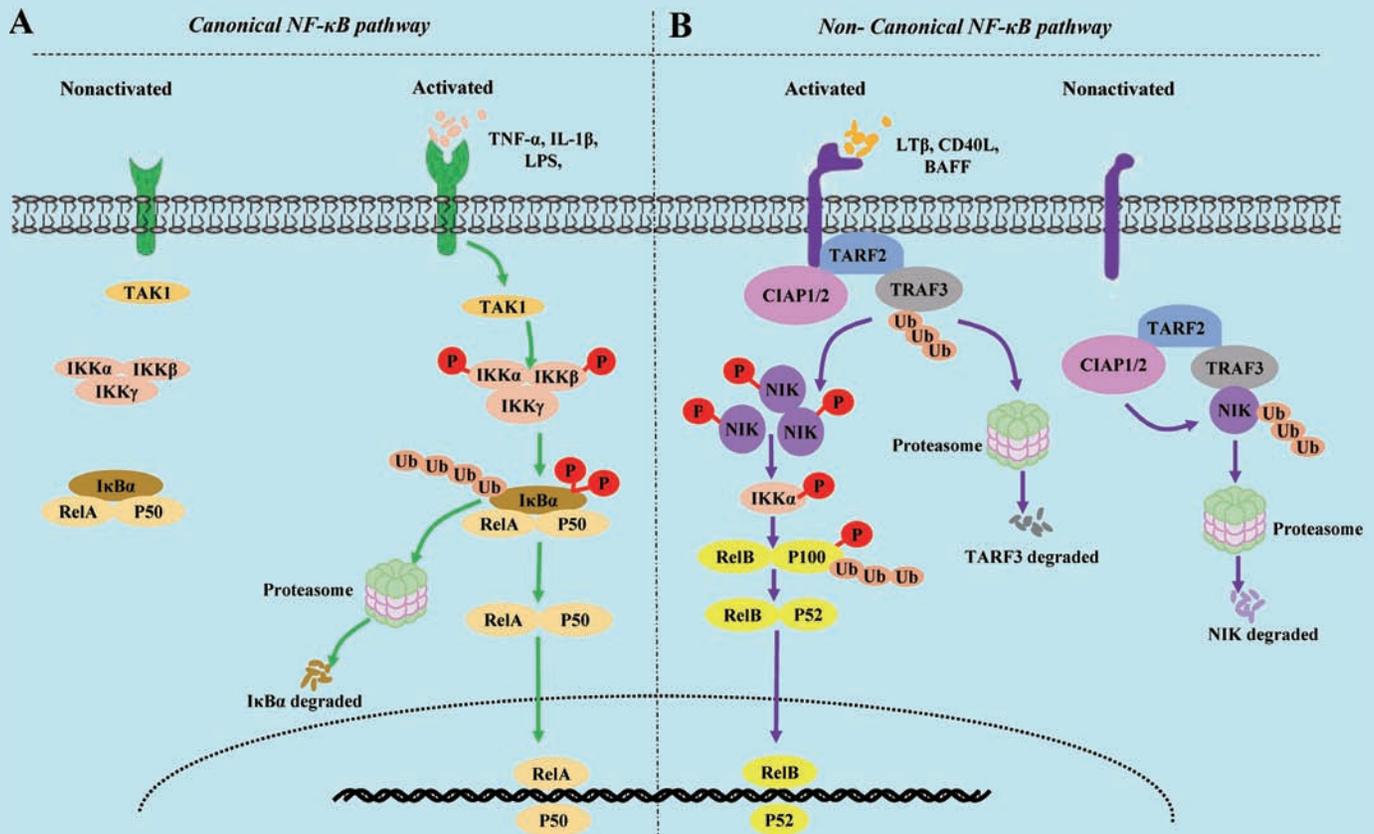


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Journal of Clinical and Translational Hepatology (JCTH, J Clin Transl Hepatol) publishes high quality, peer-reviewed studies in the clinical and basic human health sciences of liver diseases. *JCTH* welcomes submissions of articles within its topical scope including: novel discoveries in clinical and basic hepatology; liver disease mechanisms; novel techniques in research and management of liver diseases; epidemiological/environmental factors of liver diseases; role of immune system function in liver diseases; acute and chronic hepatitis; cirrhosis; genetic and metabolic liver diseases and their complications; hepatobiliary diseases; liver cancer; drug metabolism; biliary disease; peritoneal tuberculosis. *JCTH* publishes various types of articles, including original article, review, short communication, systematic review, meta-analysis, case report, methodology article, letter to the editor, and editorial.

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Recommendations and Clinical Guidance for Children with Metabolic-associated Fatty Liver Disease during the COVID-19 Pandemic

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The outbreak of coronavirus disease-2019 (COVID-19), a new disease caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in December 2019 affected most countries in the world within just a few months. By March 11, 2020, the Director-General at the World Health Organization (WHO) declared that COVID-19 can be considered a pandemic, and as of January 11, 2021, there were over 88.8 million cases of COVID-19 worldwide and 1.9 million deaths reported by WHO. In order to prevent the further spread of this epidemic outbreak, measures such as social distancing, stay-at-home orders, and school closure were executed in many countries. However, these measures also greatly reduced the opportunities for physical activity among children.¹ Based on past experience, there are indications that an increased rate of weight gain occurs during summer school vacation for overweight children.² Therefore, it is reasonable to speculate that under the influence of the COVID-19 epidemic, long-term school closures may significantly increase the risk of weight gain in obese children. For children with metabolic-associated fatty liver disease (MAFLD),^{3,4} formerly known as nonalcoholic fatty liver disease, weight reduction through lifestyle changes (caloric restriction and physical activity) is considered as the first step in reversing disease progression, which makes managing MAFLD in children quite difficult during the ongoing COVID-19 pandemic. In addition, several studies found a significant correlation between a higher risk of severe illness from COVID-19 and metabolic disorders (e.g. diabetes, obesity, MAFLD).⁵⁻⁷

Keywords: COVID-19; MAFLD; Children.

Abbreviations: COVID-19, coronavirus disease-2019; DHA, docosahexaenoic acid; MAFLD, metabolic-associated fatty liver disease; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; WHO, World Health Organization.

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Challenges and risks

Reduced physical activity

Due to the closure of schools, public swimming pools, football stadiums and other sports venues, outlets for physical activities in children have decreased significantly, especially for urban children, due to the small venues available in the living area. While this helps to avoid cross-infection in the community, staying at home frequently equates to less physical activity.

Sedentary and increased electronic screen exposure time

Due to the decrease in social activities, many children tend to spend more time watching television shows and playing video games, which also leads to less physical activity.

Unhealthy diet

Due to the impact of the epidemic, travel restrictions in many places have reduced shopping behavior, while limited transportation has caused a reduction in the distribution of supplies; collectively, these have led to an increased intake of unhealthy food (frozen meat, fast-food delivery service, etc.), and reduced intake of the healthier fresh vegetables and fruits. More exposure time to electronic screens and less social activities outside the house also leads to increased consumption of snacks, such as potato chips, and many children will experience a higher calorie intake in their diet.

Response measures and recommendations

To the clinicians

MAFLD is often described as a disease without symptoms and has a time scale of decades. Therefore, clinical visits for pediatric MAFLD patients are not recommended at this time. Whether COVID-19 may affect the progression of MAFLD is unknown. This is because the evolution of COVID-19 in the

near future is uncertain and specialized medicine to treat SARS-CoV-2 infection is still in the early stage of development. MAFLD-cirrhosis children may require a clinical visit every 3-6 months, although it is recommended to avoid going to the hospital if possible.

There are presently no approved pharmacologic drugs for the treatment of MAFLD, but numerous drugs for fatty liver have entered phase II or phase III clinical trials. Although studies have shown that vitamin E and docosahexaenoic acid (commonly known as DHA) have a certain role in improving liver steatosis in children, they are known to not be effective in reducing alanine aminotransferase. Probiotics might prove to be an effective treatment strategy.⁸ In addition, for children with MAFLD, the safety of drugs should be considered, especially the risk of side effects affecting growth and development.

To the children with MAFLD and their guardians

For this population, the following are important guidelines:

- a) Choose safe and ventilated places for physical activities and avoid crowded or indoor places, such as swimming pools. For children with restrictions to sports venues, it is recommended to choose physical activities, such as skipping, that do not require a specific location. Once outside of the home, it is recommended to wear masks on the road and wash hands frequently.
- b) Guardians should spend more time with children and engage in physical activities to reduce the exposure time to electronic products, such as television programs and video games.
- c) Guardians should provide children with a healthy diet, especially of fresh vegetables and fruits, and reduce the consumption of fatty food and high-calorie snacks, such as potato chips and carbonated beverages.
- d) Since current studies suggest that abnormal liver function and obesity seem to predict poor clinical evolution in patients with COVID-19, guardians are recommended to keep note of the child's weight during the course of social distancing and be aware of the negative consequences of weight gain toward MAFLD.

Summary

It is estimated that the prevalence of MAFLD is 7.6% in non-obese children and 34.2% in obese children.⁷ Because more and more adult MAFLD patients need liver transplantation due to liver cirrhosis, it is necessary to intervene resolutely in children with MAFLD in the early stages of life in order to reverse or delay the progress of the disease. This necessity is further emphasized when taking into consideration that even milder forms of liver damage or dysfunction have shown a relation to worse outcomes in COVID-19 patients.⁹

As we have previously reported, obesity increases the risk for severe COVID-19 illness,⁶ especially among MAFLD patients, for whom the risk is more than 6-fold.¹⁰ Therefore, prevention of SARS-CoV-2 infection in conjunction with weight control in pediatric MAFLD patients (who are usually already obese) during the COVID-19 epidemic is very important, especially when considering that the SARS-Cov-2 virus may coexist with humans for a long time to come and that the current global spread has not yet been effectively contained.

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Conflict of interest

The authors have no conflict of interests related to this publication.

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Systematic Review and Meta-analysis of Circulating Fetuin-A Levels in Nonalcoholic Fatty Liver Disease

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Abstract

Background and Aims: Accumulated studies have reported the key role of circulating fetuin-A in the development and progression of nonalcoholic fatty liver disease (NAFLD) but the results have not been consistent. In this study, we performed a systematic review and meta-analysis to explore the relationship between circulating fetuin-A level and the development and classification of NAFLD. **Methods:** The PubMed, EMBASE, and Cochrane Library databases were searched to obtain the potentially relevant studies up to May 2020. Standardized mean differences (SMD) and 95% confidence intervals of circulating fetuin-A levels were extracted and summarized. Sensitivity, subgroup analysis and meta-regression analysis were performed to investigate the potential heterogeneity. Association of circulating fetuin-A level with classification of NAFLD was also reviewed. **Results:** A total of 17 studies were included, composed of 1,755 NAFLD patients and 2,010 healthy controls. Meta-analysis results showed that NAFLD patients had higher circulating fetuin-A level (SMD=0.43, 95% confidence interval [CI]: 0.22–0.63, $p<0.001$) than controls. Subgroup analysis indicated that circulating fetuin-A level was markedly increased in adult NAFLD patients (SMD=0.48, 95% CI: 0.24–0.72, $p<0.001$) and not in pediatric/adolescent patients compared to controls. Circulating fetuin-A level was markedly increased in ultrasound-proven NAFLD pediatric/adolescent patients (SMD=0.42, 95% CI: 0.12–0.72, $p=0.007$), other than in the liver biopsy-proven NAFLD pediatric/adolescent patients. Body mass index might be the influencing factor to the heterogeneity in adult patients. Circulating fetuin-A level was not associated with the classification of NAFL vs. nonalcoholic steatohepatitis (NASH). Whether the circulating fetuin-A level was associated with the development of fibrosis remains controversial. **Conclusions:** Circulating fetuin-A level was significantly higher in NAFLD patients and was not associated with the classification of NAFL vs. NASH. Whether the circulating fetuin-A level was associated with the development of fibrosis remains controversial.

Keywords: Nonalcoholic fatty liver disease; Fetuin-A; Meta-analysis; Fibrosis.

Abbreviations: ARHQ, Agency for Healthcare Research and Quality; BMI, body mass index; CI, confidence interval; HCC, hepatocellular carcinoma; NAFL, nonalcoholic fatty liver; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NOS, Newcastle-Ottawa scale; SD, standard deviation; SMD, standardized mean differences.

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Introduction

Nonalcoholic fatty liver disease (NAFLD) has become one of the most common chronic liver diseases in recent years, and the overall prevalence of NAFLD is approximately 25% in the world.^{1,2} There is a broad spectrum of NAFLD, which ranges from nonalcoholic fatty liver (NAFL) to nonalcoholic steatohepatitis (NASH), fibrosis, cirrhosis, and even hepatocellular carcinoma (HCC).^{3,4} NAFLD is the hepatic manifestation of metabolic syndrome and is affected by many risk factors, such as obesity, hyperglycemia, type 2 diabetes, and hypertriglyceridemia usually.⁵ However, under certain conditions, mostly during genetically-determined NAFLD (such as in carriers of the *TM6SF2* E167K or *PNPLA3* I148M gene polymorphism), NAFLD does not show association with the metabolic syndrome and an increased risk of cardiovascular disease.⁶

Liver biopsy remains the gold standard for diagnosis and histological assessment of NAFLD, but the obvious defects (e.g., invasiveness, inter-observer differences, sampling error) cannot be ignored.^{7,8} In clinical practice, imaging methods such as ultrasonography, computed tomography, controlled attenuation parameter and magnetic resonance have been used widely for diagnosing NAFLD.^{9–13} In addition, many studies have been conducted to explore the valuable serum biomarkers for early diagnosis and progression of NAFLD. Several serum biomarkers, such as alanine aminotransferase, aspartate aminotransferase, gamma-glutamyltranspeptidase, cytokeratin-18 and fibroblast growth factor 21, have been researched in some studies and their potential to serve as the biomarkers in clinical diagnosing of NAFLD have been mentioned.^{14–18}

Fetuin-A, also known as the 2-Heremans-Schmid glycoprotein, is a phosphorylated glycoprotein and a member of the fetuin group of serum binding proteins that are synthesized primarily by hepatocytes.¹⁹ As an endogenous inhibitor of tyrosine kinase, fetuin-A can trigger insulin resistance in the target tissues, such as liver and skeletal muscle.^{20,21} Pal *et al.*²² reported that fetuin-A acts as an endogenous ligand for toll-like receptor 4 and could enhance both insulin resistance and inflammation response. High serum fetuin-A was also found to strongly interact with the high levels of free fatty acids to induce insulin resistance in rodents,

which was then observed in large human studies; moreover, the relationships of fetuin-A with fatty acids, to determine insulin resistance, were particularly strong in patients with NAFLD.^{23,24} Fetuin-A is also known to inhibit transforming growth factor- β 1 signaling, which promotes fibrotic changes in many tissues, including liver and arteries; therefore, fetuin-A could prevent fibrotic changes in organs.^{25,26} Recently, fetuin-A has been regarded as a potential link molecule between obesity, insulin resistance, and coronary heart disease,^{27–31} and was found to be strongly related to several parameters such as circulating lipid levels (non-esterified free fatty acids and triglycerides), glucose tolerance, circulating pro-inflammatory and anti-inflammatory factors, and interleukin-6.³² Fetuin-A can also induce low-grade inflammation and repress adiponectin production in animals and humans, playing an important role in the pathogenesis of insulin and possessing a pro-adiposity effect.^{33,34} Accumulated lines of evidence have reported the significant association between circulating fetuin-A level and the development and progression of NAFLD, but the results have been inconsistent. Additionally, there has been no definite conclusion as to whether circulating fetuin-A can reflect the grading of NAFL vs. NASH and advanced fibrosis.

