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Established and emerging factors affecting the progression of nonalcoholic fatty liver disease

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List of abbreviations: AAT, alpha-1 antitrypsin; AATD, alpha-1 antitrypsin deficiency; AST, aspartate aminotransferase; BMI, body mass index; CVD, cardiovascular disease; DIOS, dysmetabolic iron overload syndrome; DNL, de novo lipogenesis; FFA, free fatty acid; FIB-4, Fibrosis-4; FS, fibrosis stage; GCKR, glucokinase regulatory protein; GWAS, genome-wide association studies; HCC, hepatocellular carcinoma; HDL, high-density lipoprotein; HSD17B13, 17β-hydroxysteroid dehydrogenase 13; IL-6, interleukin-6; IL-28B, interleukin-28B; JAK-STAT, janus kinase-signal transducer and activator of transcription proteins; JNK, c-Jun NH_2-terminal kinase; KLF6, Kruppel-like factor 6; LDL, low-density lipoprotein; LYPLAL1, lysophospholipase like 1; MARC1, mitochondrial amidoxime-reducing component 1; MBOAT7, membrane bound O-acyltransferase domain-containing 7; MCP-1, monocyte chemoattractant protein-1; MERTK, MER protocol-oncogene, tyrosine kinase; MRI, magnetic resonance imaging; NAFL, NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NASH CRN, NASH Clinical Research Network; NF-κβ, nuclear factor-κβ; PEth, phosphatidylethanol; Pi, proteinase inhibitor; PNPLA3, patatin-like phospholipase domain-containing 3; ROS, reactive oxygen species; SERPINA1, serine proteinase inhibitor 1; SNP, single-nucleotide polymorphism; T2DM, type 2 diabetes mellitus; TM6SF2, transmembrane 6 superfamily 2; TG, triglycerides; TNF-α, tumor necrosis factor-α; UCP2, uncoupling protein 2; VLDL, very low-density lipoprotein.

Abstract

Non-alcoholic fatty liver disease (NAFLD) has become the most common chronic liver disease affecting approximately 25% of the global population. Although a majority of NAFLD patients will never experience liver-related symptoms it is estimated that 5-10% will develop cirrhosis-related complications with risk of death or need for liver transplantation. NAFLD is closely associated with cardiovascular disease and components of the metabolic syndrome. However, NAFLD is not uncommon in lean individuals and may in these subjects represent a different entity with separate pathophysiological mechanisms involved implying a higher risk for development of end-stage liver disease. There is considerable fluctuation in the histopathological course of NAFLD that may partly be attributed to lifestyle factors and dietary composition. Nutrients such as fructose, monounsaturated fatty acids, and trans-fatty acids may aggravate NAFLD. Presence of type 2 diabetes mellitus seems to be the most important clinical predictor of liver-related morbidity and mortality in NAFLD. Apart from severity of the metabolic syndrome, genetic polymorphisms and environmental factors, such as moderate alcohol consumption, may explain the variation in histopathological and clinical outcome among NAFLD patients.

Keywords: End-stage liver disease, Liver-related complications, Hepatocellular carcinoma, Fibrosis progression

1. Introduction

Accumulation of lipids (steatosis) is the most common histopathologic hepatic alteration/finding globally. Hepatic triglyceride content can be accurately measured non-invasively with magnetic resonance imaging (MRI) [1,2]. Using a cut-off of 5.56% the prevalence of fatty liver among 2,287 individuals included in the Dallas Heart Study was 31% [3] and the global prevalence has been estimated to 25% [4]. However, recently it was shown that more subjects with biopsy-proven steatosis could be diagnosed with MRI using a cut-off of 3% [5] which implies that the prevalence of fatty liver has probably been underestimated in previous studies.

In most individuals with hepatic steatosis the underlying cause is alcohol overconsumption or nonalcoholic fatty liver disease (NAFLD) [6]. NAFLD is considered the hepatic manifestation of the metabolic syndrome [7]. Initially NAFLD was considered a benign disease but these days the progressive potential of NAFLD is indisputable with 5-10% of subjects progressing to cirrhosis, end-stage liver disease or hepatocellular carcinoma (HCC) [8]. Although the vast majority of NAFLD patients will never experience liver-related complications the high prevalence of NAFLD entails a significant public health issue with a high disease and economic burden and an increased need of liver transplantation [4].

NAFLD entails a spectrum of histopathological features that ranges from simple steatosis via establishment of inflammation and hepatocellular injury, i.e. non-alcoholic steatohepatitis (NASH), with or without fibrosis to cirrhosis with risk for development of end-stage liver disease or HCC [9-10]. An overview of the putative mechanisms involved in progression of NAFLD is shown in Fig. 1.

NAFLD is independently associated with cardiovascular disease (CVD) and type 2 diabetes mellitus (T2DM) [11-14]. Moreover, there is a collinearity between the severity of NAFLD and the components of the metabolic syndrome [15-17]. There is considerable fluctuation (*i.e.*

progression and regression) of inflammation and fibrosis in NAFLD [18-19]. Particularly inflammation is highly dynamic, partly attributed to lifestyle factors that are difficult to completely account and control for in clinical trials. These include weight change, dietary composition and alcohol consumption. Although inflammation and hepatocellular injury, i.e. NASH, is considered a progressive form of NAFLD, follow-up studies have shown that hepatic fibrosis is the only independent predictor of clinical disease progression. There are 14 studies with paired biopsies in NAFLD [20-33], including in total 996 patients with a median follow-up time ranging from 1.8 to 13.8 years (Table 1). Whether NASH precedes fibrosis in NAFLD is disputed. NASH correlates with presence of fibrosis, but this does not mean that NASH equals the prediction of fibrosis progression. To date, there is no objective evidence that presence of NASH at baseline, correlates with progression of fibrosis.

