
Model 3				
SMI	0.63 (0.39 – 1.02)	0.061	0.92 (0.76 – 1.12)	0.401
	Women			
	Normal-weight (n=840)	***P*-value**	**Overweight (n=1789)**	***P*-value**
Model 1				
SMI	**0.37 (0.23 – 0.59)**	**<0.001**	0.90 (0.75 – 1.09)	0.291
Model 2				
SMI	**0.39 (0.23 – 0.64)**	**<0.001**	0.98 (0.80 – 1.20)	0.846
Model 3				
SMI	**0.48 (0.29 – 0.80)**	**0.005**	1.08 (0.87 – 1.33)	0.485

Results are given as OR (95%CI) for NAFLD as outcome stratified by sex and BMI. Results in **bold** reflect significant findings with a *P*-value <0.010. **Model 1:** adjusted for age, study cohorts, weight and height. **Model 2:** in addition: HOMA-IR, and triglycerides. **Model 3:** in addition: AGR. **Additional adjustments** for confounding by education level, physical activity, alcohol intake, energy intake, ALT, and smoking resulted in negligible changes in odds ratio (<5%).

Abbreviations: AGR: android-fat-to-gynoid-fat ratio; BMI: body mass index; CI: confidence interval; HOMA-IR: homeostasis model assessment of insulin resistance; NAFLD: non-alcoholic fatty liver disease; OR: odds ratio; SMI; skeletal muscle index.

women, nonetheless these findings did not hold after further adjustment (*Supplementary Table 6*). In total, 1548 participants (33.6%) had either missing data on grip strength or gait speed. None of the participants had missing data on both variables. Sensitivity analysis using imputed values on grip strength and gait speed resulted in reclassification of 24 participants and yielded similar results to the original data (data not shown).

Of the 1623 individuals with NAFLD, 1126 (69.4%) had data available on LSM. NASH prevalence was low (total population: (n=115) 10.2%, normal-weight: (n=10) 8.5%, and overweight: (n=105) 10.4%). The small number of cases in the normal-weight (3 in men and 7 in women) hampered the possibility to stratify by BMI. *Supplementary Table 7* shows that SMI was associated with lower NASH prevalence in women, but this was no longer significant after correction for multiple testing (OR 0.48, 95%CI 0.25–0.92, *P*=0.027).

Discussion

In this large Western population-based study we examined the association between muscle mass, fat mass, fat distribution and NAFLD, using DXA-scans and hepatic imaging, both highly reliable measuring methods. Moreover, this is the first study that examines the sarcopenia definition, as proposed by the EWGSOP consensus, in association with NAFLD. We made several novel observations. First, we showed that incremental skeletal muscle mass was consistently associated with lower NAFLD prevalence and severity in normal-weight women (using different approaches). This association was independent of metabolic confounders, and importantly, independent of fat distribution. A similar association was seen in normal-weight men, but significance did not hold after correction for multiple testing. Prevalence of pre-sarcopenia and sarcopenia was low, but most prevalent in normal-weight NAFLD. However, in multivariable analysis neither sarcopenia nor pre-sarcopenia was associated with NAFLD, most likely because of the relative high SMI in this elderly community dwelling population. Second, high fat mass appeared a better predictor for NAFLD prevalence than low muscle mass. In particular, android fat mass had a significant association with higher NAFLD prevalence in all subgroups, whereas gynoid fat tended to be associated with lower NAFLD prevalence. Likewise, AGR as proxy for fat distribution was the strongest predictor for NAFLD.

Recently, low skeletal muscle mass emerged as potential risk factor for steatosis independent of age and BMI.[380-384] Most of these studies were carried out in Korean populations.[380-382,384] However, as ethnicity is important in the evaluation of body composition,[12] it is difficult to generalize these results to Western populations. Asian individuals carry proportionately higher fat mass for a given BMI than Caucasians. To date, only two studies have been performed in Western populations.[371,385] Both studies found that the prevalence of pre-sarcopenia increased with fibrosis severity in NAFLD. But direct comparison to these studies is hampered by the different study populations (abovementioned studies included advanced steatohepatitis patients with cirrhosis prevalence of 33.3% and 15.6% without healthy control group).[371,385] This is in contrast to our study in which we targeted a different study population, i.e. an unselected presumably healthy population in which only 10% of the participants with steatosis had coinciding elevated LSM. Furthermore, low skeletal muscle mass alone has been systematically referred to as *sarcopenia* in these previous reports. However, loss of strength or performance is strictly needed to make the actual diagnosis of sarcopenia.[374] A recent small study examined actual sarcopenia univariately with NAFLD and found that sarcopenia was actually associated with lower instead of higher NAFLD prevalence.[386] Yet, this finding is in line with our univariate results in which SMI was also associated with higher prevalence of NAFLD. While the previous study did not perform a multivariable analysis, we found that this association changed after adjustment for weight.

Another commonly used proxy for skeletal muscle mass is ALM-fraction.[370,371,380-384] We chose to use SMI as advised by the EWGSOP consensus guidelines, but we did perform additional analyses on ALM-fraction, and found that both ALM fraction and SMI were equally good in predicting the prevalence of NAFLD if only adjusted for weight and height. We hypothesize that the explanation lies in the fact that most phenotypes associated with sarcopenia, are typically related to frailty and poor nutritional status (and hence to a low BMI),[374] whereas NAFLD is typically related to adiposity and over-nutrition (and thus to a high BMI).[13] We would therefore like to pose that ALM-fraction might be more clinically applicable than SMI for the (univariate) assessment of pre-sarcopenia in NAFLD as it already takes into account the confounder weight. In our study, neither pre-sarcopenia nor sarcopenia was independently associated with NAFLD. We believe this is due to the low number of (pre)sarcopenic cases. Indeed, the median SMI was relatively high in our population. As a consequence, (pre)sarcopenia in the obese and overweight was rare (prevalence of 0.4% and 5% respectively). This could explain the lack of SMI being associated with overweight NAFLD. As for muscle function, grip strength has been analysed previously in a large Asian cohort that found an inverse association between grip strength (relative to body weight) and NAFLD.[387] In our study, incremental grip strength was associated with lower NAFLD prevalence in men only, however this association did not hold significance after multivariable adjustment.

Interestingly, the association between SMI and NAFLD was more pronounced in women than in men. This is in contrast to previous studies in which associations are generally more pronounced in men. However, there were fewer men than women in this study (n=1980 vs n=2629) and, as said before, SMI was relatively high in our community-dwelling population. Therefore, we cannot fully exclude the possibility that differences in sex-stratified analyses resulted, in part, from power issues. Nevertheless, there are obvious differences in sex hormones between men and women that could have affected the association between SMI with NAFLD. As our study concerns an elderly population, there is a presumed relative reduced testosterone, a strong anabolic hormone, in men.[388] Nonetheless, absolute testosterone in elderly men is generally still higher than in women.[389]Also, relative oestrogen is decreased in women because of the menopause,[388] and fat mass-to-fat free mass ratio is much higher in women than in men.[377] Furthermore, apart from the differences in sex hormones, other signalling hormones such as growth hormone could have also affected the relation between body composition and NAFLD.[317] In addition, inflammation has been suggested to play a major role in sarcopenia; myokines secreted by skeletal muscle mass, for instance, antagonize the pro-inflammatory and metabolic effects of adipocytes from fat tissue.[383,390] This could explain the hypothesized synergistic effect of low skeletal muscle mass and adiposity together. Moreover, it is known that visceral fat, in particular, impairs adipocyte function and adipocytokine secretion, which can lead to an increase in pro-inflammatory cytokines such as interleukin-6 and tumour necrosis factor-alpha.[391]

This could explain the attenuating effect of AGR between body components with NAFLD, which is particularly interesting as AGR was much higher in men than in women.
A major strength of our study is the use of a large population-based cohort with access to a great number of reliably measured traits. Additionally, body composition was determined by the gold standard (DXA-scan) and we used the recommended EWGSOP definition to determine the presence of pre-sarcopenia and sarcopenia.[374] Moreover, we included only individuals with a European background to exclude bias due to racial differences in body composition. And lastly, we performed several sensitivity analyses to test the robustness of our conclusions. Nonetheless, several limitations need mentioning. First, the cross-sectional design of this study makes it impossible to draw conclusions on the cause-effect relation between body composition and NAFLD. Second, the gold standard for diagnosis of NAFLD is liver biopsy rather than US. However, performing an invasive liver biopsy in presumed healthy individuals is unethical. Moreover, US is a widely used screening tool that yields high sensitivity and specificity for moderate and severe steatosis.[50] Third, missing data on gait speed was substantial (32.6%). Yet, grip strength, as indicator for muscle strength, was present in almost all participants (>99%). Moreover, sensitivity analyses using imputed data on gait speed showed similar results to the main non-imputed analysis, suggesting no under or overestimation of sarcopenia. Fourth, we had no information on possible fat infiltration in the muscle, which could have affected the quality of muscle mass. Previous studies have suggested that, in older women particularly, there is quite a proportion of fat within the quadriceps muscle, this infiltration was related to gait speed.[392] However, in our study was gait speed not associated with NAFLD, neither in men nor in women.
In summary, skeletal muscle mass as assessed by multiple proxies was consistently associated with NAFLD in normal-weight women. However, fat mass was a better predictor for NAFLD probability in both sexes. In particular, android-fat-to-gynoid-fat ratio was strongly associated with NAFLD. This is in line with android fat being associated with higher odds and gynoid fat being associated with lower odds for NAFLD. These findings, if confirmed by others, add to the rationale of resistance training in order to replace fat mass by lean mass (regardless of simultaneous weight loss) as an easily accessible, inexpensive, and targeted approach for individuals with NAFLD.[393]

Supplementary Methods: details on the imputation process

Missing values of analysis variables were 0.04–18.1%. Following the advice of White et al.,[394] 74 imputed datasets were created using the R-package mice.[323] The analyses were performed in each dataset. The results presented are pooled over the multiple analyses using Rubin's rules in order to take into account the added uncertainty due to the missing data.[254] Imputation was performed before exclusion of subjects due to alcohol abuse or non-European background. Participants with missing values on these 'exclusion variables' were included only if the imputed values fulfilled the inclusion criteria for alcohol consumption in at least >80% of the imputed datasets, and ethnicity was imputed as European background in at least >95% of the imputed datasets. The difference in cut-off is based on the fact that ethnicity is purely an exclusion criterion, whereas alcohol consumption was evaluated as predictor in the regression models.

Supplementary Methods: details on the multiple imputation process

	Multiple imputation
Software used	R version 3.4.2
Imputation method and key settings	Fully conditional specification (Markov chain Monte Carlo method); maximum iterations: 50
No. of imputed data sets created	74
Analyses variables	diabetes mellitus, hypertension, smoking status, total cholesterol, diastolic and systolic blood pressure, high density lipoprotein cholesterol, energy intake, platelets, waist circumference, haemoglobin, haematocrit, heart rate, waist/hip ratio, aspartate transaminase; alanine transaminase; homeostasis model assessment of insulin resistance, triglycerides, insulin, gamma-glutamyl transferase, alkaline phosphatase, bilirubin, alcohol consumption, creatinine, glomerular filtration rate, grip strength, physical activity, gait speed, spleen size, glucose, education level, and ancestry, liver stiffness measurements, IQR of liver stiffness
Auxiliary variables	android and gynoid fat mass, height, skeletal muscle index, weight, age, amiodarone, antihypertensives, systemic corticosteroid use, sex, Rotterdam Study cohorts, steatosis, and tamoxifen
Treatment of not normally distributed variables	Predictive mean matching
Treatment of normally distributed variables	Linear regression
Treatment of binary/categorical variables	(Proportional odds) Linear Regression
Population	For the imputation we excluded outliers, unreliable FFQs and missing data on DXA-scans. Imputed population (n=5.714).

Supplementary Table 1: Imputation Characteristics

	Original Data (n=4609)	Imputed data* (n=4609)
Demographics		
Age (years)	69.3 (9.2)	-
Female (%)	57.0	-
Education Level (%)		
Low	49.2	49.3
Intermediate	29.6	29.6
High	21.1	21.1
Smoking status (%)		
Never	35.1	34.5
Past/Current	64.9	65.5
Alcohol (units/d)	0.47 [0.06, 1.22]	0.50 [0.07, 1.28]
Physical Activity	39.8 [15.8, 76.5]	38.9 [15.5, 75.6]
Caloric Intake (kcal/day)	2114 (730)	2132 (737)
Physical examination		
Height (cm)	168.8 (9.5)	-
Weight (kg)	78.5 (14.4)	-
BMI (kg/m^2)	27.5 (4.2)	-
underweight	0.3	-
normal	28.7	
overweight	47.3	
obese	18.4	
morbid obese	5.3	
Gait speed (m/s)	1.21 (0.20)	1.19 (0.21)
Hand grip strength (kg)	28.0 (10.3)	28.0 (10.3)
Android fat mass (kg)	2.5 [1.8, 3.2]	-
Gynoid fat mass (kg)	3.8 [3.1, 4.8]	-
Android/Gynoid ratio		
men	0.79 [0.69, 0.91]	-
women	0.53 [0.45, 0.63]	-
Total fat mass (kg)	27.3 [22.2, 33.1]	-
Body fat (%)	36.3 (7.4)	-
ALM (kg)	21.5 (5.0)	-
SMI (kg/m^2)	7.5 (1.2)	-
Biochemistry		
AST (U/L)	24 [21, 28]	24 [21, 28]
ALT (U/L)	18 [15, 24]	18 [15, 24]
GGT (U/L)	23 [17, 34]	23 [17, 34]
Platelets (*10^9/L)	261 [222, 305]	261 [222, 306]
HOMA-IR	2.6 [1.7, 4.1]	2.6 [1.7, 4.1]

Supplementary Table 1 (*continued*)

	Original Data (n=4609)	Imputed data* (n=4609)
Total Cholesterol (mmol/l)	5.4 (1.1)	5.4 (1.1)
HDL-C (mmol/l)	1.5 (0.4)	1.5 (0.4)
Triglycerides (mmol/l)	1.3 [1.0, 1.7]	1.3 [1.0, 1.7]
Comorbidities		
Metabolic Syndrome (%)	53.6	53.6
- WC	44.2	44.2
- Triglycerides	46.8	46.5
- HDL-C	45.9	45.6
- Blood pressure	83.4	83.4
- Fasting Plasma Glucose	47.2	47.2
Diabetes Mellitus (%)	13.2	13.1
Hypertension (%)	72.8	72.8
NAFLD	35.2	-

* Imputed data is based on pooled data from 74 imputations. Data is presented as mean (SD), median [P25-P75] or percentage. ¥ Physical activity in metabolic equivalent task hours/week. – Represents no missing values.

Supplementary Table 2: Population Characteristics across NAFLD strata in normal and overweight individuals

	Total population			*Normal-weight*			*Overweight*		
	No NAFLD n=2986	NAFLD n=1623	*P*-value*	No NAFLD n=1178	NAFLD n=161	*P*-value*	No NAFLD n=1808	NAFLD n=1462	*P*-value*
Demographics									
Age (years)	69.3 (9.5)	69.3 (8.5)	0.887	69.2 (9.9)	68.8 (8.5)	0.615	69.5 (9.3)	69.36 (8.51)	0.787
Female (%)	58.0	55.3	0.078	63.3	58.4	0.259	54.5	54.9	0.852
Education Level (%)									
Low	46.4	54.5	<0.001	46.0	48.4	0.117	46.6	55.2	
Intermediate	30.3	28.4		27.9	32.8		31.9	27.9	<0.001
High	23.3	17.1		26.1	18.8		21.5	16.9	
Smoking status (%)									
Never	36.9	30.3	<0.001	38.7	35.1	0.038	35.7	29.7	<0.001
Past/Current	63.1	69.7		61.3	64.9		64.3	70.3	
Alcohol (units/d)	0.50 [0.07, 1.26]	0.50 [0.07, 1.28]	0.986	0.49 [0.05, 1.22]	0.61 [0.17, 1.57]	0.243	0.52 [0.09, 1.33]	0.49 [0.06, 1.27]	0.996
Physical activity¥	42.5 [16.8, 79.7]	32.7 [13.5, 67.3]	<0.001	45.5 [18.7, 81.6]	38.4 [18.1, 80.2]	0.919	39.7 [15.3, 77.0]	31.8 [13.0, 66.2]	0.001
Caloric Intake (kcal/day)	2154 (740)	2092 (729)	0.015	2180 (746)	2205 (707)	0.656	2137 (736)	2080 (730)	0.044
Biochemistry									
AST (U/L)	24.0 [21.0, 28.0]	25.0 [21.0, 29.0]	<0.001	24.0 [21.0, 28.0]	25.0 [21.0, 29.0]	0.101	24.0 [21.0, 27.0]	25.0 [21.0, 29.0]	<0.001

Supplementary Table 2 (*continued*)

	Total population			*Normal-weight*			*Overweight*		
	No NAFLD n=2986	**NAFLD n=1623**	***P*-value***	**No NAFLD n=1178**	**NAFLD n=161**	***P*-value***	**No NAFLD n=1808**	**NAFLD n=1462**	***P*-value***
ALT (U/L)	17.0 [14.0, 22.0]	22.0 [17.0, 29.0]	<0.001	17.0 [14.0, 21.0]	20.0 [16.0, 27.0]	<0.001	18.0 [14.0, 22.0]	22.0 [17.0, 29.0]	<0.001
GGT (U/L)	21.0 [15.0, 30.0]	28.0 [20.0, 41.0]	<0.001	19.0 [14.0, 27.0]	26.0 [19.0, 37.0]	<0.001	22.0 [16.0, 32.0]	28.0 [20.0, 41.0]	<0.001
Platelets (*10^9/L)	259 [221, 304]	266 [225, 308]	0.040	265 [229, 312]	274 [230, 315]	0.371	255 [218, 298]	265 [224, 308]	0.002
HOMA-IR	2.15 [1.50, 3.10]	4.03 [2.74, 6.09]	<0.001	1.72 [1.27, 2.41]	2.66 [1.73, 4.22]	<0.001	2.48 [1.76, 3.53]	4.20 [2.87, 6.26]	<0.001
Total Cholesterol	5.46 (1.10)	5.36 (1.13)	0.003	5.53 (1.08)	5.54 (1.04)	0.919	5.42 (1.11)	5.34 (1.13)	0.055
HDL-C	1.54 (0.42)	1.32 (0.37)	<0.001	1.65 (0.45)	1.42 (0.40)	<0.001	1.46 (0.39)	1.31 (0.36)	<0.001
Triglycerides	1.16 [0.90, 1.53]	1.55 [1.17, 2.06]	<0.001	1.07 [0.83, 1.38]	1.29 [0.93, 1.86]	<0.001	1.23 [0.96, 1.61]	1.57 [1.20, 2.08]	<0.001
Comorbidities									
Metabolic Syndrome (%)	41.9	75.2	<0.001	25.9	44.7	<0.001	52.3	78.5	<0.001
- WC	29.9	70.4	<0.001	3.7	11.8	<0.001	47.0	76.9	<0.001
- Triglycerides	39.1	60.1	<0.001	31.2	48.4	<0.001	44.7	61.6	<0.001
- HDL-C	39.0	57.8	<0.001	32.0	48.4	<0.001	43.9	59.1	<0.001
- Blood Pressure	79.4	90.6	<0.001	72.4	78.9	0.100	84.0	91.9	<0.001
- Fasting plasma glucose	36.7	66.5	<0.001	25.5	44.0	<0.001	43.8	69.0	<0.001
Diabetes Mellitus (%)	7.6	17.7	<0.001	5.2	14.5	<0.001	9.3	24.3	<0.001
Hypertension (%)	67.5	82.3	<0.001	59.3	67.1	0.069	72.9	84.0	<0.001

Pooled data based on 74 imputations is presented as mean (SD), median [P25-P75] or percentage. *P-value is calculated using analyses of variance, Kruskall-Wallis, or Chi-squared test. ¥ Physical activity in metabolic equivalent task hours/week.

Supplementary Table 3: Substitution Analyses

	Men			
	Normal-weight n=499	***P*-value**	**Overweight n=1481**	***P*-Value**
Total lean mass *instead of variables left-below*				
Total body fat	**0.88 (0.81 – 0.94)**	**0.001**	**0.92 (0.89 – 0.95)**	**<0.001**
Trunk fat	0.92 (0.72 – 1.18)	0.505	1.02 (0.93 – 1.11)	0.748
Gynoid fat	1.62 (0.74 – 3.56)	0.231	**1.45 (1.11 – 1.88)**	**0.006**
Android fat	0.36 (0.15 – 0.85)	0.021	**0.31 (0.22 – 0.44)**	**<0.001**
Appendicular lean mass				
Trunk lean mass	0.89 (0.73 – 1.09)	0.267	1.02 (0.93 – 1.12)	0.694
Total body fat	**0.83 (0.73 – 0.94)**	**0.004**	0.93 (0.88 – 0.99)	0.018
Trunk fat	0.86 (0.66 – 1.13)	0.282	1.06 (0.95 – 1.18)	0.273

Supplementary Table 3 (*continued*)

	Men			
	Normal-weight n=499	***P*-value**	**Overweight n=1481**	***P*-Value**
Gynoid fat	1.58 (0.72 – 3.48)	0.255	**1.47 (1.12 – 1.91)**	**0.005**
Android fat	**0.30 (0.12 – 0.74)**	**0.009**	**0.32 (0.22 – 0.44)**	**<0.001**
Trunk lean mass				
Appendicular lean mass	1.12 (0.92 – 1.37)	0.267	0.98 (0.89 – 1.08)	0.694
Total body fat	0.92 (0.82 – 1.04)	0.197	**0.91 (0.87 – 0.97)**	**0.002**
Trunk fat	1.03 (0.79 – 1.34)	0.845	1.03 (0.93 – 1.14)	0.589
Gynoid fat	1.88 (0.84 – 4.22)	0.125	**1.42 (1.09 – 1.86)**	**0.010**
Android fat	0.36 (0.15 – 0.86)	0.022	**0.31 (0.22 – 0.43)**	**<0.001**
Total body fat				
Total lean mass	**1.14 (1.06 – 1.23)**	**0.001**	**1.08 (1.05 – 1.12)**	**<0.001**
Trunk lean mass	1.08 (0.96 – 1.22)	0.197	**1.09 (1.03 – 1.16)**	**0.002**
Appendicular lean mass	**1.21 (1.06 – 1.38)**	**0.004**	1.07 (1.01 – 1.14)	0.018
Trunk fat				
Gynoid fat	1.72 (0.65 – 4.55)	0.277	1.39 (1.00 – 1.92)	0.049
Android fat	0.38 (0.13 – 1.11)	0.077	**0.30 (0.20 – 0.45)**	**<0.001**
Total lean mass	1.06 (0.83 – 1.36)	0.640	0.96 (0.88 – 1.05)	0.371
Trunk lean mass	0.97 (0.74 – 1.27)	0.845	0.97 (0.88 – 1.08)	0.589
Appendicular lean mass	1.16 (0.89 – 1.51)	0.282	0.94 (0.85 – 1.05)	0.273
Gynoid fat				
Trunk fat	0.58 (0.22 – 1.55)	0.277	0.72 (0.52 – 1.00)	0.049
Android fat	**0.22 (0.09 – 0.56)**	**0.002**	**0.22 (0.15 – 0.32)**	**<0.001**
Total lean mass	0.62 (0.28 – 1.36)	0.231	**0.69 (0.53 – 0.90)**	**0.006**
Trunk lean mass	0.53 (0.24 – 1.19)	0.125	0.70 (0.54 – 0.92)	0.010
Appendicular lean mass	0.63 (0.29 – 1.39)	0.255	**0.68 (0.52 – 0.89)**	**0.005**
Android fat				
Trunk fat	2.66 (0.90 – 7.85)	0.077	**3.34 (2.21 – 5.05)**	**<0.001**
Gynoid fat	**4.55 (1.78 – 11.7)**	**0.002**	**4.64 (3.18 – 6.77)**	**<0.001**
Total lean mass	2.82 (1.17 – 6.76)	0.021	**3.21 (2.28 – 4.51)**	**<0.001**
Trunk lean mass	2.78 (1.16 – 6.68)	0.022	**3.28 (2.31 – 4.64)**	**<0.001**
Appendicular lean mass	**3.31 (1.34 – 8.15)**	**0.009**	**3.17 (2.25 – 4.47)**	**<0.001**
	Women			
	Normal-weight n=840	***P*-value**	**Overweight n=1789**	***P*-Value**
Total lean mass *instead of variables left-below*				
Total body fat	**0.85 (0.80 – 0.91)**	**<0.001**	**0.95 (0.92 – 0.98)**	**0.001**
Trunk fat	0.89 (0.78 – 1.00)	0.058	0.94 (0.89 – 1.00)	0.034
Gynoid fat	**1.82 (1.25 – 2.63)**	**0.002**	**1.65 (1.42 – 1.90)**	**<0.001**
Android fat	**0.23 (0.14 – 0.38)**	**<0.001**	**0.34 (0.28 – 0.42)**	**<0.001**

Supplementary Table 3 (*continued*)

	Women			
	Normal-weight n=840	***P*-value**	**Overweight n=1789**	***P*-Value**
Appendicular lean mass				
Trunk lean mass	0.95 (0.79 – 1.14)	0.573	0.99 (0.91 – 1.07)	0.743
Total body fat	**0.83 (0.74 – 0.93)**	**0.002**	0.94 (0.89 – 1.00)	0.037
Trunk fat	0.87 (0.74 – 1.01)	0.074	1.01 (0.93 – 1.09)	0.859
Gynoid fat	**1.73 (1.18 – 2.54)**	**0.005**	**1.71 (1.46 – 2.00)**	**<0.001**
Android fat	**0.21 (0.13 – 0.36)**	**<0.001**	**0.35 (0.28 – 0.42)**	**<0.001**
Trunk lean mass				
Appendicular lean mass	1.05 (0.88 – 1.27)	0.573	1.01 (0.93 – 1.10)	0.743
Total body fat	0.87 (0.79 – 0.97)	0.012	0.95 (0.91 – 1.00)	0.040
Trunk fat	0.94 (0.81 – 1.10)	0.459	0.94 (0.89 – 1.01)	0.079
Gynoid fat	**1.89 (1.29 – 2.76)**	**0.001**	**1.60 (1.38 – 1.86)**	**<0.001**
Android fat	**0.23 (0.14 – 0.39)**	**<0.001**	**0.32 (0.26 – 0.40)**	**<0.001**
Total body fat				
Total lean mass	**1.17 (1.10 – 1.25)**	**<0.001**	**1.06 (1.02 – 1.09)**	**0.001**
Trunk lean mass	1.15 (1.03 – 1.27)	0.012	1.05 (1.00 – 1.10)	0.040
Appendicular lean mass	**1.21 (1.07 – 1.36)**	**0.002**	1.06 (1.00 – 1.13)	0.037
Trunk fat				
Gynoid fat	**2.01 (1.28 – 3.14)**	**0.002**	**1.70 (1.42 – 2.04)**	**<0.001**
Android fat	**0.25 (0.14 – 0.45)**	**<0.001**	**0.35 (0.28 – 0.44)**	**<0.001**
Total lean mass	1.11 (0.97 – 1.25)	0.125	1.04 (0.98 – 1.10)	0.229
Trunk lean mass	1.06 (0.91 – 1.24)	0.459	1.06 (0.99 – 1.13)	0.079
Appendicular lean mass	1.16 (0.99 – 1.36)	0.074	0.99 (0.92 – 1.08)	0.859
Gynoid fat				
Trunk fat	**0.50 (0.32 – 0.78)**	**0.002**	**0.59 (0.49 – 0.70)**	**<0.001**
Android fat	**0.13 (0.07 – 0.23)**	**<0.001**	**0.21 (0.16 – 0.26)**	**<0.001**
Total lean mass	**0.55 (0.38 – 0.80)**	**0.002**	**0.61 (0.53 – 0.70)**	**<0.001**
Trunk lean mass	**0.53 (0.36 – 0.77)**	**0.001**	**0.62 (0.54 – 0.73)**	**<0.001**
Appendicular lean mass	**0.58 (0.39 – 0.85)**	**0.005**	**0.59 (0.50 – 0.68)**	**<0.001**
Android fat				
Trunk fat	**4.00 (2.22 – 7.20)**	**<0.001**	**2.85 (2.27 – 3.58)**	**<0.001**
Gynoid fat	**8.02 (4.42 – 14.6)**	**<0.001**	**4.85 (3.82 – 6.16)**	**<0.001**
Total lean mass	**4.42 (2.66 – 7.35)**	**<0.001**	**2.95 (2.41 – 3.60)**	**<0.001**
Trunk lean mass	**4.30 (2.58 – 7.16)**	**<0.001**	**3.09 (2.50 – 3.82)**	**<0.001**
Appendicular lean mass	**4.69 (2.78 – 7.92)**	**<0.001**	**2.90 (2.37 – 3.55)**	**<0.001**

Results are presented as OR (95%CI). OR is for substituting body composition parameter (bold and centred) for the left-mentioned parameters (exchange in fraction, %). Results in bold reflect significant findings with a P-value <0.010. Adjusted for age, study cohorts, weight and height (model 1).