The aim of this study was to investigate the importance of fetuin-A in the development and classification of NAFLD.

Methods

Search strategy

This systematic review and meta-analysis was conducted following a priori established protocol and was reported according to PRISMA guidelines.³⁵ Two independent observers (Shousheng Liu and Jianhan Xiao) performed a systematic search of the PubMed, EMBASE, and the Cochrane Library databases up to May 2020 and with English language restrictions. The first step for information retrieval was to gain the subject term of fetuin-A, NAFLD or NASH in the MeSH database of PubMed; meanwhile, we gained the entry terms of them, respectively. The combined results of fetuin-A, NAFLD or NASH in the three databases were obtained based on the search method of "subject term + entry terms". In addition, we examined the reference lists in relevant original research and review articles to search additional potentially eligible studies.

Inclusion and exclusion criteria

Studies that investigated the circulating fetuin-A level in patients with NAFLD were eligible for review. Studies were included in this systematic review and meta-analysis if they met the following criteria: (1) original full-text publications; (2) comparison of circulating fetuin-A level between NAFLD patients and healthy controls; (3) investigations of the effect of circulating fetuin-A level on the classification of NAFL vs. NASH or fibrosis. Studies were excluded according to the following criteria: (1) patients with other causes of liver disease (*e.g.*, viral or autoimmune hepatitis, alcoholic fatty liver disease, HCC, coronary artery disease) or in whom NAFLD co-existed with another liver disease; (2) there was overlap of patients who were included in more than one study. Quality of case-control studies were evaluated use the Newcastle-Ottawa scale (NOS) scoring system and the quality of cross-sectional studies were evaluated use the Agency for Healthcare Research and Quality (AHRQ) scoring system.³⁶ (3) Studies of low methodological quality, as defined by a NOS score ≤ 2 or AHRQ score ≤ 3 , were excluded.

Finally, (4) reviews, editorials, case reports, conference abstracts, letters to the editor, hypotheses, book chapters, and studies on animals or cell lines were excluded.

Data extraction

Available data were extracted from the full text and corresponding supplemental information by two investigators working independently (Shousheng Liu and Jianhan Xiao) and confirmed by a third reviewer (Zhenzhen Zhao). Disagreement was resolved by discussion among all researchers. The following information of each selected publication was extracted: (1) general characteristics, such as first author's name, year of publication, country where the study was carried out, study design, diagnostic methods of NAFLD, type of samples (*e.g.*, serum, plasma, blood); (2) subjects' characteristics, such as age, gender, body mass index (BMI); and (3) effect of circulating fetuin-A on the grading of NAFL vs. NASH or fibrosis. When the same population was published in several journals, we retained only the most informative article or complete study, to avoid duplication. If some necessary data were not offered in the article, the corresponding author would be contacted for the data. If no response, the following methods would be carried out: (1) data from the graphical plots were extracted to calculate the circulating fetuin-A levels by using WebPlotDigitizer (version 4.1.0, <https://apps.automeris.io/wpd/>);³⁷ (2) circulating fetuin-A levels which were expressed as median (mix-mix) or median (25–75 quartile) were transformed into the standard form of mean, according to the Cochrane book or method (Hozo, Stela Pudar *et al.*³⁸).

Quality assessment

The included studies in the systematic review and meta-analysis were independently assessed by two investigators (Shousheng Liu and Jianhan Xiao). We assessed the quality of included case-control studies based on the NOS scoring system, and cross-sectional studies based on the AHRQ scoring system. The full NOS score was 9 stars; a study that met 7 or more stars was defined as a high-quality study, less than 3 stars as low-quality and other studies were defined as moderate quality. Article quality by AHRQ was assessed as follows: low quality: 0–3; moderate quality: 4–7; and high quality: 8–11.

Statistical analysis

All statistical analyses were performed using Stata12.0 (StataCorp LP, College Station, TX, USA). The effect sizes were generated by sample sizes, mean circulating fetuin-A levels, and the standard deviation (SD), and presented as standardized mean differences (SMD) and 95% confidence intervals (95% CIs) for circulating fetuin-A levels in comparisons between groups. Given the expected heterogeneity of the outcome, a random-effect inverse-variance model was chosen for this meta-analysis.³⁹ The heterogeneity between the results of different studies was evaluated using the I^2 statistic, values of $I^2 > 50\%$ were considered to represent substantial heterogeneity. The potential moderating effects of continuous variables on between-study heterogeneity were evaluated by meta-regression analyses and subgroup-analysis. Subgroup-analysis were first conducted according to age, region, and diagnostic method of NAFLD, then the sex distribution (number of males), mean age, BMI and HOMA-IR of NAFLD patients were regarded as the potential moderators for the adult outcome of the

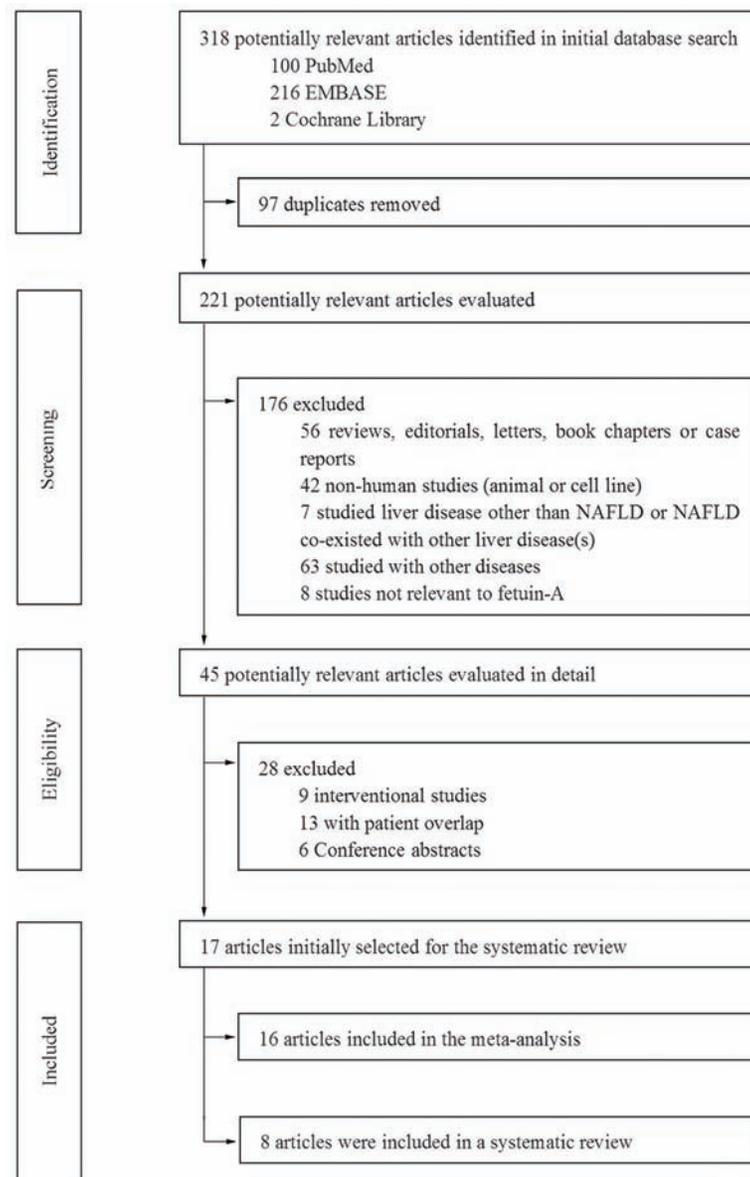


Fig. 1. Flowchart presenting the literature search process, according to the PRISMA statement.

meta-analysis when a high heterogeneity of adult NAFLD was observed. Sensitivity analysis was performed to investigate the influence of each study on the pooled measures by omitting a study each time to assess the stability of our results. A *p*-value of <0.05 was considered to indicate a statistical difference. Publication bias was assessed by funnel plot and Begg's and Egger's tests.

Results

Literature search

A total of 318 studies were retrieved initially from the three databases. After removing duplications (*n*=97), 221 studies remained for evaluation. In all, 204 studies were excluded for representing reviews, editorials, letters, book chapters

or case reports, animal or cell experiments, other liver diseases, patient overlap, conference abstracts, and so on (Fig. 1). The final dataset for the systematic review and meta-analysis comprised 17 full-text studies.^{25,27,28,40-53} Among them, 16 studies were selected to conduct the meta-analysis and 8 studies were selected to investigate the relationship between circulating fetuin-A level and the classification of NAFL vs. NASH.

Characteristics of included studies

A total of 1,755 NAFLD patients and 2,010 healthy controls were included in the 17 studies, the main characteristics of these studies are shown in Table 1. Among these studies, two were conducted with the same participants, but the former investigated the association of plasma fetuin-A level with NAFLD;⁴⁴ however, the latter study not only investi-

Table 1. Characteristics of included studies focused on circulating levels of fetuin-A in this meta-analysis and systematic review

Study (Year)	Country	Region	Study design	Age	Sample size	Tissue type	Fetuin-A measurement method	NAFLD diagnosis method	NOS, 0–9 score	AHRO, 0–11 score	Additional information
Reinehr et al. (2008)	Germany	European	Cross-sectional	Pediatric/ Adolescent	48	Serum	ELISA	Ultrasound	6		
Yilmaz et al. (2010)	Turkey	Eurasian	Case-control	Adult	174	Serum	ELISA	Liver biopsy	7		
Haukeland et al. (2012)	Norway	European	Cross-sectional	Adult	242	Plasma	ELISA	Liver biopsy	4		
Ou et al. (2012)	Chinese	Asian	Cross-sectional	Adult	510	Serum	ELISA	Ultrasound	6		(1)
Ballestri et al. (2013)	Italy	European	Cross-sectional	Adult	70	Serum	ELISA	Ultrasound	5		(2)
Dogru et al. (2013)	Turkey	Eurasian	Cross-sectional	Adult	189	Plasma	ELISA	Liver biopsy	5		(3)
Kahraman et al. (2013)	Germany	European	Cross-sectional	Adult	118	Serum	ELISA	Liver biopsy	4		
Lebensztejn et al. (2014)	Poland	European	Cross-sectional	Paediatric/ Adolescent	45	Serum	ELISA	Ultrasound	5		
Rametta et al. (2014)	Italy	European	Cross-sectional	Adult	397	Serum	ELISA	Liver biopsy	7		
Sato et al. (2015)	Japanese	Asian	Cross-sectional	Adult	295	Serum	ELISA	Ultrasound	5		
Wong et al. (2015)	Chinese	Asian	Case-control	Adult	920	Serum	ELISA	Ultrasound	5		
Celebi et al. (2015)	Turkey	Eurasian	Cross-sectional	Adult	/	Plasma	ELISA	Liver biopsy	5		(3)
Cui et al. (2017)	Chinese	Asian	Case-control	Adult	158	Serum	ELISA	Ultrasound	5		
Siraz et al. (2017)	Turkey	Eurasian	Cross-sectional	Paediatric/ Adolescent	80	Serum	ELISA	Ultrasound	6		
Pampanini et al. (2018)	Italy	European	Cross-sectional	Paediatric/ Adolescent	183	Serum	ELISA	Ultrasound	6		(1)
Mondal et al. (2018)	India	Asian	Cross-sectional	Adult	188	Serum	ELISA	Ultrasound	6		(2)
Nascimbeni et al. (2018)	Italy	European	Cross-sectional	Adult	149	Serum	ELISA	Ultrasound Liver biopsy	6		(4)

(1) All participants were prediabetic. (2) All participants were suspected cases of coronary heart disease. (3) The two studies were conducted on the same subjects, with the former study investigating the circulating fetuin-A levels between NAFLD patients and healthy controls, and the latter study investigating the circulating fetuin-A levels between NASH and NAFL patients, and fibrosis and no fibrosis patients. (4) The study investigated fetuin-A levels in 81 obese children with NAFLD diagnosed by biopsy, 79 obese children with NAFLD defined by liver ultrasonography, and 23 lean subjects. In order to make a better analysis, we made two groups: one was for comparison between obese NAFLD and non-NAFLD patients verified by ultrasound; the other one was for comparison between obese NAFLD verified by liver biopsy and characterized as lean healthy.

Abbreviation: ELISA, enzyme-linked immunosorbent assay.

gated the relationship between plasma fetuin-A level and NASH or NAFL but also investigated the association of plasma fetuin-A level and fibrosis.⁴⁷ As such, 16 studies were selected to perform the meta-analysis.

Studies in this meta-analysis included three case-control and thirteen cross-sectional designs. Enzyme-linked immunosorbent assay was used to test the serum/plasma fetuin-A level in all the studies. Liver biopsy was performed to determine the NAFLD in six studies, and ultrasound was used in other ten. Another study, conducted by Pampanini *et al.*,²⁷ diagnosed NAFLD with both liver biopsy and ultrasound in different groups; so, we regarded this study as two individual studies. Among these studies, six performed the comparison of circulating fetuin-A level between NAFL and NASH patients and eight determined the relationship of circulating fetuin-A level with liver fibrosis (Table 2).

Quality of included studies

The qualities of included case-control or cohort studies were assessed based on the NOS, and the cross-sectional studies were assessed based on the ARHQ methodology checklist.³⁶ The detailed quality scores of each study are shown in Table 1. All the studies were assessed as moderate quality. No study was eliminated due to low quality (NOS score ≤2 or AHRQ score ≤3).

Effect of circulating fetuin-A level on NAFLD

A random-effect meta-analysis was performed to investigate the effect of circulating fetuin-A level on the development of NAFLD. As the results show in Fig. 2A, the circulating fetuin-A level in NAFLD patients was significantly higher than in healthy controls, with a summarized SMD of 0.43 (95%CI: 0.22–0.63, *p*<0.001). A striking heterogeneity among included studies was observed in the comparison of circulating fetuin-A level in NAFLD patients and healthy controls; the *I*² value was 85.7% (*p*<0.001).