NAFLD patients with advanced fibrosis, as defined by stage 3-4 fibrosis, are at the highest risk of developing cirrhosis-related complications which may lead to liver transplantation or death [34-36]. The presence of advanced fibrosis, particularly cirrhosis, alters clinical management**,** including the possible initiation of surveillance for gastroesophageal varices and HCC, and consideration for treatment, including in the context of clinical trials. The burden of advanced fibrosis caused by NAFLD is projected to further increase in coming decades because of the rising prevalence of obesity [37]. Thus, this review will primarily focus on factors that have been shown to be associated with advanced fibrosis and/or affect fibrosis progression and the development of cirrhosis, end-stage liver disease, liver-related, and all-cause mortality in NAFLD (Fig. 2).

2. Body mass index (BMI) and weight change

2.1 Obesity

Overweight and obesity increases the risk of incident NAFLD substantially. The prevalence of NAFLD increases exponentially with higher BMI and reaches 57% in men and 44% in women with BMI >35 kg/m^2 when ultrasonography is used as diagnostic tool [38]. However, the sensitivity of ultrasonography is poor in low grade steatosis and thus the prevalence of NAFLD is probably even higher among obese subjects. Weight gain during young adulthood has been linked to future development of NAFLD [39}. Furthermore, weight loss, either by lifestyle intervention or bariatric surgery, is associated with resolution of NAFLD as well as insulin resistance [40-44]. In more pronounced weight loss ($\geq$10%) not only steatosis is reduced but also inflammation and fibrosis are positively affected [45].

Obesity and visceral adiposity predict development of severe liver disease in the general population [46-48]. In a prospective study by Calle *et al*, an exponential increase in the risk of HCC for every 5 unit increase in BMI was evident in males [49]. Similar findings were reported by Hagström *et al*, who studied 1.2 million men enlisted for military conscription in Sweden [50]. Subjects were followed for a mean period of 28.5 years and at the end of follow-up, 5,281 cases of severe liver disease and 251 cases of HCC were identified. Individuals who were overweight or obese at baseline had an increased hazard ratio for HCC of 1.68 (95%CI 1.09-2.57) and 4.28 (95%CI 2.25-8.15), respectively.

2.2 Lean NAFLD

Although most NAFLD patients have a BMI of $\geq$25 kg/m^2, a subset of individuals has a BMI <25 kg/m^2, which is usually denoted as lean NAFLD [51]. The prevalence of lean NAFLD ranges from 12 to 20% in different populations [52-57]. Lean NAFLD is more common in Asia (India [52], Japan [53], China [54], Korea [56]), but it has recently been recognized as an important clinical entity also among Caucasians (Greece [55], USA [57], Australia [58], Sweden [59]). A significant proportion of lean NAFLD patients are individuals manifesting

the disease with normal BMI but having excess visceral adiposity and insulin resistance. However, accumulating evidence suggests that lean NAFLD in many subjects represents a distinct pathophysiological entity with the onset of disease occurring at a lower BMI with lower levels of insulin resistance and is influenced by genetic factors, alterations in gut microbiota, and bile acids [4,58]. In a recent follow-up study [59] it was shown that lean NAFLD patients had higher risk of future development of severe liver disease compared to NAFLD patients with a BMI of $\geq$25 kg/m^2. This occurred despite the finding that lean NAFLD patients had lower prevalence of advanced fibrosis and NASH at baseline. This suggests that fibrosis progression is faster in lean NAFLD. Genetic variations, differences in dietary patterns and other lifestyle parameters that may affect fibrosis progression could possibly explain the association between lean NAFLD and the increased risk of future development of severe liver disease. Further studies are needed to elucidate these aspects.

2.3 NAFLD and weight: Summary

In summary, obesity increases the risk of NAFLD. Although there is a statistically significant increased risk for development of future end-stage liver disease including HCC, most obese subjects with NAFLD will not experience liver-related morbidity. Modest weight reduction ($\geq$5%) reduces steatosis but in order to stabilize or reduce fibrosis stage, the only histopathological feature associated with long-term outcomes of patients with NAFLD, more profound weight reduction ($\geq$10%) seems to be necessary. NAFLD in lean subjects may represent a different entity with separate pathophysiological mechanisms involved implying a higher risk for development of end-stage liver disease.

3. Physical activity

Increasing physical activity reduces intrahepatic triglyceride content and markers of hepatocellular injury in NAFLD patients independently of weight loss. However, there are

insufficient data supporting the effects of physical activity on the progression of NAFLD to NASH with advanced fibrosis, and on extrahepatic disease-related morbidity and mortality. [60-64].

4. Type 2 diabetes (T2DM)

4.1 Type 2 diabetes and NAFLD

Since 1980 the age-standardized prevalence of T2DM in adults has increased substantially [65]. Among the components of the metabolic syndrome, T2DM seems to be the most important risk factor for having NAFLD (including NASH) and the most important clinical predictor of liver-related morbidity and mortality [66-70]. NAFLD is highly intertwined with T2DM, showing a bidirectional interaction [17, 71-74] but whether NAFLD precedes or succeeds T2DM has not been clarified [75-76].