Supplementary Table 4: Comparing body composition parameter in normal and overweight across sex

	Men	
	Normal-weight n=499	**Overweight n=1481**
SMI	**0.50 (0.32 – 0.79)**	**0.75 (0.63 – 0.90)**
AIC	378	1857
ALM-fraction	**0.85 (0.76 – 0.95)**	**0.91 (0.87 – 0.96)**
AIC	379	1854
(Pre)sarcopenia		
Normal SMI	ref (1)	ref (1)
Presarcopenia	1.73 (0.84 – 3.59)	1.28 (0.63 – 2.59)
Sarcopenia	2.88 (1.26 – 6.58)	2.11 (1.12 – 3.98)
AIC	383	1864
Grip	**0.93 (0.89 – 0.96)**	0.99 (0.98 – 1.01)
AIC	373	1865
Speed	0.85 (0.15 – 4.97)	1.41 (0.71 – 2.82)
AIC	387	1864
Body fat fraction	**1.12 (1.05 – 1.20)**	**1.08 (1.05 – 1.12)**
AIC	376	1841
AGR*	**1.97 (1.41 – 2.76)**	**1.98 (1.69 – 2.32)**
AIC	372	1790
	Women	
	Normal-weight n=840	**Overweight n=1789**
SMI	**0.37 (0.23 – 0.59)**	0.90 (0.75 – 1.09)
AIC	546	2270
ALM-fraction	**0.79 (0.71 – 0.88)**	0.95 (0.89 – 1.00)
AIC	546	2267
(Pre)sarcopenia		
Normal SMI	ref (1)	ref (1)
Presarcopenia	**2.48 (1.25 – 4.95)**	2.49 (0.74 – 8.41)
Sarcopenia	1.54 (0.66 – 3.62)	0.99 (0.28 – 3.52)
AIC	560	2271
Grip	0.96 (0.91 – 1.00)	1.00 (0.98 – 1.02)
AIC	561	2271
Speed	0.78 (0.18 – 3.38)	1.60 (0.80 – 3.20)
AIC	564	2270
Body fat fraction	**1.15 (1.08 – 1.23)**	**1.05 (1.02 – 1.08)**
AIC	545	2261
AGR*	**4.81 (3.19 – 7.26)**	**3.42 (2.83 – 4.14)**
AIC	501	2083

Results are presented as OR (95%CI). Results in bold reflect significant findings with a P-value <0.010.The lowest AIC is highlighted and reflects the best predictive parameter. Adjusted for age, study cohorts, weight and height. *AGR is standardised (increase is per 1SD). Abbreviations: AGR: android-to-gynoid fat ratio; AIC: akaike information criterion; ALM: appendicular lean mass; SMI: skeletal muscle index.

Supplementary Table 5: ALM-fraction & NAFLD

		Men		
	Normal-weight (n=499)	***P*-value**	**Overweight (n=1481)**	***P*-value**
Model 1				
ALM-fraction	**0.85 (0.76 – 0.95)**	**0.003**	**0.91 (0.87 – 0.96)**	**<0.001**
Model 2				
ALM-fraction	0.88 (0.78 – 0.98)	0.021	0.95 (0.90 – 1.01)	0.090
Model 3				
ALM-fraction	0.90 (0.80 – 1.01)	0.070	0.97 (0.92 – 1.03)	0.279
		Women		
	Normal-weight (n=840)	***P*-value**	**Overweight (n=1789)**	***P*-value**
Model 1				
ALM-fraction	**0.79 (0.71 – 0.88)**	**<0.001**	0.95 (0.89 – 1.00)	0.050
Model 2				
ALM-fraction	**0.80 (0.71 – 0.90)**	**<0.001**	0.97 (0.91 – 1.03)	0.298
Model 3				
ALM-fraction	**0.84 (0.75 – 0.95)**	**0.006**	1.00 (0.94 – 1.06)	0.908

Results are presented as OR (95%CI). Results in bold reflect significant findings with a P-value <0.010. Model 1: adjusted for age, study cohorts, weight and height Model 2: in addition to model 1; HOMA-IR and triglycerides. Model 3: in addition to model 2; AGR. Additional adjustments for confounding by education level, physical activity, alcohol intake, energy intake, ALT, and smoking resulted in negligible changes in odds ratio (<5%). Abbreviations: AGR: android/Gynoid ratio; ALM: appendicular lean mass; CI: confidence interval; HOMA-IR: homeostasis model assessment of insulin resistance; NAFLD: non-alcoholic fatty liver disease; OR: odds ratio.

Supplementary Table 6: Presarcopenia, sarcopenia & NAFLD

		Men		
	Normal-weight (n=499)	***P*-value**	**Overweight (n=1481)**	***P*-value**
Model 1				
normal SMI	ref (1)		ref (1)	
presarcopenia	1.73 (0.84 – 3.59)	0.139	1.28 (0.63 – 2.59)	0.495
sarcopenia	2.88 (1.26 – 6.58)	0.012	2.11 (1.12 – 3.98)	0.022
Model 2				
normal SMI	ref (1)		ref (1)	
presarcopenia	1.51 (0.72 – 3.19)	0.276	1.19 (0.56 – 2.52)	0.657
sarcopenia	2.34 (1.00 – 5.45)	0.050	2.07 (1.06 – 4.05)	0.033
Model 3				
normal SMI	ref (1)		ref (1)	
presarcopenia	1.36 (0.63 – 2.89)	0.433	1.09 (0.50 – 2.33)	0.836
sarcopenia	2.20 (0.94 – 5.13)	0.069	1.88 (0.95 – 3.72)	0.068

Supplementary Table 6 (*continued*)

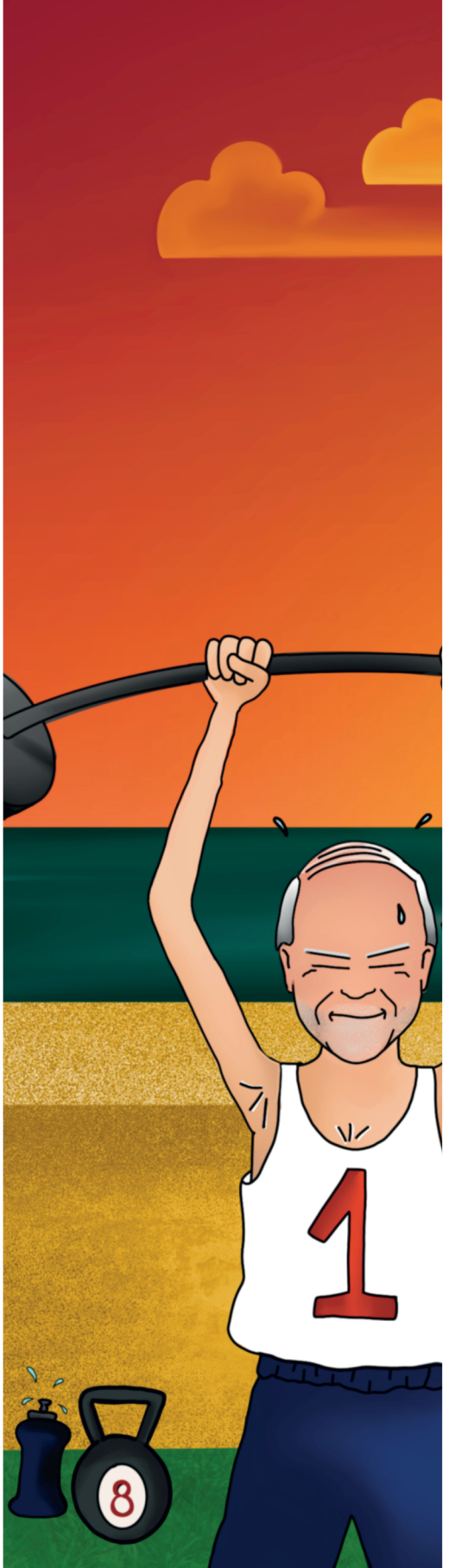
	Women			
	Normal-weight (n=840)	***P*-value**	**Overweight (n=1789)**	***P*-value**
Model 1				
normal SMI	ref (1)		ref (1)	
presarcopenia	**2.48 (1.25 – 4.95)**	**0.010**	2.49 (0.74 – 8.41)	0.143
sarcopenia	1.54 (0.66 – 3.62)	0.321	0.99 (0.28 – 3.52)	0.986
Model 2				
normal SMI	ref (1)		ref (1)	
presarcopenia	1.68 (0.79 – 3.58)	0.182	2.88 (0.74 – 11.19)	0.127
sarcopenia	1.31 (0.53 – 3.22)	0.561	0.75 (0.18 – 3.09)	0.692
Model 3				
normal SMI	ref (1)		ref (1)	
presarcopenia	1.20 (0.55 – 2.64)	0.641	2.54 (0.62 – 10.44)	0.196
sarcopenia	1.23 (0.49 – 3.07)	0.654	0.57 (0.14 – 2.41)	0.446

In men: presarcopenia: n=158 & sarcopenia n=130 & in women: presarcopenia: n=116 & sarcopenia: n=78. Results are presented as OR (95%CI). Results in bold reflect significant findings with a P-value <0.010. Model 1: adjusted for age, study cohorts, weight and height. Model 2: in addition to model 1; HOMA-IR and triglycerides. Model 3: in addition to model 2; AGR. Abbreviations: AGR: android-to-gynoid fat ratio; CI: confidence interval; HOMA-IR: homeostasis model assessment of insulin resistance; NAFLD: non-alcoholic fatty liver disease.

Supplementary Table 7: SMI & NASH

	Men		**Women**	
	Total (n=539)	**P-value**	**Total (n=587)**	**P-value**
Model 1				
SMI	1.00 (0.64 – 1.55)	0.982	0.52 (0.28 – 0.96)	0.038
Model 2				
SMI	1.02 (0.65 – 1.61)	0.922	0.47 (0.25 – 0.90)	0.023
Model 3				
SMI	1.03 (0.65 – 1.61)	0.909	0.48 (0.25 – 0.92)	0.027

In men: n=71 NASH and in women: n=44 NASH. Results are presented as OR (95%CI). Results in bold reflect significant findings with a P-value <0.010. Model 1: adjusted for age, study cohorts, weight and height. Model 2: in addition to model 1; HOMA-IR and triglycerides. Model 3: in addition to model 2; AGR. Abbreviations: AGR: android-to-gynoid fat ratio; CI: confidence interval; HOMA-IR: homeostasis model assessment of insulin resistance; NASH: non-alcoholic steatohepatitis; OR: odds ratio; SMI: skeletal muscle index.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Chapter 9

NAFLD and beneficial effects of lifestyle intervention: defining the meat of the matter

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To the editor:

It is with great interest that we read the paper of Wong et al.[329] In this large population-based study, the authors address a very important issue: is lifestyle modification as important in non-obese NAFLD as it is in obese NAFLD?

Based on their study results the answer is probably yes: 67% of the non-obese patients and 61% of the obese patients had normalisation of the intrahepatic triglyceride content on MR-spectroscopy after 12 months of lifestyle intervention. The authors subsequently focus on the relative weight loss and variably advice 3–10% (abstract and lay summary) or 5–10% (discussion and conclusion) body weight loss in order to achieve this primary end point in non-obese individuals.

What is interesting, however, is that the impact of the lifestyle intervention was independent of the achieved change in absolute body weight and in waist circumference in multivariable analysis. We would therefore like to pose the question whether the authors believe it is decrease in body weight or decrease in waist circumference or maybe another component within the lifestyle intervention that reverses NAFLD? And, if the latter is true, then what could that be?

As the authors discuss themselves, body mass index (BMI) is an imperfect measure of adiposity because it cannot distinguish between fat and muscle mass.[395] Indeed, it has been found that waist-to-hip ratio is a better predictor of severe liver disease than BMI.[396] As stated above, waist circumference was indeed independently associated with reversing non-obese NAFLD in the multivariable analysis. Yet, the actual change in waist circumference over time cannot be deducted from the paper (not shown in text, tables or figures). Also, the relative weight loss (i.e. percentage of weight reduction) was in fact not analysed in multivariable fashion at all, merely the absolute change in body weight.[329]

Another matter that caught our attention (although beyond the primary outcome of this study) was the use of the fatty liver index (FLI) for the diagnosis of NAFLD after 6 years of follow-up. As known, the FLI includes waist circumference and BMI, as well as triglycerides and gamma glutamyltransferase in its algorithm.[397] The FLI was originally developed on the basis of anthropometric parameters against ultrasound and later validated against actual intrahepatic triglyceride content. However, it is exactly because of this association between anthropometrics and NAFLD, that the choice of FLI in the context of the present study is somewhat unfortunate.[329] The authors state in their results that 'obese patients had higher FLI at year 6 compared to non-obese patients' and that 'obese patients were less likely to have an FLI below 30 than non-obese patients'. These findings are, although true, inherent to the algorithm and therefore redundant. As there is little data on the use of FLI as diagnostic tool for follow-up, it would have been interesting to compare the FLI at baseline against MR-spectroscopy in this study, reassuring the robustness of NAFLD diagnosis after 6 years.

In order to illustrate this point further, we validated the FLI against ultrasound (US) in our Western population-based cohort, stratified by BMI at cut-points 25kg/m² and 30kg/m². This analysis is an extension of a previous study from our group.[398] The present study consists of 5756 participants, of whom 57% is female and with a median age of 68.4 years, median BMI of 27.0 kg/m², and median FLI of 46.7.[248] We are aware of the fact that a validation of the FLI against US is suboptimal given the poor sensitivity of US for liver fat content below 25%. Nonetheless, US does have a good sensitivity for (clinically significant) moderate steatosis.[50]

As shown in *Table 1*, the FLI had a lower performance for the stratified groups than for the total group (AUROC of 0.69–0.75 in the stratified groups versus 0.80 in the total group; *Table 1*). Also, the sensitivity of FLI-defined steatosis (FLI>60) was poor in the lean (7.7%) and overweight (47.0%) population. Likewise, specificity of FLI-guided exclusion of steatosis (FLI<30) in the overweight (29.0%) and obese (2.1%) was poor as well. In addition, BMI itself can greatly affect FLI outcome. For instance, in a patient with a given set of clinical parameters (GGT: 29U/L, triglycerides: 1mmol/L, and waist circumference: 100cm) FLI could be 60.3 (including steatosis) when BMI is 30kg/m², 43.1 (inconclusive diagnosis) when BMI is 25kg/m², or 27.4 (excluding steatosis) when BMI is 20kg/m². This drives the point home that BMI affects diagnosis of steatosis when using FLI as surrogate diagnostic marker. Hence we advise against the use of the FLI as surrogate marker for steatosis in the context of examining the association between body composition and NAFLD.

That having said, we would like to emphasize our appreciation for the successful long-term follow-up after lifestyle treatment for NAFLD, a challenging target which has been rarely accomplished in the literature to date. We would therefore like to congratulate the authors with this elegant trial that addresses such an important issue.

Table 1: Validation of FLI against ultrasound-defined steatosis stratified by BMI

	All n = 5756	Lean n = 1667	Overweight n = 2727	Obese n = 1362
AUROC FLI	0.80 (0.79 – 0.81)	0.75 (0.72 – 0.79)	0.70 (0.68 – 0.72)	0.69 (0.66 – 0.72)
FLI 30				
Sensitivity	91.4	52.2	92.0	99.9
Specificity	46.9	83.1	29.0	2.1
FLI 60				
Sensitivity	62.8	7.7	47.0	92.6
Specificity	79.0	98.8	77.2	24.6

AUROC of FLI (with 95% confidence interval) was derived using the continuous measure. Stratification was carried out using the BMI cut-points 25kg/m² and 30kg/m². The total population comprises also participants with secondary causes for steatosis. Results on NAFLD instead of all-cause steatosis are similar (data not shown).
Abbreviations: AUROC – area under the receiver operator characteristic; BMI – body mass index; FLI – fatty liver index; NAFLD – non-alcoholic fatty liver disease.

Part V

GUT MICROBIOME & HEPATIC STEATOSIS

Chapter 10

Microbiomics, metabolomics, predicted metagenomics and hepatic steatosis in a population-based study of 1355 adults

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Abstract

Introduction Previous small studies have appraised the gut microbiome (GM) in steatosis, but large-scale studies are lacking. We studied the association of GM diversity and composition, plasma metabolites, predicted functional metagenomics and steatosis.

Methods This is a cross-sectional analysis of the prospective population-based Rotterdam Study. We used 16Sribosomal-RNA gene sequencing and determined taxonomy using the Silva-reference database. Alpha-and beta-diversity were calculated using Shannon-index and Bray-Curtis dissimilarities. Differences were tested across steatosis using PerMANOVA. Hepatic steatosis was diagnosed by ultrasonography. We subsequently selected genera using regularised regression. The functional metagenome was predicted based on the GM using KEGG-pathways. Serum metabolomics were assessed using high-throughput proton nuclear magnetic resonance. All analyses were adjusted for age, sex, BMI, alcohol, diet, and proton-pump inhibitors.

Results We included 1355 participants of which 472 had steatosis. Alpha-diversity was lower in steatosis (P=1.1·10^{-9}) and beta-diversity varied across steatosis strata (P=0.001). Lasso selected 37 genera of which three remained significantly associated after adjustment (*Coprococcus3:* β=–65; *Ruminococcus Gauvreauiigroup*: β=62; and *Ruminococcus Gnavusgroup*: β=45, *Q*-value =0.037). Predicted metagenome analyses revealed that pathways of secondary bile-acid synthesis and biotin metabolism were present and D-alanine metabolism was absent in steatosis. Metabolic profiles showed positive associations for aromatic-and branched chain amino acids and glycoprotein acetyls with steatosis and *R. Gnavusgroup*, whereas these metabolites were inversely associated with alpha-diversity and *Coprococcus3*.

Conclusion We confirmed, for the first time on a large-scale, the lower microbial diversity and association of *Coprococcus* and *Ruminococcus Gnavus* with steatosis. We additionally showed that steatosis and alpha-diversity share –opposite– metabolic profiles.

Introduction

Hepatic steatosis is the most common liver disease worldwide[280] and its presence is implicated in the development of advanced liver disease as well as cardiovascular disease.[182] Indeed, at present hepatic cirrhosis due to alcoholic and non-alcoholic fatty liver disease together is the number one indication for liver transplantation,[399] and its co-incidence with other liver diseases, such as viral hepatitis, worsens disease severity.[400] Aetiology of hepatic steatosis is multifactorial, but unhealthy lifestyle habits, such as excessive alcohol consumption in alcoholic fatty liver disease and an unhealthy diet in non-alcoholic fatty liver disease (NAFLD), are key in the development of this disease.[17] Aside from these well-known risk factors, there is an increasing interest in the contribution of the gut microbiome to steatogenesis.[401] The gut microbiome refers to the collective genomic content of microbiota in the intestinal tract consisting of over 100 trillion microbes, being mainly bacteria (>99%).[402] These gut microbes are crucial for life as they perform essential functions such as energy harvest, host nutrition, and immunomodulation.[403]

Hepatic steatosis is closely related to obesity.[6] There is a myriad of studies that demonstrate the importance of the gut microbiome in the development and progression of obesity.[404] In addition, Le Roy and colleagues have shown that diet-induced steatosis in mice was directly dependent on the gut microbial composition. Furthermore, the authors showed that the steatosis phenotype was transmissible via the gut microbiome in germ-free mice.[405] Subsequent studies in humans tried to link the presence of steatosis with specific bacteria, but these endeavours to define a so-called 'core microbiome' in steatosis have been highly inconsistent. This inconsistency is possibly due to the limited sample size, heterogeneous study populations (paediatric vs adult), different phenotypes (simple steatosis vs steatofibrosis), and diverse analytical approaches of these studies.[406-417] Importantly, most of these studies did not correct for important confounders such as diet, body mass index (BMI), and alcohol consumption, making it hard to draw inferences on the independent association between microbes and steatosis.

The close connection between the gut microbiome and the liver is referred to as the 'gut-liver axis' – as microbiome-derived metabolites and other bacterial products can easily reach the liver via the portal vein.[401] Zhu et al. for instance proposed that ethanol-producing bacteria in the gut microbiome of children contributed to the pathogenesis of steatosis, mimicking alcoholic fatty liver disease.[409] In addition, it has been shown that gut microbiota are the major driver of circulating lipid levels such as triglycerides and other fatty acids that are known risk factors for steatosis.[6,418]

In this large epidemiologic cohort study, we aimed to characterize the composition of the gut microbiome in individuals with steatosis. In addition, we examined predicted functional pathways of the gut microbiome in steatosis and we assessed the metabolic profiles of steatosis and the gut microbiome by means of high-throughput proton Nuclear Magnetic Resonance (NMR) metabolomics.

Subjects and methods

Study Population

This study is embedded in the Rotterdam Study, a prospective cohort study in Ommoord, a suburb in the city of Rotterdam, the Netherlands. A detailed description on study design and rationale can be found in a recent overview paper.[248] This study is cross-sectional, all analyses are performed in cohort III visit 2 (May 2012 to June 2014), in which participants were asked to collect faecal samples for microbiome analyses. Inclusion criteria for participation in the current study were availability of gut microbiome and ultrasound data and age of 45 years or above at initial visit. The Rotterdam Study has been conducted in accordance with the Declaration of Helsinki and has been approved by the institutional review board (Medical Ethics Committee) of the Erasmus Medical Centre and by the review board of The Netherlands Ministry of Health, Welfare and Sports. All participants provided written informed consent.

Liver imaging

Abdominal ultrasound was performed by an experienced nurse ultrasonographist (PvW) using the Hitachi HI VISION 900. The definition of hepatic steatosis was overt hyperechogeneity of the liver parenchyma as compared to that of the kidney.[104] We dichotomized hepatic steatosis, because of the poor performance for the detection of mild steatosis and known subjective ultrasonographic grading of steatosis.[419] We analysed a subset of participants in which the steatosis was unlikely to be secondary, i.e. ruling out excessive alcohol consumption, steatogenic drugs, and viral hepatitis. The remaining group was eligible for the diagnosis non-alcoholic fatty liver disease (NAFLD). In addition, liver stiffness measurements (LSM) were carried out in all participants using transient elastography (Fibroscan®, EchoSens, Paris, France). Those with steatosis and elevated LSM (of 8 kilopascals [kPa] or higher) were assumed to have more advanced fatty liver disease, i.e. (non-alcoholic) steatofibrosis (NASF).[54] The use and reliability criteria of transient elastography have been published in more detail previously.[78]

Faecal samples

Participants were instructed to collect faecal samples in sterile tubes at home in a standardized fashion. Participants were also asked to disclose recent antibiotic use (past twelve months), journeys abroad (past month), and probiotic use (past three months). Samples were then returned to the Erasmus MC through postal mail. Upon arrival, samples were recorded and stored at –20°C. An aliquot of approximately 300mg was homogenized in

stool stabilizing buffer according to the manufacturer's protocol (Arrow Stool DNA; Isogen Life Science, De Meern, the Netherlands). Homogenized samples were bead in lysing Matrix B tubes containing 0.1mm silica beads (MP Biomedicals®, LLC, Bio Connect Life Sciences BV, Huissen, the Netherlands). Samples were then centrifuged and the supernatant was subjected to automated DNA isolation according to the manufacturer's protocol (Arrow; DiaSorin S.P.A., Saluggia, Italy). Isolated DNA was then stored at –20°C.

Metadata

All covariates used in this study were derived by an extensive home interview, by drawing fasting blood samples, by automated linkage with the local pharmacy and by completion of an external-validated 389-item food frequency questionnaire. Detailed information on these covariates can be found in the *Supplementary Methods*.

Gut microbiome data set generation

For a detailed description of the gut microbiome dataset generation, we refer to the recent paper of *Radjabzadeh et al*.[420] In short, 16S ribosomal RNA (rRNA) gene amplification of variable regions 3 and 4 was used to sequence all samples by the Illumina MiSeq® platform v3. The sequence data were rarefied at 10,000 reads per sample and reads were clustered into operational taxonomic units (OTU) with 97% similarity. These OTUs were classified using the RDP classifier (version 2.12)[421] and Silva rRNA database project® (v128).[422] It has been demonstrated that quality-filtering 16S amplicon sequence reads can greatly improve accuracy of microbial community analysis, we therefore used a cut-off threshold excluding OTUs with a total read count of less than 0.005% of the total reads.[423] Alpha diversity was calculated at this step. OTUs presented in less than 1% of the samples were also removed in order to harmonize the dataset.

Functional metagenome

In order to predict bacterial function in steatosis, we used Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt (v.1.1.0)) algorithm.[424] We then used HUMAnN2 (v0.99) to identify Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways.

Metabolite assessment

All metabolites were quantified using EDTA plasma samples by high-throughput proton NMR metabolomics (Nightingale Health, Helsinki, Finland). Details on this methodology

have been published previously.[425] We quantified metabolic measures, including various sizes of lipoproteins, serum lipids, various fatty acids, branched-chain amino-acids (BCAAs), aromatic amino acids (AAAs) and glycolysis-related metabolites. In this study we analysed 146 metabolic measures.

Statistical analyses

Imputation analyses and participant characteristics

We first excluded participants of whom sequencing failed due to technical issues and those with duplicate samples, samples with unknown travel time or over 3 days of travel time between collection and analysis, and participants with recent antibiotic use. Thereafter, we excluded participants that had no ultrasound data available or that had unreliable food frequency questionnaires (i.e. <500 or >7500 kilocalories/day). Missing values in the meta-data were imputed using multiple imputation under the fully conditioned specification to reduce bias due to missing data. We created 50 imputed datasets using the R Package mice. The results from these analyses were pooled using Rubin's rules in order to take into account the added uncertainty due to the missing of data. The *Supplementary Method* contains a detailed description of the imputation process and references of the statistical methods described in this method section.
Characteristics of the study population after imputation were described across steatosis strata. Differences were appraised using analyses of variance (ANOVA) for approximately normally distributed variables, Kruskall-Wallis tests for continuous non-normally distributed data, and Chi-squared tests for categorical data.