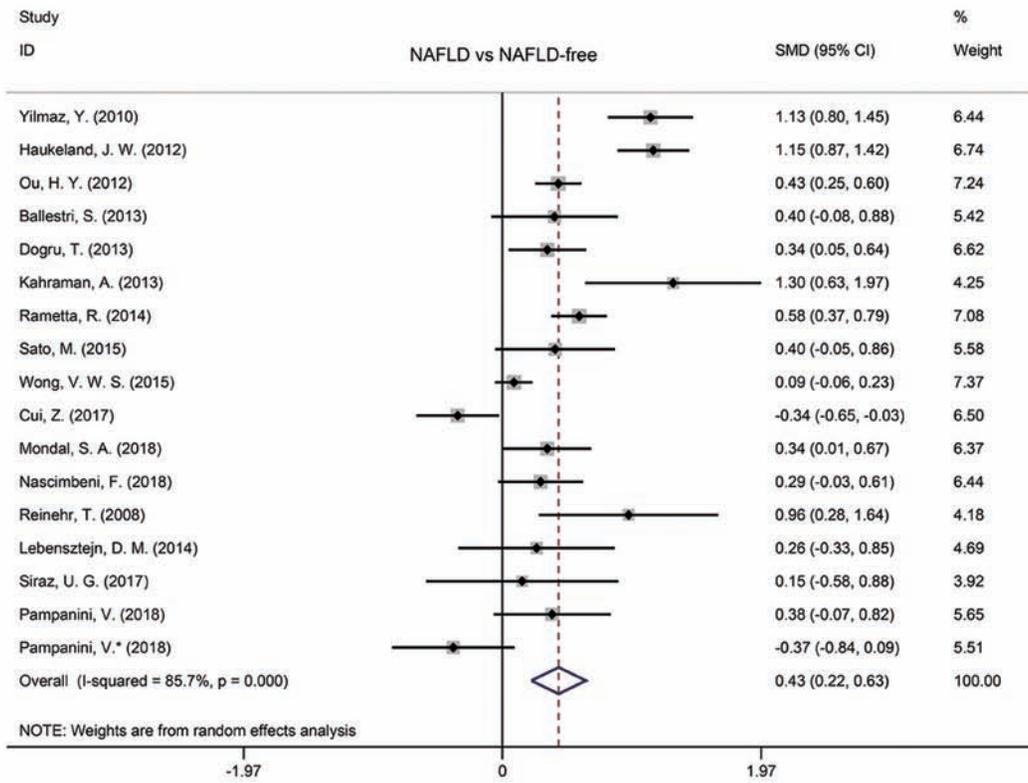
Subgroup analysis based on age implied that the circulating fetuin-A level of NAFLD patients was significantly elevated in adults (SMD=0.48, 95% CI: 0.24–0.72, *p*<0.001) and no obvious difference was observed in pediatric/adolescent patients (SMD=0.25, 95% CI: -0.18–0.67, *p*=0.256) (Fig. 2B). Based on age, we also performed subgroup analysis according to the region of subjects and diagnostic method of NAFLD, respectively. As shown in Fig. 3A, the circulating fetuin-A level in adult NAFLD patients was increased among Europeans (SMD=0.71, 95% CI: 0.35–1.07, *p*<0.001), and no significant differences were observed in the Eurasians (SMD=0.73, 95% CI: -0.04–1.50, *p*=0.062) nor Asians (SMD=0.18, 95% CI: -0.08–0.43, *p*=0.174). In the pediatric/adolescent group, there was no significant difference of circulating fetuin-A level between NAFLD patients and controls of European populations (SMD=0.27, 95% CI: -0.25–0.79, *p*=0.303) and Eurasian populations (SMD=0.15, 95% CI: -0.58–0.88, *p*=0.688). Subgroup analysis results according to NAFLD diagnosis method (ultrasound vs. liver biopsy) are shown in Fig. 3B. In adults, the level of circulating fetuin-A was higher in both ultrasound-proven NAFLD patients and liver biopsy-proven NAFLD patients than in healthy controls (SMD=0.21, 95% CI: 0.01–0.42, *p*<0.001; SMD=0.86, 95% CI: 0.51–1.21, *p*<0.001, respectively). Interestingly, the circulating fetuin-A level in ultrasound-proven NAFLD pediatric/adolescent patients was significantly increased compared to pediatric/adolescent controls (SMD=0.42, 95% CI: 0.12–0.72, *p*=0.007), but no difference was observed between the liver biopsy-proven NAFLD pediatric/adolescent patients and healthy

Table 2. Relationship of circulating fetuin-A levels with the progression of NAFLD

Studies	Sample sizes	NASH vs. NAFL				Fibrosis vs. No fibrosis			
		Correlation	Diagnosis method	Data	Logistic regression analysis	Correlation	Diagnosis method	Data	Logistic regression analysis
Yilmaz et al. (2010)	99	Ns	Liver biopsy	Not shown	Positive correlation	Liver biopsy	Graphic results	Consistent	
Haukeland et al. (2012)	111	Ns	Liver biopsy	Graphic results	Ns	Liver biopsy	Not shown	/	
Kahraman et al. (2013)	109	Ns	Liver biopsy	Detailed data	Negative correlation	Liver biopsy	Not shown	/	
Rametta et al. (2014)	137	Ns	Liver biopsy	Graphic results	Ns	Liver biopsy	Not shown	/	
Celebi et al. (2015)	105	Ns	Liver biopsy	Detailed data	Ns	Liver biopsy	Detailed data	/	
Sato et al. (2015)	275	/	/	/	Negative correlation	serology	Detailed data	Consistent	
Pampanini et al. (2018)	81	Ns	Liver biopsy	Detailed data	Ns	Liver biopsy	Graphic results		
Mondal et al. (2018)	46	/	/	/	Positive correlation	FibroScan	Detailed data	Consistent	

Abbreviation: Ns, not significant.

A



B

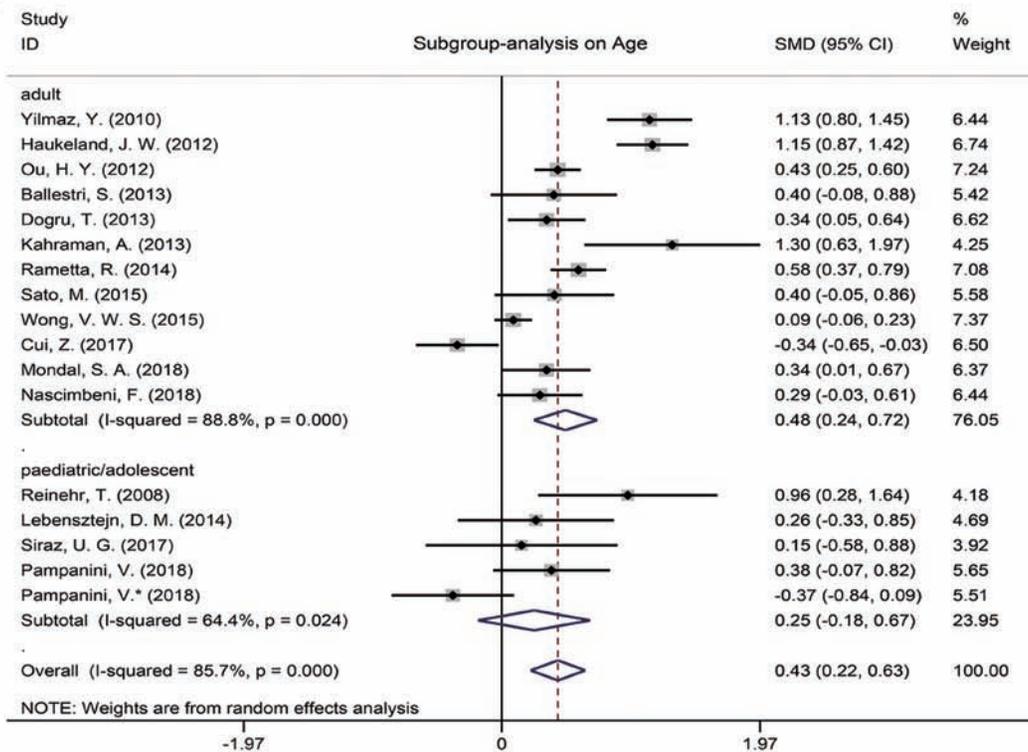


Fig. 2. Meta-analysis and influence of age. (A) Meta-analysis of the circulating fetuin-A levels in the included NAFLD patients compared to healthy controls. (B) Subgroup analysis on the difference of circulating fetuin-A levels between the included NAFLD patients and healthy controls based on age.

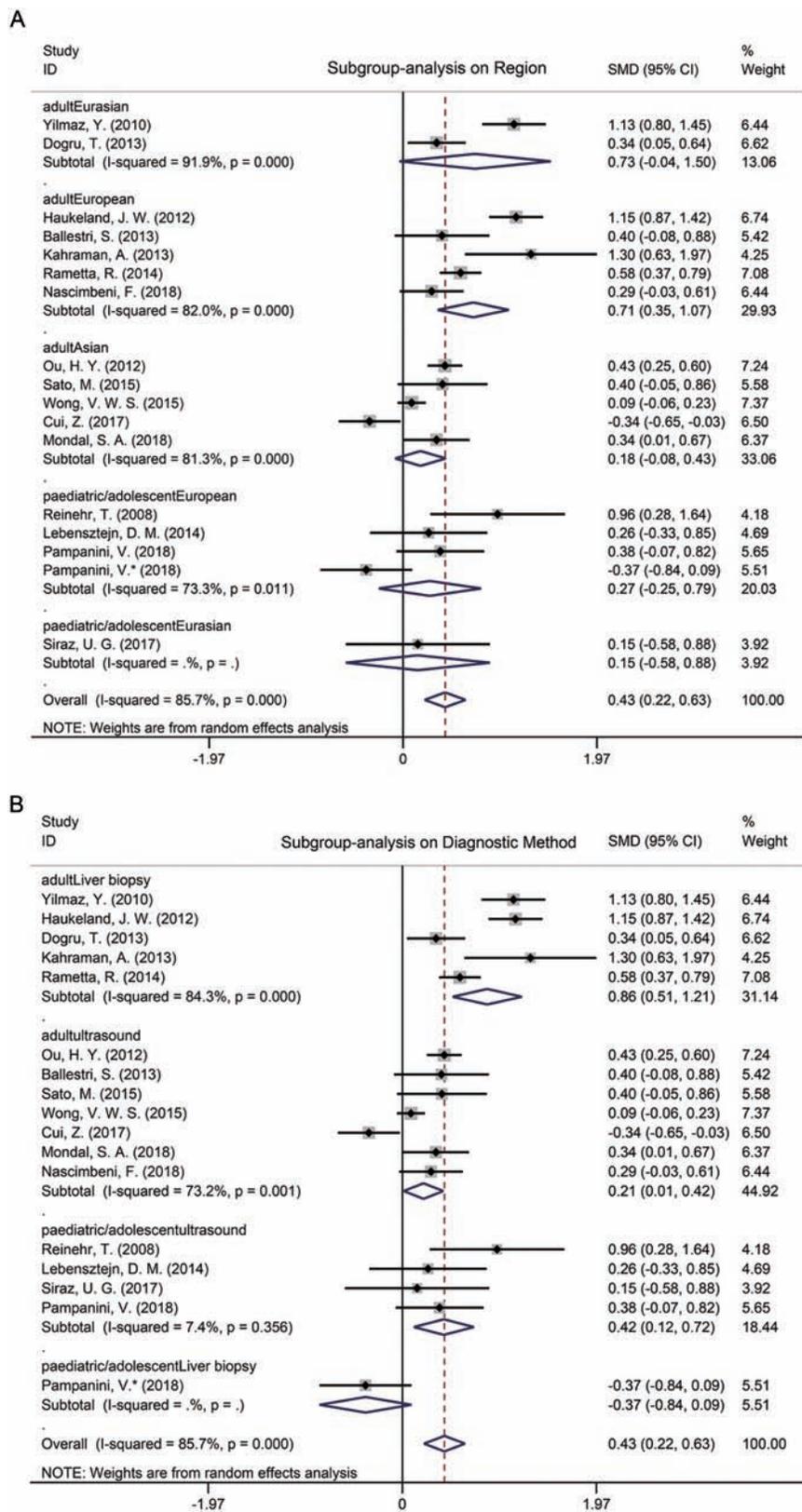


Fig. 3. Influence of region and diagnostic method. (A) Subgroup analysis on the difference of circulating fetuin-A levels between the included NAFLD patients and healthy controls based on region. (B) Subgroup analysis on the difference of circulating fetuin-A levels between the included NAFLD patients and healthy controls based on diagnostic method.

Table 3. Demographic and clinical data of patients with NAFLD and healthy controls among the adults

Studies	Group	Size, n	Males, %	Age in years, mean (SD)	BMI in kg/m ² , mean (SD)	HOMA-IR
Yilmaz <i>et al.</i> (2010)	NAFLD	99	46.0	47.0 (9.0)	30.7 (4.9)	3.80 (0.40)
	Control	75	51.0	47.0 (8.0)	27.5 (4.3)	1.40 (0.30)
Haukeland <i>et al.</i> (2012)	NAFLD	111	60.0	46.5 (11.6)	30.5 (4.3)	2.21 (1.14)
	Control	131	44.0	43.3 (3.0)	23.9 (3.0)	1.40 (0.77)
Ou <i>et al.</i> (2012)	NAFLD	255	56.0	61.1 (10.3)	26.7 (3.1)	1.21 (0.12)
	Control	255	60.0	62.1 (11.3)	23.3 (2.8)	0.58 (0.16)
Ballestri <i>et al.</i> (2013)	NAFLD	29	69.0	64.5 (10.5)	29.2 (5.0)	1.50 (0.325)
	Control	41	68.3	70.6 (12.7)	25.8 (3.1)	1.40 (0.350)
Dogru <i>et al.</i> (2013)	NAFLD	115	100.0	31.0 (5.2)	28.4 (2.97)	3.35 (2.18)
	Control	74	100.0	28.0 (5.2)	24.0 (2.65)	1.22 (0.62)
Kahraman <i>et al.</i> (2013)	NAFLD	108	23.0	41.9 (0.9)	53.3 (1.1)	Na
	Control	10	50.0	32.5 (5.5)	23.9 (1.2)	Na
Rametta <i>et al.</i> (2014)	NAFLD	137	77.4	49.7 (12.1)	26.9 (3.4)	2.50 (2.80)
	Control	260	80.0	47.7 (12.1)	25.1 (2.8)	1.30 (0.20)
Sato <i>et al.</i> (2015)	NAFLD	275	55.0	56.4 (6.9)	26.5 (3.6)	Na
	Control	20	65.0	61.0 (7.0)	22.2 (2.6)	Na
Wong <i>et al.</i> (2015)	NAFLD	263	54.0	51.0 (9.0)	25.3 (4.0)	2.50 (0.37)
	Control	657	37.4	47.0 (11.0)	21.3 (3.1)	1.10 (0.15)
Cui, Xuan, and Yang (2017)	NAFLD	79	73.0	42.0 (10.8)	26.0 (3.0)	3.27 (2.18)
	Control	79	73.0	40.0 (12.0)	22.0 (2.0)	1.81 (1.80)
Mondal <i>et al.</i> (2018)	NAFLD	46	57.0	49.5 (12.2)	27.5 (6.2)	1.10 (0.26)
	Control	142	66.0	46.2 (12.7)	25.7 (4.8)	1.10 (0.10)
Nascimbeni <i>et al.</i> (2018)	NAFLD	80	79.0	70.0 (7.0)	28.0 (3.8)	1.80 (3.05)
	Control	69	77.0	73.0 (8.2)	25.0 (2.5)	1.40 (1.87)
R ² (%)			15.95	-10.90	41.60	-13.12
β			0.297	0.995	1.058	0.041
p value			0.097	0.693	0.023	0.812

Abbreviation: NA, not applicable.

controls (SMD=-0.37, 95% CI: -0.84-0.09, $p=0.116$). In addition, heterogeneity in the pediatric/adolescent patients with ultrasound diagnosis was markedly lower ($I^2=7.4%$, $p=0.356$) than the overall heterogeneity in the remaining pediatric/adolescent patients ($I^2=64.4%$, $p=0.024$) (Figs. 2B and 3B).