The prevalence of T2DM in NAFLD patients ranges from 45% to 75% in hospital-based studies and from 30% to 60% in population-based studies [77]. In a recently published meta-analysis, the global prevalence of NAFLD among patients with T2DM was estimated to 55%, while the European prevalence was even higher (68%). The global prevalence of NASH among individuals with T2DM was 37%, while the prevalence of advanced fibrosis in patients with NAFLD and T2DM was 17% [78]. Moreover, other reports indicate the additive risk of both NASH and T2DM, resulting in a worse metabolic profile and a higher risk of CVD [79-80].

In a systematic review and meta-analysis by Bellestri *et al*, a twofold increase in the risk of incident T2DM was reported in NAFLD patients [81]. Similar results were reported by Chen *et al* [82]. The relationship between biopsy-proven NAFLD and the future risk of developing T2DM has also been studied. In a long-term follow-up study [29] of 129 NAFLD patients 8.5% had T2DM at baseline. After a mean follow-up time of 13.7 years, 53% was diagnosed with

T2DM or impaired glucose tolerance. In an extended follow-up of the same cohort, 71% was diagnosed with T2DM or impaired glucose tolerance after a mean follow-up of 19.8 years [8]. McPherson *et al*. also showed association between NAFLD and future development of T2DM [83]. At baseline the prevalence of T2DM was 48%, which increased to 65% after a median follow-up of 6.6 years.

Hitherto 14 dual biopsy studies have been conducted in NAFLD patients with an overall T2DM prevalence of 43% (Table 1) [20-33]. In these studies, presence of T2DM did not predict fibrosis progression. Nevertheless, overall mortality [34,68] and liver-related morbidity [68] are increased in patients with NAFLD and concomitant T2DM. Moreover, patients with T2DM seem to have an increased mortality in the presence of concomitant NAFLD [84].

In a retrospective study including 148 subjects undergoing liver biopsy, diabetes was more prevalent in patients with liver-related clinical outcomes compared to patients without end-stage liver disease (62.5% vs. 27.4%) [85]. Moreover, in a recent study by Vilar-Gomez *et al*, T2DM was shown to be a strong negative predictor of transplantation free survival (HR 3.33, 95%CI 1.69–6.54) and liver-related outcomes (sHR 2.82, 95%CI 1.54–5.15 for decompensation and sHR 4.72, 95%CI 2.13–10.45 for HCC) [86].

4.2 Type 2 diabetes and hepatocellular carcinoma (HCC)

The relationship between T2DM and HCC is well established [87-89]. Patients with T2DM, without viral hepatitis or alcohol overconsumption, have a twofold increased risk of developing HCC compared to patients without diabetes (aHR 2.13, 95%CI 1.99-2.28) [90]. Moreover, T2DM seems to be an independent risk factor for developing HCC, mainly attributed to NAFLD [90-91]. Dyson *et al* reported a 1.8-fold increase in mortality from HCC between 2000 and 2010. This was mainly attributed to increasing prevalence of NAFLD, which nowadays is the most common chronic liver disease associated with HCC [92].

The collinearity between NAFLD and T2DM is high and therefore it is hard to assess if the increased risk of HCC is secondary to T2DM itself, or its hepatic manifestation (*i.e.* NAFLD). Therefore, more studies are needed depicting whether NAFLD patients with T2DM have an increased risk of HCC compared to NAFLD patients without T2DM.

5. Hypertension

In a meta-analysis [93] of 11 cohort studies (411 patients) assessing paired liver biopsy specimens to estimate the rates of fibrosis progression in NAFLD patients it was found that the presence of hypertension (odds ratio, 1.94; 95% CI, 1.00–3.74) at the time of baseline biopsy was associated with the development of progressive fibrosis.

6. Dyslipidemia

Hypertriglyceridemia is common in NAFLD patients [94]. Increased non-HDL cholesterol levels are associated with NASH, which correlates with presence of fibrosis and is likely to impact on risk of developing future end-stage liver disease [95-96]. However, when cirrhosis develops due to NAFLD the severity of the initial hyperlipidemia is often blunted because of hepatic biosynthetic failure [97-99], which parallels the disappearance of hepatic steatosis in NASH cirrhosis [100].

7. Sarcopenia

The strongest evidence to prove an independent association of NAFLD with sarcopenia, i.e, the loss of muscle mass and/or strength, was given in a study from Korea [101], in which this association was investigated in a large cohort of both obese and non-obese subjects (n = 15,132). A strong inverse relationship of the skeletal muscle index, a measure for muscle mass, with NAFLD was found. Accordingly, the prevalence of NAFLD in sarcopenic subjects

was higher compared to non-sarcopenic subjects, irrespective of the presence of obesity or the metabolic syndrome. Studies evaluating the association of fibrosis in NAFLD with sarcopenia have found a significant relationship between fibrosis and sarcopenia [101-106], regardless if significant fibrosis was defined non-invasively or based on biopsy. However, most studies assessing sarcopenia in NAFLD included mainly Asian patients. Hence, extrapolating these results to other than Asian groups may be problematic because of differences in body composition between ethnicities.

8. Age

Cross-sectional studies have demonstrated increasing age to be associated with more severe fibrosis in NAFLD. However, this may reflect the cumulative sum of metabolic exposures and longer duration of the disease in these populations [67, 107-108]. In contrast, longitudinal studies have not consistently demonstrated age to impact the rate of fibrosis progression [93].

