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]

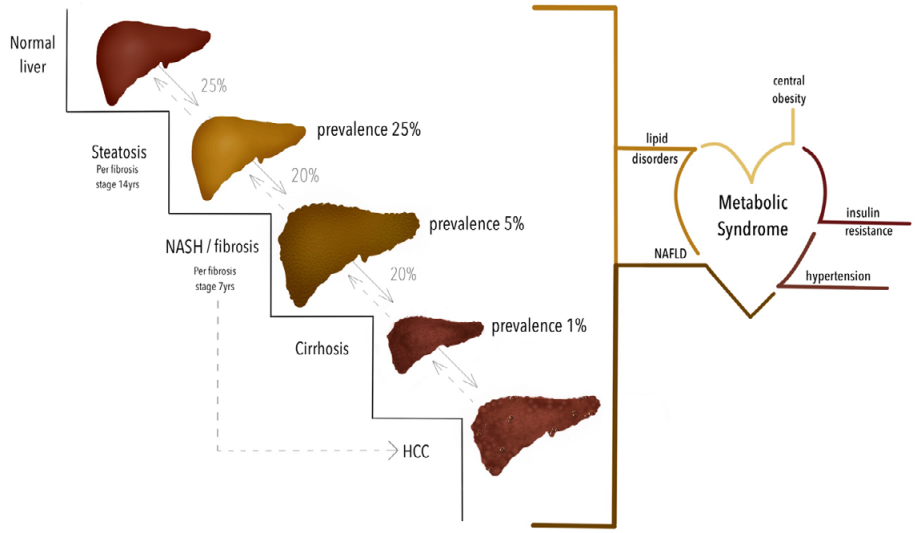

Figure 1: Natural History of NAFLD

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.

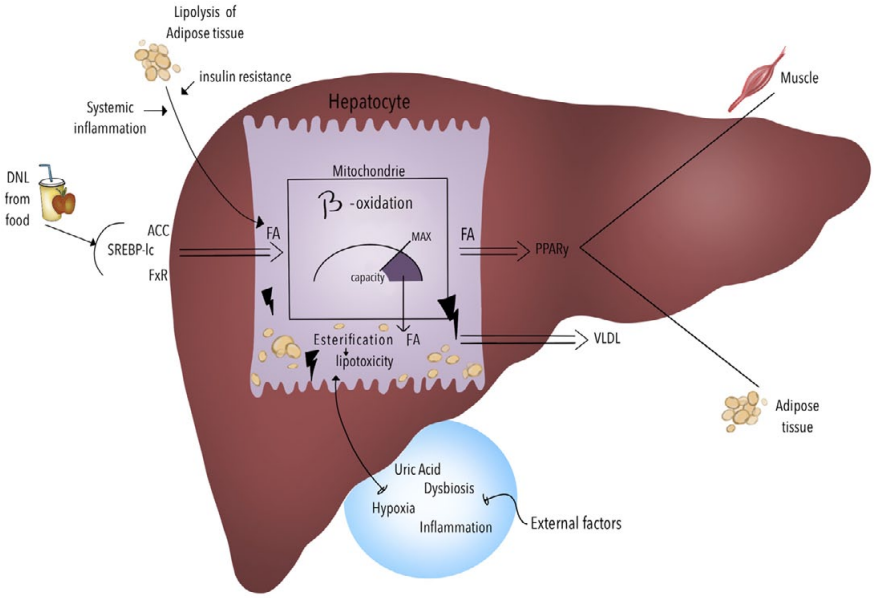

Figure 2: Pathophysiology of NAFLD

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.

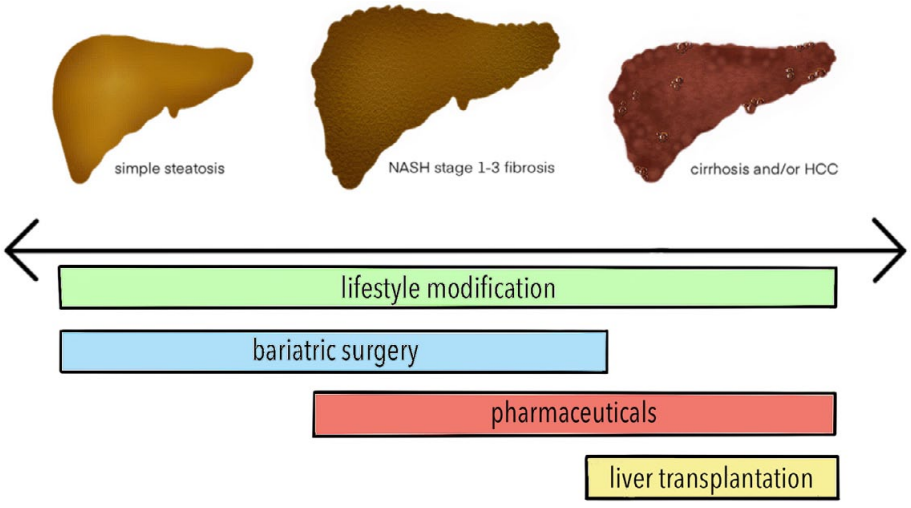

Figure 3: Treatment options for NAFLD

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.