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.

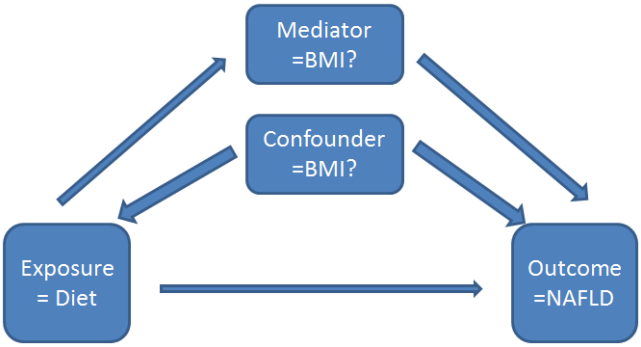

Figure 1: The possible roles of BMI as covariate

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.