9. Alcohol

Alcohol overconsumption is the leading cause of end-stage liver disease in the Western world [109]. A putative crucial factor for the course of NAFLD is the impact of the quantity, pattern, and duration of alcohol consumption. Weekly alcohol consumption in excess of 210 g for men and 140 g for women is not compatible with diagnosis of NAFLD and excludes subjects from NAFLD research studies [110]. However, these arbitrary thresholds are based on levels above which the risk of cirrhosis is higher and have not been specifically shown to influence NAFLD [111]. On the other hand, the most common cause of mortality and morbidity in NAFLD patients is CVD [29,112] and NAFLD and CVD share many common risk factors. There is evidence for beneficial effects of modest alcohol consumption on risk of metabolic syndrome and insulin resistance [113], which are crucial factors of the NAFLD disease process.

9.1 Moderate alcohol consumption: The evidence for protection

A meta-analysis of mostly cross-sectional studies concluded that moderate alcohol consumption was associated with a 23% reduction in the prevalence of fatty liver disease [114]. In a prospective Japanese study of subjects without liver disease at baseline alcohol consumption was associated with decreased incidence of steatosis [115]. Moreover, moderate alcohol consumption did not induce fatty liver in healthy individuals when hepatic triglyceride content was measured prospectively with proton magnetic resonance spectroscopy in a randomized study [116].

In a cross-sectional study of adult patients with biopsy-proven NAFLD, after exclusion of heavy and binge drinkers, modest alcohol consumption was associated with 34% less hepatocellular ballooning and 44% lower risk of liver fibrosis compared with nondrinkers [117]. Similar results were shown in a Swedish study of 120 NAFLD patients with biopsy-proven NAFLD in which a maximum of 13 drinks per week was associated with lower fibrosis stage [118]. However, increased levels of phosphatidylethanol (PEth) in blood were associated with higher stages of fibrosis. This may indicate that more pronounced alcohol consumption, contrary to modest consumption, is harmful in NAFLD or that assessment of alcohol consumption through questionnaires is prone to error.

Currently, twelve studies have assessed the impact of alcohol on histopathology in NAFLD (Table 2) [117-128]. Robust conclusions cannot be drawn since study design varies and particularly since the definition of moderate alcohol consumption is not consistent. However, type of alcohol and pattern of consumption seem to affect the histopathological course of NAFLD. Generally, consumption of moderate amounts of alcohol (< 70 g/week) is associated with a lower rate of NASH and fibrosis, especially if wine is consumed in a non-binge pattern.

Results regarding the effect of alcohol consumption on survival in NAFLD patients have been conflicting [115,117]. Recently, 4,568 subjects with NAFLD from the National Health and Nutrition Examination Survey were evaluated. Consumption of 7 g to 21 g alcohol per day decreased the risk of overall mortality by 41% compared with not drinking [129]. Since NAFLD patients are more likely to die from CVD than liver disease these results are in accordance with previous studies showing that modest alcohol consumption is associated with decreased risk of cardiovascular disease mortality [130]. However, a major weakness of the aforementioned study [129] is that the diagnosis of NAFLD was based on a biochemical model and not on imaging or histology.

9.2 Moderate alcohol consumption: The evidence for detrimental effects

In some studies, moderate alcohol consumption is associated with a more advanced histopathological stage. Binge drinking (occasional consumption of > 60 g ethanol in males and > 48 g in females) may be harmful since it is associated with higher fibrosis stages. In a histopathological study from Sweden, 71 NAFLD patients were followed for an average of almost 14 years and it was shown that heavy episodic drinking was associated with increased risk of progression of fibrosis [119]. Further evidence for a potentially harmful effect of moderate alcohol consumption on the progression of NAFLD comes from a recently published longitudinal study, in which it was concluded that NAFLD patients with moderate alcohol consumption were less likely to experience spontaneous improvement in liver histology [120].

The largest study assessing the effect of alcohol on the severity of NAFLD was recently reported by Chang *et al* [131]. They studied the effect of moderate alcohol consumption on non-invasive liver fibrosis indices in 58,927 Korean adults with NAFLD and low fibrosis scores who were followed for a median of 8.3 years. The authors concluded that moderate alcohol consumption was associated with deterioration of non-invasive markers of fibrosis. Fibrosis stage is the best predictor of future liver-related morbidity and overall mortality in NAFLD [34-

36]. Thus, their study may indicate that modest alcohol consumption is harmful in subjects with NAFLD. However, a major weakness of using non-invasive fibrosis markers is that, although they are excellent in ruling out significant fibrosis, their ability to confirm advanced fibrosis is limited when liver biopsy is used as the reference method. Thus, worsening of fibrosis indices does not necessarily imply that liver fibrosis has progressed during follow-up.

Increasing evidence suggests an additive, or even a synergistic, effect between alcohol consumption and BMI for the development of HCC [132]. In a recent Japanese study of 301 patients with biopsy-proven NAFLD, patients with modest drinking had significantly higher risk of developing HCC compared with nondrinkers [121].

9.3 NAFLD and alcohol: Summary

In summary, most studies indicate that modest alcohol consumption is associated with decreased risk for development of fatty liver disease and moderate drinking may be associated with increased survival in NAFLD patients. Emerging evidence indicates an additive risk of BMI and alcohol for the development of HCC in NAFLD. There are conflicting results regarding the role of alcohol for fibrosis progression in established NAFLD. Further studies are needed before well-grounded advice can be given to NAFLD patients regarding modest alcohol consumption.