Description of the microbiome

The median relative abundance of all phyla was evaluated. In addition, we examined the Firmicutes-to-Bacteroidetes-ratio (FBR). Richness and alpha-diversity, as determined by Shannon and Inverse-Simpson indices, were calculated. Difference in alpha-diversity between steatosis strata was visualized using violin plots and evaluated using Wilcoxon rank sum test with continuity correction. The beta-diversity was studied using principal coordinate analysis applied to Bray-Curtis dissimilarity using the function "capscale" from the R package vegan on genus level.[426] Sample clustering of genera was tested for association with steatosis, using permutational multivariate analysis of variance (PerMANOVA, n=999) on Bray-Curtis dissimilarities. We evaluated the percentage of explained variation in the gut microbiome variability by steatosis (r^2= 0.0030) and NAFLD (r^2= 0.0029).
In order to investigate the association between steatosis and all genera simultaneously, logistic regression was performed. To prevent overfitting regression coefficients of the gen-

era were regularized using lasso.[427] This technique extends ordinary regression by imposing penalty terms on the regression coefficients resulting in shrinkage towards zero, such that coefficients of covariates that do not sufficiently contribute to the model are set to zero. To additionally take into account the compositional nature of the microbiome we followed the approach of Lin et al., which is implemented in the R package zeroSum.[428,429] For this selection of genera, relative abundances were log-transformed (log10[1+genus]) and the model was adjusted for batch-effect and travel time of samples. The degree of penalization was determined by 10-fold cross-validation, which was repeated 20 times in each imputed dataset to average out effects of random sampling. The degree of penalization resulting in the on average smallest cross-validation error per imputed dataset was then applied in the analysis of that respective dataset. Genera with coefficients different from zero in at least half of the imputed datasets were selected as predictor variables in the final logistic (unrestricted) regression model.

To take into account potential confounding we considered the following covariates (based on previous studies)[6] for additional adjustment of the logistic regression for steatosis: age, sex, BMI, education level, energy intake, alcohol units, smoking status, dietary quality scores, physical activity, diabetes, proton-pump inhibitor (PPI) use, serum triglycerides and high-density lipoprotein (HDL) cholesterol. The selection of covariates on the basis of previous studies in which these covariates were associated with steatosis.[6] Due to the limited number of steatosis cases, however, we were only able to include a subset that we expected to have the highest confounding potential: age, sex, BMI, technical variables, alcohol intake, dietary quality scores and PPI-use. This selection was based on pathophysiological background knowledge. Models were evaluated for multicollinearity using the variable inflation factor. Predicted probabilities for steatosis from this model were plotted against the relative abundance of selected significant bacteria to facilitate the interpretation of the results.

Predicted functional metagenomics

Predicted metagenomics pathways were tested for significance by performing logarithmic linear discriminant analysis (LDA) in linear discriminant analysis effect size with the significance level of 0.05 and the logarithmic LDA score threshold equal to 2.0.

Metabolic profiles

The metabolic profile (as exposure) was derived using linear or logistic regression analyses for steatosis, alpha-diversity, and significant genera while adjusting for abovementioned covariates plus the technical variable: metabolic batch. The adjusted effect estimates were plotted in a heatmap. We dealt with zeros in metabolic measures by adding half of the

minimum non-zero values to all values, we then transformed (log10) and standardized all metabolic measures (n=146). As most of the metabolites we studied concern lipid-related measures, sensitivity analyses excluding participants with lipid-lowering medication was performed.

NAFLD analyses

We performed a subgroup analysis on the associations between the microbiome, metabolites, and predicted metagenomics, excluding all participants with potential secondary causes for steatosis (i.e. NAFLD and NASF). Although we have included alcohol intake as covariate in the multivariable models, we would like to see if the gut microbiome, metabolic profile and predicted functional metagenomic in NAFLD is different from the overall steatosis group, or whether similar pathways and bacteria are involved.

Statistical Significance

Adjusted *P*-values (referred to as *Q*-values) were calculated using the Benjamini & Hochberg correction taking together all genera or metabolites within the same model,[430] to account for the inflated type I error that arises due to multiple testing. All computations were done using R version 3.5.2 (R Core Team [2018]), the package Vegan version 2.5.2 [2018], and the package ZeroSum version 1.1.1.

Results

Participant Characteristics

The flowchart of the study is depicted in Figure 1. In total, 1739 out of 2440 individuals participated (71.3%). The final study population comprised 1355 participants. Population characteristics (Supplementary Table 1) were as follows: mean age was 62.4 (5.9) years, mean BMI was 27.4 (4.5), 57.8% was female, 34.8% had steatosis, and the majority was of European descent (96.7%). Characteristics of the steatosis strata are presented in Table 1.

Description of the microbiome

We identified 11 different phyla in our dataset. The relative abundance of these phyla is illustrated in *Figure 2* and median relative abundances are presented in *Supplementary Table 2*. The most prevalent phylum was *Firmicutes* (81.2%), followed by *Bacteroidetes* (8.6%), *Actinobacteria* (2.7%), and *Proteobacteria* (1.4%). Phyla *Verrucomicrobia*, *Tenericutes*,

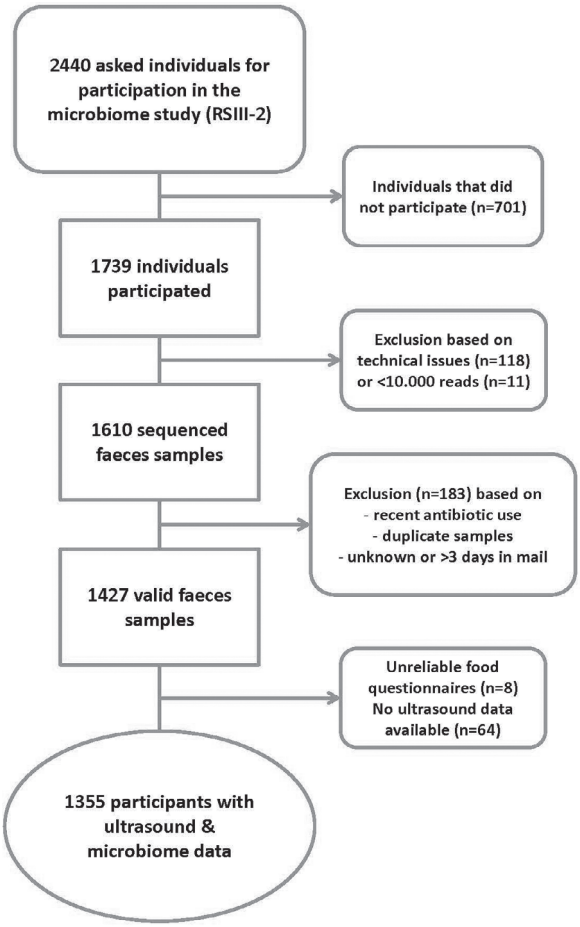


Figure 1: Flowchart of the included study population

Proteobacteria and *Euryarchaeota* were less prevalent in steatosis. FBR was not different across steatosis strata (*Figure 3*; *P*=0.335). Richness and alpha-diversity were significantly lower in steatosis (*Figure 3, P-values* $\leq 2.0 \cdot 10^{-6}$). The principle coordinate analyses plots are depicted in *Supplementary Figure 1*. Although in the plots the variation between the two groups was not clearly visible, PerMANOVA showed that sample clustering was significantly different for steatosis (P<0.001), taking into account the sequentially added technical variables, age, BMI, and sex. Lasso regularized regression identified 37 genera. These genera are listed in *Supplementary Table 3* with their median relative abundances. In subsequent multivariable logistic regression analyses for steatosis including these 37 genera; three of them had a statistically significant association (*Table 2*). *Coprococcus3* was inversely associated with steatosis, whereas *Ruminococcus Gauvreauiigroup* and *Ruminococcus Gnavusgroup* were positively associated with steatosis. Relative abundances were

Table 1: Population Characteristics

	No steatosis n=883 (65.2%)	Steatosis n=472 (34.8%)	*P*-value*
Age (years)	62.19 (5.92)	62.69 (5.73)	0.136
Female (%)	60.9	51.9	0.002
European (%)	96.1	97.9	0.191
Education Level (%)			0.095
Low	40.1	43.3	
Intermediate	27.3	29.9	
High	32.6	26.9	
Smoking status (%)			0.030
Never	36.7	31.8	
Past or Current	63.3	68.2	
Alcohol (units/day)	0.81 [0.16, 1.92]	0.91 [0.20, 2.18]	0.514
Physical Activity (METh/wk)	50.85 [22.85, 84.85]	41.45 [17.50, 76.59]	0.019
Energy intake (kcal/day)	2276 [1906, 2725]	2214 [1831, 2738]	0.972
Dietary Quality Score (0–15)	7 [6, 8]	7 [6, 8]	0.047
BMI (kg/m^2)	26.12 (3.90)	29.86 (4.49)	<0.001
Waist-to-hip ratio	0.86 [0.80, 0.93]	0.94 [0.88, 1.00]	<0.001
HOMA-IR	2.10 [1.49, 3.01]	3.94 [2.62, 5.95]	<0.001
Total Cholesterol (mmol/L)	5.63 (1.10)	5.49 (1.12)	0.035
HDL-Cholesterol	1.60 (0.47)	1.33 (0.37)	<0.001
Triglycerides	1.16 [0.90, 1.55]	1.58 [1.19, 2.08]	<0.001
Diabetes Mellitus (%)	5.6	18.2	<0.001
Hypertension (%)	52.3	69.2	<0.001
Liver stiffness measurements (kPa)†	4.40 [3.60, 5.40]	4.90 [3.90, 6.10]	<0.001
LSM≥8.0 kPa n (%)	17 (1.9)	32 (6.8)	<0.001
Lipid-lowering drug use	24.2	32.8	<0.001
Proton-pump inhibitor use	15.5	21.8	<0.001
Microbiome batch #0/1	78.8 / 21.2	78.8 / 21.2	1.000
Time in mail (%)			0.777
1 day	64.8	66.3	
2 days	29.8	28.0	
3 days	5.4	5.7	
Secondary causes for steatosis n (%)‡	145 (20.1)	83 (22.7)	0.378
Viral hepatitis	5 (0.6)	3 (0.6)	1.000
Excessive alcohol use	128 (17.6)	74 (20.2)	0.328
Steatogenic drug use	14 (1.6)	7 (1.5)	1.000
NAFLD§ n (%)	-	283 (60.0)	-

Data are presented as median value (P25-P75), as mean value (SD) or as percentage.*P-value is assessed using ANOVA, Kruskall-Wallis or Chi-squared tests. †n=1195 reliable measurements (n=783 for no steatosis and n=412 for steatosis), ‡data for n=1085 participants, § data for n=857 participants.

Abbreviations: HOMA-IR: homeostasis model of insulin resistance; kcal: kilocalories; kPa: kilopascals; LSM: liver stiffness measurement; METeqh/wk: metabolic equivalent task hours per week; NAFLD: non-alcoholic fatty liver disease

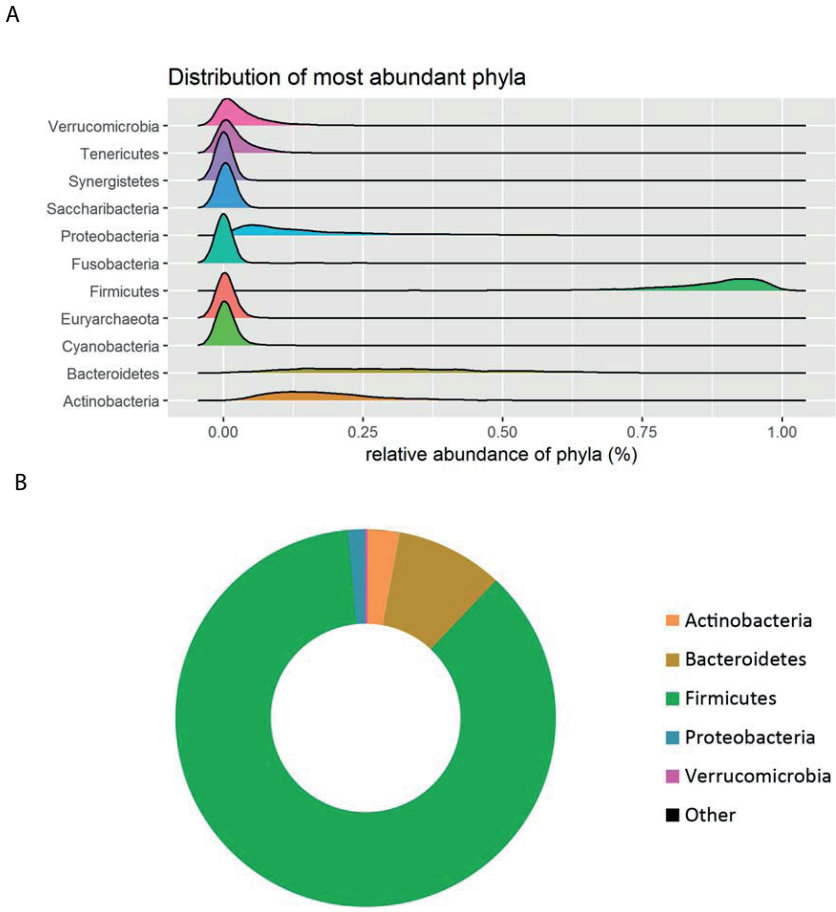


Figure 2: Relative abundance of phyla in the total population

A) This figure depicts the distribution of the relative abundance per phylum (using squared root transformation). The relative abundance of all phyla together add up to one. On the y-axis, the relative abundance is shown. On the x-axis the distribution of the relative abundance in the total population is shown. B) This donut plot depicts the median relative abundance per phylum in percentages. The relative abundance of all phyla here add up to 100%. Please see Supplementary Table 2 for the absolute median percentages and the P25-P75.

plotted across steatosis strata (*Supplementary Figure 2A*). There was no evidence for sex differences nor for interaction of these genera with steatosis. The predicted probability of steatosis across relative abundances of these genera, for exemplary men and women with fixed BMI values (while fixing all other covariates in the model to reference values) were plotted (*Supplementary Figure* 3).

Figure 3: Visualization of richness and alpha-diversity and FBR using violin plots across steatosis strata.

Violin plots of the distribution of A-B) alpha-diversity as assessed by the Shannon-index and Inverse Simpson, C) microbial richness, and D) log-transformed Firmicutes-to-Bacteroidetes-ratio by steatosis strata. Violin plots have additional value to boxplots since they show the full distribution of the data, whereas boxplots only show a summary. But the basic idea is similar. The statistical difference between strata was assessed using Wilcoxon rank sum test with continuity correction and was as follows: A) Shannon: P= 1.1·10–9; B) Inverse Simpson: P= 2.0·10–6; C) Microbial Richness: P=7.3·10–9 and; D) Firmicutes-to-Bacteroidetes-ratio: P=0.335.
Abbreviations: InvSimpson: Inverse Simpson; logFBratio: log-transformed Firmicutes-to-Bacteroidetes-ratio.

Predicted functional metagenome

Predicted analyses revealed four pathways that were present and two that were absent in steatosis (*Figure 4*). The most present pathways were that of "Secondary bile acid biosynthesis" (ko00121) and that of "biotin (vitamin H or B7) metabolism" and acetyl-CoA carboxylase (ko00780). Another bile acid-related pathway "Taurine and hypotaurine" (ko00430) was absent in steatosis as well as "D-alanine metabolism" (ko00473).

Metabolic profiles

Figure 5 A-E shows the independent metabolic profiles of steatosis, alpha diversity, and the three significant genera. The top-hits for steatosis were BCAAs isoleucine and leucine. Both were independently associated with higher steatosis prevalence (*Figure 5E*). In addition, valine (also a BCAA), alanine (a non-essential amino-acid), and the AAAs tyrosine and phenylalanine were significantly associated with higher steatosis prevalence. The acute

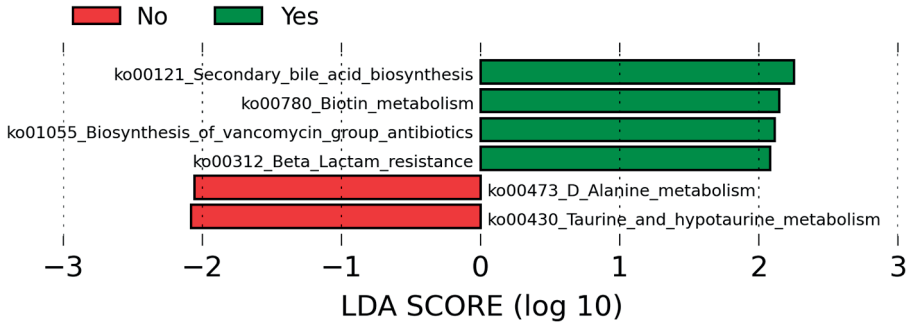


Figure 4: Predicted metagenomics pathways for steatosis.
This figure depicts six metabolic pathways that were either present (Yes in green) or absent in steatosis (No in red). The predicted metagenomics are based on 16S rRNA gene sequencing data from our study cohort. Linear discriminant effect size was calculated and shown on the x-axis based on PICRUSt. A metabolic pathway was considered significant if the false discovery rate was lower than 0.05.

phase reaction marker glycoprotein acetyls was positively associated with steatosis (*Figure 5E*). Furthermore, multiple significant positive associations were detected for very low-density lipoproteins (VLDL) particles of all sizes (*Figure 5A*), saturated fatty acids, mono-unsaturated fatty acids and total triglycerides (*Figure 5D*). Positive associations were found for large and extra-large HDL particles, whereas there was an inverse association for small HDL particles (*Figure 5C*). Additionally, effect estimates for steatofibrosis were plotted, and we found that glucose and lactate were significantly associated with both steatosis and steatofibrosis. Glutamine had a significant inverse association with steatofibrosis, but not with steatosis (*Figure 5E*).

The metabolic profiles of richness and alpha-diversity mirrored those of steatosis, having the –often significant– opposite direction with metabolites (*Figure 5*). As for the significant genera, the only significant association found was that of *Ruminococcus Gnavusgroup* with glycoprotein acetyls (*Figure 5E*).

The sensitivity analysis of metabolic profiles, excluding participants with lipid-lowering medication, showed attenuated but largely similar results (*Supplementary Figure 4*).

NAFLD analyses

The subgroup NAFLD included 857 participants of which 283 (33%) had NAFLD. Results on differences in relative abundance of phyla, FBR, and alpha-diversity resembled across NAFLD strata resembled steatosis (*Supplementary Table 4* and *Supplementary Figure 5*). Beta-diversity was also significantly different across NAFLD strata (sequentially adjusted PerMANOVA: P=0.003). Similar significant results were found for multivariable regression analysis in NAFLD (*Supplementary Table 5*). Relative abundance of these genera associated with NAFLD and NASF were plotted in *Supplementary Figure 2C* and *2D*. Relative

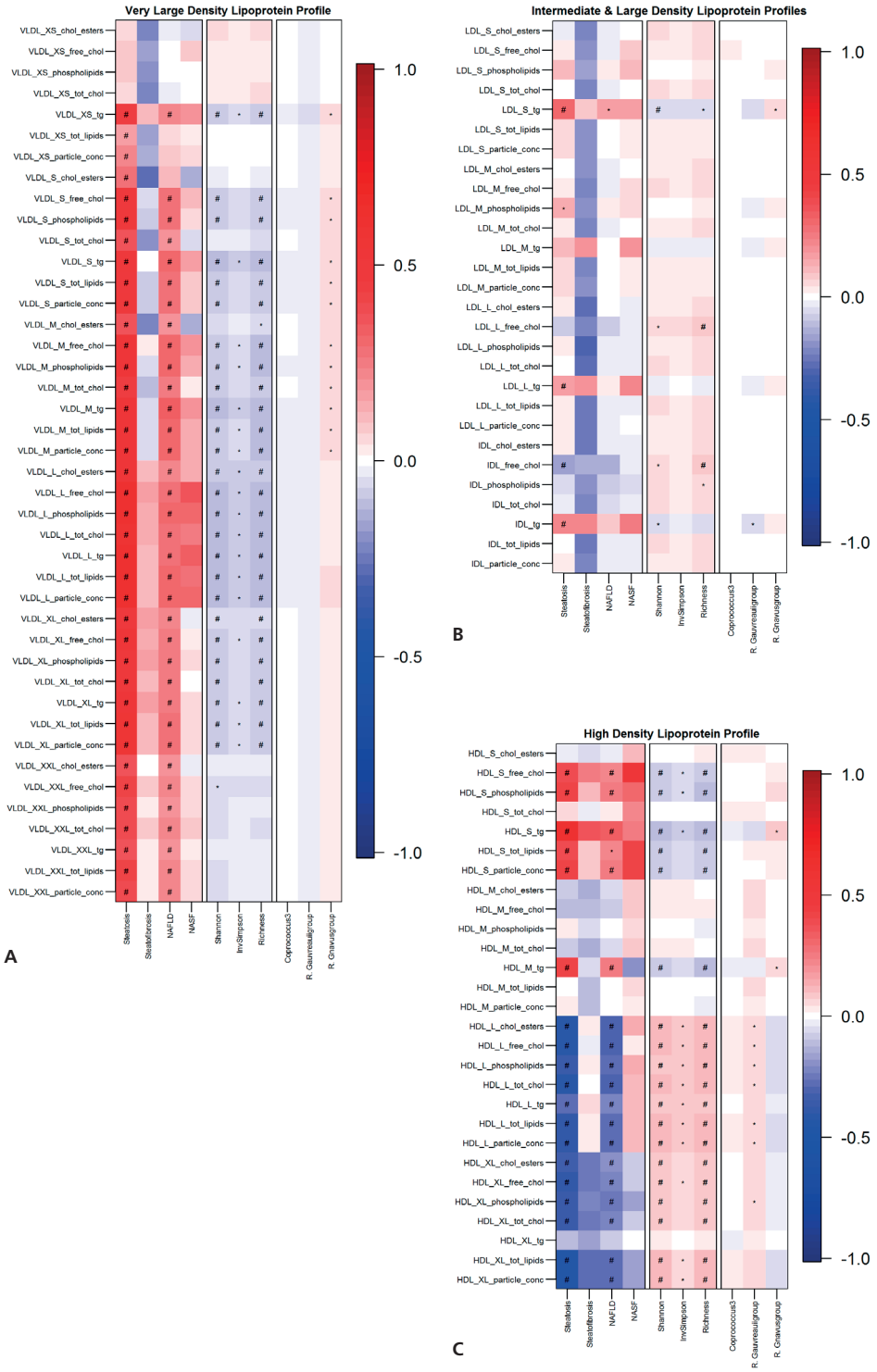
Table 2: Multivariable logistic regression analysis of lasso-selected genera with steatosis as dependent variable

Phylum			Variables	**β**	**95%CI**	**P-value**	**Q-value**
Class	Order	Family					
			intercept	−5.48	-	-	-
			batch 0	ref			
			batch 1	−0.002	−0.33, 0.33	0.992	-
			Time in mail	−0.02	−0.24, 0.21	0.884	-
			age (per year)	0.004	−0.02, 0.03	0.708	-
			sex (women)	−0.35	−0.63, −0.07	**0.013**	-
			BMI (per kg/m^2)	0.21	0.18, 0.25	**<0.001**	-
			alcohol (per unit)	0.11	0.01, 0.21	**0.025**	-
			PPI use	0.33	−0.02, 0.67	0.060	-
			dietary quality - score	−0.03	−0.11, 0.06	0.530	-
Actinobacteria							
Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	*Bifidobacterium*	−2.87	−12.33, 6.59	0.552	0.796
Coriobacteriia	Coriobacteriales	Coriobacteriaceae	*Collinsella*	31.36	−11.09, 73.81	0.148	0.399
Bacteroidetes							
Bacteroidia	Bacteroidales	Bacteroidaceae	*Bacteroides*	−1.07	−8.30, 6.16	0.771	0.839
		BacteroidalesS247gr	*Unknowngenus*	−17.76	−51.24, 15.71	0.298	0.537
		Prevotellaceae	*Prevotella9*	−2.12	−9.87, 5.62	0.591	0.810
		Rikenellaceae	*Alistipes*	−2.24	−28.27, 23.79	0.866	0.871
Firmicutes							
Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	−1.91	−20.13, 16.31	0.837	0.871
		Streptococcaceae	*Streptococcus*	−5.10	−13.44, 3.24	0.231	0.494
		Christensenellaceae	*ChristensenellaceaeR7gr*	−9.89	−23.39, 3.62	0.151	0.399
		Clostridiaceae	*Clostridiumsensustricto1*	−3.07	−21.01, 14.87	0.737	0.839
			Blautia	−2.53	−9.79, 4.74	0.495	0.796

			Butyrivibrio	−7.13	−29.88, 15.62	0.539	0.796
			Coprococcus3	**−65.26**	**−106.78, −23.74**	**0.002**	**0.037**
			Dorea	−4.55	−25.34, 16.23	0.668	0.823
			Fusicatenibacter	−4.81	−20.98, 11.35	0.559	0.796
			Lachnoclostridium	−4.65	−31.85, 22.56	0.738	0.839
		Lachnospiraceae	*LachnospiraceaeND3007gr*	−73.91	−140.92, −6.90	0.031	0.227
			Eligensgr	−18.68	−54.40, 17.03	0.305	0.537
			R. Gauvreauiigr	**61.72**	**24.90, 98.54**	**0.001**	**0.037**
			R. Gnavusgr	**44.68**	**15.23, 74.14**	**0.003**	**0.037**
			Halliigr	−2.19	−16.08, 11.71	0.758	0.839
Clostridia	Clostridiales		*Rectalegr*	1.93	−6.83, 10.68	0.666	0.823
		Peptostreptococcaceae	*Romboutsia*	−21.16	−44.15, 1.84	0.071	0.377
			Faecalibacterium	−6.28	−13.75, 1.19	0.100	0.399
			RuminococcaceaeNK4A214gr	−19.80	−73.21, 33.60	0.467	0.786
			RuminococcaceaeUCG002	−15.73	−34.73, 3.27	0.104	0.399
		Ruminococcaceae	*RuminococcaceaeUCG014*	−6.84	−15.74, 2.07	0.132	0.399
			Ruminococcus1	−5.92	−29.58, 17.74	0.624	0.823
			Ruminococcus2	15.99	3.03, 28.95	0.016	0.144
			Subdoligranulum	−7.10	−18.95, 4.75	0.240	0.494
			Coprostanoligenesgr	13.35	−6.10, 32.79	0.178	0.440
		Erysipelotrichaceae	*Catenibacterium*	−2.36	−30.88, 26.16	0.871	0.871
		Acidaminococcaceae	*Phascolarctobacterium*	19.20	−6.53, 44.92	0.144	0.399
Negativicutes	Selenomonadales	Veillonelaceae	*Megamonas*	20.95	−18.77, 60.67	0.301	0.537
Proteobacteria							
	Aeromonadales	Succinivibrionaceae	*Succinivibrio*	21.54	−7.41, 50.50	0.145	0.399
Gammaproteo-bacteria			*EscherichiaShigella*	−6.54	−12.86, −0.23	0.042	0.261
	Enterobacteriales	Enterobacteriaceae	*Klebsiella*	−14.68	−38.38, 9.02	0.225	0.494

Logistic regression analyses with steatosis as dependent variable. All genera (log10(1+genus)) are analyzed simultaneously. Results are given as regression coefficient (95%CI), nominal *P*-value and *Q*-value-corrected for multiple comparisons using Benjamini & Hochberg.

Abbreviations: BMI: body mass index; PPI: proton-pump inhibitor.