To further investigate the cause of heterogeneity in adult NAFLD patients, we performed univariate, random-effects meta-regression analysis to test whether the continuous variables, including sex distribution (percentage of males), mean age, BMI and HOMA-IR of NAFLD patients, could explain the high heterogeneity among studies. As the results show in Table 3 and Fig. 4, BMI was the significant influencing factor of the meta-analysis ($R^2=41.60$, $\beta=1.058$, $p=0.023$), and the other tested variables did not show moderating effects.

Sensitivity and publication bias analyses

A leave-one-out sensitivity analysis was conducted to evaluate the stability of this meta-analysis (Fig. 5A). Each study included in our meta-analysis was evaluated one-by-one, to reflect the effect of pooled SMDs. The overall statistical significance did not change when any single study was omitted at one time. Therefore, the data presented in this meta-analysis is relatively stable and credible. Publication bias in this meta-analysis was confirmed by Egger's test, the

results showed no significant publication bias ($p=0.152$). In addition, no significant publication biases were observed in the adult population Egger's test ($p=0.275$) and in the pediatric/adolescent population Egger's test ($p=0.450$). Funnel plots of effect size vs. standard error were symmetrical ($p>0.05$) (Fig. 5B).

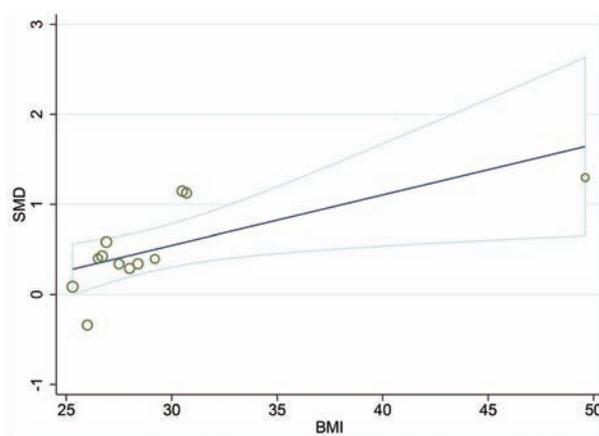


Fig. 4. Meta-regression analysis for the effect of BMI on the NAFLD patients and healthy controls.

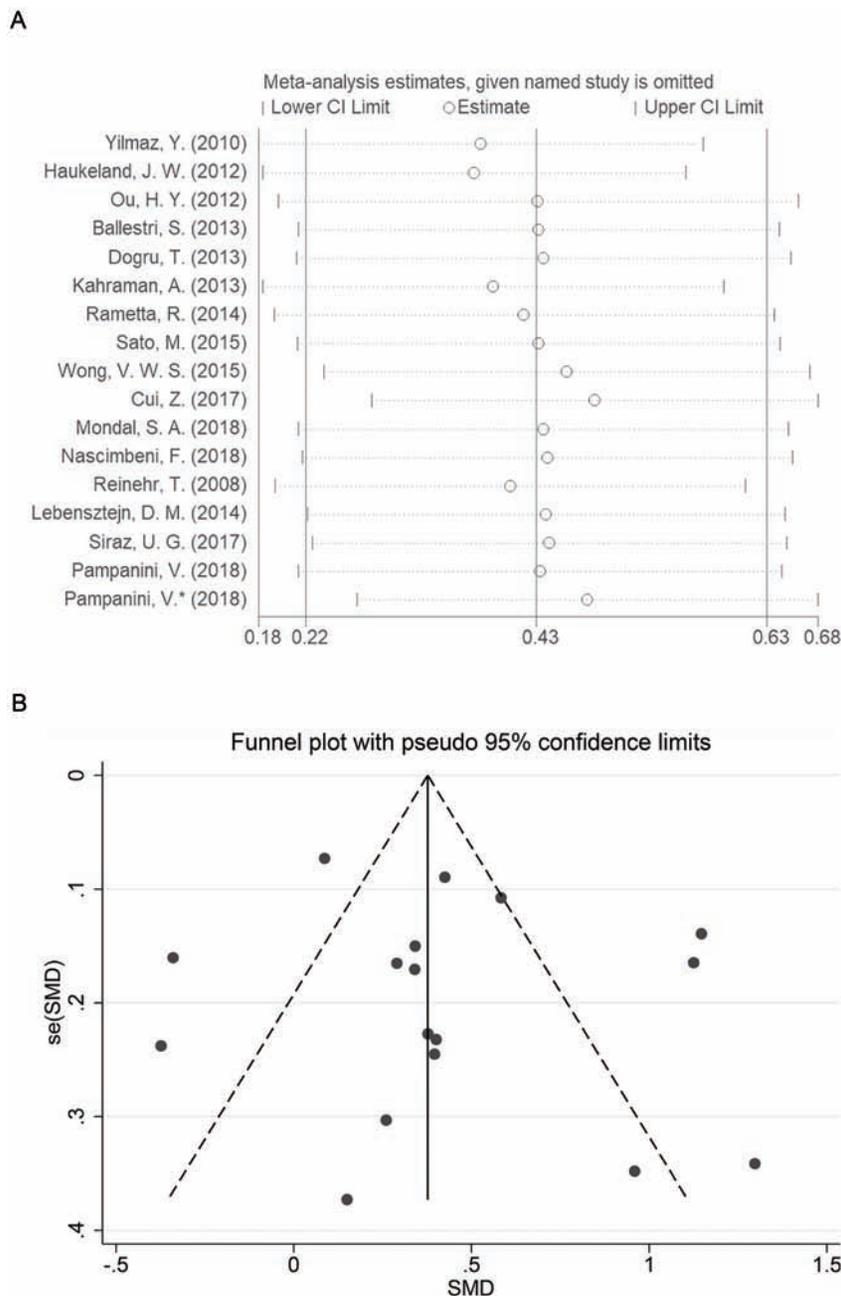


Fig. 5. Sensitivity analysis and forest plotting. (A) Sensitivity analysis of the summary odds ratio coefficients on the difference of circulating fetuin-A levels between NAFLD patients and healthy controls. (B) Forest plots on the difference of circulating fetuin-A levels between NAFLD patients and healthy controls.

Effect of fetuin-A levels on grading of NAFLD

In order to investigate whether circulating fetuin-A level can be used as a potential diagnostic biomarker for the classification of NAFLD, all the available information of circulating fetuin-A level on the classification of NAFLD were collected. As shown in Table 2, there were no significant differences of circulating fetuin-A level in the classification of NAFL vs. NASH in liver biopsy-proven NAFLD patients. In the liver biopsy-proven fibrosis patients, results from four studies suggested there was not association between circulating fetuin-A level and the development of fibrosis, two stud-

ies showed that circulating fetuin-A levels were negatively correlated with fibrosis, and one study showed a positive correlation. In addition, one study found that circulating fetuin-A level was negatively correlated with serology-proven fibrosis, and another one study showed positive correlation in FibroScan-proven fibrosis (Table 2).

Discussion

NAFLD has become one of the most prevalent chronic liver diseases in the world and the major cause of liver-related

morbidity and mortality.¹ Up to now, liver biopsy remains the recognized gold standard for the diagnosis of NAFLD. In consideration of the defects of biopsy, several imaging and serological diagnosis methods have been developed. Fetuin-A is a member of the fetuin group of serum binding proteins that are primarily synthesized by the hepatocytes.⁵⁴ Some studies have investigated the circulating fetuin-A level between NAFLD patients and healthy controls in adult or pediatric/adolescent populations, but the results have been inconsistent. In this study, we conducted a meta-analysis to summarize the circulating fetuin-A levels in NAFLD patients and healthy controls, and a systematic review to determine the correlation of circulating fetuin-A level with the classification of NAFLD. As the results show, the circulating levels of fetuin-A in patients with NAFLD were significantly higher than in healthy controls for the included subjects. There was no difference of circulating fetuin-A level between NAFL and NASH patients. In addition, the relationship of circulating fetuin-A level with fibrosis remains unclear. Sensitivity analysis suggested that our meta-analysis was stable, and no significant publication bias was observed.

In this meta-analysis, the included studies were carried out from a different region in adult and pediatric/adolescent patients, and the diagnostic methods of NAFLD were also different. A marked heterogeneity was observed ($I^2=85.7$, $p<0.001$) when the circulating fetuin-A levels were analyzed for the patients with NAFLD and healthy controls. In order to explore the potential factors which contribute to the heterogeneity, subgroup analysis based on the different variables was conducted. In the adults, circulating fetuin-A level was significantly increased in patients with NAFLD compared to healthy controls, with a high heterogeneity ($I^2=88.8$, $p<0.001$). Subsequently, the region of subjects and diagnostic methods of NAFLD were considered for analysis of the origin of heterogeneity. In the adults, circulating fetuin-A levels in Europeans were increased in patients with NAFLD but not in Eurasians and Asians, which suggested that region may be one of the influencing factors that contributes to the heterogeneity.

The circulating level of fetuin-A in patients with NAFLD did not vary according to the different diagnostic methods. Furthermore, meta-regression analysis suggested that BMI was the significant influencing factor to the heterogeneity. The reason why BMI contributes to the heterogeneity may be due to the incomplete correlation between BMI and the risk of NAFLD. Usually, subjects with metabolically healthy obesity, which is predominantly characterized by low liver fat content, may possess low risk of developing NAFLD, and the body fat distribution (more strongly than BMI) determines NAFLD in the general population; even patients with newly developed lipodystrophy can strongly develop NASH.⁵⁵⁻⁵⁷

The data of circulating fetuin-A level in NAFLD in pediatric/adolescent patients were relatively insufficient in that only four studies were included in this meta-analysis. We analyzed the pediatric/adolescent data using five individual studies, due to the study which was conducted by Pampiani *et al.*²⁷ that recruited two independent cohorts in which NAFLD was diagnosed with ultrasound and liver biopsy, respectively. There was no significance difference of circulating fetuin-A levels between patients with NAFLD and healthy controls. It is noteworthy that the high circulating fetuin-A level was observed in the ultrasound-diagnostic NAFLD in pediatric/adolescent patients. Notably, the heterogeneity of this subgroup analysis was very low ($I^2=7.4\%$, $p<0.001$). The circulating fetuin-A levels in biopsy-proven NAFLD and healthy controls in pediatric/adolescent patients was tested for the first time and no obvious difference of circulating fetuin-A levels was found between the two groups. The different fetuin-A levels in ultrasound-diagnostic NAFLD compared to biopsy-proven NAFLD in pediatric/adolescent patients may due to the difference of diagnostic results of

NAFLD. In other words, some ultrasound-diagnostic NAFLD may belong to healthy controls when diagnosed by biopsy. In order to illuminate this query, more studies should be conducted to investigate the circulating fetuin-A level in liver biopsy-proven NAFLD in pediatric/adolescent patients.

Fetuin-A possesses a pro-inflammatory role and is down-regulated during inflammation; expression of fetuin-A is also inversely correlated with the level of C-reactive protein, which is a well-known marker of systemic inflammation.^{33,47} Sato *et al.*²⁵ reported that fetuin-A might promote insulin resistance and inhibit NAFLD progression, but whether circulating fetuin-A level is positively or negatively correlated with the classification of NAFLD remains controversial. Our results have suggested that circulating fetuin-A level is not related with the classification of NAFL vs. NASH, as no significant change of fetuin-A level was found between the two groups. In consideration of the previous controversial results, more studies should be conducted to investigate the relationship of fetuin-A with the markers of inflammation. Besides, there was not a definite conclusion as to whether circulating fetuin-A level was associated with fibrosis due to the positive, negative, and unrelated correlations that have been reported. In order to clarify the significant role of circulating fetuin-A level on the diagnosis of fibrosis, more attention should be paid to the relationship of circulating fetuin-A level with fibrosis in different regions and ethnicity groups.

Our study had several limitations. First, considerable heterogeneity among studies limits the reliability of the results. Although we performed subgroup and meta-regression analyses to investigate some potential sources of heterogeneity, the high levels of heterogeneity cannot be reasonably explained in adult NAFLD. Second, due to the lack of detailed data on fetuin-A in the classification of NAFLD in some studies, we just reviewed the change of fetuin-A rather than making a quantitative analysis. Finally, all these studies are the cross-sectional or case-control epidemiological design, and the dynamic changes of circulating fetuin-A level in NAFL or NASH patients were unclear.

Conclusions

In summary, the circulating fetuin-A level was significantly higher in NAFLD patients than in healthy controls in adults and no difference was observed in pediatric/adolescent patients. BMI might be the risk factor that affects the stability of meta-analysis in adults. In pediatric/adolescent patients, ultrasound-proven NAFLD patients possess a markedly higher circulating fetuin-A level than healthy controls and there were no differences in biopsy-proven NAFLD patients. Our results suggest that circulating fetuin-A level could be regarded as a potential serum biomarker for the early diagnosis of NAFLD. Diagnostic values and differences of ultrasound and biopsy in pediatric/adolescent should be further studied to illuminate the significant effect of circulating fetuin-A level on pediatric/adolescent NAFLD. In addition, association of circulating fetuin-A level with fibrosis should be studied further.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Study concept and design (SL, YX), data collection (SL, JX, ZZ, MW, and YW), analysis of data (SL, JX, and ZZ), drafting and writing of the manuscript (SL and JX), revision of the manuscript (YX).

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HbA_{1c} as a Biomarker of Non-alcoholic Fatty Liver Disease: Comparison with Anthropometric Parameters

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Abstract

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Background and Aims: Multiple non-invasive methods including radiological, anthropometric and biochemical markers have been reported with variable performance. The present study assessed glycosylated hemoglobin (HbA_{1c}) as a biomarker to predict non-alcoholic fatty liver disease (NAFLD) and its severity, compared with body mass index (BMI), waist to hip ratio (WHR) and waist circumference (WC) **Methods:** This case control study included 450 individuals, including 150 cases and 300 age- and gender-matched controls recruited from the Dow Radiology Institute on the basis of radiological findings of fatty infiltration on abdominal ultrasound through convenient sampling. BMI, WHR and WC were measured according to standard protocols. HbA_{1c} was determined by turbidimetric inhibition immunoassay **Results:** Among the cases and controls, 66% and 32% had HbA_{1c} levels higher than 5.7% respectively. HbA_{1c} and BMI were significantly associated with NAFLD [crude odds ratio (cOR)=4.12, 2.88, 2.25 (overweight) and 4.32 (obese)]. WC was found to be significantly associated with NAFLD for both genders (cOR in males=5.50 and females=5.79, $p<0.01$). After adjustment for other parameters, HbA_{1c} and WC were found to be significantly associated with NAFLD (aOR=3.40, $p<0.001$) along with WC in males (aOR=2.91, $p<0.05$) and in females (aOR=4.28, $p<0.05$). A significant rise in severity of hepatic steatosis was noted with increases in HbA_{1c}, BMI and WC. HbA_{1c} possessed a positive predictive value of 76% for the study population [0.76, confidence interval (CI): 0.715-0.809], 70.6% for males (0.706, CI: 0.629-0.783) and 80% for females (0.80, CI: 0.741-0.858). **Conclusions:** Higher than normal HbA_{1c} and WC measurements possess a more than 70% potential to predict NAFLD. It is the single risk factor that is strongly associated with NAFLD after adjustment for indices of body measurements. HbA_{1c} may be presented as a potential biomarker for NAFLD in examination with other anthropometric measures in the adult population.