10. Genetics

10.1 Genome-wide association studies

Genome-wide association studies (GWAS) have identified numerous gene loci associated with NAFLD. The two most extensively studied polymorphisms are the patatin-like phospholipase domain-containing 3 (PNPLA3) and the transmembrane 6 superfamily 2 (TM6SF2). PNPLA3 codes for adiponutrin, a protein involved in lipid remodeling, and the gene product of TM6SF2 is involved in VLDL secretion from hepatocytes. Both genes have in several studies been associated

with hepatic steatosis, NASH and advanced fibrosis [133-136]. Moreover, the rs738409 c.444 C>G, p.lle148Met polymorphism in the PNPLA3 gene has been associated with increased risk for development of HCC in NAFLD [137-139].

The gene locus rs641738 at the membrane bound O-acyltransferase domain-containing 7 (MBOAT7) has also been associated with NAFLD [140], but this association has recently been questioned [141]. In some studies, the genes lysophospholipase like 1 (LYPLAL1), glucokinase regulatory protein (GCKR), and PP1R3B have been associated with NAFLD but further confirmatory studies are needed [142-143].

Interestingly, several genes protective of hepatic steatosis and fibrosis have recently been reported, the mitochondrial amidoxime-reducing component 1 (MARC1), the 17β-hydroxysteroid dehydrogenase 13 (HSD17B13), the LPIN1, the uncoupling protein 2 (UCP2), the interleukin-28B (IL-28B), the Kruppel-like factor 6 (KLF6), and the MER protocol-oncogene, tyrosine kinase (MERTK) [144].

10.2 HFE, ferritin, and iron

The dysmetabolic iron overload syndrome (DIOS), which corresponds to a mild increase of hepatic and total body iron content, is a common finding among subjects with the metabolic syndrome and NAFLD. The pathogenesis of DIOS is related to altered regulation of iron transport associated with steatosis, insulin resistance, and subclinical inflammation, often in the presence of predisposing genetic factors. Hyperferritinemia has been reported in 58% [145] and approximately one third of NAFLD patients have stainable hepatic iron [146-147]. However, in many NAFLD patients elevated ferritin reflects subclinical inflammatory state, not iron overload. Thus, association between hyperferritinemia and severity of NAFLD may not reflect detrimental effects of iron in NAFLD.

The relationship of serum ferritin with severity of NAFLD has been examined in several studies [148-151]. In a study by the NASH Clinical Research Network (NASH CRN), 628 patients with biopsy-proven NAFLD were included [151]. Patients with a ferritin higher than 1.5 times and 2.5 times the upper limit of normal had a 1.67 and 2.46-fold increased risk of advanced fibrosis, respectively. Moreover, Hagström *et al* showed that patients with biopsy-proven NAFLD with higher levels of ferritin had a long-term increased risk of death [152]. Although the association between ferritin and advanced fibrosis has been corroborated by several study groups [150-151,153], the use of ferritin for predicting presence of advanced fibrosis in NAFLD is low [153].

In a study by Valenti *et al*, 587 biopsy-proven NAFLD patients were enrolled to investigate the effects of iron and HFE in NAFLD [154]. They reported that hepatocellular iron accumulation was associated with a higher risk of fibrosis stage >1 (aOR 1.7, 95%CI 1.2-2.3) compared to patients without histopathological siderosis. Although, there was no significant association between presence of specific HFE genotypes and the severity of fibrosis, one third of patients with HFE mutations had hepatocellular iron deposits.

Early case studies of iron depletion through phlebotomy showed decreased insulin resistance [155], reduction of steatosis [156], and liver enzymes in serum [156-157]. However, in a phase 2 clinical trial [158] and a randomized controlled trial [159], phlebotomy had no effect on liver enzymes, hepatic triglyceride content, insulin resistance or histopathological features of NAFLD. However, none of the studies evaluated fibrosis progression and end-stage liver disease as endpoints.

10.3 Alpha-1 antitrypsin deficiency

Alpha-1 antitrypsin deficiency (AATD) caused by mutations in the gene serine proteinase inhibitor 1 (*SERPINA1*) previously known as proteinase inhibitor (*Pi*) is most common in

Scandinavia and North America. In a meta-analysis by Serres *et al*, the global prevalence for heterozygotic *SERPINA1* mutations (MS and MZ) was 3.4% and for homozygotic mutations (ZZ, ZS and SS) 0.8% [160].

While the presence of the ZZ genotype portends a high risk of future liver disease, the role of MZ in liver disease remains controversial [161-162]. In a study by Regev *et al*, 651 patients with known chronic liver disease, of whom 26% had NAFLD, were tested for alpha-1 antitrypsin (AAT) phenotypes [163]. Although they did not find any association between heterozygous Z state and the presence of chronic liver disease, the presence of the MZ state was more common in NAFLD patients with decompensated liver disease. Strnad *et al.* included 1,148 patients with biopsy proven NAFLD [164]. Of patients with cirrhosis, 13.8% had the Z variant present, compared to 2.4% of those without fibrosis. The Z variant was associated with increased risk for cirrhosis (aOR 7.3, 95%CI 2.2-24.8). These studies may indicate that the MZ state is a risk factor for progression of fibrosis in NAFLD but further confirmatory studies are needed.

11. Nutrients

In subjects with the metabolic syndrome excessive dietary carbohydrate and fat intake have been associated with the occurrence of insulin resistance as well as NAFLD [165-167]. Carbohydrates are important stimuli of hepatic de novo lipogenesis and are more likely to directly contribute to NAFLD than dietary fat [168]. Especially fructose has been linked as a major substrate for de novo hepatic lipogenesis and fibrosis [169]. Sugar-sweetened beverages increased liver and visceral fat over a 6-month period as compared to milk and water [170]. Moreover, fructose has been associated with increased oxidative stress in the liver [171].