Very Large Density Lipoprotein Profile
VLDL_XS_chol_esters
VLDL_XS_free_chol
VLDL_XS_phospholipids
VLDL_XS_tot_chol
VLDL_XS_tg
VLDL_XS_tot_lipids
VLDL_XS_particle_conc
VLDL_S_chol_esters
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VLDL_XXL_free_chol
VLDL_XXL_phospholipids
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VLDL_XXL_tot_lipids
VLDL_XXL_particle_conc
Steatosis
Steatofibrosis
NAFLD
NASF
Shannon
InvSimpson
Richness
Coprococcus3
R. Gauvreauiigroup
R. Gnavusgroup
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Intermediate & Large Density Lipoprotein Profiles
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LDL_S_free_chol
LDL_S_phospholipids
LDL_S_tot_chol
LDL_S_tg
LDL_S_tot_lipids
LDL_S_particle_conc
LDL_M_chol_esters
LDL_M_free_chol
LDL_M_phospholipids
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Steatosis
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NAFLD
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InvSimpson
Richness
Coprococcus3
R. Gauvreauiigroup
R. Gnavusgroup
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B
High Density Lipoprotein Profile
HDL_S_chol_esters
HDL_S_free_chol
HDL_S_phospholipids
HDL_S_tot_chol
HDL_S_tg
HDL_S_tot_lipids
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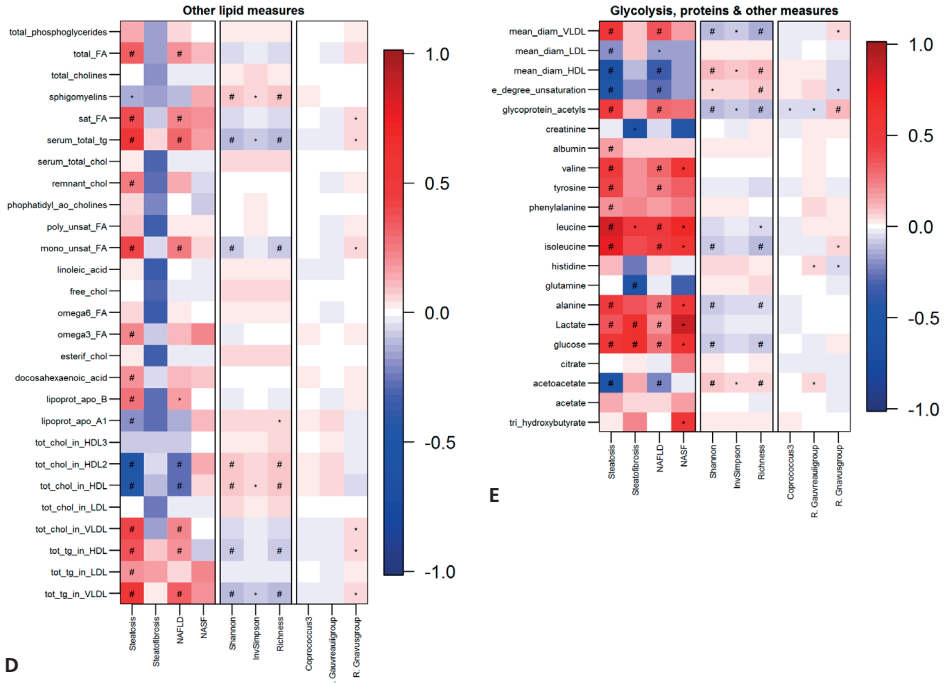


Figure 5: Metabolic profiles of steatosis, alpha-diversity and lasso-selected genera.
The colors in columns represent the standardized betas of the metabolites (as exposure) with steatosis, steatofibrosis, NAFLD, NASF, alpha-diversity and the significant genera (as various outcomes) in separate multivariable logistic regression models. The betas were adjusted for covariates: sex†‡, age†‡, technical variables†‡, BMI†‡, Dietary Quality-Score, PPI use, and alcohol units†. We corrected for multiple testing using Benjamini & Hochberg, significant Q-values are depicted by #, nominally significant values by *. †Steatofibrosis (n=32) and ‡NASF (n=14) were adjusted for less covariates in the multivariable models because of risk of overfitting.
Abbreviations: BMI: body mass index; chol: cholesterol; conc: concentration; diam: diameter; FA: fatty acids; HDL: high-density lipoprotein; IDL: intermediate density lipoprotein; InvSimpson: Inverse Simpson; L: large; LDL: low-density lipoprotein; M: medium; NAFLD: non-alcoholic fatty liver disease; NASF: non-alcoholic steatofibrosis; PPI: proton-pump inhibitor; S: small; sat: saturated; tg: triglycerides; tot: total; VLDL: very low density lipoprotein; (X)XL: (extra) extra large

abundance of *Coprococcus3* was markedly lower in NASF as compared to simple steatosis. Predicted metagenomics in NAFLD largely overlapped the results for steatosis for "secondary bile acid biosynthesis" (ko00121), "biotin metabolism" (ko00780) and "D-alanine metabolism" (ko00473), as depicted in *Supplementary Figure 6*. Lastly, the metabolic profile for NAFLD was almost identical to that of steatosis, as was that of NASF compared to steatofibrosis (*Figure 5*).

Discussion

Over the last decade, the role of the gut microbiome in human health has gained global interest. The liver, which receives approximately 70 percent of the blood outflow from the gut, is a preeminent example of the cross talk between our other genome –the gut microbiome– and the rest of the body. There have been many studies that appraised the gut microbiome in steatosis.[406-417] However, results were rather inconsistent, which is not surprising given the relatively small sample size of the studies (maximum n=156), heterogeneous study designs and the fact that the gut microbiome is highly variable between individuals. With our study we had the unique possibility to examine the diversity and composition of the gut microbiome and its associated metabolites and predicted metagenomics in the context of (non-alcoholic) steatosis and its advanced subtype steatofibrosis in a large population-based cohort of 1355 adults.

We have made several interesting observations. First, we confirmed that the gut microbiome of individuals with steatosis was less diverse than without steatosis. Second, the composition of the steatotic gut microbiome was significantly different from that of the controls. Thirty-seven genera contributed to this difference of which *Coprococcus3*, *Ruminococcus Gauvreauiigroup*, and *Ruminococcus Gnavusgroup* remained independently associated with steatosis after adjustment for confounders, such as BMI and diet, and multiple testing. Third, the composition of the gut microbiome in steatosis was predicted to contribute to secondary bile acid synthesis and biotin metabolism and to counteract D-alanine metabolism. Fourth, the metabolomic top-hits for steatosis included higher BCAAs, AAAs, glycoprotein acetyls and a detrimental lipid profile. Interestingly, incremental alpha-diversity was associated with lower BCAAs, glycoprotein acetyls and a favourable lipid profile, and hence had an opposite metabolic profile than steatosis.

Steatosis and obesity are tightly linked, especially in the context of NAFLD.[17] It has been proposed that a high ratio of Firmicutes-to-Bacteroidetes contributes to higher energy harvest in obese individuals.[431] In our study there was an eminent median relative abundance of *Firmicutes* in the total population, but no difference in FBR across steatosis or NAFLD strata. However, in line with our results, other reports could not always confirm this finding. Therefore it is unlikely that this ratio is solely responsible for obesogenic shifts in the microbiome.[432]

At genus level, lasso penalty selected 37 genera from four different phyla that were associated with steatosis. Three of these bacteria, all from the *Lachnospiraceae* family, remained significantly associated with steatosis. The anaerobic *Coprococcus3* was associated with lower steatosis prevalence, which is consistent with previous reports on the gut microbiome and hepatic steatosis.[409,415-417,433,434] Looking at the unadjusted relative abundance of *Coprococcus3*, this was markedly lower as well, particularly in NASF. Indeed, an elegant study of Hoyles et al. found that the genus Coprococcus was associated with less hepatocyte

ballooning as assessed by liver biopsy.[417] And although there was no significant association with the NMR metabolomics for this genus in our study, *Coprococcus3* was nominally significantly associated with lower glycoprotein acetyls. Indeed, the genus Coprococcus has previously been described to be lower in inflammatory bowel disease, confirming its anti-inflammatory profile.[435] Interestingly, other studies revealed that Coprococcus was associated with lower fasting glucose[433] and higher microbial richness.[436]

The two other significant genera *Ruminococcus Gauvreauiigrou*p and *Ruminococcus Gnavusgroup* are –although within the same family as *Coprococcus3*– associated with higher prevalence of steatosis. *R. Gauvreauiigroup* has not been described in relation to steatosis nor to other phenotypes before. It is known, however, that this genus is a strict anaerobe that produces mainly acetate as by-product of glucose fermentation.[437] *R. Gauvreauiigroup* was nominally significant associated with acetoacetate, but not with acetate. In contrast, *R. Gnavusgroup* has been described in relation with steatosis before.[417] A significant association of *R. Gnavusgroup* with acute phase reaction marker – glycoprotein acetyls. This is in line with the current knowledge that presence of *R. Gnavusgroup* has been previously described in individuals with lower microbial richness,[436] atherosclerotic cardiovascular disease,[438] and inflammatory bowel disease.[439]

Most of the other 34 lasso-selected genera have been previously described in relation to liver traits. For example, an interesting study of Boursier et al. found that Ruminococcus was positively and Prevotella was negatively associated with biopsy-proven fibrosis in NAFLD.[412] In our study, *Ruminococcus2* showed a trend towards a positive association with steatosis (*P*-value=0.016). Of note, there are many different Ruminococcus species which can express different functions. Another study showed a reduction of Prevotella after an animal-based diet and an increase after a high in fibre diet.[440] In our study, *Prevotella9* had a non-significant inverse association with steatosis. The previous study also showed that an animal-based diet was associated with a significant increase in gene expression among beta-lactamases. This is also in line with our study, in which we found an enriched beta-lactam resistance pathway (ko00312) in steatosis. This is of particular interest as our group recently showed that a diet high in animal protein was independently associated with liver steatosis.[344]

Other enriched predicted functional pathways in (non-alcoholic) steatosis included those involved in secondary bile acid biosynthesis (ko00121) and biotin metabolism (ko00780). Biotin is an essential cofactor of biotin-dependent carboxylases, such as acetyl-CoA carboxylase, which is implicated in the biosynthesis of fatty acids and hepatic steatosis.[441] Interestingly, a recent paper from Jiao et al. found that both absolute serum bile acids as well as percentage of secondary bile acids was elevated in the gut of non-alcoholic steatohepatitis patients.[442] However, in that study different bile acid-related pathway, 'taurine and hypo-taurine' (ko00430), was enriched. Nonetheless, a number of differences between their study and ours exist, such as sample size (n=27 vs n=1355), ethnicity (Asian vs European), and disease stage (non-alcoholic steatohepatitis vs NAFLD) that could have

contributed to the different findings and hampers direct comparison. Lastly, D-alanine metabolism (ko00473), a pathway that concerns the transformation from L-alanine to D-alanine, was predicted to be absent in steatosis and NAFLD. A lowered D-alanine metabolism and subsequent accumulation of L-alanine could lead to an increase in aspartate, glutamate and alanine – metabolites that were indeed significantly upregulated in steatosis and NAFLD in our study.

Several studies have already provided insights into the molecular signature of steatosis.[443] We found a particular increase in bacterial biosynthetic potential for BCAAs isoleucine and leucine, AAAs tyrosine and phenylalanine, and glycoprotein acetyls. Our results are in line with a large Finnish population-based study that found that circulating lipids, fatty acids, and amino acids –similar to our study– were associated with and preceded the development of hepatic steatosis in young adults.[443] Interestingly, a previous study shows that BCAAs tend to be higher in males than in females.[444] However, we have included sex as covariate in our multivariable model to control for potential confounding. Nevertheless, we cannot completely exclude the possibility of sex differences in the triad metabolomics, sex and steatosis. In addition, glycoprotein acetyls (mainly alpha 1), a composite marker of the most abundant acute phase proteins in the circulation, was positively associated with (non-alcoholic) steatosis and steatofibrosis in our study. Interestingly, this marker has been associated with various other inflammatory and metabolic comorbidities,[445] and with an overall reduced life expectancy.[446] Intriguingly, a large study of Wurtz et al. has shown that aberrations in BCAAs and AAAs precede insulin resistance –key in the development of steatosis– whereas glutamine levels were inversely related to insulin resistance and risk of type II diabetes.[445] Indeed, glutamine was inversely associated with steatofibrosis in our study. Intriguingly, alpha-diversity had an opposite metabolic profile compared to steatosis. Whether this may infer a role for gut microbial richness in the metabolic profile of steatosis is reserved for future longitudinal studies to discover.

The use of microbiomics, predicted metagenomics, and serum metabolomics combined with reliable liver imaging in the context of a large population-based study allowed us to perform a unique in-depth analysis on the gut-liver axis in (non-alcoholic) fatty liver disease. Nonetheless, the results of this study have to be interpreted in light of the following limitations. First, to optimize participation rate, we chose to collect stool samples via postal mail, but sample collection at room temperature and subsequent travel time can affect the composition and diversity of the samples. Second, we (as many others) examined the microbiome of faecal samples, which does not necessarily reflect the microbial variation throughout the whole gut. Also, we used 16S rRNA sequencing, which has the capacity to capture a broad shift in microbial diversity, but metagenomic approaches are in general more precise. Third, this study is cross-sectional by design and hence we cannot make inferences on the cause-effect relations. Fourth, assessing all bacteria in one regression model is challenging due to overfitting. Regularization methods, such as the use of Lasso

penalties are gaining popularity in the field of microbiomics, but as far as we know, we are the first study to use this method in context of the steatosis phenotype. However, overfitting might still have taken place in the phenotypes steatofibrosis and NASF because of the little number of cases. Fifth, replication in another large cohort with similar phenotype data does not exist (to our knowledge), but is needed to confirm our findings. Sixth, the linear regression analyses between the three genera and metabolites should be interpreted with caution because of the zero-inflated distribution (which was less prominent after log-transformation). Lastly, the gold standard for the diagnosis of steatosis and steatofibrosis is a liver biopsy. However, performing a liver biopsy in presumed healthy individuals is ethical debatable. We therefore used LSM, which has been shown to be a reliable non-invasive proxy for fibrosis. In addition, ultrasound is not a perfect measure for steatosis either, it has a low sensitivity for the presence of mild steatosis. However, we chose to dichotomize steatosis into present or absent instead of grading steatosis, because the sensitivity for moderate/severe steatosis is good. In addition, ultrasound is easy-to perform, widely available and inexpensive.

We hope future large-scale studies were to confirm our findings. Then, future studies on a more fundamental level over the course of time can test the causal inferences between the gut microbiome, circulating metabolites and steatosis. Also it would be interesting to further look at the differences between the various forms of steatosis: is the gut microbiome and its metabolites a common denominator in the pathophysiology behind steatosis? Ultimately, such information could help developing precision medicine in steatosis. In a large-scale setting the combination use of elastography and controlled attenuation parameter would be suitable to investigate steatosis and steatofibrosis non-invasively. In addition, it would be interesting if future studies could combine genetics, microbiomics and metabolomics. It is known that certain host genetic variants predispose an individual towards microbiome dysbiosis, which could also be an important factor in lipid metabolism and steatosis.[447] For example, it is known that polymorphisms PNPLA3-I148M and TM6SF2 are associated with an increased risk of NASF by means of interfering lipid pathways.[32-34] It would be interesting to see if associations still hold true when correcting for these kind of intrinsic covariates.

In conclusion, this work offers a comprehensive understanding of microbial diversity, composition, metabolomics and predicted metagenomics with steatosis, NAFLD, and its advanced subtype steatofibrosis in a community-dwelling population. We confirmed the lower microbial diversity and association of previously described 'beneficial' *Coprococcus* and 'harmful' *Ruminococcus Gnavus* with steatosis. Moreover, our study showed an exact opposite association between NMR metabolites and steatosis versus NMR metabolites and alpha-diversity, suggesting that they share –opposite– metabolic profiles. If future large-scale studies were to confirm our findings, our results could help developing precision medicine in steatosis.

Supplementary Files

Supplementary Methods

Metadata

An extensive home interview was carried out by professionally trained assistants, to obtain information on education level, smoking habits, and physical activity (using the LASA Physical Activity Questionnaire, expressed in metabolic equivalent of task (MET)hours/week).[299] Information on medication use (lipid lowering drugs, anti-diabetic medication, PPIs, amiodarone, systemic corticosteroids, methotrexate, and tamoxifen) was retrieved using automated linkage with the local pharmacy with which 98% of the participants was registered. Semi-quantitative (389-item) food frequency questionnaires were used to extract information on alcohol consumption, energy intake, and dietary quality score. This score contained the following components on intake of: I) vegetables (≥200g/day), II) fruit (≥200g/day), III) whole-grain products (≥90g/day), IV) legumes (≥135g/week), V) unsalted nuts (≥15g/day), VI) fish (≥100g/week), VII) dairy (≥350g/day), VIII) tea (≥150mL/day), IX) whole grains ≥50% of total grains, X) unsaturated fats and oils ≥ of total fats, XII) red and processed meat <300g/week, XIII) sugar-containing beverages (≤150mL/day), XIV) alcohol (≤10 g/day), and XV) salt (≤6 g/day). The food questionnaire was filled in 5.5 years prior to ultrasound and fecal evaluation, but a previous paper from our group showed that dietary consumption and specifically alcohol consumption was generally stable over time.[321] Excessive alcohol consumption was defined as ≥ 20 grams per day for women and ≥ 30 grams per day for men. Height (m) and weight (kg) were measured during the visit at the research centre, subsequent BMI was calculated (kg/m^2). Automatic enzyme procedures were used to measure blood lipids and glucose. Automatic immunoassays were used to measure insulin, hepatitis B surface antigen and anti-hepatitis C virus (Roche Diagnostic®, GmbH, Mannheim, DE). We calculated the homeostasis model assessment of insulin resistance (HOMA-IR), i.e. fasting glucose (mmol/dl) times fasting insulin (mU/L) divided by 22.5,[106] to proxy degree of insulin resistance in participants. Diabetes was diagnosed if the participant used anti-diabetic drugs and/or fasting glucose was 7.0 mmol/L or above. Hypertension was defined as the use of drugs for elevated blood pressure and/or elevated blood pressure, being either a systolic pressure of ≥ 140 mmHg or a diastolic pressure of ≥ 90 mmHg.

Statistical Analysis

Imputation analyses and participant characteristics

Missing values in the metadata (ranging from 0.00% to 19.26% per variable) were imputed using multiple imputation under the fully conditioned specification to reduce bias

due to missing data.[301] We created 50 imputed datasets using the R Package mice.[323] The results from these analyses were pooled using Rubin's rules in order to take into account the added uncertainty due to the missing of data.[254]

Description of the microbiome

The beta-diversity, which reflects the between-individual variability, was studied using principal coordinate analysis (PCoA) applied to Bray-Curtis dissimilarity using the function "capscale" from package Vegan[448] on genus level.

To additionally take into account the compositional nature of the microbiome we followed the approach of Lin et al.[428], which is implemented in the R package zeroSum.[449]

Predicted functional metagenomics

Predicted metagenomics pathways were tested for significance by performing logarithmic linear discriminant analysis (LDA) in linear discriminant analysis effect size with the significance level of 0.05 and the logarithmic LDA score threshold equal to 2.0.[450]

Statistical Significance

Adjusted *P*-values (referred to as *Q*-values) were calculated using the Benjamini & Hochberg correction[430] taking together all genera or metabolites within the same model, to account for the inflated type I error that arises due to multiple testing. All computations were done using R version 3.5.2 (R Core Team [2018]), the package Vegan version 2.5.2 [2018],[448] and the package ZeroSum version 1.1.1.[428,449]

Detailed information on the imputation process

	Multiple imputation
Software used	R version 3.5.1
Imputation method and key settings	Fully conditional specification (package mice version 3.1.0)*;* maximum iterations: 20
No. of imputed data sets created	50
Analyses variables	age; sex; steatosis; steatogenic medication; BMI; proton-pump inhibitors; time in mail; batch; 22 principle components of genera; smoking status; diabetes mellitus; high-density lipid cholesterol; glucose; energy intake; physical activity; waist-hip ratio; Dutch dietary guideline score; triglycerides; alcoholic units; education level
Auxiliary variables	Fibroscan probe; anti-diabetic drugs; liver stiffness (with IQR); alanine aminotransferase; total cholesterol; homeostasis model assessment of insulin resistance; systolic blood pressure; diastolic blood pressure; platelet count; spleen size; gamma-glutamyl transferase; insulin
Treatment of not normally distributed continuous variables	Predictive mean matching
Treatment of normally distributed variables	Linear regression
Treatment of binary/categorical variables	(proportional odds) Logistic regression
Population	For the imputation we used reliable FFQs. We included only participants with ultrasound data. Missing FFQs-related covariates (e.g. diet quality, energy intake) were imputed.

Supplementary Table 1: Imputed and Original characteristics

	Original data	Imputed data
Age (years)	62.36 (5.86)	*no missing*
Female (%)	57.8	*no missing*
European (%)	96.7	96.7
Education Level (%)		
Low	41.2	41.2
Intermediate	28.2	28.2
High	30.6	30.6
Smoking status (%)		
Never	35.4	35.0
Past or Current	64.6	65.0
Alcohol (units/d)	0.83 [0.17, 2.05]	0.83 [0.17, 2.05]
Physical Activity (METeq/wk)	49.00 [21.00, 82.73]	49.00 [21.00, 82.78]
Energy intake (kcal/day)	2254 [1887, 2734]	2251 [1882, 2734]
Dietary Quality Score (0–15)	7.00 [6.00, 8.00]	7.00 [6.00, 8.00]
BMI (kg/m^2)	27.42 (4.49)	*no missing*
Waist-to-hip ratio	0.89 [0.82, 0.96]	0.89 [0.82, 0.96]
HOMA-IR	2.56 [1.70, 4.08]	2.56 [1.70, 4.08]
Total Cholesterol (mmol/L)	5.58 (1.11)	5.58 (1.11)
HDL-Cholesterol	1.51 (0.45)	1.51 (0.45)
Triglycerides	1.29 [0.96, 1.74]	1.30 [0.96, 1.74]
Diabetes Mellitus (%)	10.0	10.0
Hypertension (%)	58.3	58.3
Liver stiffness measurements (kPa)*	4.55 [3.70, 5.60]	4.55 [3.70, 5.60]
Lipid-lowering drug use	27.2	*no missing*
Proton-pump inhibitor use	17.7	*no missing*
Microbiome batch 0/1	78.8 / 21.2	*no missing*
Time in mail (%)		*no missing*
1 day	65.3	
2 days	29.2	
3 days	5.5	
Steatosis n (%)	472 (34.8)	*no missing*
Viral hepatitis†	8 (0.6)	*not imputed*
Excessive alcohol use‡	202 (18.5)	*not imputed*
Steatogenic drug use	21 (1.5)	*not imputed*

*Liver stiffness not yet filtered for reliability here. †Data available for 1338 participants. ‡Data available for 1094 participants.

Abbreviations: HOMA-IR: homeostasis model of insulin resistance; kcal: kilocalories; kPa: kilopascals; LSM: liver stiffness measurement; METeqh/wk: metabolic equivalent task hours per week; NAFLD: non-alcoholic fatty liver disease

Supplementary Table 2: Relative abundance of phyla between steatosis strata

	Total population (n=1355)	No steatosis (n=883)	Steatosis (n=472)	*P*-value*
Verrucomicrobia	$3.1{\cdot}10^{-2}$ (0 – 0.21)	$3.4{\cdot}10^{-2}$ (0 – 0.23)	$2.1{\cdot}10^{-2}$ (0 – 0.16)	0.040
Tenericutes	0 (0 – $8.7{\cdot}10^{-2}$)	$1.1{\cdot}10^{-2}$ (0 – 0.10)	0 (0 – $4.6{\cdot}10^{-2}$)	<0.001
Synergistetes	0 (0 – 0)	0 (0 – 0)	0 (0 – 0)	0.982
Saccharibacteria	0 (0 – $1.1{\cdot}10^{-2}$)	0 (0 – $1.1{\cdot}10^{-2}$)	0 (0 – $1.1{\cdot}10^{-2}$)	0.148
Proteobacteria	1.4 (0.33 – 5.3)	1.4 (0.37 – 5.8)	1.2 (0.29 – 4.4)	0.035
Fusobacteria	0 (0 – 0)	0 (0 – 0)	0 (0 – 0)	0.239
Firmicutes	81.2 (69.3 – 88.6)	81.2 (69.6 – 88.4)	81.1 (69.1 – 88.8)	0.693
Euryarchaeota	0 (0 – 0)	0 (0 – $1.0{\cdot}10^{-2}$)	0 (0 – 0)	0.008
Cyanobacteria	0 (0 – 0)	0 (0 – 0)	0 (0 – 0)	0.227
Bacteroidetes	8.6 (3.1 – 17.9)	8.8 (3.4 – 17.7)	8.0 (2.8 – 18.0)	0.323
Actinobacteria	2.7 (1.2 – 5.3)	2.8 (1.2 – 5.1)	2.6 (1.2 – 5.5)	0.998

Relative abundance (%) median (P25-P75) are given. * *P*-value based on Wilcoxon Rank Sum test comparing steatosis with no steatosis per phylum.