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Abbreviations: aOR, adjusted odds ratio; AUC, area under the ROC curve; BMI, body mass index; CI, confidence interval; cOR, crude odds ratio; DUHS, Dow University of Health Sciences; FLD, fatty liver disease; HbA_{1c}, glycosylated hemoglobin; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; ROC, receiver operating characteristic; TAG, triacylglycerol; Type II DM, type II diabetes mellitus; WC, waist circumference; WHR, waist to hip ratio.

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Introduction

Non-alcoholic fatty liver disease (NAFLD) refers to a condition wherein excess fat accumulates in the liver of people with no history of significant alcohol consumption.¹ Fat molecules are deposited in the form of triacylglycerols (TAGs) in hepatocytes. Hepatic steatosis refers to fatty change in hepatocytes and is largely a benign condition, while, not in a small number of patients, it may trigger an immune response and lead to non-alcoholic steatohepatitis (NASH) followed by cirrhosis and cancer.² NAFLD is alarmingly increasing around the globe. The estimated global prevalence of NAFLD ranges from 6.3-33% among the general population, varying among and within populations.³ The prevalence is highest among obese (57%) and diabetic (90%) populations.⁴ A rising trend of prevalence of NAFLD has been observed in line with obesity, at a rate of 25%.⁵ Sedentary lifestyle, dyslipidemia and metabolic syndrome are also well documented risk factors for NAFLD,⁴ along with other risk factors, such as hepatitis B and C virus infection, Wilson's disease, and chronic blood and kidney diseases.

High blood glucose levels non-enzymatically form glycosylated hemoglobin (HbA_{1c}) as an irreversible reaction. Once formed, HbA_{1c} remains in circulation for 2-3 months; hence, it has been identified as the marker for diabetes diagnosis and control. According to the American Association of Clinical Endocrinologists, an acceptable level of HbA_{1c} in diabetics is <6.5% and reflects good metabolic control;⁶ although, tight control is recommended to avoid increased risk of hypoglycemia, but the level of <6.5% is considered as acceptable in this study. Obesity and diabetes have been reported as strong predictors of NAFLD.⁷ Therefore, it may be assumed that patients with NAFLD have increased levels of HbA_{1c} as well.

On the contrary, recently emerging data suggest that HbA_{1c} may be raised in the absence of diabetes. Chen *et al.*,⁸ in 2020, reported that after multiple adjustments HbA_{1c} serves as a risk factor for NAFLD, with a significant odds ratio of 1.58 in metabolically-intact patients. The South Asian countries have reported a prevalence of 13.9% of NAFLD in the adult healthy population that excludes obesity and diabetes.⁹ It is suggested that inter-individual biological differences may also contribute to the elevation of HbA_{1c}, apart from high blood sugar.^{10,11}

Similarly, various indices of body measurements, such as body mass index (BMI), waist to hip ratio (WHR), and obesity status, have been linked with insulin resistance, type II diabetes mellitus (Type II DM),¹² and NAFLD. About 20–35% of lean NAFLD cases have been reported from rural areas of some Asian countries.¹³ Waist circumference (WC),

on the other hand, has emerged as the physical measure associated significantly with NAFLD.¹⁴ The debate on the relevance of various body weight measurements, including BMI, WHR and WC, has generated much data with conflicting observations regarding their significance as the risk factor for NAFLD.^{15,16}

Although liver biopsy is the gold standard for the diagnosis and grading of severity of hepatic steatosis and NASH, due to its invasive nature and cost/risks, alternate non-invasive and cost-effective methods have been widely searched and reported.¹⁷ Radiological diagnoses and grading is widely recommended by nearly all associations for study of liver diseases, such as the European Association for the Study of the Liver, the Italian Association for the Study of the Liver, and the American Association for the Study of Liver Diseases.¹⁸ The non-invasive protocols to diagnose NAFLD cannot equate to the gold standard liver biopsy, but they may help in detecting early steatosis in hepatocytes.

NAFLD is among the most common cause of chronic liver disease.¹⁷ The diagnosis protocol of NAFLD basically addresses pathological/radiological evidence of fatty infiltration, liver biochemistry, and history of alcohol consumption and other chronic diseases. There is dire need of identifying biochemical markers with significant discriminatory performance for NAFLD diagnosis. The present study was, therefore, designed to measure and find the association of HbA_{1c} as a novel biomarker for the diagnosis of NAFLD patients identified through abdominal ultrasound.

Methods

This case control study was conducted at the Dow University of Health Sciences (DUHS). Diagnosis of fatty liver disease (referred to here as FLD) was based on ultrasonographic evidence of fatty infiltration of hepatocytes.¹⁸ Individuals of both genders in the adult age group, undergoing upper abdominal ultrasonography at the Department of Radiology at the DUHS, were recruited for the study. Those having FLD on ultrasound were identified as cases, while people showing no fatty infiltration were included as controls. Informed consent was obtained after explaining the study procedures and outcomes; those who refused to be included were dropped out. Considering the prevalence of the condition in Pakistan,⁶ sample size was calculated by OpenEpi as 104 (52 each in the case and control groups). However, to improve the strength of our study, the total sample size was increased to 450, with case-to-control ratio of 1:2. The participants were recruited through convenient sampling (150 cases and 300 age- and gender-matched controls). The severity of steatosis was graded on the basis of fatty infiltration found on ultrasonography, from grades (1-3) as follows: grade 1 had minimal infiltration, with echogenicity slightly increased; grade 2 had moderate infiltration, with echogenically obscured portal vessel walls; and grade 3 had heavy fatty infiltration.¹⁹

For the purpose of standardization, subjects undergoing ultrasonography (by two trained sonologists) were included in the study. Patients with chronic liver disease, tumors, acute hepatitis, Wilson's and kidney diseases, known NAFLD, and those having history of alcohol consumption were excluded. History regarding presenting complaints, comorbidities, lifestyle, dietary intake, and medication were recorded on structured proforma. Detailed physical examination was carried out. Height in meters and weight in Kg was recorded for BMI (reported as Kg/m²). WC and WHR were measured by standard methods.²⁰ Blood samples were collected in the fasting state, with samples in appropriate bar-coded containers, for estimation of HbA_{1c} by turbidimetric inhibition immunoassay and expressed as percentage (%). The value

of 5.7% or below was taken as normal.²¹ The study was approved by the institutional review board of DUHS (IRB-447/DUHS/-14) and funded by the Higher Education Commission of Pakistan.

Statistical analysis

Data was analyzed using SPSS version 21.0 and STATA 14. Chi-square, ANOVA and binary logistic regression were used for analysis. Frequencies and proportions were generated for all categorical variables, study participants' characteristics and body weight measurements with NAFLD. These were compared using the chi-square (χ^2) test, while mean differences for anthropometric measures with steatosis severity grades were assessed using ANOVA. Binary logistic regression analyses (univariate and multivariate) were used to assess the factors associated with NAFLD occurrence. Results of regression were reported as crude odds ratio (cOR) and adjusted odds ratio (aOR) with 95% confidence interval (CI). Receiver operating characteristic (ROC) curve was plotted to compare each variable with NAFLD and to find the valid predictive value of HbA_{1c} to diagnose NAFLD. A *p* value <0.05 was taken as significant.

Results

Baseline characteristics are given in Table 1. Females dominated the sample, with 56% among cases and 60.3% among controls. The mean age of the study sample was 43.96 ± 11.06 years.

Table 2 shows the variations of HbA_{1c}, BMI, WHR, WC and frequency of known diabetics (type II DM) among the various grades of NAFLD. We found that 40% of individuals with type II DM had grade III steatosis, while 23.7% and 10.9% were among grades II and I respectively. Only 7.7% of diabetic people within the study sample did not have FLD.

Odds for HbA_{1c} were significantly high [cOR=4.12 (CI: 2.72-6.25)] and were consistently high after adjusting with history of type II DM and the indices of body measurements [BMI, WHR and WC of 3.40 (CI: 2.19-5.26); in males, 2.08 (CI: 1.06- 4.11) and in females, 5.20 (CI: 2.79-9.68)] (Table 3). BMI was significantly associated with NAFLD; however, after adjustment with type II DM and HbA_{1c}, the odds of BMI were found to retain significance in obese individuals only. Further, after stratification of data on the basis of gender, it became insignificant in males. In both genders, WHR was found to be not significant. Odds for HbA_{1c} and WC were found to consistently be significant in the total study sample as well as in both genders (Table 3).

ROC curve analysis demonstrated a valid positive prediction value for HbA_{1c} in comparison with WC, and HbA_{1c} for a binary outcome (NAFLD) (Fig. 1A, 1B) in both genders. The area under the curve (AUC) was 76% for HbA_{1c} in the overall study population (0.76, CI: 0.715-0.809), being 70.6% for males (0.706, CI: 0.629-0.783) and 80% for females (0.80, CI: 0.741-0.858).

Discussion

Baseline characteristics of the study population are given in Table 1. Age- and gender-matched controls exhibited more than 5.7% HbA_{1c} in 66% and 32% of controls and cases respectively. The other significant presentation was a higher BMI in more than 80% and 70% of controls and cases respectively, which is consistent with others reports.^{22,23} Generally, BMI and central obesity are higher in Asian populations.²⁴ We also confirmed a female preponderance (89.4

Table 1. Baseline characteristics among cases and controls, n=450

Variables		Cases	Controls	p-value
Age, years	Mean ± SD	44.68 ± 10.62	43.61 ± 11.27	0.333
Gender, n (%)				0.378
	Male	66 (44.0)	119 (39.7)	
	Female	84 (56.0)	181 (60.3)	
HbA _{1c} , n (%)				<0.001
	≤5.7%	51 (34.0)	204 (68.0)	
	>5.7%	99 (66.0)	96 (32.0)	
Diabetes status, n (%)				<0.001
	Yes	29 (19.3)	23 (7.7)	
	No	121 (80.7)	277 (92.3)	
BMI, n (%)				<0.001
	Underweight/Normal	18 (12.0)	93 (31.0)	
	Overweight	45 (30.0)	103 (34.3)	
	Obese	87 (58.0)	104 (34.7)	
WHR, n (%)				
Male	<0.9	9 (13.6)	47 (39.5)	<0.001
	≥0.9	57 (86.4)	72 (60.5)	
Female	<0.85	16 (19.0)	43 (23.8)	0.391
	≥0.85	68 (81.0)	138 (76.2)	
Waist circumference, n (%)				
Male	<90 cm	7 (10.6)	47 (39.5)	<0.001
	≥90 cm	59 (89.4)	72 (60.5)	
Female	<80 cm	3 (3.6)	32 (17.7)	0.002
	≥80 cm	81 (96.4)	149 (82.3)	

vs. 96.4), with higher WC presenting with NAFLD. Attempts are in progress to develop non-invasive methods to predict NAFLD, one being the assessment of HbA_{1c} to detect hepatic steatosis. A cross-sectional study found a significant association of HbA_{1c} with NAFLD in an elderly Chinese population.²⁵ Similarly, a significant correlation was found between HbA_{1c} and NAFLD in an adult Korean population, again in a cross-sectional setting.²⁶ However, the cross-

sectional design only identifies the prevalence of a factor at a certain point of time. A longitudinal study, on the other hand, suggested that HbA_{1c} may contribute to the development of NAFLD.⁸ Chen *et al.*⁸ further expressed the need for more studies to test the impact of HbA_{1c} on development of NAFLD.

The present study found a significant association of HbA_{1c} with NAFLD in a case control design, which strengthens the

Table 2. Variations in HbA_{1c} and indices of body measurements with severity of steatosis

	No fatty liver	Fatty liver			p-value
		Grade I	Grade II	Grade III	
HbA _{1c} (Mean ± SD)	5.54 ± 0.89	6.21 ± 1.25	6.90 ± 1.85	7.49 ± 2.22	<0.001
BMI (Mean ± SD)	28.38 ± 6.15	29.86 ± 5.92	32.19 ± 5.29	32.36 ± 3.63	<0.001
WHR-Male (Mean ± SD)	0.91 ± 0.11	0.94 ± 0.04	0.96 ± 0.06	0.98 ± 0.09	0.038
WHR-Female (Mean ± SD)	0.90 ± 0.10	0.91 ± 0.07	0.92 ± 0.07	0.95 ± 0.10	0.772
WC-Male (Mean ± SD)	94.16 ± 16.4	101.10 ± 11.0	105.63 ± 14.2	111.2 ± 8.9	<0.001
WC-Female (Mean ± SD)	95.12 ± 14.3	100.38 ± 11.2	101.61 ± 12.2	108.3 ± 6.4	0.003
Diabetes, n (%)					<0.001
Yes	23 (7.7)	7 (10.9)	18 (23.7)	4 (40.0)	
No	277 (92.3)	57 (89.1)	58 (76.3)	6 (60.0)	

Table 3. Associations of HbA_{1c}, BMI, history of type II DM, WHR and WC with NAFLD

Variables	cOR (95% CI)		NAFLD		NAFLD-Male		NAFLD-Female	
	aOR (95% CI)	p-value	aOR (95% CI)	p-value	aOR (95% CI)	p-value	aOR (95% CI)	p-value
HbA_{1c}								
≤ 5.7%	1	1	1	1	1	1	1	1
> 5.7%	4.12 (2.72-6.25)	<0.001	3.40 (2.19-5.26)	<0.001	2.08 (1.06-4.11)	0.033	5.20 (2.79-9.68)	<0.001
Diabetes status								
No	1	1	1	1	1	1	1	1
Yes	2.88 (1.60-5.19)	<0.001	1.64 (0.86-3.090)	0.127	1.08 (0.40-2.86)	0.877	2.21 (0.88-5.52)	0.089
BMI								
Underweight/Normal	1	1	1	1	1	1	1	1
Overweight	2.25 (1.22-4.17)	0.009	1.79 (0.94-3.40)	0.074	1.65 (0.66-4.07)	0.278	1.02 (0.36-2.89)	0.962
Obese	4.32 (2.42-7.71)	<0.001	3.30 (1.80-6.050)	<0.001	1.73 (0.70-4.26)	0.230	2.90 (1.11-7.58)	0.029
WHR								
Male								
WHR <0.9	1	-	-	-	1	-	-	-
WHR ≥0.9	4.13 (1.87-9.13)	<0.001	-	-	2.03 (0.81-5.09)	0.128	-	-
Female								
WHR <0.85	1	-	-	-	-	-	1	-
WHR ≥0.85	1.32 (0.69-2.52)	0.392	-	-	-	-	0.72 (0.32-1.61)	0.432
WC								
Male								
WC <90 cm	1	-	-	-	1	-	-	-
WC ≥90 cm	5.50 (2.31-13.07)	<0.001	-	-	2.91 (1.06-7.94)	0.037	-	-
Female								
WC <80 cm	1	-	-	-	-	-	1	-
WC ≥80 cm	5.79 (1.72-19.52)	0.005	-	-	-	-	4.28 (1.10-16.61)	0.035

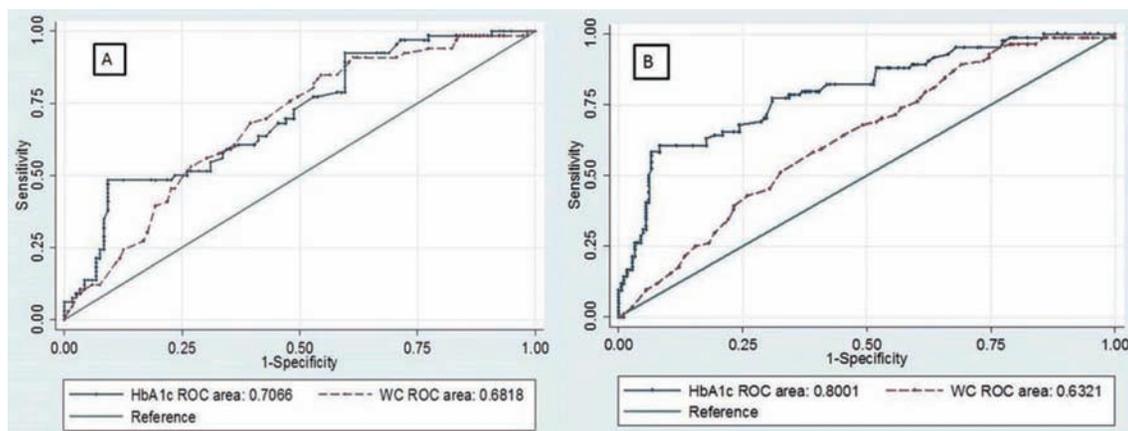


Fig. 1. ROC curve of HbA_{1c} and WC in males (A) and females (B) in relation to NAFLD.

results to be interpreted as a potential predictor of the disease retrospectively. This association was positive both in diabetic, non-diabetic, obese, and lean persons, indicating that those who have HbA_{1c} higher than 5.7% are 4-times more prone to developing fatty liver disease (cOR=4.12, $p<0.001$). Recently, HbA_{1c} has been reported as risk factor for NAFLD, with an odds ratio of 1.58 ($p<0.004$).⁸ Another study also showed that presence of NAFLD presents a higher risk of glycemic progression and incident diabetes.²⁷ Therefore, existing data until now suggests that NAFLD and glycemic derangement coexist, but the conflict remains regarding the cause and effect relationship of HbA_{1c} and NAFLD.