A higher dietary lipid intake with a higher omega-6 to omega-3 polyunsaturated fatty acids ratio and a higher intake of saturated fat have also been associated with hepatic inflammation and NAFLD [172]. Thus, an increase in the intake of monounsaturated fatty acids may modify the

progression of NAFLD. Furthermore, trans-fatty acids are implicated in the metabolic syndrome, as they are strongly associated with an increase in hepatic inflammatory processes, cholesterol, and plasma triglycerides, as well as a reduction in HDL cholesterol [173]. A previous animal study demonstrated the presence of a positive correlation between the increased intake of trans-fatty acids from oxidized oils and hepatic inflammation [173].

The importance of dietary vitamin intake and decreased hepatic triglyceride content have been addressed in several studies. One of these studies assessed the role of vitamin A in the pathogenesis of NAFLD [174]. It was shown that hepatic steatosis may be reduced by retinoic acid. Another study by Kwok *et al.* [175], reported that NAFLD patients had lower serum levels of vitamin D, suggesting that vitamin D deficiency may play a role in the pathogenesis of NAFLD.

12. Conclusions

NAFLD is present in approximately 25% of the population globally. The natural history and impact on patient morbidity and mortality is widely divergent. Presence of high age, metabolic factors, such as T2DM, obesity, and hypertension, influence the severity of underlying liver histology and, thus, are likely to impact on risk of developing cirrhosis and HCC. Recent studies have emphasized the importance of fibrosis stage in determining future mortality risk. NAFLD patients with the aforementioned risk factors should undergo assessment with at first hand non-invasive methods and, in case of indeterminant results, liver biopsy in order to evaluate severity of fibrosis. Genetic polymorphisms, particularly in the PNPLA3 and TM6SF2 genes, influence severity of NAFLD and may be implemented in the diagnostic work-up of NAFLD patients in the future. Lifestyle factors, such as dietary composition and moderate alcohol consumption, may also influence the progression of NAFLD. However, further studies are needed before solid lifestyle advice can be given to patients.

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Table 1. NAFLD studies with paired biopsies.

Authors, year [ref.]	n	Follow-up time, years (median (range))	Baseline		
			NASH	T2DM	Predictors of fibrosis progression
Lee et al., 1989 [20]	13	3.3 (1.2-6.9)	100%	54%	None
Powell et al., 1990 [21]	13	3 (1.5-6.5)	100%	46%	None
Teli et al., 1995 [22]	12	7.6-16	0%	N/A	N/A
Ratziu et al., 2000 [23]	14	5 (1.5-15)	29%	N/A	N/A
Evans et al., 2002 [24]	7	7 (5.5-14)	N/A	43%	N/A
Harrison et al., 2003 [25]	22	5.7 (1.4-15.7)	41%	41%	AST
Fassio et al., 2004 [26]	22	4.3 (3-14.3)	100%	36%	BMI
Adams et al., 2005 [27]	103	3.2 (0.7-21.3)	93%	42%	BMI, FS, T2DM
Hui et al., 2005 [28]	17	6.1 (3.8-8)	35%	24%	None
Ekstedt et al., 2006 [29]	68	13.8 (10.3-16.3)	49%	9%	None
Wong et al., 2010 [30]	52	3	33%	50%	LDL
Pais et al., 2013 [31]	70	3.4 (1-12)	64%	35%	N/A
McPherson et al., 2015 [32]	108	6.6 (1.3-22.6)	75%	48%	FIB-4
Sanyal., 2019 [33]	475	1.8	71%	68%	FS

Abbreviations: NASH, nonalcoholic steatohepatitis; T2DM, type 2 diabetes mellitus; AST, aspartate aminotransferase; BMI, body mass index; LDL, low-density lipoprotein; FIB-4, Fibrosis-4 (biochemical score for prediction of liver fibrosis); FS, fibrosis stage; N/A, not applicable.

Table 2. Studies assessing the impact of alcohol on histopathology in NAFLD.

Authors, year [ref.]	**Sample size**	**Diagnosis of NAFLD**	**Study design**	**Definition of moderate alcohol consumption**	**Assessment of alcohol consumption**	**Focus/highlight**	**Outcome**
Dixon et al., 2001 [122]	105	Liver biopsy	Cross-sectional	<200 g/week	Clinical interview + questionnaire	Liver histology	No significant difference in NASH after adjusting for insulin resistance and diabetes
Cotrim et al., 2009 [123]	132	Liver biopsy	Cross-sectional	<280 g/week	Clinical interview	Liver histology	No difference in liver histology
Ekstedt et al., 2009 [119]	71	Liver biopsy	Cohort	< 140 g/week	Clinical interview + questionnaire	Fibrosis progression	Binge drinking was associated with higher fibrosis stage
Ascha et al., 2010 [124]	195	Cirrhosis (liver biopsy or symptoms of portal hypertension)	Cohort	<168 g/week	Not stated	HCC	Alcohol consumption as a risk factor for HCC
Dunn et al., 2012 [117]	582	Liver biopsy	Cross-sectional	<140 g/week	Questionnaire	Liver histology, steatohepatitis	Less steatohepatitis and fibrosis in moderate consumers
Kwon et al., 2014 [125]	77	Liver biopsy	Cross-sectional	<40 g/week	Questionnaire	Liver histology, lifetime consumption	Higher rate of advanced fibrosis in low/no consumption group
Sookoian et al., 2016 [126]	266	Liver biopsy	Cross-sectional/mendelian randomization	210 g/week (male) 140 g/week (female) + gene carriers	Clinical interview	Genetic carriers as a measure of alcohol consumption, no protective association of moderate consumption on histology	Higher rate of steatosis and inflammatory changes in non-carriers (i.e. drinkers)