Supplementary Table 3: Relative abundance of selected genera between steatosis strata

Phylum *Class* *Order* *Family* *Genus (median % (P25-P75))*	No steatosis (n=883)	Steatosis (n=472)
Actinobacteria		
Actinobacteria *Bifidobacteriales* *Bifidobacteriaceae* *Bifidobacterium*	1.36 (0.36 – 3.40)	1.32 (0.27 – 3.72)
Coriobacteriia *Coriobacteriales* *Coriobacteriaceae* *Collinsella*	0.09 (0.01 – 0.39)	0.12 (0.01 – 0.54)
Bacteroidetes		
Bacteroidia *Bacteroidales* *Bacteroidaceae* *Bacteroides*	3.97 (1.51 – 9.48)	3.96 (1.18 – 8.63)
Bacteroidia *Bacteroidales* *BacteroidalesS247group* *unknowngenus*	0 (0 – 0.13)	0 (0 – 0.05)
Bacteroidia *Bacteroidales* *Prevotellaceae* *Prevotella9*	0 (0 – 0.75)	0 (0 – 0.72)
Bacteroidia *Bacteroidales* *Rikenellaceae* *Alistipes*	0.43 (0.14 – 1.09)	0.33 (0.07 – 1.13)

Supplementary Table 3 (*continued*)

Firmicutes		
Bacilli *Lactobacillales* *Lactobacillaceae* *Lactobacillus*	0.01 (0 – 0.09)	0.01 (0 – 1.01)
Bacilli *Lactobacillales* *Streptococcaceae* *Streptococcus*	0.40 (0.12 – 1.47)	0.46 (0.14 – 1.98)
Clostridia *Clostridiales* *Christensenellaceae* *ChristensenellaceaeR7group*	1.58 (0.55 – 3.85)	0.81 (0.19 – 2.29)
Clostridia *Clostridiales* *Clostridiaceae1* *Clostridiumsensustricto1*	0.35 (0.04 – 1.38)	0.18 (0.02 – 1.05)
Clostridia *Clostridiales* *Lachnospiraceae* *Blautia*	8.40 (4.77 – 14.27)	10.0 (5.52 – 16.64)
Firmicutes		
Clostridia Clostridiales Lachnospiraceae Butyrivibrio	0 (0 – 0.03)	0 (0 – 0)
Clostridia Clostridiales Lachnospiraceae Coproccocus3	0.60 (0.27 – 1.08)	0.63 (0.23 – 1.11)
Clostridia Clostridiales Lachnospiraceae Dorea	1.61 (0.94 – 2.74)	1.93 (0.98 – 3.20)
Clostridia Clostridiales Lachnospiraceae Fusicatenibacter	1.68 (0.77 – 3.02)	1.70 (0.69 – 3.22)
Clostridia Clostridiales Lachnospiraceae Lachnoclostridium	0.55 (0.32 – 0.99)	0.70 (0.34 – 1.37)
Clostridia Clostridiales Lachnospiraceae LachnospiraceaeND3007group	0.42 (0.13 – 0.82)	0.33 (0.12 – 0.61)
Clostridia Clostridiales Lachnospiraceae Eligensgroup	0.26 (0.05 – 0.82)	0.15 (0.01 – 0.56)
Clostridia Clostridiales Lachnospiraceae R. Gauvreauiigroup	0.36 (0.07 – 0.81)	0.42 (0.08 – 0.96)

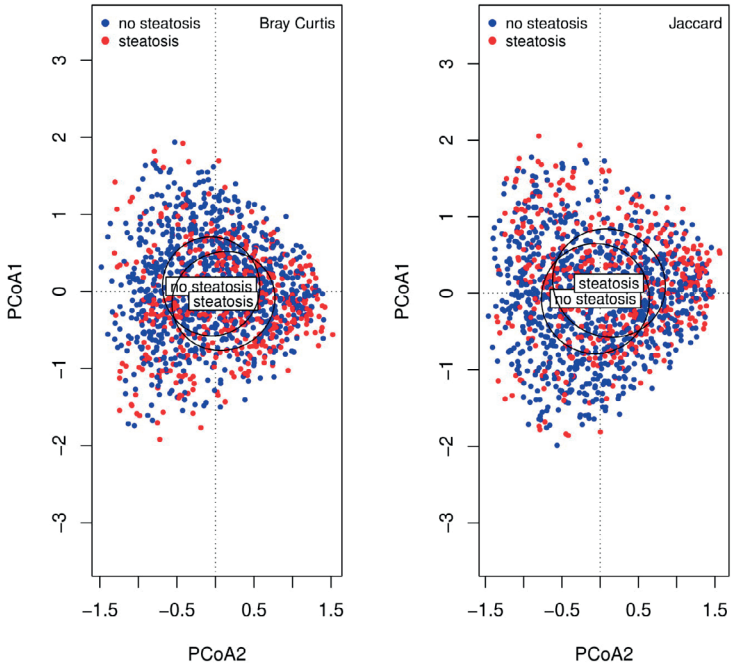
Supplementary Table 3 (*continued*)

Clostridia Clostridiales Lachnospiraceae R. Gnavusgroup	0 (0 – 0.04)	0 (0 – 0.08)
Clostridia Clostridiales Lachnospiraceae Halliigroup	2.26 (1.21 – 4.18)	2.84 (1.36 – 5.11)
Clostridia Clostridiales Lachnospiraceae Rectalegroup	3.08 (1.29 – 6.25)	4.10 (1.81 – 7.95)
Clostridia Clostridiales Peptostreptococcaceae Romboutsia	4.71 (0.08 – 1.46)	3.33 (0.03 – 1.17)
Clostridia Clostridiales Ruminococcaceae RuminococcaceaeNK4A214group	0.42 (0.14 – 0.91)	0.26 (0.05 – 0.68)
Firmicutes		
Clostridia *Clostridiales* *Ruminococcaceae* *RuminococcaceaeUCG002*	1.48 (0.58 – 3.20)	0.93 (0.29 – 2.20)
Clostridia *Clostridiales* *Ruminococcaceae* *RuminococcaceaeUCG014*	2.08 (0.33 – 4.84)	0.83 (0.11 – 3.25)
Clostridia *Clostridiales* *Ruminococcaceae* *Ruminococcus1*	0.65 (0.22 – 1.53)	0.51 (0.09 – 1.27)
Clostridia *Clostridiales* *Ruminococcaceae* *Ruminococcus2*	1.76 (0.60 – 3.25)	1.51 (0.35 – 3.78)
Clostridia *Clostridiales* *Ruminococcaceae* *Subdoligranulum*	2.59 (1.43 – 4.47)	2.35 (1.23 – 4.43)
Clostridia *Clostridiales* *Ruminococcaceae* *Coprostanoligenesgroup*	1.61 (0.95 – 2.75)	1.51 (0.78 – 2.61)
Erysipelotrichia *Erysipelotrichales* *Erysipelotrichaceae* *Catenibacterium*	0 (0 – 0)	0 (0 – 0)
Negativicutes *Selenomonadales* *Acidaminococcaceae* *Phascolarctobacterium*	0.11 (0 – 0.74)	0.06 (0 – 0.65)

Supplementary Table 3 (*continued*)

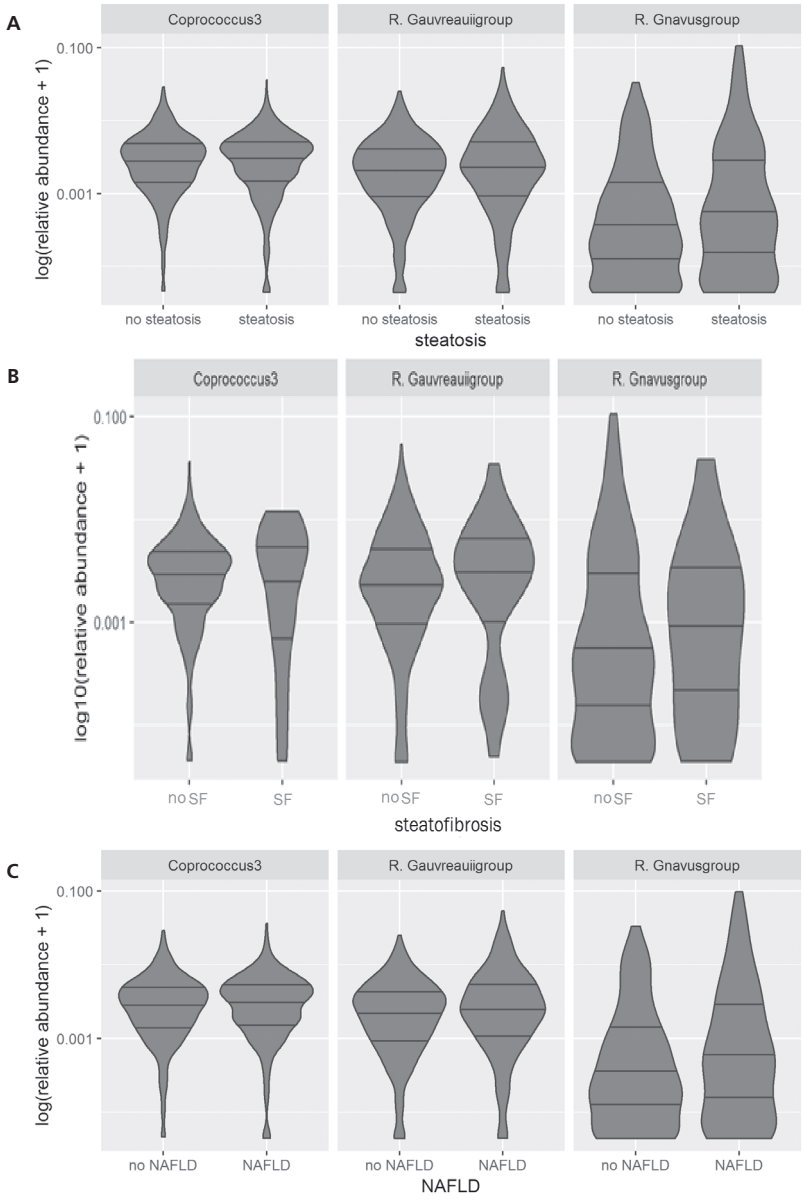
Negativicutes *Selenomonadales* *Veillonelaceae* *Megamonas*	0 (0 – 0)	0 (0 – 0)
Proteobacteria		
Gammaproteobacteria *Aeromonadales* *Succinivibrionaceae* *Succinivibrio*	0 (0 – 0)	0 (0 – 0)
Gammaproteobacteria *Enterobacteriales* *Enterobacteriaceae* *EscherichiaShigella*	0.45 (0.04 – 4.23)	0.28 (0.03 – 2.56)
Gammaproteobacteria *Enterobacteriales* *Enterobacteriaceae* *Klebsiella*	0 (0 – 0)	0 (0 – 0)

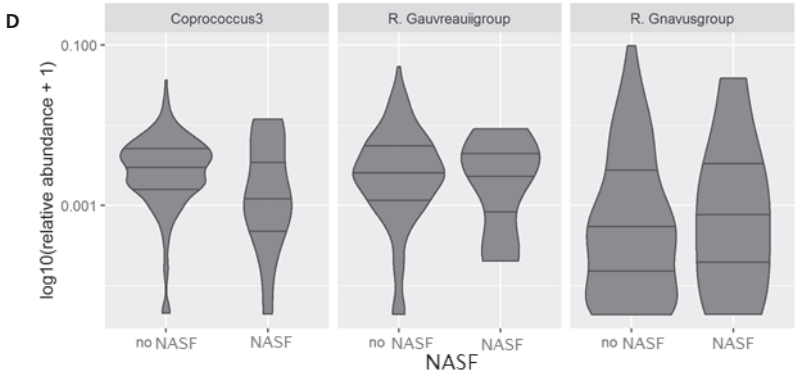
Relative abundance (%) median (P25-P75) are given for the group with steatosis and without steatosis.



Supplementary Figure 1: Principle Coordinate Analysis Plots

The Principle Coordinate Analyses using Bray-Curtis dissimilarity distances (quantitative mode on the left) and Jaccard similarity distances (binary mode on the ride). The blue dots reflect cases without steatosis and the red dots those with steatosis. Both plots show the group centroids (black circles). For the statistical analyses we used PerMANOVA adjusted for the covariates age, sex, BMI and technical covariates. PerMANOVA on both distances showed significant variation between the two groups (R2=0.003; p<0.001 for Bray-Curtis and R2=0.002; p<0.001 for Jaccard).

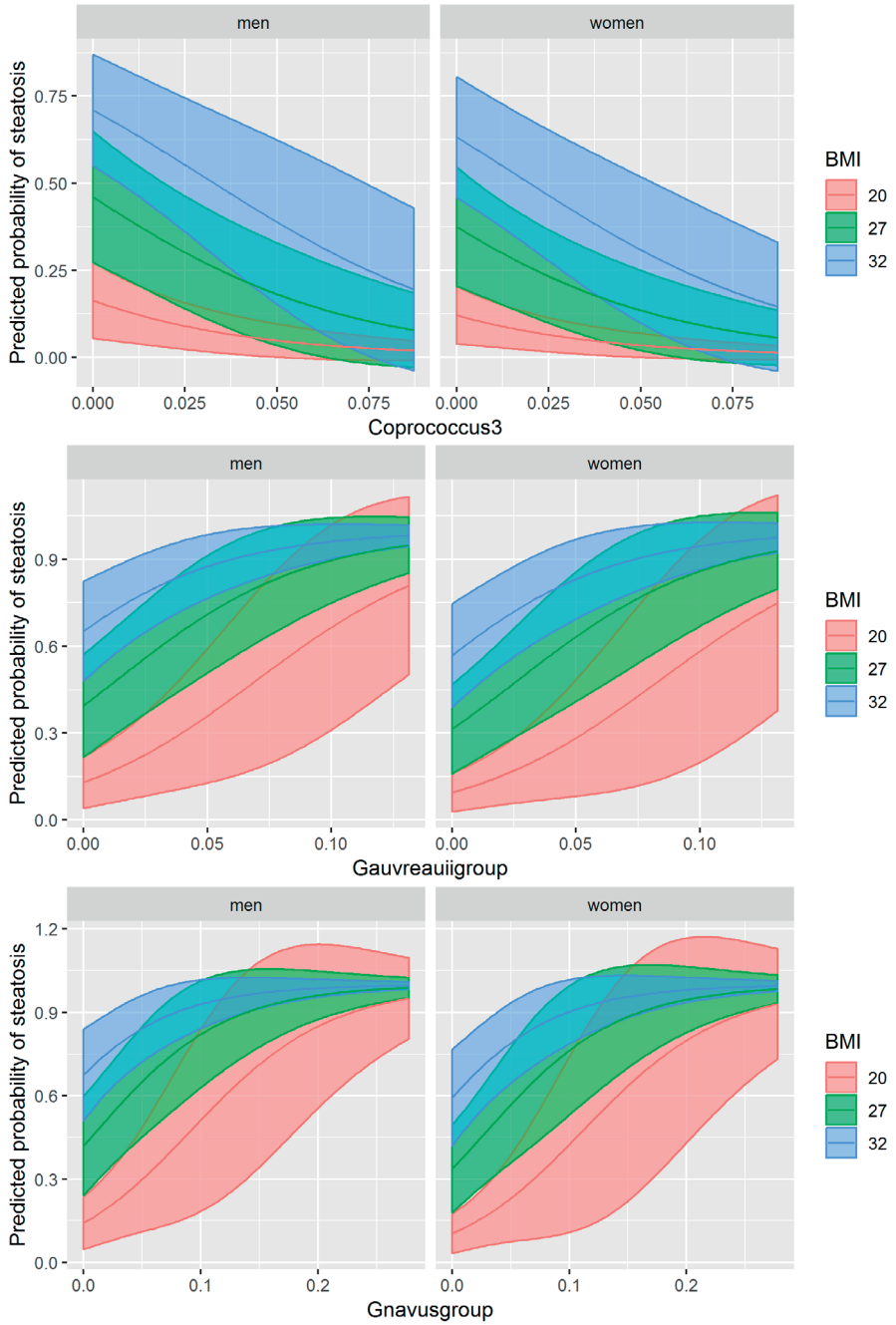

A
Coprococcus3
R. Gauvreauiigroup
R. Gnavusgroup
log(relative abundance + 1)
0.100
0.001
no steatosis
steatosis
steatosis
B
Coprococcus3
R. Gauvreauiigroup
R. Gnavusgroup
log10(relative abundance + 1)
0.100
0.001
no SF
SF
steatofibrosis
C
Coprococcus3
R. Gauvreauiigroup
R. Gnavusgroup
log(relative abundance + 1)
0.100
0.001
no NAFLD
NAFLD
NAFLD



Supplementary Figure 2: Plotted relative abundance for steatosis-associated genera across (A) steatosis strata, (B) steatofibrosis strata, (C) NAFLD strata, and (D) NASF strata

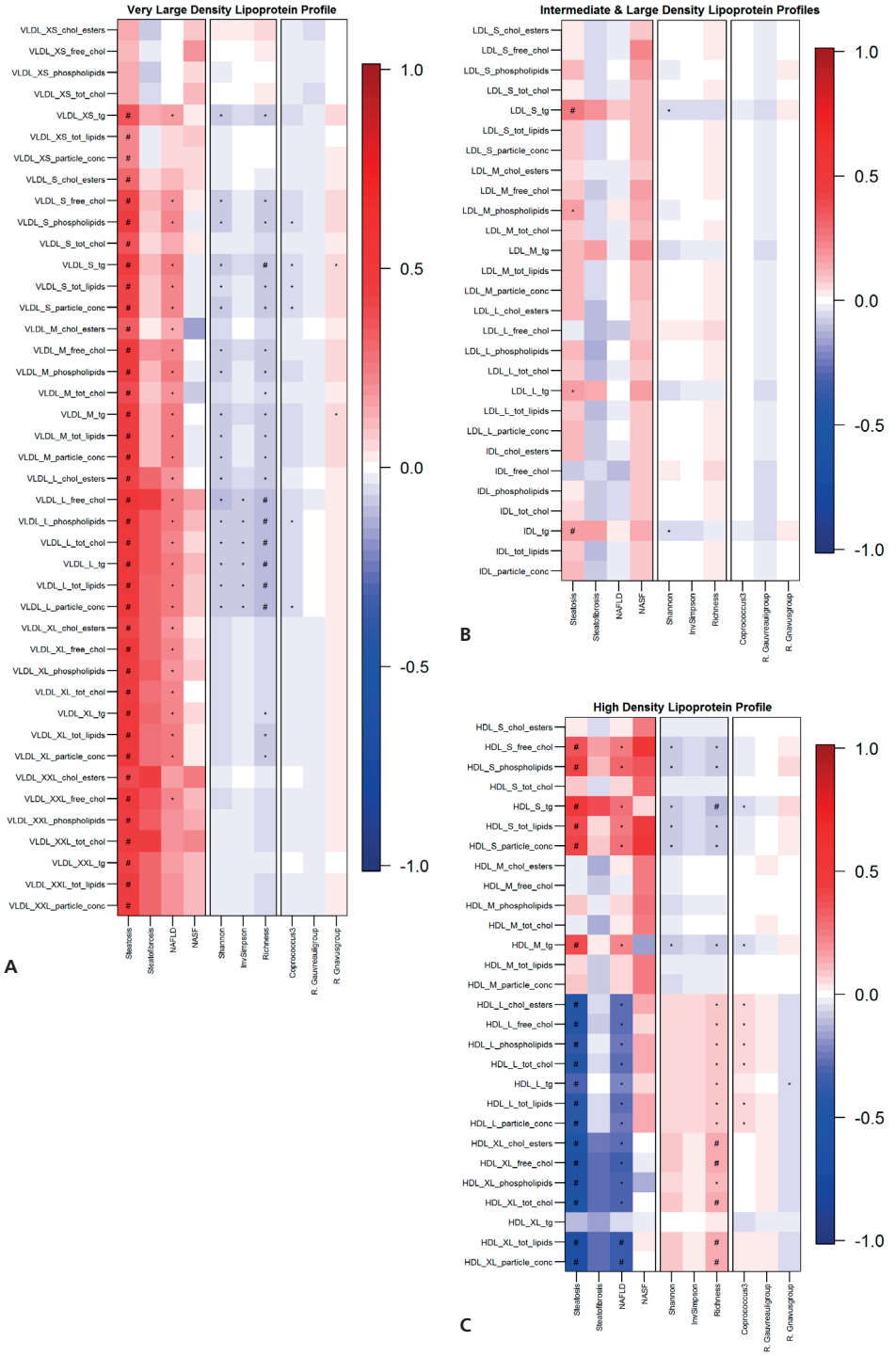
The log10 transformed non-zero values of the significant lasso-selected genera are depicted across A) steatohepatitis, B) NAFLD, and C) NASH strata using violin plots. The log-transformed non-zero values of the significant lasso-selected genera are depicted across steatosis strata using violin plots.

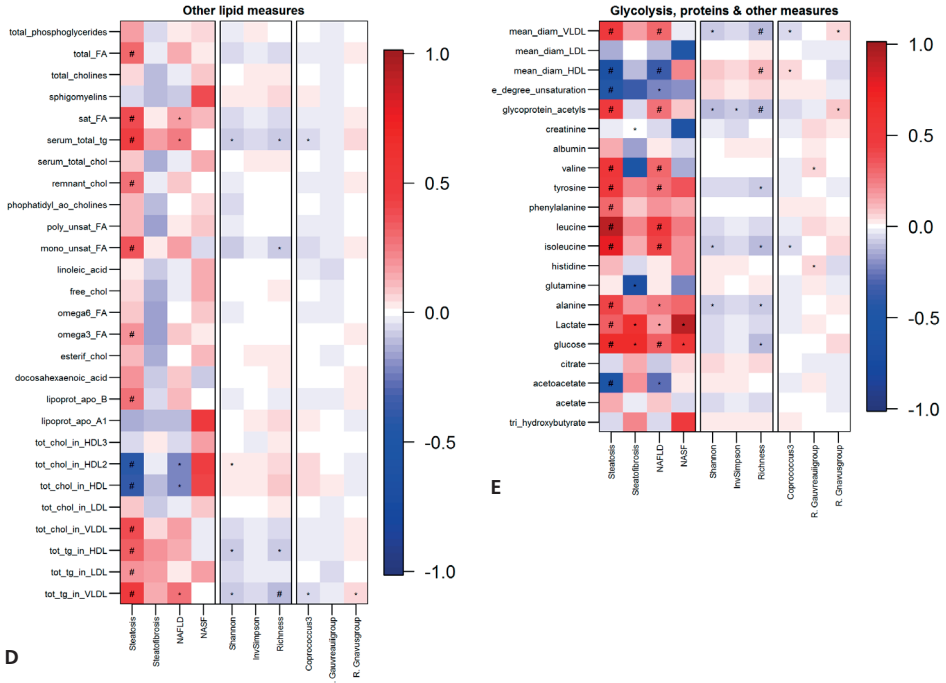
Abbreviations: NAFLD: non-alcoholic fatty liver disease; NASH: non-alcoholic steatohepatitis.



Supplementary Figure 3: Predicted probability of steatosis for relative abundance of Coprococcus3, R Gauvreauiigroup, and R Gnavusgroup

A-C: Predicted probability for exemplary patients plotted by sex and BMI (two significant variables), based on the multivariable model as shown in table 2. On the y-axis: the predicted probability (in %) for steatosis with 95%CI. On the x-axis: the relative abundance of the genus. Abbreviations: BMI: body mass index.


Very Large Density Lipoprotein Profile
VLDL_XS_chol_esters
VLDL_XS_free_chol
VLDL_XS_phospholipids
VLDL_XS_tot_chol
VLDL_XS_tg
VLDL_XS_tot_lipids
VLDL_XS_particle_conc
VLDL_S_chol_esters
VLDL_S_free_chol
VLDL_S_phospholipids
VLDL_S_tot_chol
VLDL_S_tg
VLDL_S_tot_lipids
VLDL_S_particle_conc
VLDL_M_chol_esters
VLDL_M_free_chol
VLDL_M_phospholipids
VLDL_M_tot_chol
VLDL_M_tg
VLDL_M_tot_lipids
VLDL_M_particle_conc
VLDL_L_chol_esters
VLDL_L_free_chol
VLDL_L_phospholipids
VLDL_L_tot_chol
VLDL_L_tg
VLDL_L_tot_lipids
VLDL_L_particle_conc
VLDL_XL_chol_esters
VLDL_XL_free_chol
VLDL_XL_phospholipids
VLDL_XL_tot_chol
VLDL_XL_tg
VLDL_XL_tot_lipids
VLDL_XL_particle_conc
VLDL_XXL_chol_esters
VLDL_XXL_free_chol
VLDL_XXL_phospholipids
VLDL_XXL_tot_chol
VLDL_XXL_tg
VLDL_XXL_tot_lipids
VLDL_XXL_particle_conc
Steatosis
SteatoFibrosis
NAFLD
NASF
Shannon
InvSimpson
Richness
Coprococcus3
R. Gauvreauiigroup
R. Gnavusgroup
1.0
0.5
0.0
-0.5
-1.0
A
Intermediate & Large Density Lipoprotein Profiles
LDL_S_chol_esters
LDL_S_free_chol
LDL_S_phospholipids
LDL_S_tot_chol
LDL_S_tg
LDL_S_tot_lipids
LDL_S_particle_conc
LDL_M_chol_esters
LDL_M_free_chol
LDL_M_phospholipids
LDL_M_tot_chol
LDL_M_tg
LDL_M_tot_lipids
LDL_M_particle_conc
LDL_L_chol_esters
LDL_L_free_chol
LDL_L_phospholipids
LDL_L_tot_chol
LDL_L_tg
LDL_L_tot_lipids
LDL_L_particle_conc
IDL_chol_esters
IDL_free_chol
IDL_phospholipids
IDL_tot_chol
IDL_tg
IDL_tot_lipids
IDL_particle_conc
Steatosis
SteatoFibrosis
NAFLD
NASF
Shannon
InvSimpson
Richness
Coprococcus3
R. Gauvreauiigroup
R. Gnavusgroup
1.0
0.5
0.0
-0.5
-1.0
B
High Density Lipoprotein Profile
HDL_S_chol_esters
HDL_S_free_chol
HDL_S_phospholipids
HDL_S_tot_chol
HDL_S_tg
HDL_S_tot_lipids
HDL_S_particle_conc
HDL_M_chol_esters
HDL_M_free_chol
HDL_M_phospholipids
HDL_M_tot_chol
HDL_M_tg
HDL_M_tot_lipids
HDL_M_particle_conc
HDL_L_chol_esters
HDL_L_free_chol
HDL_L_phospholipids
HDL_L_tot_chol
HDL_L_tg
HDL_L_tot_lipids
HDL_L_particle_conc
HDL_XL_chol_esters
HDL_XL_free_chol
HDL_XL_phospholipids
HDL_XL_tot_chol
HDL_XL_tg
HDL_XL_tot_lipids
HDL_XL_particle_conc
Steatosis
SteatoFibrosis
NAFLD
NASF
Shannon
InvSimpson
Richness
Coprococcus3
R. Gauvreauiigroup
R. Gnavusgroup
1.0
0.5
0.0
-0.5
-1.0
C



Supplementary Figure 4: Metabolic profiles of steatosis, alpha-diversity and lasso-selected genera after exclusion of participants that use lipid-lowering medication (n=986)

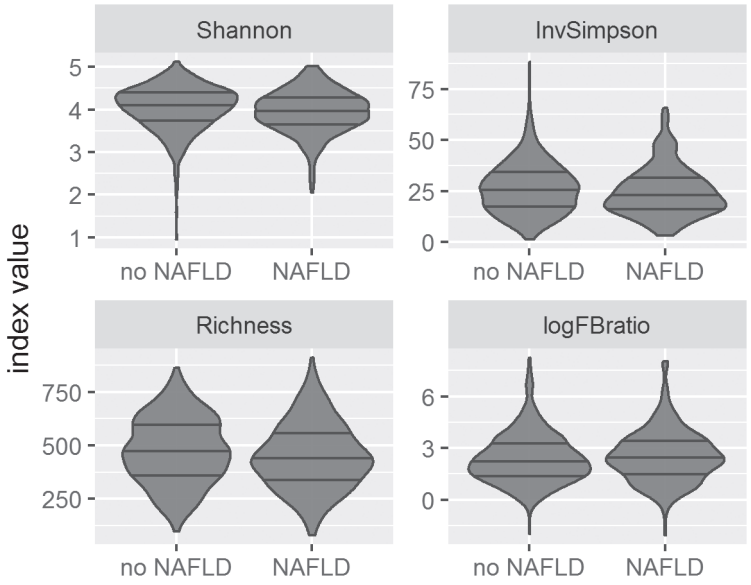
The colors in columns represent the standardized effect estimates (betas) of the metabolites with steatosis, steatohepatitis, NAFLD, NASH, alpha-diversity and significant genera. The betas were adjusted for covariates in model 1 (sex†‡, age†‡, technical variables†‡, BMI†‡, Dietary Quality-Score, PPI use, alcohol units†). We corrected for multiple testing using Benjamini & Hochberg, significant Q-values are depicted by #, nominally significant values by *. †Steatohepatitis adjustment, ‡NASH adjustment.

Abbreviations: BMI: body mass index; chol: cholesterol; conc: concentration; diam: diameter; FA: fatty acids; HDL: high-density lipoprotein; HOMA-IR: homeostasis model of insulin resistance; IDL: intermediate density lipoprotein; InvSimpson: Inversed Simpson; L: large; LDL: low-density lipoprotein; M: medium; PPI: proton-pump inhibitor; S: small; sat: saturated; tg: triglycerides; tot: total; VLDL: very low density lipoprotein; (X)XL: (extra) extra large

Supplementary Table 4: Relative abundance of phyla between NAFLD strata

	No NAFLD (n=574)	NAFLD (n=283)	*P*-value*
Verrucomicrobia	$3.2{\cdot}10^{-2}$ (0 – 0.24)	$2.3{\cdot}10^{-2}$ (0 – 0.18)	0.637
Tenericutes	$1.0{\cdot}10^{-2}$ (0 – 0.10)	0 (0 – $4.3{\cdot}10^{-2}$)	<0.001
Synergistetes	0 (0 – 0)	0 (0 – 0)	0.584
Saccharibacteria	0 (0 – $1.1{\cdot}10^{-2}$)	0 (0 – $1.1{\cdot}10^{-2}$)	0.232
Proteobacteria	1.5 (0.34 – 6.1)	1.1 (0.29 – 3.5)	0.018
Fusobacteria	0 (0 – 0)	0 (0 – 0)	0.121
Firmicutes	81.2 (70.2 – 88.3)	82.3 (72.1 – 88.4)	0.274
Euryarchaeota	0 (0 – $7.6{\cdot}10^{-3}$)	0 (0 – 0)	0.146
Cyanobacteria	0 (0 – 0)	0 (0 – 0)	0.581
Bacteroidetes	8.6 (3.3 – 17.5)	7.5 (2.9 – 16.2)	0.150
Actinobacteria	2.9 (1.2 – 5.3)	2.8 (1.2 – 6.1)	0.796

Relative abundance (%) median (P25-P75) are given. * *P*-value based on Wilcoxon Rank Sum test comparing steatosis with no steatosis per phylum.
Abbreviations: NAFLD: non-alcoholic fatty liver disease.