Ultrasonography has recently been largely discussed as a useful bedside diagnostic tool to detect hepatic steatosis.²⁸ The need for non-invasive tools/biomarkers has also become more important, with numerous drug trials underway, some having reached phase IV. Follow-up of these cases is not feasible with multiple biopsies. The ultrasonographic fatty liver indicator is reported to be able to identify mild hepatic steatosis that correlates with histological findings of severity and metabolic parameters.²⁹ In concordance with this, our results also identified ultrasound as a reliable tool to detect hepatic steatosis and its severity. Ballestri *et al.*²⁹ also correlated ultrasonographically -detected steatosis with all parameters of glycemic control, except HbA_{1c}.

The present study confirms that the severity grades of steatosis correlate significantly with HbA_{1c} levels as well. Among the body measurements, BMI and WC showed stronger correlation with severity grades of steatosis than WHR in both genders (Table 2). Others have reported that ultrasonographic techniques have been improved over the last decade, but there is still a dire need to develop a combination of pre-test probability based on anthropometric variables and/or biochemical biomarkers with various ultrasonographic techniques³⁰ which can be applied to the liver biopsy scoring system.³¹ Our results confirm that HbA_{1c} and WC along with ultrasonographic evidence of steatosis can detect early fatty change in hepatocytes satisfactorily (Table 2).

HbA_{1c} is produced in direct proportions to the duration and episodes of high blood glucose concentrations.³² HbA_{1c} may also vary due to biological differences among individuals, apart from hyperglycemic episodes.³³ Hyperglycemic episodes, in addition to the production of advanced glycation end-products also affect lipid metabolism and result in increased synthesis of TAGs that tend to deposit in various tissues of the body, including liver. TAG deposition in adipose tissue increases BMI, while in liver parenchyma it leads to fatty liver. Type II DM has been strongly linked with fatty deposition in liver and HbA_{1c} may be causally associated with NAFLD.³⁴ On the other hand, obesity in the absence

of type II DM also relates to increased fat content in body tissues. Higher BMI has been associated with insulin resistance and increases in HbA_{1c}.³⁵ However, in addition to this, our study confirms increase in HbA_{1c} levels results in more than 3-times chance of NAFLD development independent of diabetes mellitus.

Studies have demonstrated variable results when comparing effect of age, gender, BMI, and obesity. There are opinions that BMI is not a good indicator of chronic disease association, as compared to abdominal fatness³⁶ (central obesity, represented by WC). Excess abnormal fat predisposes to obesity-related disease, regardless of total body fat. The present study found both BMI and WC to be significantly different ($p<0.001$) with presence of NAFLD in both genders (Table 1), while WHR was significantly different only among males. All of these indices were significantly associated with NAFLD (Table 3). However, when it was adjusted for other parameters, this association became weaker, whereas association with WC remained significant both in males and females (aOR 2.91 and 4.28 respectively, $p<0.001$). This indicates that abdominal obesity is more associated with presence of NAFLD. Even the patients who are lean develop fatty liver if they have central obesity.³⁷ Both of these conditions are associated with insulin resistance and, hence, high HbA_{1c} may be a common link between NAFLD and type II DM/central obesity.

The results of this study also depicted that, as compared to male patients, females had higher central obesity (Table 1) and NAFLD in concordance to results reported by Dai *et al.*,³⁸ who also found increased measures of BMI and WC in NAFLD patients. The present study demonstrates that NAFLD can be predicted by a combination of HbA_{1c} and WC both in males (AUC=0.706 and 0.681 respectively) and in females (AUC=0.800 and 0.632 respectively). This is in concordance to others who claimed that a combination of age, sex, WC, alanine aminotransferase, HbA_{1c}, and HOMA-IR with an AUC of 0.87 can best predict NAFLD.³⁹ With these data, it is tempting to suggest that investigation of HbA_{1c} and central obesity may predict the presence of NAFLD in otherwise healthy individuals.

Conclusions

HbA_{1c} level is significantly associated with presence of NAFLD. Higher than normal HbA_{1c} levels possess greater than 70% potential to predict NAFLD. WC is the second most associated factor with NAFLD. HbA_{1c} is the single risk factor that is strongly associated with NAFLD after adjust-

ment for BMI, WHR and WC. HbA_{1c} may be presented as a novel potential biomarker for NAFLD examined with WC in the adult population.

Limitations

Liver biopsy was not performed owing to its invasive nature, with no justification for the test in controls. Secondly, ultrasonography of liver may not identify cases of NAFLD with early changes; therefore, some of the potential cases may have been grouped as controls.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Conception and design of the study (MM, ZH), recruitment and examination of the patients (MM), collection of the data (MM), performance of the assays (MM), analysis of the data (MM, ZH), and writing of the paper (MM, ZH).

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A Deep Look into the Program of Rapid Tumor Growth of Hepatocellular Carcinoma

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Abstract

Background and Aims: Great efforts have been made towards increasing our understanding of the pathogenesis involved in hepatocellular carcinoma (HCC), but the rapid growth inherent to such tumor development remains to be explored. **Methods:** We identified distinct gene coexpression modes upon liver tumor growth using weighted gene coexpression network analysis. Modeling of tumor growth as signaling activity was employed to understand the main cascades responsible for the growth. Hub genes in the modules were determined, examined *in vitro*, and further assembled into the growth signature. **Results:** We revealed modules related to the different growth states in HCC, especially the fastest growth module, which is preserved among different HCC cohorts. Moreover, signaling flux in the cell cycle pathway was found to act as a driving force for rapid growth. Twenty hub genes in the module were identified and assembled into the growth signature, and two genes (*NCAPH*, and *RAD54L*) were tested for their growth potential *in vitro*. Genetic alteration of the growth signature affected the global gene expression. The activity of the signature was associated with tumor metabolism and immunity in HCC. Finally, the prognosis effect of the growth signature was reproduced in nine cancers. **Conclusions:** These results collectively demonstrate the molecule organization of rapid tumor growth in HCC, which is a highly synergistic process, with implications for the future management of patients.

Keywords: Hepatocellular carcinoma; Tumor growth; Coexpression network; Cell cycle; Metabolism.

Abbreviations: CNV, copy number variation; GEO, Gene Expression Omnibus; GS, gene significance; KEGG, Kyoto Encyclopedia of Genes and Genomes; LIHC, liver hepatocellular carcinoma; ME, module eigengene; MM, module membership; NES, normalized enrichment score; OS, overall survival; PCA, principal components analysis; SCNA, somatic copy-number alternations; sh, short hairpin; ssGSEA, single-sample gene-set enrichment analysis; TCGA, The Cancer Genome Atlas; TMB, tumor mutational burden; WGCNA, weight gene coexpression network analysis.

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Introduction

Hepatocellular carcinoma (HCC) represents a leading cause of cancer-related death worldwide.¹ Currently, though several staging systems can stratify the HCC patients into appropriate risk categories, a great deal of divergence remains within each risk group, due to the molecular heterogeneity of tumor cells and microenvironment. One way to describe the progression of HCC is tumor growth, since it might hold for the long-held view that rapidly growing tumors are more likely to metastasize and become lethal than slow-growing tumors;² although, the fundamental question regarding the ability of tumor cells to rapidly grow remains to be answered.

In fact, tumor cells adapt to changing environmental conditions and profoundly shape the dependencies of individual cells. For instance, through aerobic glycolysis, cancer cells produce energy by taking up glucose at much higher rates than other cells, while, at the same time, using a smaller fraction of the glucose for energy production. This allows cancer cells to function more like fetal cells, promoting extremely rapid growth.³ However, the underlying molecular basis of the intertwined interactions among tumor immunology, oncogenic signaling, and tissue/biochemical context, upon tumor growth remains largely unknown.⁴

Currently, a robust gene coexpression network for the mining of hub genes that drive pivotal signaling pathways in terms of large-scale gene expression profiles can be built through weighted gene coexpression network analysis (WGCNA).³ Previous studies have applied WGCNA to provide functional explanations of systems biology, proposing candidate therapeutic targets or diagnostic biomarkers for cancer in recent years.⁵ For example, Zhao *et al.*⁶ utilized WGCNA to investigate the relationships underlying the molecular and clinical characteristics of cholangiocarcinoma. However, a robust WGCNA network for cancer growth has not yet been established.

In the present study, the HCC transcriptome and tumor growth-related modules were explored by WGCNA. Focusing on rapid tumor growth, the integrative functional analy-

Table 1. Hub genes in the HCC rapid growth module

Top10 GS	GS.Fastest	p.GS.Fastest	Top10 K.in	K.in	Top10 MM	MM.green	p.MM.green
<i>CCNA2</i>	0.5795	7.97E-16	<i>CDCA5</i>	51.7516	<i>CCNB1</i>	0.9639	2.53E-93
<i>NDC80</i>	0.5733	1.89E-15	<i>CCNB1</i>	50.1223	<i>CDCA5</i>	0.9611	8.49E-91
<i>CDCA8</i>	0.5700	2.99E-15	<i>PRC1</i>	50.1064	<i>PRC1</i>	0.9503	1.69E-82
<i>CENPE</i>	0.5580	1.48E-14	<i>CDK1</i>	48.4094	<i>CDK1</i>	0.9492	9.73E-82
<i>KIF11</i>	0.5576	1.55E-14	<i>EXO1</i>	47.6809	<i>EXO1</i>	0.9478	7.54E-81
<i>H2AFX</i>	0.5571	1.66E-14	<i>BIRC5</i>	46.9120	<i>DLGAP5</i>	0.9431	6.61E-78
<i>RAD54L</i>	0.5534	2.69E-14	<i>DLGAP5</i>	46.5064	<i>CENPF</i>	0.9404	2.24E-76
<i>NCAPH</i>	0.5520	3.20E-14	<i>CENPF</i>	44.2346	<i>BIRC5</i>	0.9354	1.11E-73
<i>RFC4</i>	0.5495	4.42E-14	<i>PTTG1</i>	44.0655	<i>PTTG1</i>	0.9352	1.32E-73
<i>HJURP</i>	0.5491	4.61E-14	<i>CDCA8</i>	43.8984	<i>NUF2</i>	0.9350	1.83E-73

sis was expanded to the levels of the growth signature, associated molecular events, and corresponding modulations.

Methods

Data preparation

The transcription profile of HCC was downloaded from the Gene Expression Omnibus (commonly referred to as GEO) with accession number GSE54236, which includes 78 primary HCC tumor samples representing the different speeds of tumor growth.⁷ Briefly, patients underwent two computed tomography scans 6 weeks apart to determine tumor volumes and HCC doubling time, which ranged from 30 to 621 days and were divided into the following quartiles: ≤53 days ($n=19$), 54–82 days ($n=20$), 83–110 days ($n=20$), and ≥111 days ($n=19$). Based on these quartiles, tumor growth was classified into slow, fast, faster, and fastest states, respectively. Low and non-expressed genes were removed by selecting probes with a mean expression in the top 50% of all probes. Next, genes with expression variance above average level were selected. Different probes targeting the same gene were collapsed. These steps finally resulted in 5511 genes to infer coexpression networks.

In addition, GSE14520, GSE25097, GSE62232, GSE36376 datasets were obtained from the GEO database. RNA-seq expression profiles from nine cancer types, including adrenocortical carcinoma, kidney renal clear cell carcinoma, kidney renal papillary cell carcinoma, brain lower grade glioma, liver hepatocellular carcinoma (LIHC), lung adenocarcinoma, mesothelioma, pancreatic adenocarcinoma, and sarcoma, were obtained from The Cancer Genome Atlas (commonly known as TCGA) database. Detailed information about the datasets is shown in Supplementary Table 1.

Coexpression network construction

We constructed the coexpression network using the WGCNA package.⁸ Briefly, the steps included: (1) defining the similarity matrix; (2) choosing the soft threshold, β , and inferring the adjacency matrix; (3) defining the topological overlap matrix; (4) performing hierarchical clustering; (5) performing the dynamic tree cut method to identify the modules; and (6) computing the module eigengene (ME) of each module. The ME can be considered as a representative of the gene expression profiles in a module. The average-linkage hierarchical clustering method was employed

to cluster the MEs of all modules, and the modules with high similarity were merged to obtain the coexpression network.⁹ Another tool, the CEMITool package, was used to validate the gene modules, as described previously.¹⁰

The module preservation statistic Zsummary was used to assess the overlap between network modules,⁸ which takes into account the overlap in module membership (MM), the density (mean connectivity) and connectivity (sum of connections) patterns of modules. A module was considered not being preserved if $Zsummary < 2$, moderately preserved if $2 \leq Zsummary < 10$, and highly preserved if $Zsummary \geq 10$.