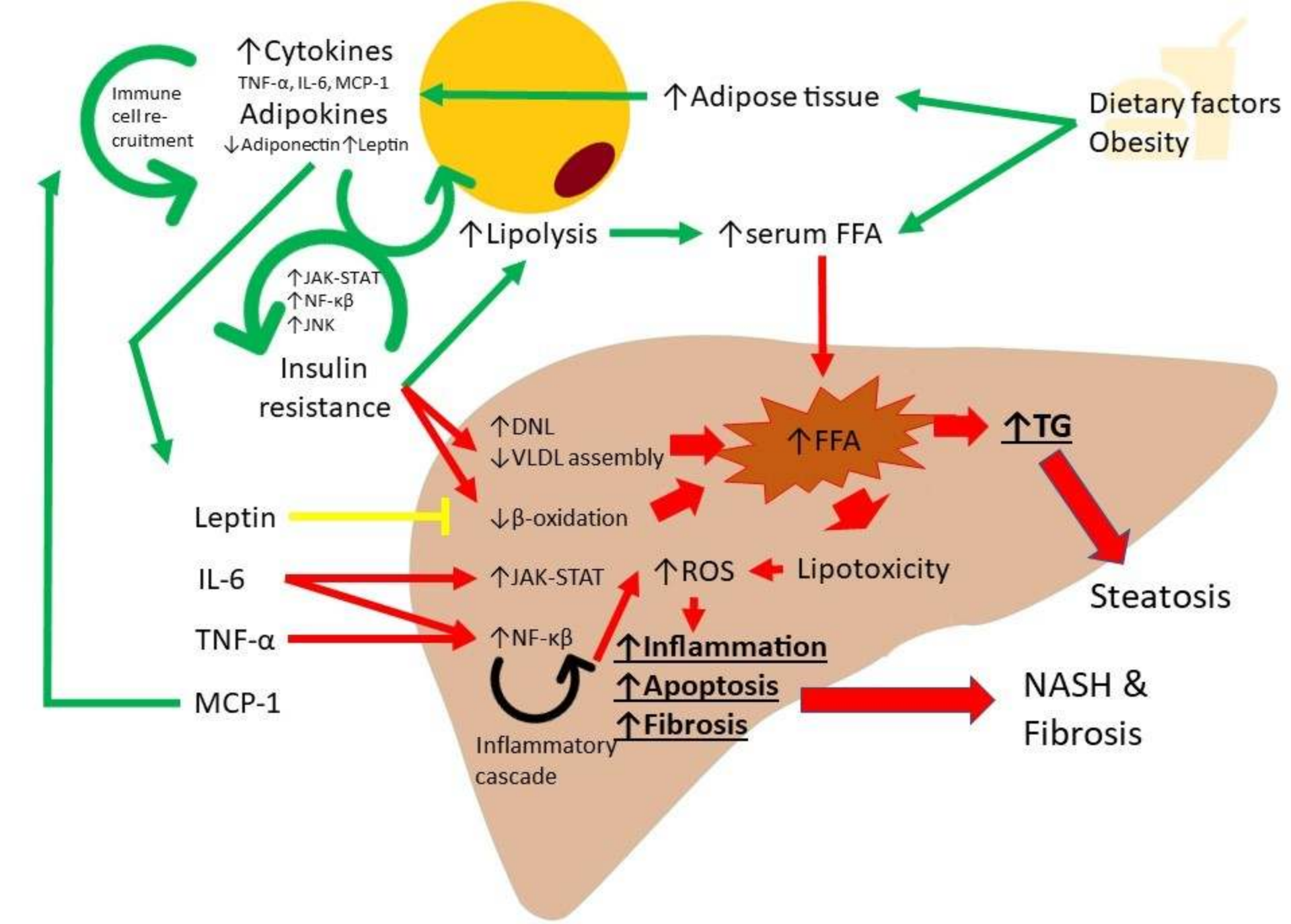
Hagström et al., 2017 [118]	120	Liver biopsy	Cross-sectional	168 g/week	Questionnaire + PEth	Liver histology	Reduced risk of fibrosis in moderate consumers. Elevated PEth levels increased risk of significant fibrosis
Ajmera et al., 2018 [120]	285	Liver biopsy	Cohort	<140 g/week	Clinical interview + questionnaire	Liver histology	Greater reduction in steatosis and NASH in non-drinkers
Yamada et al., 2018 [127]	178	Liver biopsy	Cross-sectional	≤140 g/week	Questionnaire	Liver histology	Lower fibrosis score in moderate consumers
Mitchell et al., 2018 [128]	187	Liver biopsy	Cross-sectional	210 g/week (male) 140 g/week (female)	Clinical interview + questionnaire	Fibrosis, binge-drinking, type of alcohol	Less fibrosis among subjects consuming wine <70 g/w, and in non-binge drinkers
Kimura et al., 2018 [121]	301	Liver biopsy	Cohort	<140 g/week	Clinical interview + questionnaire	HCC	Higher incidence of HCC plus prevalence of cirrhosis in moderate consumers

Abbreviations: HCC, hepatocellular carcinoma; NASH, non-alcoholic steatohepatitis; PEth, phosphatidylethanol.