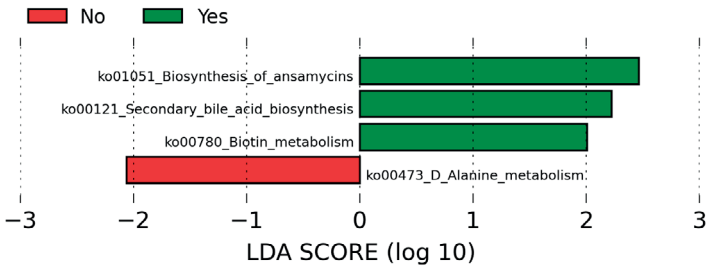


Supplementary Figure 5: Alpha-diversity and Firmicutes-to-Bacteroidetes-ratio in NAFLD
Violin plots of the distribution of A-B) alpha-diversity as assessed by the Shannon-index and Inverse Simpson, C) microbial richness, and D) log-transformed FBR across steatosis strata. The statistical difference between strata was assessed using Wilcoxon rank sum test with continuity correction and was as follows (Shannon: P= 0.003; Inverse Simpson: P= 0.021; Richness: P=0.015 and; FBR: P=0.876).
Abbreviations: InvSimpson: Inversed Simpson; logFBratio: log-transformed Firmicutes-to-Bacteroidetes-ratio.

Supplementary Table 5: Multivariable logistic regression analysis of genera that were associated with steatosis (n=1355) and are now tested against steatosis and NAFLD as dependent variable (n= 857).

	Steatosis 472 / 1355		NAFLD 283 / 857	
	β	95%CI	β	95%CI
Coprococcus3	–51.76	–89.98, –13.54	–43.85	–90.29, 2.58
Gauvreauiigroup	64.29	31.03, 97.56	61.71	22.17, 101.25
Gnavusgroup	52.12	24.22, 80.02	50.26	18.51, 82.0

Logistic regression analyses with steatosis or NAFLD as dependent variable, both adjusted for age, sex, BMI, alcohol, PPI-use, dietary quality and technical variables (batch and time in mail). All genera (log10(1+genus)) are analyzed simultaneously. Results are given as regression coefficient (95%CI).
Abbreviations: BMI: body mass index; PPI: proton-pump inhibitor; NAFLD: non-alcoholic fatty liver disease.



Supplementary Figure 6: Predicted metagenomics pathways for NAFLD (n=857)
Predicted functional composition of metagenomes based on 16S rRNA gene sequencing data in our study cohort. Linear discriminant effect size-based on the PICRUSt dataset revealed six significant (FDR<0.05) metabolic pathways that were predicted to be present in NAFLD (Yes in green) and that were predicted to be absent in NAFLD (No in red).

Part VI

CORRESPONDENCE

Chapter 11

Reply to: Herbal tea consumption and the liver – all is not what it seems!

Louise J.M. Alferink, Jessica C. Kiefte-de Jong,
Sarwa Darwish Murad

J Hepatol. 2018 Mar;68(3):613–614

Reply to

We would like to thank Philips and Augustine for their valuable comments on our study on coffee and tea consumption in relation to liver health in the general population.[130,451] In our study we show that both coffee as well as herbal tea are associated with lower liver stiffness independent of many potential confounding factors. In their letter, Philips and Augustine raise the valid concern that papers like ours may convey a general message that consumption of tea containing herbal extracts or other complex mixtures is evidently beneficial and safe. Naturally, this can be a dangerous assumption. Consumption of unknown and non-FDA approved herbal extracts, concentrated infusions, or oral preparations of green tea can indeed, occasionally, cause hepatotoxicity and even acute liver failure.[452] We understand that, especially in Asian countries such as India (where the authors work) and China, consumption of herbal compounds may be more widespread, may be part of traditional medicinal remedies[453] and may involve complex and potentially hazardous preparations.[454]

Therefore, it is important to understand the study population we have examined. The participants in our study were nearly all community dwelling elderly Caucasians, who generally follow a traditional Dutch diet. Due to limitations of the Food Frequency Questionnaire, we could not provide more detailed information on the exact type of herbal tea consumed.[98,99] However, it is well-known that consumption of herbal tea in The Netherlands is typically limited to mainstream, pre-packaged, and commercially available herbal teas, such as chamomile, rooibos and mint tea http://www.rivm.nl/Onderwerpen/V/Voedselconsumptiepeiling/). Moreover, in our study, consumption of herbal tea was infrequent: only one-third of the participants consumed herbal tea with a median of 0.80 cups per day (IQR 0.45 – 1.16). Studies who have reported on herbal preparations and liver injury generally reported ingestion of much higher dosages and unknown mixtures.[452] Although we cannot exclude the possibility that some of our participants may have consumed unknown herbal preparations or extracts, it is important to emphasize that liver enzymes were normal in 99% of all participants.

Another concern raised by Philips and Augustine relates to our discussion about the existing literature on green tea and liver health. They particularly question the validity of the papers we cited, in particular, the cross-sectional study by Imai et. al. that concluded that higher green tea consumption (even over 10 cups per day) was associated with lower prevalence of cardiovascular disease and lower serum transaminases.[95] Philips et al. argue that, after this paper was published, no other studies have confirmed their findings. However, as Philips and Augustine themselves state in their letter, our study is the first to examine in depth the relation of tea intake and liver health in a large general population. As a result, we discussed prior studies on this topic, even those with conclusions that conflict with ours. Namely that we show that green tea was not associated with liver health.[130]

A final issue raised by the authors of this letter is the potential toxicity of green tea extract itself. Indeed, cases of severe drug-induced liver injury associated with consumption of green tea *extracts or concentrated infusions* have been reported (https://livertox.nih.gov/GreenTea.htm). It is important, however, to realise these rare occurrences are almost exclusively related to the use of green tea supplements, extracts or concentrated infusions rather than the use of regular beverage consumption. Also, there is the issue of potential toxic contamination, as the composition of these herbal products is not always known and tested.[454] We did not examine extracts or supplements but rather tea consumption itself, which is generally believed to be safe at moderate, regular, and habitual use (NIH).[455]

To conclude, we thank Philips and Augustine for their valuable comments. As we have clearly stated in the conclusions of our paper, we agree that it is premature to make any firm recommendations on herbal tea consumption and liver health. Undoubtedly, more studies are needed to study the cause-effect relation between consumption of coffee, herbal tea, and liver health. Moreover, as with any scientific study, caution is always warranted when translating study results to clinical practice recommendations.

Chapter 12

Reply to: Association between beverage consumption and liver fibrosis

Louise J.M. Alferink, Jessica C. Kiefte-de Jong,
Sarwa Darwish Murad

J Hepatol. 2018 May;68(5):1096–1098.

Reply to

Thank you for the opportunity to reply to the letter by Huang et al.[456] The authors of this letter posed two main questions, which we will address consecutively. First, they questioned why we categorised subtypes of tea (no vs any) differently from coffee consumption (no, moderate, and frequent). This is simply related to the small number of participants with frequent tea consumption, being N=91 with frequent green tea and

Table 1:

	log-transformed LSM		LSM≥8 kPa	
	beta (95%CI)	P-value	OR (95%CI)	P-value
		Model 1*		
Herbal Tea				
no	0 (ref)	**<0.001**	1 (ref)	**0.025**
moderate	**–0.097 (–0.128; –0.067)**		0.64 (0.41 – 1.01)	
frequent	**–0.109 (–0.163; –0.055)**		0.51 (0.21 – 1.20)	
Green Tea				
no	0 (ref)	0.093	1 (ref)	0.437
moderate	–0.028 (–0.060; 0.005)		0.87 (0.54 – 1.42)	
frequent	–0.029 (–0.098; 0.039)		0.73 (0.26 – 2.04)	
Black Tea				
no	0 (ref)	**0.006**	1 (ref)	0.273
moderate	–0.028 (–0.057; 0.001)		0.83 (0.55 – 1.24)	
frequent	**–0.051 (–0.089; –0.013)**		0.76 (0.45 – 1.30)	
		Model 3†		
Herbal Tea				
no	0 (ref)	**0.003**	1 (ref)	0.369
moderate	**–0.056 (–0.087; –0.026)**		0.74 (0.43 – 1.27)	
frequent	–0.040 (–0.093; 0.014)		0.86 (0.30 – 2.45)	
Green Tea				
no	0 (ref)	0.142	1 (ref)	0.579
moderate	0.017 (–0.016; 0.050)		1.31 (0.76 – 2.25)	
frequent	0.043 (–0.025; 0.111)		0.88 (0.23 – 3.29)	
Black Tea				
no	0 (ref)	0.502	1 (ref)	0.951
moderate	–0.012 (–0.041; 0.017)		0.90 (0.53 – 1.54)	
frequent	–0.011 (–0.049; 0.028)		1.20 (0.45 – 3.20)	

* Model 1: adjusted for coffee consumption, other subtypes of tea consumption, and energy intake. Significant results are marked in bold.

† Model 3 (i.e. full adjustment): adjusted for tea or coffee, energy intake, BMI, gender, age, steatosis, ALT, excessive alcohol intake, current or former smoking, HOMA-IR, soda consumption, cream and sugar use, DHDI, physical activity, lipid-lowering drugs and anti-diabetic drugs. Significant results are marked in bold.

N=162 with frequent herbal tea consumption, which would hamper the ability to perform robust multivariable analyses within subcategories. To illustrate this further, we show in *Table 1* the results of our multivariable models when using these subcategories. As can be seen, results of model 1 (adjusted only for coffee consumption, other subtypes of tea and energy intake) are very similar to our original dichotomised analyses.[130] However, results diminished (i.e. lower betas with a wide range of confidence intervals) after further adjustment for socio-demographic and lifestyle covariates in subsequent models and hence, we acknowledge that due to the low numbers, the results may not be extrapolated to populations with high herbal tea consumption. We therefore stand by our more stable, original analyses of dichotomised subtypes of tea.[130] For coffee intake, Huang et al. then conducted an unadjusted Chi-square test on our data and concluded that by dichotomising coffee intake into any vs. no, the association with significant liver fibrosis no longer existed. We cannot but disagree with using simple unadjusted Chi-square testing for making such strong inferences on complex data that can be subject to confounding. In either way, it is not surprising that pooling moderate and frequent consumption into one category with a wide range of consumption (from less than 1 up to 8.5 cups per day) would diminish the effect. There probably is a dose-effect relation which is supported by: a) our significant results for only frequent consumption, and b) a significant *P* for trend in all our coffee analyses. Also, although food and beverage categorisation reflect actual real life consumption more reliably than continuous data,[457] continuous coffee consumption *was* associated with LSM≥8kPa ($OR_{increase\ per\ cup}$ 0.84, 95%CI 0.72–0.96, *P*=0.014). This is further attested by a recent large umbrella-review of meta-analyses on coffee and human health which concluded that there was evidence of a non-linear association between coffee consumption and health outcomes with the largest relative risk reduction at three to four cups a day.[125]

Table 2

	log-transformed LSM		**LSM≥8 kPa**	
	beta (95%CI)	P-value	OR (95%CI)	P-value
		Model 3†		
Coffee				
no	0 (ref)	**0.001**	1 (ref)	**0.005**
moderate	–0.026 (–0.083; 0.032)		0.75 (0.34 – 1.68)	
frequent	**–0.066 (–0.120; –0.012)**		**0.39 (0.18 – 0.87)**	
Herbal Tea				
no	0 (ref)	**<0.001**	1 (ref)	0.274
any	**–0.053 (–0.082; –0.024)**		0.75 (0.45 – 1.26)	

†Model 3 (i.e. full adjustment): adjusted for tea or coffee, energy intake, BMI, gender, age, steatosis, ALT, excessive alcohol intake, current or former smoking, HOMA-IR, soda consumption, cream and sugar use, DHDI, physical activity, lipid-lowering drugs and anti-diabetic drugs. Significant results are marked in bold.

Secondly, the authors questioned whether we had accounted for the use of lipid-lowering and anti-diabetic drugs as potential confounders. In addition to the many other confounders we already adjusted for in our analysis, we agree that these drugs, could possibly additionally confound the relation between coffee, tea, and liver health. We therefore obtained detailed information on medication use from automated linkage to pharmacies with which 98% of the participants were registered. The most important results on the association of beverage consumption and liver stiffness additionally adjusted for lipid-lowering and anti-diabetic drugs, are depicted in *Table 2*. As can be seen, these results are nearly identical to the results from our original analyses, and hence, the abovementioned drugs do not seem to additionally confound the observed associations.

In conclusion, in this brief reply we demonstrated that (1) further categorisation of subtypes of tea led to comparable, but less stable results; we endorsed that (2) dichotomisation of coffee consumption was not associated with liver health, possibly because of a dose-responsive effect of coffee; and we showed that (3) both lipid-lowering and anti-diabetic drugs did not further confound our observed associations on coffee, tea, and liver stiffness.

Chapter 13

Animal protein intake and hepatic steatosis in the elderly – Authors' response

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Gut. 2020 Jan;69(1):189

Response

We would like to thank *Tang and Mann* for their interest in our study in which we showed that animal protein intake was independently associated with higher prevalence of non-alcoholic fatty liver disease (NAFLD) in an overweight, predominantly aged Caucasian population.[344,458]

Rightfully so, the authors express their concerns regarding the generalisability of our findings towards younger patients with clinically significant non-alcoholic steatohepatitis (NASH).[458] Indeed, our results only pertain to our study population, consisting of an elderly and presumably healthy population in which the vast majority will have NAFLD, not NASH. Nonetheless, we believe that aiming for a healthy lifestyle, such as adherence to a healthy diet, is key across the entire spectrum of NAFLD and is not reserved for advanced disease only.[13] Also, *Tang et al.* question the clinical significance of NAFLD as a liver disease at the age of 70 years, but patients with NAFLD are, in fact, twice as likely to die from cardiovascular disease than from liver disease itself.[17] As cardiovascular disease is a clinically relevant disease in the elderly, we do believe that NAFLD embodies a relevant trait in our population with a mean age of 70 years.

Other remarks that are put forward in the letter were, in fact, already elaborated on in the discussion of our paper.[344,458] Briefly, energy intake as assessed by a semi-quantitatively food-frequency questionnaire is not reliable because it is known to be underreported.[278] This under-reporting does generally not affect the energy-adjusted macronutrient composition of the diet. In addition, we extensively adjusted for energy intake in various ways[457] to account for extraneous variation and potential measurement error. Second, we have not studied mechanistic pathways underlying our findings. We have merely discussed two already established hypotheses to explain the association between animal protein and NAFLD. Future studies are needed to explore these hypotheses in depth before conclusions can be drawn. Finally, in contrast to the impression by *Tang and Mann,* our main findings are not that surprising at all. Mounting evidence from other large population studies (with an average age of about 55 years) support our results on animal protein being associated with higher prevalence of NAFLD.[247,352] Also, the presumable detrimental effect of mono and disaccharides is thought to be only true in presence of excess caloric intake or related to specific food sources such as sugar-containing beverages, for which we already corrected in our analyses.[459]

In conclusion, our population-based study gives insight into the correlation between macronutrients and NAFLD. However, mechanistic and (randomised) intervention studies are ultimately needed to explore causality before recommendations can be made.

Part VII

DISCUSSION

Chapter 14

Summary & Discussion

Rationale behind this dissertation

At present, approximately 3.5% of global mortality is due to liver disease. This number is greater than deaths due to classical health treats such as human immunodeficiency virus or tuberculosis. The spectrum of liver disease ranges from mild (steatosis or hepatitis without fibrosis) to severe liver injury (cirrhosis and hepatocellular carcinoma).[7] Fortunately, not every patient with a liver disease will die from this condition, mostly due to competing mortality risks and possibility of reversal of liver injury. In the last decades, alongside the obesity pandemic, non-alcoholic fatty liver disease (NAFLD) has become a global health burden. The prevalence of this disease is currently estimated to be 25% worldwide[7] and as such NAFLD has surpassed other classical liver diseases and became the most prevalent liver disease in adults. Alarmingly, the same is also true for children[363], in which over 20% of the NAFLD patients already have (pre)diabetes.[18] The most prevalent cause of death in all patients with NAFLD, however, is not liver related but cardiovascular.[17]

Studying the effect of lifestyle on the development of liver disease requires a large study cohort. Fortunately, with the non-invasive diagnostic tools that are available today, we were able to study liver disease in a large elderly population, the so-called Rotterdam Study. In this dissertation entitled "Lifestyle and liver disease in the general population" we focussed on the rather mild spectrum of liver disease, i.e. steatosis and/or fibrosis in which non-invasive lifestyle measures might play a preventing or reversing role. In the second part we examined the role of coffee consumption in attenuating or preventing liver disease. In part III, we studied the role of diet in relation to NAFLD. In part IV we focussed on the connection between the different aspects of body composition and NAFLD. Finally, in part V we studied the gut microbiome and its predicted function in hepatic steatosis.

Summary & Discussion

Part II: Coffee & liver health

The idea to study the connection between coffee, tea, and liver health emerged from previous studies that found an association between coffee and tea with lower all-cause and cause-specific mortality by means of attenuating risk factors associated with the metabolic syndrome.[125] Moreover, preliminary evidence existed for a possible protective effect of coffee and tea specifically on liver health.[460,461]

In **Chapter 3** we showed that consumption of 3 or more cups of coffee per day was inversely associated with liver stiffness (measured by Fibroscan®, using ≥8 kilopascals as

proxy for fibrosis), but not with liver steatosis. Of all the different subtypes of tea, only herbal tea (not green nor black tea) was associated with lower liver stiffness. With this study we were the first and largest to examine both coffee and tea with liver fibrosis and steatosis. However, inherent to the epidemiological study design, we could only speculate on the pathophysiological mechanisms behind these associations. We therefore carried out a systematic review on the experimental evidence behind the association between coffee and liver health in **Chapter 4**. Constituents in coffee that had been most studied were polyphenols, caffeine and coffee lipids. We found several pathways. The anti-steatotic effect of coffee could be effectuated by increased fatty acid beta-oxidation and decreased fatty acid synthesis. Also, coffee had an observed positive effect on the glutathione content in the liver, which is known to reduce oxidative stress and consequently lower steatosis and fibrosis. The anti-fibrotic effect of coffee was achieved by lowering transcription growth factor beta. Also, caffeine itself seemed to have a direct antagonizing effect on adenosine A2A receptors, which inhibits the hepatic stellate cell activation. Lastly, both fibrosis and carcinogenesis were supressed by coffee through the modulation of apoptosis, transcription factors, and extracellular signal-regulated protein kinases.

The population-based study on coffee and herbal tea has drawn quite some attention in the scientific field, resulting in three letters to the editor.[462-464] We replied to two of these letters, which can be found in **Chapter 11 and 12**. One of the main questions expressed in these letters was related to our finding of herbal tea being beneficial for liver health, which surprised the scientific community. Indeed, there is ample evidence that many herbal infusions and extracts could be hepatotoxic rather than therapeutic.[465] We fully agree that herb consumption is not without hazard and could even lead to drug-induced-liver injury, especially when using non-regulated herbal ingredients. Importantly, however, is that the consumption of herbal tea in our elderly population was low (median 0.8 cup/day) and typically limited to pre-packaged FDA approved teas such as mint, chamomile or Rooibos. These types are not known to be hepatotoxic.

Part III: Diet & NAFLD

Diet plays an important role in both the development and regression of NAFLD. Previous studies have showed that losing 5% or more of the body weight is effective in reversing steatosis and that losing 10% or more in reversing fibrosis, even in non-obese patients.[240,329,466] On the other hand, there is also evidence that NAFLD may reverse upon conversion to a healthy diet without co-occurrence of weight loss or reduction in energy intake.[285] In part III, we therefore studied the role of dietary quality in NAFLD independent of energy intake in more detail.

In **Chapter 5**, we examined the association between macronutrient intake and NAFLD. We found that, in particular high animal protein intake was associated with greater prevalence of NAFLD in overweight participants. In addition, we found a trend towards lower prevalence of NAFLD in participants with high mono-and disaccharide consumption. However, the latter association was not independent from metabolic confounders.

Although a plant-based diet, rather than an animal-based diet, is also in line with the well-advocated Mediterranean diet, this study was found to be quite provocative and seemingly contradictive to previous studies. It thus caused discussion in both semi-scientific and scientific media. Historically, carbohydrates were believed to be the main harmful macronutrient associated with NAFLD.[467] However, in most studies, it is hard to separate the effect of fructose-containing sugars from that of other dietary factors such as energy intake (which is often high in sugar-rich food items).[468] In addition, most studies examined soft drinks as food item embodying the macronutrient subtype 'mono-and disaccharides', whereas fruit for example also belongs to this group. Also, there was discussion on the finding whether animal protein would be harmful for steatosis. Indeed, a small study by others implied a beneficial instead of a harmful effect on liver health.[262] On the other hand, there is a growing mountain of evidence from large-scale studies on the harmful effect of a diet rich in animal-protein with several health outcomes including liver disease.[260,352] Nonetheless, one of the main messages from our study was that there were no specific substitution effects in macronutrient replacement analyses, which underlines the ongoing need of a diverse, rather than a restrictive diet.

A letter to the editor was written in response to our paper by *Tang and Mann* expressing their concern on the overall relevance of our results given the use of an elderly population, and the possibly limited generalizability of our data towards younger patients with NASH.[469] Our response to this letter can be found in **Chapter 13**. Naturally, caution of generalisability is always warranted, but we believe that our results are relevant even though it concerns elderly people with NAFLD. Although these elderly with (simple) steatosis will likely not progress to end-stage liver disease in their life time, NAFLD patients are twice as likely to die from cardiovascular disease than from liver disease itself, which makes our findings still clinically relevant.[240]

Which pathophysiological mechanism is specifically responsible for the association between animal protein intake and NAFLD is still not fully elucidated. However, we hypothesized that subclinical metabolic acidosis induced by high diet-dependent acid load (DAL) in animal-protein rich diets could play a role.[274] We sought to examine this hypothesis in a spin-off study that can be found in **Chapter 6**. The rationale behind this hypothesis is that a diet rich in food items that supply acid precursors (such as sulfuric acid from meat and

fish) and low in food items that supply base precursors (such as citrate and bicarbonate from vegetables and fruits) lead to a homeostatic disturbance in acid-base balance. There are several (validated) algorithms that proxy the dietary (renal) acid load which we used in this study. Participants in this study appeared to have a relative alkaline diet compared to other study populations. The main finding was that DAL was independently associated with NAFLD. This association, however, was not linear. The highest probability of NAFLD (37%) was found for an acidic diet and the minimum predicted probability of NAFLD (29%) for an alkaline diet, supporting our hypothesis.

Interestingly, macronutrient subtype mono-and disaccharides were inversely correlated to DAL, whereas macronutrient subtype animal protein was positively associated with DAL. Opponents of the DAL-hypothesis may argue that it is just another way to score adherence to a healthy diet. This argument we cannot completely exclude on the basis of our study, but the association between DAL and NAFLD was not fully explained by dietary quality score either. Nevertheless, it would be interesting to do future research using acid-base biomarkers such as urinary ammonium instead of the algorithms we used, to study low-grade metabolic acidosis more objectively.[470]

A consistent limitation of the previous two studies was their cross-sectional design, which hampered us to draw conclusions on cause-effect relations. Therefore, we performed a longitudinal study in **Chapter 7** exploring the effect of long-term adherence to dietary patterns and the risk of developing incident NAFLD over time. After a follow-up period of 4.5 years, most participants had regression of NAFLD (30%) whereas only 5% developed incident NAFLD. We selected three well-known *a-priori* healthy dietary pattern scores, i.e. the Mediterranean Diet Score, the World Health Organization (WHO) score and the Dutch Dietary Guidelines. We found that adherence to the WHO-score was particularly associated which regression of NAFLD. In addition, we identified five factor-analysis derived *a-posteriori* dietary pattern scores, i.e. *vegetable & fish*, *red meat & alcohol*, *traditional*, *salty snacks & sauces* and *fats & sweets*. Only adherence to the *traditional* pattern -characterised by a high intake of vegetable oils & stanols, margarines & butters, potatoes, whole grains, and sweets & desserts- was associated with the regression of NAFLD. All analyses were adjusted for BMI, which is of particular importance as BMI over time depended on the adherence to these dietary patterns. Interestingly, the dietary patterns that were associated with regression of NAFLD had a macronutrient composition that was mostly plant-based, high in fibre and low in fat. Which is in line with our initial cross-sectional results from **Chapter 5** and **6**.

Part IV: Body composition & NAFLD

Apart from diet, body composition of its own, in particular adiposity, is strongly related to NAFLD as well. At the same time, it is known that not all obese individuals have NAFLD and *vice versa*. Indeed, we know that BMI is a suboptimal measure of adiposity. Recently, emerging evidence has suggested that sarcopenia also independently contributes to the development of NAFLD too.[370,371] These studies, however, predominantly originated from Asia, in which non-obese NAFLD is much more prevalent than in Western countries.[12]

In **Chapter 8** we therefore studied the independent association of the different components of the body (fat and muscle mass) with NAFLD, stratified by sex and BMI. In this study we showed that incremental skeletal muscle mass was consistently associated with lower NAFLD prevalence and severity in normal-weight women. A similar observation was made in men, though this did not hold true after multiple testing correction. Also, we showed that high fat mass was a better predictor of NAFLD than low muscle mass. In particular android fat mass (collected around the waist) was associated with higher NAFLD prevalence, whereas gynoid fat mass (collected around the hips) was associated with lower prevalence of NAFLD. Likewise, amongst all body composition parameters android-to-gynoid fat mass ratio was the best predictor for NAFLD. We did not find an association between sarcopenia (low skeletal muscle mass plus low physical performance/strength) and NAFLD. Possibly, this was because of the relatively high median skeletal muscle mass and rare occurrence of sarcopenia in our study population.

The importance of making careful adjustments for body composition in these types of analyses is emphasized in **Chapter 9**. In this chapter we write a letter to the editor in response to the original paper of *Wong et al.* that addressed the important issue whether lifestyle modification is as important in non-obese NAFLD as it is in obese NAFLD.[329] In their study of 154 participants, even in the non-obese, relative weight loss upon lifestyle modification of at least 3% was advised. As follow-up measure, the authors used the Fatty Liver Index (FLI) to diagnose NAFLD and concluded that obese patients had a higher FLI than non-obese patients. The FLI is a non-invasive algorithm composed of waist circumference, BMI, triglycerides and gamma glutamyltransferase. In our letter, we show that the sensitivity and specificity of FLI stratified by BMI is limited because of the fact that BMI is incorporated in the same formula. We hence advocate against the use of the FLI-algorithm in the context of analyses on body composition and NAFLD.