Identification of hub genes and growth signature

Hub genes (genes that are highly interconnected with the nodes of the module) are of functional importance. MM was defined as the correlation between the ME and gene expression values. The MM measure is highly related to intramodular connectivity (K.in).⁸ Highly connected intramodular hub genes tend to have high MM values to the respective module. In short, the larger the MM value of the gene, the higher the correlation between the gene and a given module. In addition, the gene significance (GS) was defined as mediated p -value of each gene ($GS = \lg P$) in the linear regression between gene expression and the clinical traits.⁵

We used the network screening function based on GS (representing the correlation between the gene and a given clinical trait) and MM or K.in (representing the correlation between the gene and a given module) in the WGCNA package to directly identify the top hub genes in the fastest growth module, and further assembled them into the growth signature. The growth activity was quantified by applying the single-sample gene-set enrichment analysis (ssGSEA).⁶ We defined the growth signature as either high or low by using median cut-off.

Functional annotation

Functional annotations of the gene sets were performed using webgestalt¹¹ or Enrichr.¹²

Pathway activity computation

Using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways as templates for signal simulation, a ca-

nonical circuit was defined as any possible route the signal can traverse to be transmitted from a particular input to a specific output node.¹³ The computation of the signal intensity across the different circuits of the entire pathways was performed by the Hipathia program.¹³ In addition, the growth signature associated cancer pathway activity in pancreatic cancer was performed using GSCALite.¹⁴

Modeling genetic-gene expression

Multi-layered profiles for DNA copy numbers, mRNA expression, and mutations in the LIHC data were obtained from the Xena portal (<http://xena.ucsc.edu/>). A linear modeling approach that measured the association of expression levels was used on a gene-by-gene basis with a number of potential predictors, including gene mutations or genomic alterations, as previously described.¹⁵ Somatically acquired mutations and genomic alterations were presented by using Maftools¹⁶ and encoded as being present/absent. Linear expression models were fit with the Limma package.¹⁷ For the expression of gene *k* in patient *i*, Y_{ik} is fitted by the following equation:

$$Y_{ik} = \sum_{j=1} X_{ij} \beta_{jk} + \beta_{0k} + \varepsilon$$

X_{ij} is the mutation matrix for patient *i* and mutation *j*, with entries $X_{ij}=1$ denoting patient *i* has a mutation *j* and 0 otherwise. The coefficients β_{jk} measure the expression change in gene *k* induced by the presence of a mutation *j*. The entry β_{0k} implies the baseline expression level of gene *k*.¹⁵

Immune and metabolism signatures

For each tumor sample, ESTIMATE was used to assess tumor purity.¹⁸ The cytolytic activity or the interferon score of the local immune infiltrate was calculated as previously described.¹⁹ Gene signatures of 28 tumor-infiltrating lymphocytes, including CD8 or CD4 T cells, B cells, natural killer cells, as well as markers from multiple types of oncoimmunology-containing genes were referenced in a prior study.²⁰ The ssGSEA method was utilized to quantify the enrichment levels of metabolism-related signatures based on Reactome (<https://reactome.org>) gene-sets.

Cellular experiments

The human liver cancer cell line HepG2 was purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM (Gibco, Life Technologies, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco), and used within 20 passages after culture.

Lentivirus production was performed as previously described.²¹ The targeting sequences of short hairpin (sh)RNAs were as follows: 5'-CACCGAACCAACCACTTTAA-3' for sh-NCAPH#1, 5'-ACTGACTCACCTCGCTTATTG-3' for sh-NCAPH#2; and 5'-CTTTGTAATCATCCAGCTCTA-3' for sh-RAD54L#1, 5'-TGGTCTGGGTGACTCTTAG-3' for sh-RAD54L#2. Knock-down efficiency was verified by quantitative PCR. The Cell Counting Kit-8 (Dojindo Molecular Technologies, Kumamoto, Japan) assays were used to measure cell proliferation.

Statistical analysis

Experimental data are represented as the average \pm stand-

ard deviation. Unless otherwise indicated, the Student's two-tailed unpaired *t*-test was used to determine statistical significance. The significance threshold was set at 0.05. For survival analysis, the LIHC data were analyzed with the GEPIA database.²² Samples were split into high and low groups based on the median value. Kaplan-Meier survival analysis was calculated using the log rank test, with a *p* value for significance of <0.05.

Results

Rapid growth module in HCC

In defining the gene clusters involved in HCC growth, 11 distinct gene modules were explored using WGCNA, as shown in Fig. 1A.

Module stability was verified by repeating network construction and module identification on expression data that consists of resampled sets of the original dataset or alternatively by another tool (the CEMITool) running the same dataset¹⁰ (Supplementary Fig. 1). The results proved the robustness of module assignments.

Next, we evaluated the relationship between each module and the growth status. Notably, the cirrhotic features of modules (red, blue, yellow, brown) and the aggressive proliferative HCC features of modules (magenta and green for the faster and fastest state respectively) were identified (Fig. 1B and C). Network features such as GS, MM and K.in²³ of each module in different growth states were computed (Supplementary Table 2).

Moreover, we narrowed down our analyses to the fastest HCC growth. Module green (referred to as the growth module hereafter) was the best, as reflected by its strongly positive correlations to the fastest tumor growth (Fig. 1B–D).

Signal fluxes in the rapid growth module

To ask the question of whether the growth module was highly preserved across independent HCC datasets, external validation was performed. Using five different HCC cohorts, the growth module showed a higher preservation statistics summary than expected by random chance using bootstrapping procedures (Fig. 2A). Thus, the growth module was deemed to hold promise in independent tumor profiles from different patient cohorts. For function enrichment, the cut-off set with the false discover rate of <0.01, cell cycle, DNA replication, Fanconi anemia, *etc.* constituted the main KEGG pathways in the growth module (Fig. 2B).

To decompose the KEGG pathway into detail, a canonical circuit was defined as any possible route the signal can traverse to be transmitted from a particular input to a specific output node.¹³ Effector nodes at the end of the circuits trigger specific functions in the cell. Using gene expressions as proxies of node activation values, computation of the signal intensity across the different circuits of the pathways was performed by canonical circuit activity analysis to compute the transmission of the signal along the network.¹³ Thus, we estimated the level of activity of subpathways (signaling circuits) using the Hipathia program,¹³ and detected several pathways with perturbed activity in the growth module (Supplementary Fig. 2).

Focusing on the cell cycle pathway, five effector circuits were deemed ultimately responsible for the functions of DNA replication and cell cycling. These circuits were highlighted in the fastest state, one of them ending in the node including *RB1*, one including *RAD21*, one containing *TFDP1* and *E2F4*, one ending in the node with protein genes for

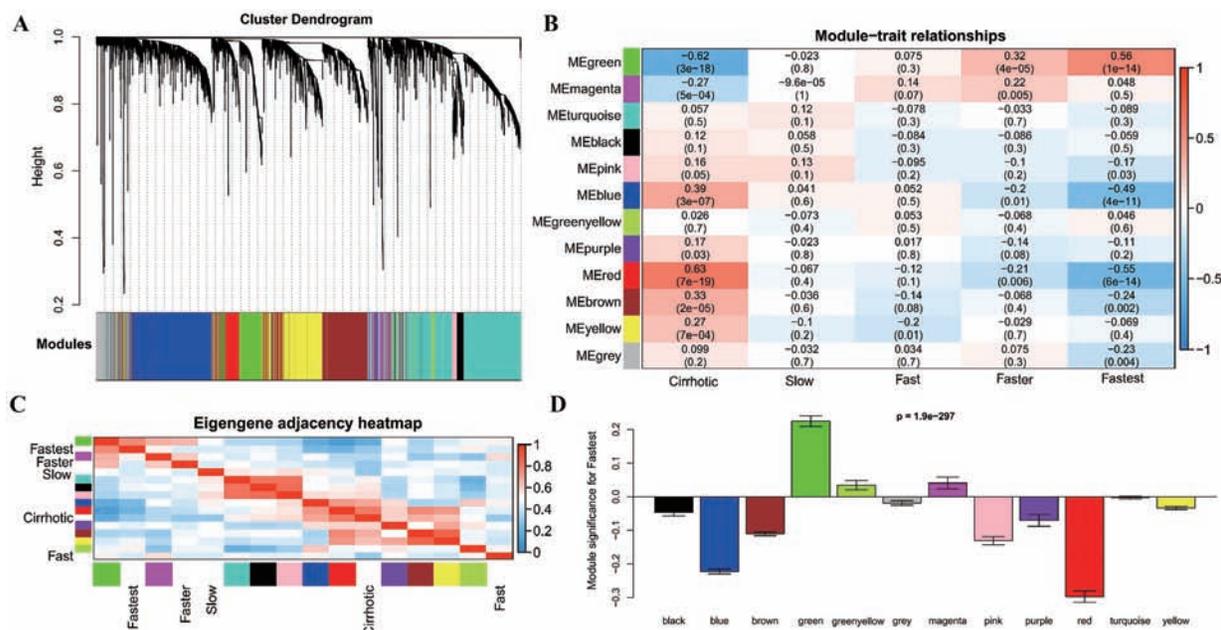


Fig. 1. Growth-related modules in HCC. (A) Clustering dendrograms of all genes, with dissimilarity based on topological overlap, together with assigned module colors. (B) Correlation between module eigengene and tumor growth state of HCC. (C) Heatmap representation of the module-module relationship. (D) Module significance of each module in the fastest growth state of HCC. The higher the mean GS in a module is, the more significantly associated with tumor growth the module will be.

CDC45, *MCM7*, *MCM6*, *MCM5*, *MCM4*, *MCM3* and *MCM2*, and the last one ending in the node with protein genes for *ORC* and *MCM* (Fig. 2C). Indeed, most nodes in the effector circuits have adverse outcome in the LIHC cohort (Supplementary Fig. 3). These results clearly suggested the signaling in the green module as providing multiple routes and broader activity to promote cell cycle progression, thus accelerating tumor growth.

Rapid growth program in HCC

Next, we examined the hub genes in the growth module. Hub genes,⁵ including the top 10 GS, K.in or MM genes, are shown in Fig. 3A and Table 1. Among them, 20 genes were identified.

There was step-wise activation of all these genes that accompanied increased speed of tumor growth (Fig. 3A and Supplementary Fig. 4). The trend was clearly consistent and coordinated. As expected, all these hub genes were involved in the advanced prognosis of HCC, as evidenced by the results from our survival analysis (Supplementary Fig. 4).

Since the top 20 hub genes were densely interacted by protein-protein interaction analysis,²⁴ we categorized them among the rapid growth signature (Fig. 3B) and applied the ssGSEA algorithm to infer the growth activity for each sample.

Recent advances in molecular biomarkers of HCC have indicated various oncogenes and tumor suppressor genes.²⁵ Indeed, growth^{high} patients in our study were more likely to show higher expression of many known adverse prognostic biomarkers, such as *AFP*, *DCP1A*, *GPC3*, *MDK*, *MCM6*. In contrast, growth^{low} patients were likely to show higher expression of tumor suppressor genes, such as *GPR155* and *IFIT3* (Fig. 3C).

Furthermore, we found that rapid growth activity has a bad survival prognosis, both in LIHC and GSE14520 cohorts (Fig. 3D). These findings suggested a negative regulation relationship between the HCC growth program and HCC

prognosis.

Next, we reasoned that genes in the signature would have a higher degree of association with cell proliferation. To test this hypothesis, we selected genes (*NCAPH* and *RAD54L*) for experimental validation. As expected, knockdown of *NCAPH* or *RAD54L* significantly suppressed the proliferation of HepG2 cells (Fig. 3E).

Somatic mutations and copy number alterations of the growth signature

We next investigated the growth signature at the genomic level. By focusing on the somatic non-silent mutation or copy number variation (commonly known as CNV) genes (Fig. 4A), the principal components analysis (commonly known as PCA) was computed to maximize the stability of the components. The first two principal components, respectively, account for 13.6% and 11.2% of the total variability in gene expression; the first 20 principal components cumulatively explain 67% of the variance (Fig. 4B). Notably, overlaying the status of the genetic alterations of hub genes (growth^{mut}) on to the first two principal components demonstrated that mutations or CNV alterations correlated with general gene expression profiles (Fig. 4C).

The transcriptome was globally perturbed by growth^{mut}, with expression levels of 6,565/15,569 (42%) genes significantly associated with at least one genetic change of the hub genes ($r > 0.3$, false discovery rate of < 0.001). For instance, genetic change of *NUF2* co-occurred with other hub genes' alteration, which led to 1069 genes' differential expression (Fig. 4D–E). The observed variability can be largely explained by the presence of other hub genes' alteration leading to strong up-regulation of *NUF2* mRNA (Fig. 4D). The expression changes of the growth^{mut}-related genes are summarized in Supplementary Table 3.

To understand the mechanism underlying growth^{mut}, webgestalt¹¹ analysis showed that growth^{mut}-related genes were enriched in the KEGG pathways of cell cycle, oocyte

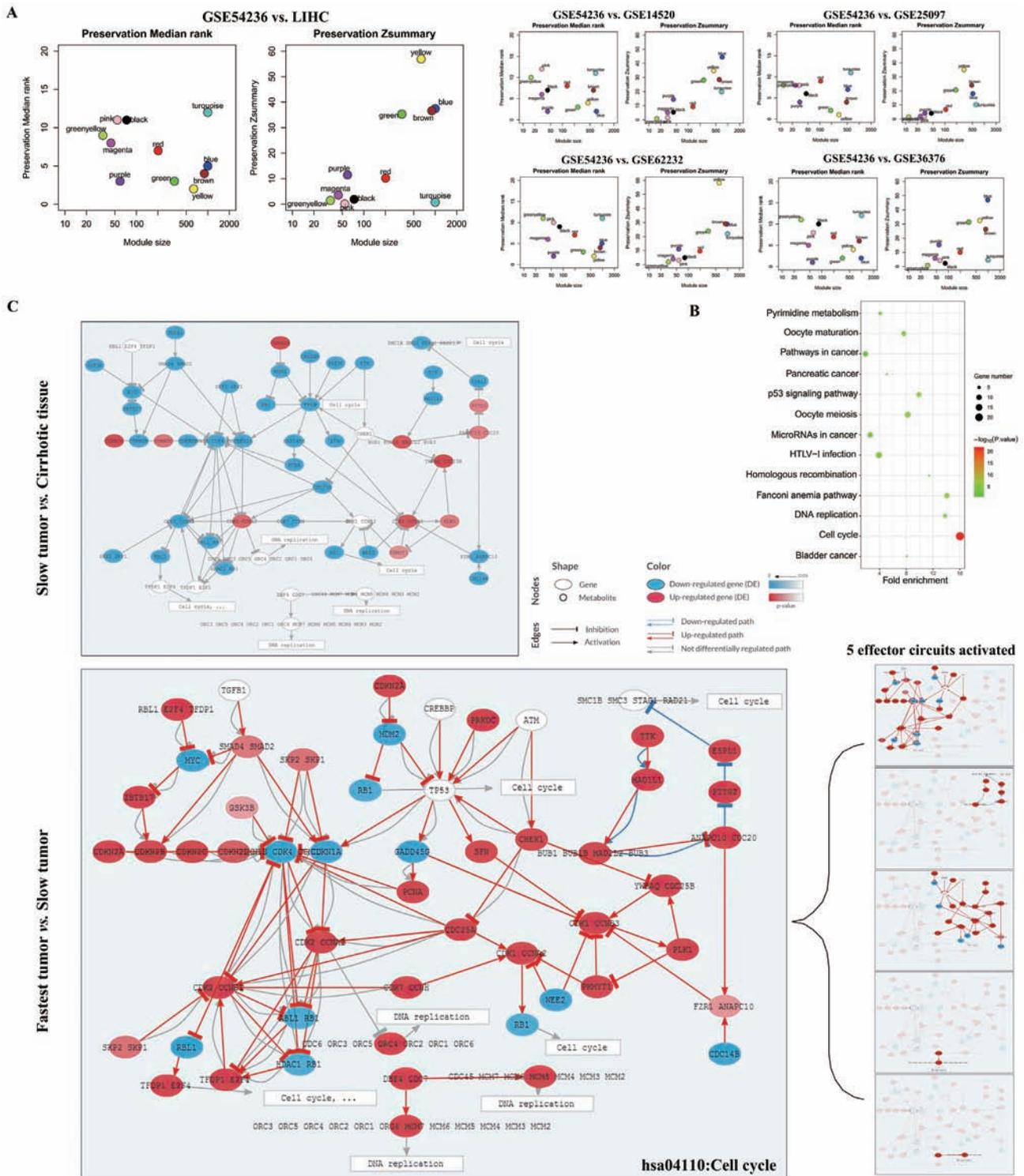


Fig. 2. Signaling events in the rapid growth module. (A) Preservation of growth modules between different datasets. Each module is represented by the color-code and label. The left panel shows the composite statistic of preservation median rank. High median ranks indicated low preservation. The right panel shows preservation of the Zsummary statistic. (B) Enrichment of KEGG pathways in the green module. (C) The cell cycle pathway (hsa04110). The upper panel indicates the comparison between slow tumors and the adjacent cirrhotic tissues. The lower panel compares the fastest tumors with the slow tumors. Genes in red represent genes that were up-regulated, and blue represents genes that were down-regulated. The right panel highlights five effector circuits for the function output.

meiosis and DNA replication, *etc.* (Fig. 4G). Given that oncogenic signaling accelerates cell cycle progression,²⁶ these

data indicated that growth^{mut} cancers can amplify growth signaling to maintain cell proliferation.