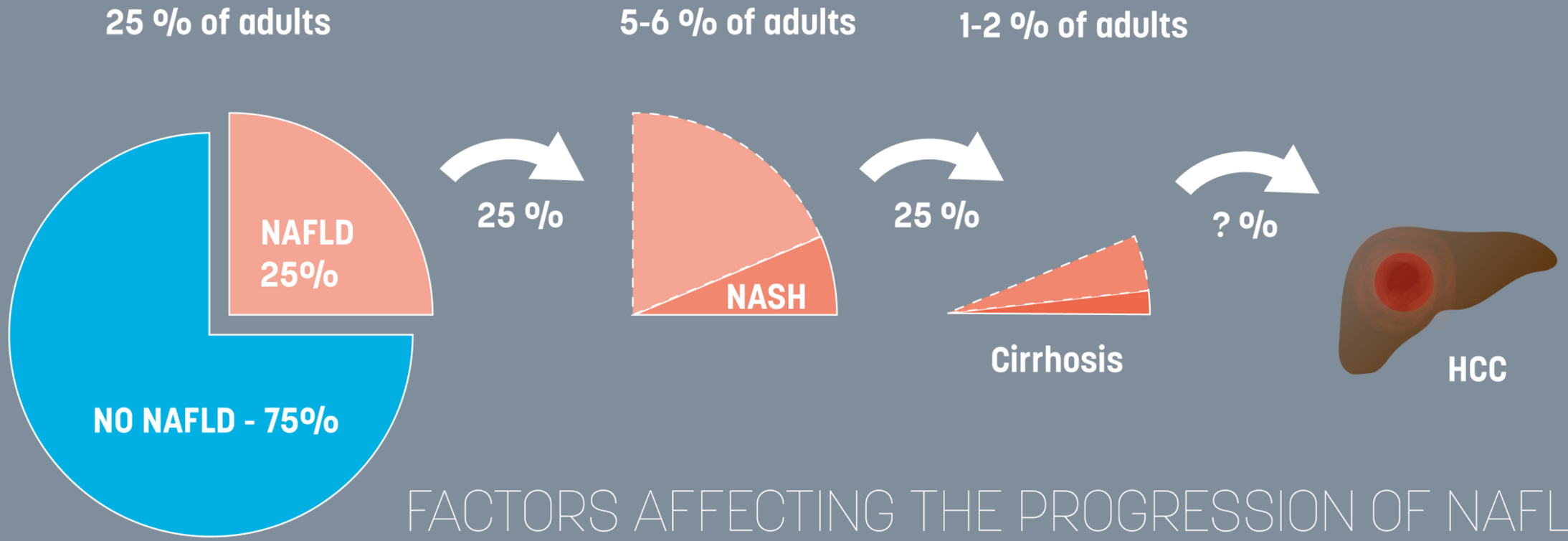
Figure 1. Multiple hit model for the development of steatosis, inflammation and fibrosis. Dietary factors, together with obesity lead to increased levels of serum FFAs and development of insulin resistance through multiple factors. Secondary to obesity a subsequent adipocyte proliferation takes place, with augmentation of insulin resistance and increased levels of proinflammatory cytokines (TNF-α, IL-6, and MCP-1) with decreasing or desensitized adipokines (adiponectin and leptin). The dysregulated adipokine and cytokine balance creates and maintains an inflammatory cascade and vicious circle and maintains the insulin resistance state. In the liver, insulin resistance amplifies de novo lipogenesis (DNL), decreases VLDL assembly and disrupts β-oxidation. The net sum, together with previously mentioned causes of raised serum FFA, is increased hepatic FFA influx. This leads to synthesis and accumulation of TG and toxic levels of FFAs. High levels of FFAs in the presence of reduced β-oxidation causes lipotoxicity and subsequently generation of ROS. This process is further enhanced in the presence of cytokines and attracted immune cells caused by the inflammatory milieu, inducing inflammation and cellular repair systems with secondary fibrosis.

Abbreviations: DNL, de novo lipogenesis; FFA, free fatty acid; IL-6, interleukin-6; JAK-STAT, janus kinase-signal transducer and activator of transcription proteins; JNK, c-Jun NH_2-terminal kinase; MCP-1, monocyte chemoattractant protein-1; NASH, non-alcoholic steatohepatitis; NF-κβ, nuclear factor-κβ; ROS, reactive oxygen species; TG, triglycerides; TNF-α, tumor necrosis factor-α.

Figure 2. The relative distribution of NAFLD, NASH, cirrhosis, and hepatocellular carcinoma and factors influencing progression of NAFLD. Prevalence data are from [4] and [71].


↑Cytokines
TNF-α, IL-6, MCP-1
Adipokines
↓Adiponectin ↑Leptin
Immune cell re-cruitment
↑Adipose tissue
Dietary factors
Obesity
↑Lipolysis
↑serum FFA
↑JAK-STAT
↑NF-κβ
↑JNK
Insulin resistance
↑DNL
↓VLDL assembly
↑FFA
↑TG
Leptin
↓β-oxidation
IL-6
↑JAK-STAT
↑ROS
Lipotoxicity
Steatosis
TNF-α
↑NF-κβ
↑Inflammation
↑Apoptosis
↑Fibrosis
NASH & Fibrosis
MCP-1
Inflammatory cascade

Global prevalence



FACTORS AFFECTING THE PROGRESSION OF NAFLD

Diabetes, Weight gain, Hypertension, Lean NAFLD, Alcohol,
Genetic polymorphisms, Nutrients