Part V: Gut microbiome & hepatic steatosis

One of the most temporary topics of today is the gut microbiome, our 'new DNA'. Because of the anatomic connection between the gut and the liver -via the portal vein-, and the explicit relation between external (lifestyle) factors and gut microbiome composition, the gut microbiota has been proposed to be involved in NAFLD pathophysiology.[471]

In **Chapter 10** we performed the largest study to date on the composition and diversity of the gut microbiome and metabolomics in relation to steatosis. We showed that lower microbial alpha-diversity was associated with higher prevalence of steatosis. In addition, the composition of the gut microbiome was significantly different in participants with steatosis than those without. Thirty-seven genera contributed to this difference of which *Coprococcus3*, *Ruminococcus Gauvreauiigroup*, and *Ruminococcus Gnavusgroup* remained associated with steatosis after extensive adjustment for important confounders, such as BMI and diet, and after multiple testing correction.

The metabolomic top-hits for steatosis included higher branched-chain amino-acids (BCAAs), higher aromatic amino acids, higher glycoprotein acetyls (an acute-phase protein) and a detrimental lipid profile. Interestingly, incremental alpha-diversity was associated with lower BCAAs, lower glycoprotein acetyls and a favorable lipid profile, and hence had an opposite metabolic profile than steatosis. *Ruminococcus Gnavusgroup* was also significantly associated with glycoprotein acetyls, which is in line with previous findings that presence of *R. Gnavusgroup* was found in individuals with lower microbial richness, and atherosclerotic cardiovascular disease. *Coproccocus3,* which had a beneficial association with steatosis, was associated with lower fasting glucose and higher microbial richness.

Furthermore, the composition of the gut microbiome in steatosis was predicted to contribute to secondary bile acid synthesis, which confirmed the findings of a recent paper of *Jiao et al*. which found increased secondary bile acid synthesis in NAFLD.[442] Although no causal inferences can be made, our results do suggest a role for alpha-diversity in explaining metabolic differences between individuals with steatosis and those without.

Methodological Considerations

Study Design

The Rotterdam Study commenced in 1989 and our Hepatology department joined the Rotterdam Study in 2009. At that time three different cohorts had already been included in this study (RS I, RS II and RS III). All inhabitants of the suburb Ommoord of 45 or 55

years and older were asked to participate. Response rates were 78%, 67% and 65% for the three cohorts respectively.

Although participation rate is quite reasonable, in fact on the higher end in comparison to other large-scale epidemiological cohorts,[472] we cannot exclude the possibility of selection bias. But, we adjusted our analyses for many sociodemographic confounders such as age and education level whenever possible. Theoretically, the prevalence of liver disease amongst non-responders could differ from the responders. However, the interpretation of our findings would only be altered if the association between exposure and outcome would be entirely different in the non-responder population compared to the responders. Given the use of an unselected population, this is rather unlikely.[473,474]

Also, observational studies are subject to confounding and bias. However, the large size, prospective nature, and the large number of recorded potential confounders in the Rotterdam Study makes it possible to overcome most biases.[475] Nevertheless, particularly in light of studying lifestyle as exposure, associations may still be affected by residual confounding, i.e. confounding that takes place by unknown and often unmeasured factors despite adjusting for a great number of known confounders.

Given the predefined setting, our population consisted of elderly people predominantly of European origin (>95%). Therefore, in most studies we discussed that caution should be taken before extrapolating our findings to other, younger or more racially mixed, populations.

Most of the studies in this thesis are of cross-sectional design, which makes it impossible to draw conclusions on causality. Even prospective studies do not conclusively prove causal relations.[476] However, I believe that, epidemiologic studies work complementary in revealing possibly new relationships that can further be studied in more detail by experimental or fundamental studies.

Dietary data

Collection of high quality nutritional data is challenging and has several aspects that need mentioning. In the Rotterdam Study nutritional data was collected retrospectively using semi-quantitative food frequency questionnaires. The advantage of this method is that it is relatively inexpensive, easy and validated. On the downside, it relies on memory which could lead to differential measurement error (recall bias) or non-differential measurement error (typically over- or underreporting).[251] Recall bias is thought to influence results only if it would occur more often in the diseased or the non-diseased group, which is unlikely.

Non-differential measurement error, however, is likely to have occurred in our study. In fact, the finding that overweight participants had lower median energy intake than non-overweight participants in **Chapter 5** is exemplary for this bias. Fortunately, there are methods to deal with this type of measurement error. For example, by adjusting for energy intake using the nutrient density method or the residual method.[302] In our papers we used either of these methods to account for the extraneous variation in energy intake and potential measurement error. A disadvantage of these correcting methods is that associations between energy-dense food and the outcome can be attenuated when using adjustments for energy intake.[345] We therefore adjusted for energy intake after defining the dietary patterns in **Chapter 7**.

Furthermore, from a statistical point of view it is generally better to analyse data continuously instead of categorically in order not to lose information. However, semi-quantitative food frequency questionnaires are better in picking up patterns of consumption instead of examining absolute intake. Moreover most questionnaires have been validated using ranking instead of absolute comparisons. Therefore, dietary data are actually more accurate and informative when analysed categorically. A letter to the editor was written in response to **Chapter 4** which was related to this issue. The authors questioned why we categorized coffee into non-drinkers, moderate and frequent consumers. In a response, however, we showed that continuous coffee consumption was associated with elevated liver stiffness as well (**Chapter 12**). It is therefore unlikely that the information lost by categorization had a significant impact on our results.

Finally, part of our study population completed the FFQ prior to abdominal ultrasound, because we had no dietary data available at visit II of cohort 3. We therefore used the dietary data of cohort 3 visit I, which was 5.5 years earlier. Because dietary data are known to be stable over time, we assumed that dietary habits in this elderly population would be rather constant.[109] This assumption was indeed justified by a paper of *Schoufour et al.* who showed that dietary quality was rather stable for all individuals over a follow-up period of 20 years in the Rotterdam Study.[321] In addition, we performed sensitivity analyses in our studies, stratifying by cohort, showing this did not change our results.

BMI as covariate

BMI is a very important covariate in NAFLD research, but it is challenging to determine whether BMI is an actual confounder when looking at the principles of causal inference. A confounder is an underlying factor that affects both the exposure (e.g. diet) and the outcome (e.g. NAFLD) variable, causing a false association. As a result, a confounder cannot be in the pathway of the association as an intermediate. As BMI is highly influenced by

diet, it is more likely that BMI is a mediator in the pathway between diet and NAFLD than a confounder.[477] We therefore chose to adjust for BMI in a separate model or as sensitivity analysis in the studies of part III.

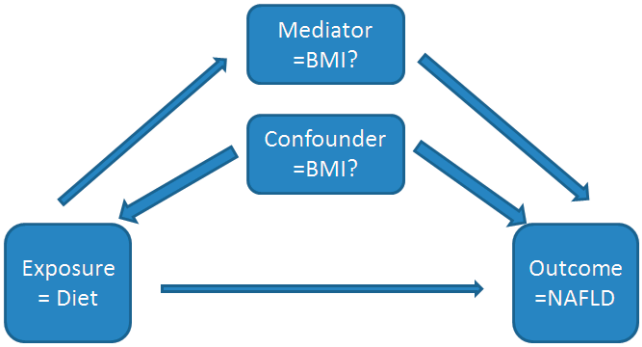

Figure 1: The possible roles of BMI as covariate

In part IV, BMI was a challenging covariate too. One of our research questions was to study the association between skeletal muscle index (kg/m²) and NAFLD. Adjusting for BMI (kg/m²) as all-encompassing body measure was therefore not possible. We thus came up with the idea to borrow the concept of substitution analyses from nutritional analyses and use it in body composition analyses. This way we could pose the hypothesis on "what if all of body component X is substituted by component Y?" without having to adjust for BMI as covariate.

Ultrasound & transient elastography

The golden standard for the diagnosis of liver fibrosis and NASH is a liver biopsy rather than ultrasound or transient elastography.[49] The use of liver stiffness as proxy for liver fibrosis in the general population has therefore been a general topic of debate. But a liver biopsy is burdensome, and more importantly, not without risk of complication. There is an estimated 0.5% of haemorrhagic complications.[47] Therefore, the performance of liver biopsies in the general asymptomatic population is ethically debatable. The cut-off values we used to diagnose elevated liver stiffness (or fibrosis) originate from smaller, disease-specific populations.[79] Hence extrapolating these cut-offs to the general population could have led to spectrum bias in our study. However, as there are no good correlative studies on degree of elastography and degree of fibrosis on histology in unselected and asymptomatic populations, we do not have a better alternative as of yet.

The use of ultrasound as diagnostic tool for NAFLD can be debated as well because of its low sensitivity for liver fat content below the threshold of 20%, and subjective estimation

of steatosis severity.[50] It is however questionable to what extent presence of steatosis less than 20% is clinically relevant. Furthermore, we have deliberately not categorized steatosis into mild, moderate or severe, but instead chose to dichotomize this outcome measure in no steatosis vs steatosis (regardless of the stage). In that setting, ultrasound yields quite satisfactory sensitivity and specificity. In addition, in contrast to magnetic resonance or histology, ultrasound is inexpensive, widely available and harmless which makes it ideal for screening purposes.

Microbiome

The gut microbiome is highly variable and dependent on environmental factors such as diet and demography, and on personal factors such as ethnicity and age.[478] Its composition has been showed to change even after a couple of days on a different diet.[440] Moreover, the scientific community is not unanimous on the gold standard approach for gut microbiome analyses. Analysis of the gut microbiome is so challenging because of the many variable factors.

First of all, there are several methods for the collection of gut microbial RNA. We used fecal stool samples from which we isolated RNA because it is easy to obtain on a large-scale and has been widely used in microbiome literature. However, fecal stools do not necessarily reflect the microbial variation throughout the gut as well as mucosal biopsies.[479] In addition, the handling of the stool samples is a delicate matter. We were compelled to collecting the thousands of stool samples via postal mail and allowed a maximum of 3 days travel time in order to exclude microbial overgrowth. However, ideally, direct freezing of stool samples at –80 degrees Celsius is desired.[480,481] In addition, because of the large-scale study, not all samples were analysed in the same round/batch which could have caused technical artifacts.[482] We therefore took the batch effect into account as potential confounder in our analyses. Also, we used 16S rRNA sequencing, which has the advantage to being both quick and thorough and thus suitable for large scale analyses to capture shifts in microbial diversity. On the downside, it is not as precise as metagenomic approaches such as qPCR. Furthermore, subsequent (arbitrary) choices in the analysis pipeline, such as the cut-off value of minimal reads (10.000 in our study) and clustering to operational taxonomic units and subsequent classification of those units brings along some uncertainty. Also, our gut microbial data shows great differences in abundance and the large number of zeros of the various bacteria. One can question what is the best analysis tool? To date, most studies still use ordinary logistic regression analysis. We chose to use regularized lasso penalties that take into account the compositional nature of the microbiome and the large amounts of zeros.[428] Lastly, as with all genetic studies, replication is highly relevant and, according to some, even essential. Unfortunately in our case, no other large cohort with similar pheno-

type data was available for replication. Either way, one could argue that the added value of replicating data on gut microbiome in a different population with different environmental factors would probably be limited.

With all abovementioned limitations in microbiome analyses, it could be discussed whether it would be better to focus on the metabolomic signature of phenotypes instead of the gut microbial composition itself.

Personal view on future directions

First of all, I would like to spend a few words on proposition number seven of this thesis, i.e. "NAFLD is a benign condition". One might think; why would you write an entire thesis on a potentially benign condition? That is because I do not believe NAFLD is as benign as it sounds. It is an important risk factor in the development of both metabolic syndrome and severe liver disease. Just like obesity is a risk factor in the development of diabetes, and hypertension in the development of cardiovascular disease. Only because these diseases are at the mild side of the disease spectrum does not mean they are harmless. I therefore believe that NAFLD is a risk factor that ultimately belongs in the first-line cardiovascular risk assessment and management.

It is worrisome that NAFLD incidence increases already in children.[363] Because of the future that lays ahead of them, they have enough time to actually develop clinically relevant advanced liver disease and ultimately liver failure. Further research in the population-based applicability of non-invasive tools such as transient elastography and controlled attenuation parameter is needed so they can be used in first-line care centres to stratify young patients at high risk of developing advanced liver disease. Fortunately, endeavours are currently made to validate the Fibroscan in the general population by the international Liverscreen consortium.[483] At the same time, we need to be aware to over-referral in high-risk populations, a real challenge for health-care workers and policy-makers alike.[283]

In this thesis we focused mainly on dietary quality and previous studies already showed the importance of dietary quantity. Interestingly, there might be a third aspect which could effectuate metabolic health: dietary timing. Intermitted fasting for example is a concept that restricts the time in which one eats with or without restriction of calories. The rationale behind this diet is that time of feeding needs to be synchronized with circadian biology. The master biological clock is the suprachiasmatic nucleus in the hypothalamus that reacts on light stimuli. However, other 'clocks' have been found in peripheral tissues such as the liver, that react on feeding stimuli. Desynchronization of this rhythm (for example in humans that work with shifts) is associated with increased risk of cardiometabolic disease

and cancer.[484] The idea of circadian timing in therapeutics is not new, it has also been proposed to increase therapeutic efficacy of drugs, so-called chronotherapy.[485] Interestingly, a 5-week randomised cross-over, isocaloric and eucaloric strictly controlled feeding trial in eight prediabetic men showed that early time-restricted feeding lowered insulin levels, blood pressure, and oxidative stress levels. Moreover, it increased fasting triglyceride levels which was hypothesized to be caused by re-esterification following lipolysis in the liver.[486] Moreover, there are human trials that suggest that consumption of the largest caloric meals early in the day while reserving the fasting window for early in the evening offers an additional benefit to serum lipid composition and glycemic control.[487]

To date however, there has only been one published abstract of a cross-sectional study from the NHANES cohort that analysed timing of eating with NAFLD as assessed by ultrasound. The authors found that more meals per day lowered the odds of steatosis and that skipping morning and midday meals were associated with higher odds of steatosis independent of the total amount of daily calories.[488] This study, however has not yet been published as full article. I believe, given the background data on metabolic syndrome and rationale, it is of interest to study this topic in relation to NAFLD in the future.

In part IV we showed that BMI is an imperfect measure for adiposity and that adiposity itself is a strong predictor of NAFLD. But not all adipose individuals are metabolically unhealthy and not all normal-weight individuals are metabolically healthy.[489] In fact, there actually is a subgroup of obese individuals (estimated prevalence of 12–35%) that is obese without metabolic comorbidities, the so-called 'healthy obese'.[490] Possible underlying molecular mechanisms are to be found in the capacity and type of adipocytes. Generally, subcutaneous adipose tissue is metabolically beneficial through their increased adipogenesis and browning potential. Visceral fat on the other hand is associated with upregulation of pro-inflammatory cytokines and chemokines. In addition, inflammation of adipocytes are thought to relate to adipocyte dysfunction and promote systemic insulin resistance and low-grade inflammation.[391] I suggest to embark on more studies in these 'healthy obese' to better understand the natural history of NAFLD and the course towards developing metabolic comorbidities or disease progression.

The gut microbiome is a hot scientific topic. And although there are lots of unanswered questions on analysis and interpretation (see methodological considerations), I believe the gut microbiome is the new area of research in both therapeutic and diagnostic strategies coming decades. Although the underlying mechanisms are not entirely clarified, the gut microbiome is already successfully used as therapeutic strategy in patients with recurrent *Clostridium Difficile* infection by means of faecal microbiota transplantation. It is thought to stimulate toll-like receptors, to inhibit growth of excessive species and to compete with niche exclusion.[491] At this moment, endeavours are underway to use faecal microbiota

transplantation in patients with liver cirrhosis and therapy-resistant hepatic encephalopathy.[492] Perhaps in a couple of years, faecal microbiota transplantation might be a therapeutic option in NAFLD as well. And if not therapeutic, I believe the gut microbiome and its related metabolites could have a (non-invasive) diagnostic function in clinical screening and/or follow-up of NAFLD (severity).[493,494]

Recently, an interesting dissertation "Glucocorticoids and obesity" from *Wester et al.* was published. The authors discuss that small variations in serum glucocorticoid concentration, due to psychosocial stress or local glucocorticoid use as measured by hair cortisol, may influence cardiometabolic health.[495] Interestingly, their data suggests that local glucocorticoid therapy, such as inhalation corticosteroids or topical agents, could be causative in the onset of obesity and associated cardiometabolic traits. To date, no study has examined the association between local glucocorticoid use and NAFLD. I propose to study local glucocorticoid use in NAFLD as well, especially given the widespread use of these agents.

As the title of this dissertation implies, we mainly focussed on 'lifestyle' as factor in liver disease. Nonetheless, hundreds of studies are currently carried out to ultimately launch a pharmaceutical agent for the treatment of NASH. From an ethical point of view, is the use of pharmaceutical agents justifiable in the case of lifestyle-driven diseases? The market for approved drugs in NASH is estimated to be worth about 20 to 35 billion dollars per year by 2025.[496] If we had to choose to either spend money on the development of a pharmaceutical solution to cure a small number of (advanced) NASH patients or spend the same amount of money on large-scale prevention programs to reduce incidence of NAFLD, what would be the best choice? In addition, could the presence of a pharmaceutical cure take away the motivation to improve a sedentary lifestyle? Five phase 3 drug trials are currently being conducted or completed. Meanwhile, very few randomized-controlled lifestyle trials have been done. Some of these drugs seem promising, but the effectiveness in all these agents is thus far less than 50%. A recent 18-month interim analysis of the promising agent Obeticholic acid was published. This FXR agonist has the therapeutic goal to improve fibrosis histology without worsening other NASH features OR to resolve NASH altogether. In the treatment group 23% of the patients achieved this primary treatment goal compared to 12% in the placebo group (p=0.0002).[497] Although this interim analysis is promising, Obeticholic acid has worrisome side effects as well. It causes a rise in atherogenic LDL-cholesterol, triglycerides and a reduction in large and medium sized HDL-sub particles.[498] Fortunately, this side effect is reversed after a couple of weeks upon discontinuation of the agent. However, the most important cause of mortality in NASH patients is not related to liver disease, but to cardiovascular disease,[17] it is therefore of great importance to follow-up on clinical events in these trials to see if this unfavourable lipid profile lead to any clinical events.

Meanwhile, could government regulations that promote a healthy lifestyle and discourage a sedentary lifestyle help to further prevent the rise of obesity and diabetes? For example, with sugar taxes or regulations that prevent discount on unhealthy food items? On the other hand, research also points out that especially the lower socioeconomic families are disproportionally affected by these measures, encouraging socioeconomic inequality.[499]

As discussed extensively throughout the dissertation, our results do not imply causality. Fundamental and intervention trials are needed to make progress on the topic lifestyle and liver disease. I believe lifestyle intervention trials should be encouraged beyond the small dietary randomized controlled trials[330] and larger-scale trials with vitamin E treatment that are available today.[63] Given the body of evidence, the use of coffee as nutraceutical could be used in a small intervention trial (caffeinated vs decaffeinated or filtered vs unfiltered) as first step towards lifestyle-based therapeutics. Meanwhile, dietary recommendations that are in agreement with an acid-base balanced or plant-based diet are generally considered beneficial for health and adherence to such a diet while awaiting the results of future studies seems justifiable.

Furthermore, in clinical practice more attention should be paid to body composition beyond BMI to assess adiposity, such as skeletal muscle mass (e.g. using bio-electrical impedance) and waist-to-hip ratio. Also, there is enough rationale of resistance training in order to replace fat mass by muscle mass (regardless of weight loss) in patients with NAFLD.

Conclusion

In summary, there is still much to be investigated before we are able to effectively prevent and treat NAFLD. However, given the many endeavours by scientists, clinicians and nutritionists all over the world, the future appears bright and hopeful.

Chapter 15

Nederlandse Samenvatting

Rationale voor dit proefschrift

Vandaag de dag is 3.5% van de wereldwijde mortaliteit te wijten aan leverziektes, een getal groter dan mortaliteit als gevolg van bijvoorbeeld HIV of tuberculose. Het spectrum leverziektes varieert van mild (vervetting of hepatitis zonder fibrose) tot ernstig (cirrose of hepatocellulair carcinoom).[7] Gelukkig overlijdt niet iedere patiënt met een leverziekte ook daadwerkelijk aan deze ziekte, want de meeste leveraandoeningen zijn (deels) reversibel. Bovendien zijn er andere –concurrerende– gezondheidsrisico's, met name cardiovasculair, waaraan patiënten kunnen overlijden. De meest voorkomende doodsoorzaak bij patiënten met *non-alcoholic fatty liver disease* (NAFLD), is inderdaad niet lever-gerelateerd, maar cardiovasculair.[17] NAFLD is de meest voorkomende leverziekte wereldwijd, met een geschatte prevalentie van reeds 25% onder volwassenen.[7] Nog verontrustender is dat hetzelfde geldt voor de prevalentie van NAFLD onder kinderen.[363] Ruim 20% van de kinderen met NAFLD heeft namelijk al een vorm van (pre)diabetes.[18] Deze epidemie heeft grotendeels te maken met een ongezonde (Westerse) leefstijl. Het bestuderen van het effect van leefstijl op de ontwikkeling van leverziektes vereist een grote onderzoekspopulatie. Wij konden, met non-invasieve diagnostiek, de prevalentie van leverziektes bestuderen bij een grote, oudere bevolking: de zogenaamde Rotterdam Studie. In dit proefschrift getiteld: "Liver health in the general population" hebben we ons gericht op de mildere kant van het spectrum van leverziektes, d.w.z. steatose en/of fibrose, waarbij niet-invasieve leefstijlinterventies een preventieve of therapeutische rol zouden kunnen spelen. In deel II richten we ons op de rol van koffie (en thee) als behandeling of ter voorkoming van leverziektes. In deel III hebben we de rol van voeding in relatie tot NAFLD bestudeerd. We hebben ons in deel IV gericht op het verband tussen lichaamsbouw (vet en spier) en NAFLD. En tot slot hebben we in deel V het microbioom bestudeerd in relatie tot steatose.

Samenvatting & discussie

Deel II: Koffie & De Lever

De rationale om het verband tussen koffie, thee en de gezondheid van de lever te bestuderen is voortgekomen uit eerdere studies die een verband lieten zien tussen koffie en thee met een lagere *overall* mortaliteit.[125] Deze relatie zou mogelijk te maken hebben met het gunstige effect dat koffie heeft op vele metabole factoren. Daarnaast was er voorzichtig wat bewijs voor een mogelijk beschermend effect van koffie en thee op de gezondheid van specifiek de lever.[91,460]

In **Hoofdstuk 3** laten we zien dat het consumeren van 3 of meer koppen koffie per dag geassocieerd was met een lagere leverstijfheid en fibrose (gemeten met de Fibroscan®),

maar niet met leververvetting. Van de verschillende soorten thee was alleen kruidenthee (en geen groene of zwarte thee) geassocieerd met een lagere leverstijfheid. Met deze studie zijn wij de eerste en grootste studie die zowel koffie als thee heeft onderzocht in het kader van leverfibrose en steatose in de algemene populatie. Echter, inherent aan het design van deze observationele studie, konden we slechts speculeren over de pathofysiologie achter het gevonden verband. We hebben daarom een review geschreven die de experimentele rationale voor dit verband onder de loep neemt in **Hoofdstuk 4**. De meest bestudeerde bestanddelen van koffie waren de polyfenolzuren, cafeïne en koffie-lipiden. Verschillende *pathways* lijken bij te dragen aan het gunstige effect van koffie op de lever. Het anti-steatogene effect van koffie wordt toegeschreven aan de verhoogde vetzuur β-oxidatie en verminderde vetzuursynthese in de mitochondrie. Daarnaast had koffie een positief effect op de glutathion voorraad in de lever, waarvan bekend is dat het de oxidatieve stress reduceert en daarmee steatose en fibrose in de lever verlaagt. Het anti-fibrotische effect van koffie zou ook kunnen komen door de verlaging van TGF-β. Cafeïne heeft daarnaast een direct antagonerend effect op de adenosine A2A-receptoren, die de activering van de *hepatic stellate cells* beïnvloed. Tot slot lijken zowel de fibrogenese als de carcinogenese te worden onderdrukt doordat koffie apoptose moduleert evenals transcriptiefactoren en *extracellular signal-regulating proteinkinases*.

De studie uit **Hoofdstuk 3** heeft behoorlijk wat (pseudo-) wetenschappelijke media aandacht gekregen wat resulteerde in drie *letters to the editor*.[462-464] Op twee stukken hebben wij geantwoord met een kort schrijven, welke te vinden is in **Hoofdstuk 11** en **12**. Een belangrijk issue was dat het gunstige effect van kuidenthee werd betwist in relatie tot de gezondheid van de lever. Inderdaad, er is veel wetenschappelijk bewijs dat kruideninfusies en/of kruidenextracten eerder hepatotoxisch dan therapeutisch zijn.[465] We beamen dat de consumptie van kruiden niet zonder gevaar is en zelfs kan leiden tot leverbeschadiging en acuut falen. Echter dit betreft met name de non-FDA geregistreerde kruiden. Tevens is het belangrijk om te benoemen dat de consumptie van kruidenthee in onze oudere populatie laag was (met een mediane inname van 0.8 kop/dag) en doorgaans beperkt tot voorverpakte theeën zoals munt, kamille of Rooibos. Het is niet bekend dat deze theesoorten hepatotoxisch zijn.

Deel III: Dieet & NAFLD

Dieet speelt een vitale rol in zowel de ontwikkeling van NAFLD als in de behandeling ervan. Eerdere studies lieten al zien dat gewichtsverlies van 5% of meer lichaamsgewicht een effectieve strategie is voor het omkeren van steatose. Bovendien leek gewichtsverlies van 10% of meer lichaamsgewicht al effect te hebben op de regressie van fibrose, zelfs bij niet-obese patienten.[240,241,329] Echter, er zijn ook geluiden dat het aanhouden van een gezond dieet zonder gewichtsverlies of vermindering van calorie-inname persé, regres-

sie van NAFLD kan bewerkstelligen.[59] In deel III van dit proefschrift hebben we daarom gekeken naar de rol van kwaliteit/samenstelling van voeding bij NAFLD, onafhankelijk van calorie-inname of BMI.

In **Hoofdstuk 5** hebben we het verband tussen macronutriënten en NAFLD onderzocht. We zagen dat met name een hoge inname van dierlijk eiwit geassocieerd was met een hogere prevalentie van NAFLD bij zwaarlijvige deelnemers. Daarnaast leek er een trend te bestaan tussen een hoge inname van mono- en disachariden en een lagere prevalentie van NAFLD, dit was echter niet onafhankelijk van metabole factoren. Hoewel een plantaardig (i.p.v. dierlijk) dieet ook in overeenstemming is met het veelbesproken Mediterraanse dieet, bracht deze studie behoorlijk wat discussie teweeg. Klassiek worden koolhydraten namelijk juist gezien als het belangrijkste schadelijke macronutriënt binnen de ontwikkeling van NAFLD.[243] Echter, in de meeste studies die dit fenomeen bepleiten is het moeilijk om het effect van fructose te onderscheiden van dat van andere factoren die vaak met fructose-rijke voedingsmiddelen samengaan zoals een hoge calorie-inname.[244] Daarnaast hebben veel studies frisdank genomen als vertegenwoordiger voor het macronutriënt mono-en disachariden, terwijl een product als fruit bijvoorbeeld ook tot deze groep behoort. Ook werd betwist of dierlijk eiwit eigenlijk wel een schadelijke associatie heeft met steatose. Inderdaad, er zijn ook andere wetenschappelijke wetenschappelijke studies die een tegenovergesteld resultaat vinden. Zo is er bijvoorbeeld een klein experimenteel onderzoek dat liet zien zien dat dierlijk eiwit juist gunstig is voor de gezondheid van de lever.[262] Anderzijds is er momenteel een groeiend aantal onderzoeken die het schadelijke effect van dierlijk eiwit (en met name rood vlees) aantoont op de gezondheid van de lever.[260,352] Desalniettemin moet worden benadrukt dat een belangrijke boodschap uit onze studie is dat er geen substitutie-effecten waren wanneer het ene macronutriënt door het andere werd vervangen. Dit onderstreept het belang van een gevarieerd dieet in plaats van een restrictief dieet.

Ook op deze studie is een *letter to the editor* geschreven door Tang en Mann waarin zij hun zorgen uitten over de generaliseerbaarheid en relevantie van de resultaten gezien onze oudere studie populatie.[458] In **Hoofdstuk 13** hebben we op deze brief geantwoord door uit te leggen dat er uiteraard altijd voorzichtigheid geboden is bij het generaliseren van resultaten naar andere populaties. Echter, wij zijn van mening, dat onze resultaten relevant zijn ook al betreft het een oudere populatie. Want hoewel deze ouderen met steatose waarschijnlijk geen eindstadium leverziekte meer zullen ontwikkelen, hebben zij wel een grote kans om te overlijden aan hart- en vaatziekten, één van de belangrijkste gezondheidsrisico's van NAFLD en het metabool syndroom.

De pathofysiologie achter het verband tussen dierlijk eiwit en NAFLD is nog niet opgehelderd. Een van de hypotheses is dat een hoge inname van dierlijk eiwit bijdraagt aan een hogere *dietary acid load* (DAL). Deze hypothese hebben wij verder uitgediept in een spin-off studie, te vinden in **Hoofdstuk 6**.[274] De rationale voor deze hypothese is dat

een dieet met bouwstoffen die bijdragen aan een basische belasting (zoals citraat of bicarbonaat uit groenten en fruit) zorgen voor een lagere DAL. En dat een dieet met voedselproducten die bouwstoffen bevatten voor een zuurbelasting (zoals zwavelzuur uit vlees en vis) zorgen voor een hogere DAL. Als de samenstelling van het dieet zorgt voor een abnormale DAL dan zou dit het zuur-base evenwicht in het lichaam subtiel kunnen verstoren, en daarmee kunnen bijdragen aan andere metabole ontregelingen. Een drietal (gevalideerde) algoritmen zijn ontwikkeld om deze diëtaire zuurbelasting in te schatten. De participanten in onze onderzoekspopulatie bleken een relatief alkalisch dieet te hebben als we dit vergelijken met andere onderzoekspopulaties. Ook zagen we dat DAL onafhankelijk (maar niet lineair) geassocieerd was met NAFLD. De hoogste voorspelde prevalentie van NAFLD was 37% bij een zuur dieet en de laagste voorspelde prevalentie van NAFLD was 29% bij een alkalisch dieet. Dit onderstreept onze hypothese. Het was interessant om te zien dat het macronutrient mono-en disachariden omgekeerd evenredig geassocieerd was met DAL, waar het macronutrient dierlijk eiwit juist positief geassocieerd was met DAL. Opponenten van de DAL-hypothese beweren dat het gebruiken van DAL-proxies slechts een andere manier is om de kwaliteit/gezondheid van een dieet te meten. Dit argument valt niet volledig te weerleggen o.b.v. onze studie, maar de associatie tussen DAL en NAFLD werd niet volledig weggevangen na correctie voor de voedingsscore gezond eten. Desalniettemin zou het interessant zijn om in de toekomst onderzoek te doen met acid-based biomarkers (zoals urine-ammonium) in plaats van algoritmes om de subklinisch metabole acidose objectiever te bestuderen.[470]

Eén van de belangrijkste beperkingen van de vorige twee studies was het cross-sectionele design, waardoor het niet mogelijk was causale verbanden te leggen. Daarom hebben we in **Hoofdstuk 7** een longitudinaal onderzoek uitgevoerd naar de relatie tussen het volgen van bepaalde dieetpatronen en het risico op het ontwikkelen van NAFLD over de tijd. Na een follow-up periode van 4.5 jaar bleken de meest participanten regressie te hebben van NAFLD (30%), terwijl slechts 5% incident NAFLD had ontwikkeld. We selecteerden drie bekende zogenaamde *a-priori* (reeds bestaande) dieetpatronen voor deze studie. Dit zijn de Mediterraanse dieetscore, de WHO-score en de Nederlandse voedingsrichtlijn. Het volgen van het WHO dieet bleek relatie te houden met regressie van NAFLD. Daarnaast identificeerden we vijf zogenaamde *a-posteriori* dieetpatronen (met behulp van factor analyse), namelijk: *vegetable & fish*, *red meat & alcohol*, *traditional*, *salty snacks & sauces* and *fats & sweets*. Alleen het volgen van het *traditional* dieetpatroon, gekenmerkt door plantaardige oliën en stanolen, margarines en boter, aardappelen, volgranen, snoep en desserts – was gerelateerd aan de regressie van NAFLD. Alle bovengenoemde analyses waren gecorrigeerd voor BMI. Dit is zeer van belang aangezien BMI in de loop van de tijd afhankelijk was van de mate van het volgen van bepaalde dieetpatronen. Interessant ook is dat de dieetpatronen die gerelateerd waren aan regressie van NAFLD een macronutriënten

samenstelling hadden die voornamelijk plantaardig was en daarnaast veel vezels en relatief weinig vet bevatte.

Deel IV: Lichaamsbouw & NAFLD

Buiten dieet om, weten we reeds dat lichaamssamenstelling zelf, en dan met name adipositas, ook sterk gerelateerd is aan NAFLD, Tegelijkertijd hebben niet alle zwaarlijvige personen NAFLD en vice versa. De relatie is dus niet zondermeer één op één. Eerdere studies toonden inderdaad dat BMI een suboptimale maat is voor adipositas. Recent onderzoek laat zien dat sarcopenie, een tekort aan spiermassa en –functie, naast adipositas óók bijdraagt aan de ontwikkeling van NAFLD.[370,371] Deze onderzoeken komen echter voornamelijk uit Azië, waar "*lean* NAFLD" (NAFLD bij mensen met een normaal BMI) veel vaker voorkomt dan in het Westen.[12]

In **Hoofdstuk 8** hebben daarom gekeken naar de associatie tussen lichaamsbouw (vet, spiermassa en de verhouding ervan) en NAFLD, gestratificeerd voor geslacht en BMI. In deze studie laten we zien dat een hogere skeletspiermassa geassocieerd is met een lagere prevalentie van NAFLD (en lagere leverstijfheid) bij *lean* vrouwen. Dit was onafhankelijk van het soort index dat we gebruikten voor het schatten van de relatieve skeletspiermassa. Een soortgelijke associatie vonden we ook bij *lean* mannen, echter deze associatie bleef niet overeind staan na correctie voor verschillende factoren. Ook zien dat het hebben van een hoge vetmassa een betere voorspeller was van NAFLD prevalentie dan een lage spiermassa. In het bijzonder, *android* vet (vet dat zich verzameld rond de taille) was geassocieerd met een hogere NAFLD prevalentie, terwijl *gynoid* vet (vet dat zich verzameld rond de heupen) was geassocieerd met een lagere prevalentie van NAFLD. Daaruit voortvloeiend, bleek de *android/gynoid* vet verhouding één van de beste voorspellers te zijn voor NAFLD prevalentie. Een vergelijkbare maat is de taille/heup ratio. In deze studie vonden we geen associatie tussen echte sarcopenie (lage skeletspiermassa én –functie) en NAFLD prevalentie. Maar dit zou goed kunnen komen door de relatief hoge mediane skeletspiermassa en dus het schaarse voorkomen van échte sarcopenie binnen onze populatie.

Het belang van zorgvuldige statistische correcties en analyses bij het bestuderen van lichaamsbouw wordt benadrukt in **Hoofdstuk 9**. In dit hoofdstuk hebben we een *letter to the editor* geschreven als reactie op het artikel van Wong et al. uit de Journal of Hepatology. In dit artikel wordt het belang van leefstijlinterventie benadrukt bij *lean* NAFLD.[329] Het laat zien dat in deze onderzoekspopulatie met 154 deelnemers zelfs participanten met *lean* NAFLD een voordeel hadden bij gewichtsverlies van 3% of meer. Als follow-up tool gebruikten de auteurs de *Fatty Liver Index* (FLI), een proxy om NAFLD mee te diagnosticeren. De FLI-score is een algoritme dat wordt berekend met de volgende parameters: taille-omtrek, BMI, triglyceriden en gamma-glutamyltransferase. De auteurs concludeerden dat participanten met overgewicht een hogere FLI-score hadden dan de *lean* participanten.

Dat is echter logisch gezien het feit dat BMI in het algoritme is geïncorporeerd. In ons communiceren laten we zien dat de sensitiviteit en specificiteit van FLI gestratificeerd voor BMI zeer beperkt is. We pleiten derhalve tegen het gebruikt van het FLI-algoritme bij het analyseren van de lichaamssamenstelling en NAFLD.

Deel V: Microbioom & Steatose

Eén van de meest populaire en relatief nieuwe aandachtsgebieden in wetenschappelijk onderzoek is de darmflora, ons 'nieuwe DNA'. Vanwege de anatomische verbinding tussen darm en lever (via de poortader) is er een directe relatie tussen het microbioom, de stoffen die het microbioom produceert, en de lever. Er wordt daarom gedacht dat de samenstelling van het microbioom betrokken is bij het ontstaan van lever steatose.[471]

In **Hoofdstuk 10** hebben we de diversiteit en samenstelling van het microbioom, de metabolieten en de voorspelde functie ervan onderzocht in relatie tot steatose. Het is de grootste studie naar darmflora in steatose tot nu toe. In deze studie laten we zien dat een lagere alpha-diversiteit – minder soorten en slechtere verdeling van bacteriën – is geassocieerd met een hogere prevalentie van steatose en NAFLD. Daarnaast was de samenstelling van het microbioom significant verschillend tussen deelnemers met en zonder steatose. Zevenendertig genera zouden bijdragen aan dit significante verschil waarvan *Coprococcus3, Ruminoccocus Gauvreauiigroup en Ruminococcus Gnavusgroup* onafhankelijk geassocieerd bleven na multiple testing correctie en na correctie voor belangrijke factoren zoals BMI en dieet.

Binnen de *metabolomic* analyse zagen we dat deelnemers met steatose met name hogere concentraties *branched-chain amino-acids* (BCAA's), *aromatic amino acids*, *glycoproteine acetyls* en een ongunstig lipidenprofiel hadden. Het is interessant om te zien dat het hebben van een hogere alpha-diversiteit juist geassocieerd was met lagere BCAA's, *glycoprotein acetyls* en een gunstig lipidenprofiel. Hoge alpha-diversiteit had dus een tegengesteld metabool profiel dan steatose. *Ruminococcus Gnavusgroup* had een significante associatie met *glycoprotein acetyls* (een acuut fase eiwit), dit is in lijn met eerder wetenschappelijk onderzoek waar de aanwezigheid van de *Ruminococcus Gnavusgroup* werd gevonden bij mensen met een lagere microbiële *richness* en cardiovasculaire ziekten. *Coproccocus3* had een associatie met lagere steatose prevalentie en deze bacterie is in eerdere studies geassocieerd met het hebben van een lager nuchter glucose en een hogere microbiële *richness*. De voorspelde functie van het microbioom in steatose liet zien dat de synthese van secundaire galzouten verhoogd zou zijn, wat in lijn is met de bevindingen van onderzoeksgroep Jiao et al. die ook een verhoogde secundaire galzuursynthese aantrof in NAFLD.[442]

Hoewel er geen causale verbanden kunnen worden getrokken uit deze studie, suggereren de resultaten dat er een belangrijke rol lijkt voor het microbioom en zijn alpha-diversiteit

bij het verklaren van metabole verschillen tussen mensen met en zonder (niet-alcoholische) steatose.

APPENDICES

Chapter 16

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Chapter 17

Abbreviations

AAA	Aromatic Amino Acids
ACC	acetyl-CoA carboxylase
AGR	android-to-gynoid ratio
AIC	Akaike Information Criterion
ALM	appendicular lean mass
ALT	alanine aminotransferase
AMPK	adenosine monophosphate-activated protein kinase
ANOVA	analysis of variance
A:P	animal protein-to-potassium ratio
ARFI	acoustic radiation force impulse
AST	aspartate aminotransferase
AUROC	area under the receiver operator characteristic
BCAA	Branched-Chain Amino-Acids
BMI	body mass index
CAP	controlled attenuation parameter
CC	caffeinated coffee
CCl4	carbon tetrachloride
CD	cluster of differentiation
CI	confidence interval/ credible interval
CGA	chlorogenic acid
COX	cyclo-oxygenase
CPT	carnitine palmitoyltransferase
CTGF	connective tissue growth factor
DAL	dietary acid load
DC	decaffeinated coffee
DDG	Dutch Dietary Guideline
DHDI	Dutch healthy diet index
DM	diabetes mellitus
DNL	de novo lipogenesis
DQ	dietary quality
DXA	dual X-ray absorptiometry
E%	energy percent
Erk	extracellular signal-regulated protein kinase
EWGSOP	European working group on sarcopenia in older people
FA	fatty acid
FABP	fatty acid binding protein
FAS	fatty acid synthesis
FBR	Firmicutes-to-Bacteroidetes-Ratio
FFQ	food frequency questionnaire
FGF	fibroblast growth factor
FLI	fatty liver index
FPG	fasting plasma glucose
FXR	farnesoid X receptor
G6Pase	glucose-6-phosphatase
GFR	glomerular filtration rate
GGT	gamma glutamyltransferase
GLP	glucagon like peptide
GST	glutathione S-transferase
HCC	hepatocellular carcinoma
HDL-C	high-density lipoprotein cholesterol
HOMA-IR	homeostasis model assessment of insulin resistance
HFD	high fat diet
HSC	hepatic stellate cell
IFN	interferon
IL	interleukin
IQR	interquartile range
Kcal	kilocalories
KEAP	Kelch-like ECH-associated protein-1
KEGG	Kyoto Encyclopedia of Genes and Genomes
kPa	kilopascal
LDL	low-density lipoprotein
LSM	liver stiffness measurement
MD	Mediterranean diet
MDA	malondialdehyde
MDS	Mediterranean Diet Score
MET	metabolic equivalent of task
MMP	matrix metalloproteinase
mTOR	mammalian target of rapamycin
MUFA	mono-unsaturated fatty acid
MyD88	myeloid differentiation factor 88
NAFLD	non-alcoholic fatty liver disease
NAS	NAFLD activity score
NASH	non-alcoholic steatohepatitis
NASF	Non-Alcoholic Steatofibrosis

NEAP	net endogenous acid production
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NFS	NAFLD fibrosis score
NMR	Nuclear Magnetic Resonance
Nrf2	nuclear factor (erythroid-derived 2)-like 2
OR	odds ratio
OTU	Operational Taxonomic Unit
P25-P75	25th percentile, 75th percentile
PerMANOVA	Permutational Multivariate Analysis of Variance
PPAR	peroxisome proliferator-activated receptor
PPI	Proton-Pump Inhibitor
PRAL	potential renal acid load
PUFA	poly-unsaturated fatty acid
Q	quartile
rRNA	Ribosomal Ribonucleic Acid
RS	Rotterdam study
SD	standard deviation
SMA	smooth muscle antigen
SMI	skeletal muscle index
SREBP	sterol element binding transcription factor
TAA	thioacetamide
TE	transient elastography
Tg	triglycerides
TGF	tissue growth factor
TLR	toll like receptor
TNF	tumor necrosis factor
UGT	uncoupling protein glucuronosyl transferase
US	ultrasound
VIF	variance inflation factor
VLDL	Very Low Density Lipoprotein
WC	waist circumference
WHO	World Health Organization

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Chapter 20

PhD Portfolio

PhD student: Louise (Loes) Johanna Maria Alferink
Promotor: Prof. Herold J. Metselaar
Promotor: Prof. Jessica C. Kiefte-DeJong
Copromotor: dr. Sarwa Darwish Murad
Department: Gastroenterology and Hepatology

Oral Presentations

A Healthy lifestyle: caffeine and the liver – BASL York 2018
BibHep huisartsen voorlichting NAFLD/NASH – Erasmus MC 2018
The Gut Microbiome and Liver Steatosis in a Western Adult Population: The Rotterdam Study
– DDD Veldhoven 2018
NASH Casus – A men Not Appearing overweight with Steatosis & Hepatitis
– Leverdag – Rotterdam 2018
Post-EASL NASH/ASH update 2018 – Rosarium Amsterdam 2018
A nomogram with sarcopenia surpasses the MELD score in predicting waiting list mortality in cirrhotic liver transplant patients: a competing risk analysis in a national cohort
– ILC Paris 2018
Post-EASL NASH/ASH update 2017 – Rosarium Amsterdam 2017
BibHep huisartsen voorlichting NAFLD/NASH – Havenziekenhuis 2017
Diet – dependent acid load is associated with non-alcoholic fatty liver disease in the general population: The Rotterdam Study – UEGW Barcelona 2017 & DDD Veldhoven 2017
Animal protein is the most important macronutrient associated with non-alcoholic fatty liver
disease in overweight participants: The Rotterdam Study
– ILC Amsterdam 2017
Voeding en leverziekten: koffie en andere ingrediënten – DDD Veldhoven 2017
Coffee & herbal tea is protective of liver stiffness in the general population: The Rotterdam Study – DDD Veldhoven 2016
Impact of infections on waiting list survival in patients with end stage liver disease
– ECCMID Amsterdam 2016 & Bootcongres Groningen 2016

Poster Presentations

The gut microbiome and liver steatosis in a Western adult population: The Rotterdam Study – AASLD San Francisco 2018
Presarcopenia is associated with non-alcoholic fatty liver disease (NAFLD) in older women: The Rotterdam Study – ILC Paris 2018
Gender differences in body composition in lean and overweight non-alcoholic fatty liver disease: The Rotterdam Study – ILC Amsterdam 2017
Diet – dependent acid load is associated with non-alcoholic fatty liver disease in the general population: The Rotterdam Study – AASLD Washington 2017
Coffee & herbal tea is protective of liver stiffness in the general population: The Rotterdam Study – AASLD Boston 2016
Impact of infections on waiting list survival in patients with end stage liver disease – ILC Barcelona 2016

Prices, Bursaries & Press activity

Abstract rated top 10% of all abstracts	– AASLD San Francisco 2018
Selected in Best Poster Session	– ILC Paris 2018
Travel grant poster presentation	– ILC Paris 2018
Travel grant and overnight stay EASL – Seminar	– EASL Madrid 2017
Travel grant oral presentation	– UEGW Barcelona 2017
Travel grant oral presentation	– ILC Amsterdam 2017
Best Clinical Oral Abstract Award	– ILC Amsterdam 2017
Official Press Release macronutrient abstract	– ILC Amsterdam 2017
Early Press Release coffee & herbal tea paper	– J Hepatol. 2017

Peer reviewing activities

Liver Transplantation, Clinical Gastroenterology and Hepatology, Journal of Hepatology, European Journal of Epidemiology, Digestive and Liver Disease, Gut, Gastroenterology

Courses & workshops

SPSS basic course	– MolMed
Introduction GraphPad Prism	– MolMed

Course on R	– MolMed
Time management for PhDs	– MolMed
Writing course biomedical English	– MolMed
SNP course	– MolMed
Master class: Advances in Epidemiologic Analysis	– NIHES
The Practice of Epidemiologic Analysis	– NIHES
Fibroscan & CAP course	– EchoSens
BROK course	– NFU
Course on integrity in scientific research	– Erasmus MC
Basic Course on Excel	– MolMed
Abdominal Sonography course for beginners	– ILC Amsterdam 2017
Course Microbiomics I	– MolMed
Scientific English Writing Course	– Erasmus MC
Abdominal ultrasonography course	– Dutch liver week 2017
Biomedical English writing and communication	– Erasmus MC

Teaching

KOEL: huisartsen voorlichting NAFLD/NASH	- Erasmus MC 2018
Coaching van 6 studenten (2x [BKO] en 2x intervisie)	– Erasmus MC 2016–2019
Minor MDL: niet-alcoholische leverziekten	– Erasmus MC 2018
Onderwijsavond MDL: NAFLD basics	– Erasmus MC 2018
BibHep huisartsen voorlichting NAFLD/NASH	– Havenziekenhuis 2017
Begeleiding internationale pre-masterstudent: "Coffee & liver health"	– Erasmus MC 2016
Begeleiding master student (5 maanden): "H. Pylori & NAFLD"	– Erasmus MC 2016
Minorstudenten (10 weken): "Nutrition & NAFLD"	– Erasmus MC 2016

Attended Seminars & Conferences

33e Erasmus Liver day	– Rotterdam 2018
69e The Liver meeting (AASLD)	– San Francisco 2018
Najaarscongres Digestive Disease Days (NVGE)	– Veldhoven 2018
Annual meeting British Association for the study of the Liver	– York 2018
Monothematic: Gut-Liver axis	– Leuven 2018

Post-EASL 2018	– Amsterdam 2018
53e International Liver Conference (EASL)	– Paris 2018
EASL seminar "Controversies in End-Stage Liver Disease"	– Madrid 2017
32e Erasmus Liver day	– Rotterdam 2017
25e European United Gastroenterology Week (UEG)	– Barcelona 2017
Najaarscongres Digestive Disease Days (NVGE)	– Veldhoven 2017
Symposium: 40-jarig NVH jubileum	– Amsterdam 2017
Post-EASL 2017	– Amsterdam 2017
52e International Liver Conference (EASL)	– Amsterdam 2017
Voorjaarscongres Digestive Disease Days (NVGE)	– Veldhoven 2017
31e Erasmus Liver day	– Rotterdam 2016
67e The Liver meeting (AASLD)	– Boston 2016
Najaarscongres Digestive Disease Days (NVGE)	– Veldhoven 2016
5e Arts en Voeding congres	– Rotterdam 2016
51e International Liver Conference (EASL)	– Barcelona 2016
26e European Congress of Clinical Microbiology & Infectious Diseases (ECCMID)	– Amsterdam 2016
28e Bootcongres (NTV)	– Groningen 2016
30e Erasmus Liver day	– Rotterdam 2015

Chapter 21

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doel: niet de handdoek in de ring te gooien, maar nog even door te zetten. Tegelijkertijd hebben jullie mij ook geleerd om de écht belangrijke dingen in het leven niet uit het oog te verliezen: vrienden en familie. Jullie helpen mij te relativeren als ik in de put zit én om mezelf te blijven, met beide benen op de grond. Jullie zijn een warm, veilig en gezellig nest waar ik altijd graag terugkom (temeer vanwege moeders heerlijke kookkunsten, al zijn papa's prutjes ook best oké☺). Dank voor alles <3.

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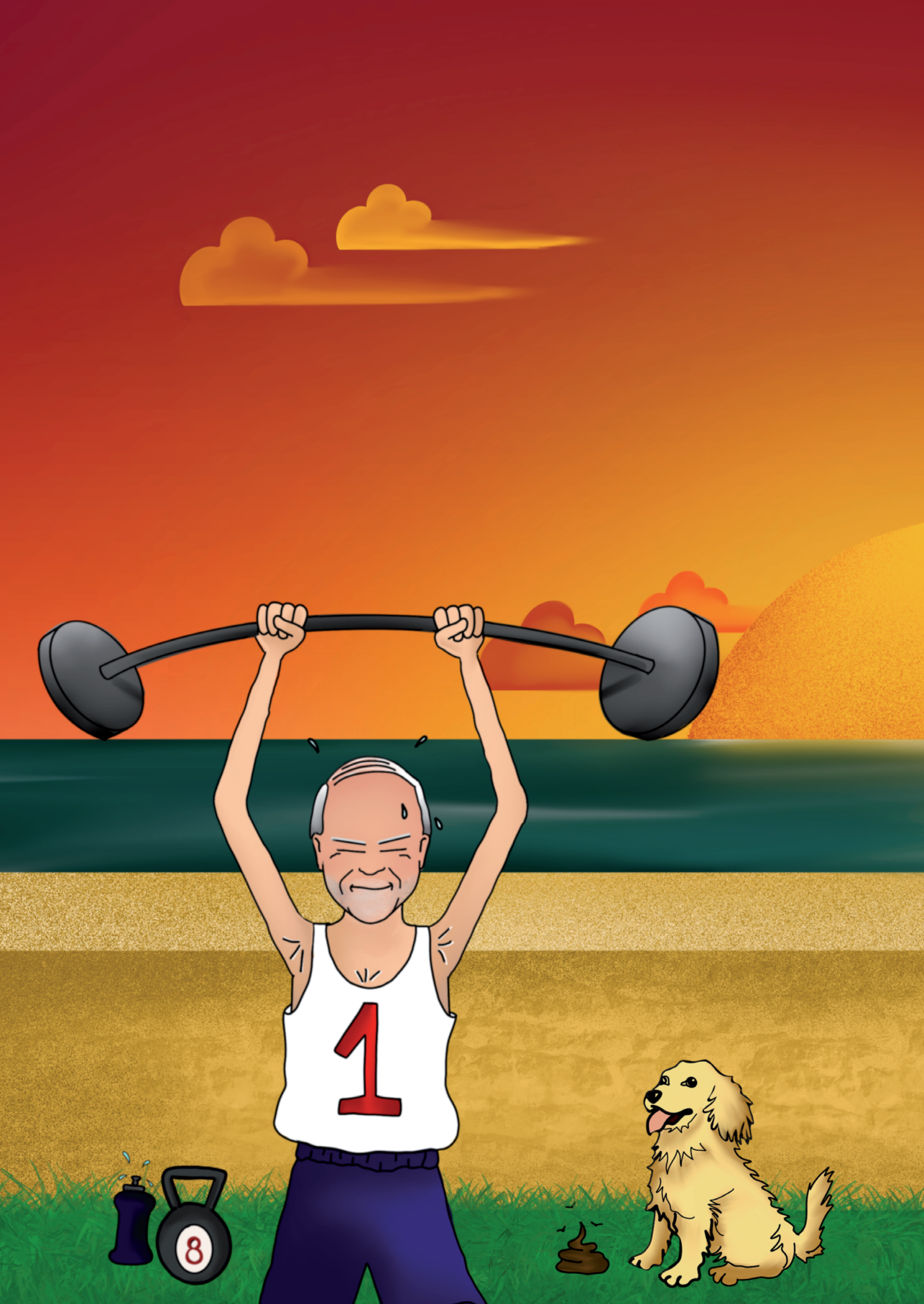
Chapter 22

About the author

Louise (Loes) J.M. Alferink was born on September 2nd 1991 in Breda, The Netherlands. In 2009 she completed her secondary school at Mgr. Frencken College in Oosterhout with Cum Laude honours. In that year she started medical school at the Erasmus University of Rotterdam. After obtaining here bachelor degree, Loes went to Uganda to do voluntary work in the Wagagai Health Clinic, Entebbe. She there founded the project healthy nutrition in children. During her masters she conducted research into infection-associated waiting list mortality in the context of liver transplantation. After her graduation in 2015, she continued doing research as a fulltime PhD-candidate on the Rotterdam Study under supervision of prof. dr. Herold J Metselaar, dr. Sarwa Darwish Murad and prof. dr. Jessica C Kiefte-deJong in the Erasmus Medical Centre in Rotterdam. Her research focusses on lifestyle & epidemiology of liver disease in the general population and resulted in this thesis. She is currently working as a resident of Internal Medicine in the Ikazia Ziekenhuis as part of the formal postgraduate training in Gastroenterology and Hepatology. She lives in Rotterdam, together with her partner Tom.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