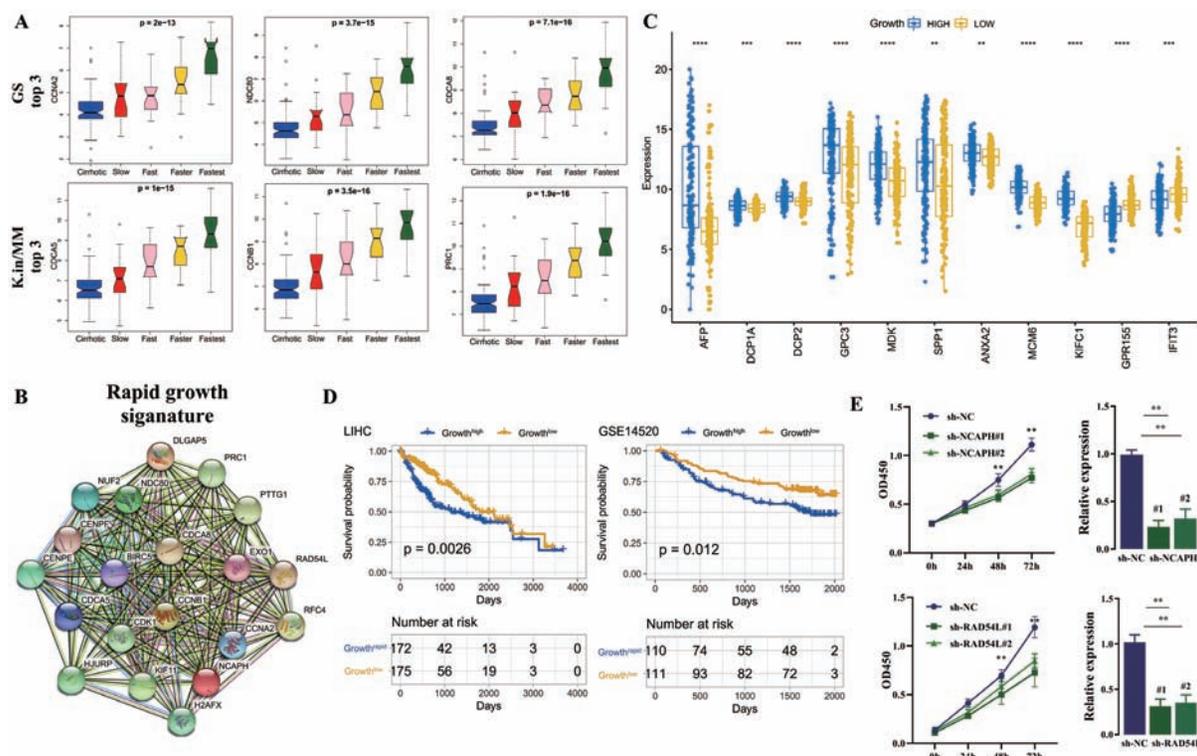


Fig. 3. Hub genes in the growth module. (A) Expression level of top three hub genes from cirrhotic, slow to the fastest growth state in HCC. (B) Highly interconnected top 20 hub genes constitute the rapid growth signature. (C) Expression of well-known biomarkers of HCC between the growth^{high} and growth^{low} group. The *p* values between the two groups were calculated by the Wilcoxon rank sum test. ***p*<0.01. (D) Kaplan-Meier survival plot of growth^{high} vs. growth^{low} in the LIHC cohort or GSE14520 cohort respectively. (E) Knockdown of *NCAPH* or *RAD54L* suppressed HepG2 cells proliferation. The right panel denotes knockdown efficiency. Student's *t* test was used to compare the differences. **p*<0.05, ***p*<0.01.

Rapid growth is associated with tumor immune-metabolism

We then explored the correlation between growth activity and tumor-infiltrating lymphocytes (Fig. 5A). We observed a significant negative correlation of growth signature with Th1 cell types and positive correlation with Th2 cell types. Next, significant negative correlations between rapid growth and natural killer cells, plasmacytoid dendritic cells or macrophages were found (Fig. 5A and Supplementary Table 4).

Moreover, dysregulation of diverse immune signatures in HCC, including HLA expression, cytokines or chemokines, and interferon response were identified between growth^{high} and growth^{low} groups (Fig. 5C). Notably, the growth signature showed strong correlation with neoantigens (*r*=0.37, *p*=1.52E-12 in LIHC; *r*=0.38, *p*=5.22E-09 in GSE14520) (Fig. 5B). In addition, tumor rapid growth showed no significant correlation with immunoinhibitors or immunostimulators, including well-known checkpoint genes (Table S4). Additionally, immune cytolytic activity or tumor burden was not significantly different between high versus low rapid growth tumors (Supplementary Fig. 5). Thus, the growth signature itself might not elicit the active immune response.

Next, we investigated the metabolic configuration according to the growth activity. Using curated metabolic gene sets from Reactome as indicators, we found rapid tumor growth was highly associated with diverse metabolism processes. For example, the rapid tumor growth was significantly positively correlated with synthesis of DNA (*r*=0.73, *p*=1.37E-58 in LIHC; *r*=0.85, *p*=2.52E-64 in GSE14520) and negatively correlated with bile acid metabolism (*r*=-0.43,

p=1.70E-17 in LIHC; *r*=-0.46, *p*=1.92E-13 in GSE14520). Detailed growth-metabolism correlations are provided in Supplementary Table 5.

The representative metabolic activity between growth^{high} and growth^{low} tumors is provided in Fig. 5D. For instance, dysregulation of cytochrome P450, xenobiotics, and biological oxidation are shown to be associated with poor prognosis. These results suggested that growth^{high} tumors tend to present an immunetolerant and metabolism reconfigured microenvironment in HCC.

Prognostic role of growth signature in pan-cancer

When extending the growth signature to the pan-cancers, we found a significant hazard ratio between the growth genes and overall survival or recurrence-free survival in multiple cancers, including adrenocortical carcinoma, kidney renal clear cell carcinoma, kidney renal papillary cell carcinoma, brain lower grade glioma, LIHC, lung adenocarcinoma, mesothelioma, pancreatic adenocarcinoma, and sarcoma (Fig. 6A). Gene expression was significantly higher in the growth^{high} group (Fig. 6D) and the growth^{high} indicated advanced prognosis in these cancers (Fig. 6B–C). The pathway relation network also indicated that the signature was mostly involved in the cell cycle in nine cancers (Fig. 6E).

To investigate the clinical implications of the growth signature, we searched for targets of candidate drugs by using the L1000 project.²⁷ The top 10 associations are presented in Fig. 6F. Palbociclib functions as a *CDK4/6* inhibitor in multiple cancers.²⁸ It also acts as a novel radiosensitizer,

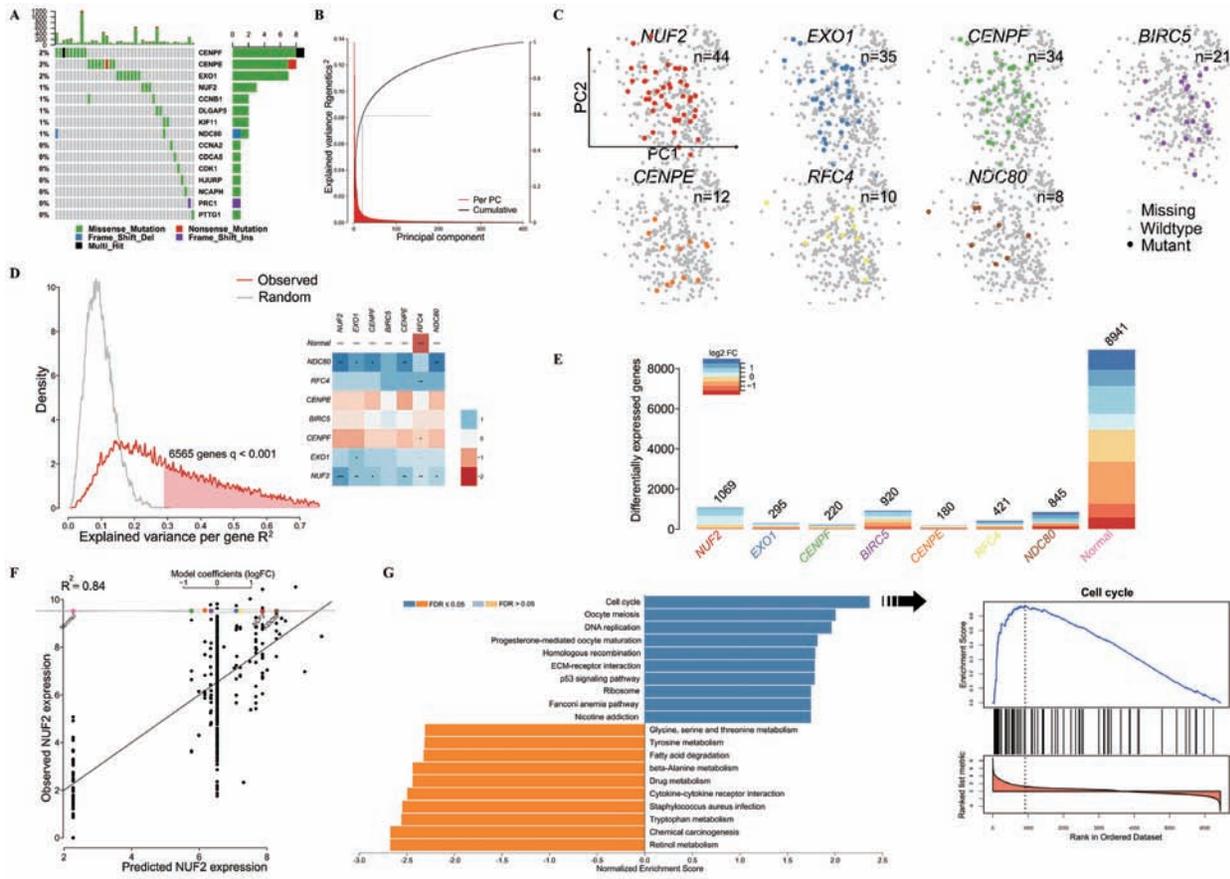


Fig. 4. Genetic change of the growth signature correlates with differential expression. (A) Overview of the somatic non-silent mutation and CNV of the growth signature using Maftools. Samples are displayed in columns, and different colors indicate the different types of somatic mutations. The bar plots show the recalibrated mutation frequencies. (B) Distribution of the variance explained by the genetic alterations across genes. (C) Scatter plot of the first two principal components of the gene expression data overlaid with mutation status. (D) Distribution of the variance explained by genetic alterations across genes. The right panel indicated the heatmap of observed pairwise mutation patterns (odds ratio). Blue color denotes preferential co-mutation/high overlap, while red color indicates mutual exclusivity. (E) Number of differentially expressed genes for each hub gene. (F) Scatter plot of expression predictions for the *NUF2* gene vs. observed expression values. The inset shows the model coefficients, indicating the predicted magnitudes of expression changes when a given alteration is present. (G) Bar plot showing the GSEA enriched KEGG pathways of growth^{mut}-affected genes. Enrichment is represented as $-\log_{10}(p \text{ value})$.

dovitinib, inducing apoptosis and overcoming sorafenib resistance in HCC through *SHP-1*-mediated inhibition of *STAT3*.²⁹⁻³² As the topoisomerase inhibitor, mitoxantrone, was widely used in clinic.³³ Canertinib is an irreversible *EGFR* inhibitor in cancers.³⁴

Discussion

The theory that patients with a rapidly growing cancer have a poor prognostic outlook has remained persistent;² thus, detailing the biological structure in the hidden layer of rapid growth is an interesting question.

As illustrated in this study, to understand the growth rate of HCC at the modular level and toward uncovering the critical drivers of the disease in a comprehensive manner, HCC transcriptomes were explored in the context of modular pattern, in which the green module was responsible for rapid tumor growth after internal and external validation. Briefly, the top enriched functional classes of this program are consistent with our existing knowledge of the cell cycle, as well as DNA repair, replication and cell proliferation in cancer.

Due to the presence of the highly interconnected top 20

hub genes, we assembled them into the growth signature. At the gene level, we demonstrated that the expression levels of all these genes were increased in coordination with the state of growth-rate. Accordingly, high expression of these genes predicted the adverse outcome of HCC. Indeed, various lines of evidence showed the involvement of these hub genes in previous studies. For example, *CCNA2* is the leading gene according to the GS rank. A recent study revealed a new poor-prognosis HCC entity and a rearrangement signature related to replication stress, due to *CCNA2* alterations.³⁵ The top K.in gene, *CDCA5*, transcribed by *E2F1*, promotes oncogenesis by enhancing cell proliferation and inhibiting apoptosis via the AKT pathway in HCC.³⁶ The top MM gene, *CCNB1* was highly expressed in the samples of recurrent HCC, which was associated with significantly reduced recurrence-free survival.³⁷ In addition, the vast majority of genes within the signature, such as *RFC4*, *HJURP*, *ECT2*, *KIFC1*, *NUSAP1*, *CDK1*, *PRC1*, *KIF4A*, etc., contribute to the pathogenesis of HCC, as reported previously.³⁸⁻⁴³ At the time of preparation of this paper, little is known about *NCAPH* and *RAD54L* in HCC. In this study, however, we found that knockdown of *NCAPH* and *RAD54L* expression is associated with growth inhibition.

Genetic background and in particular genomic alteration

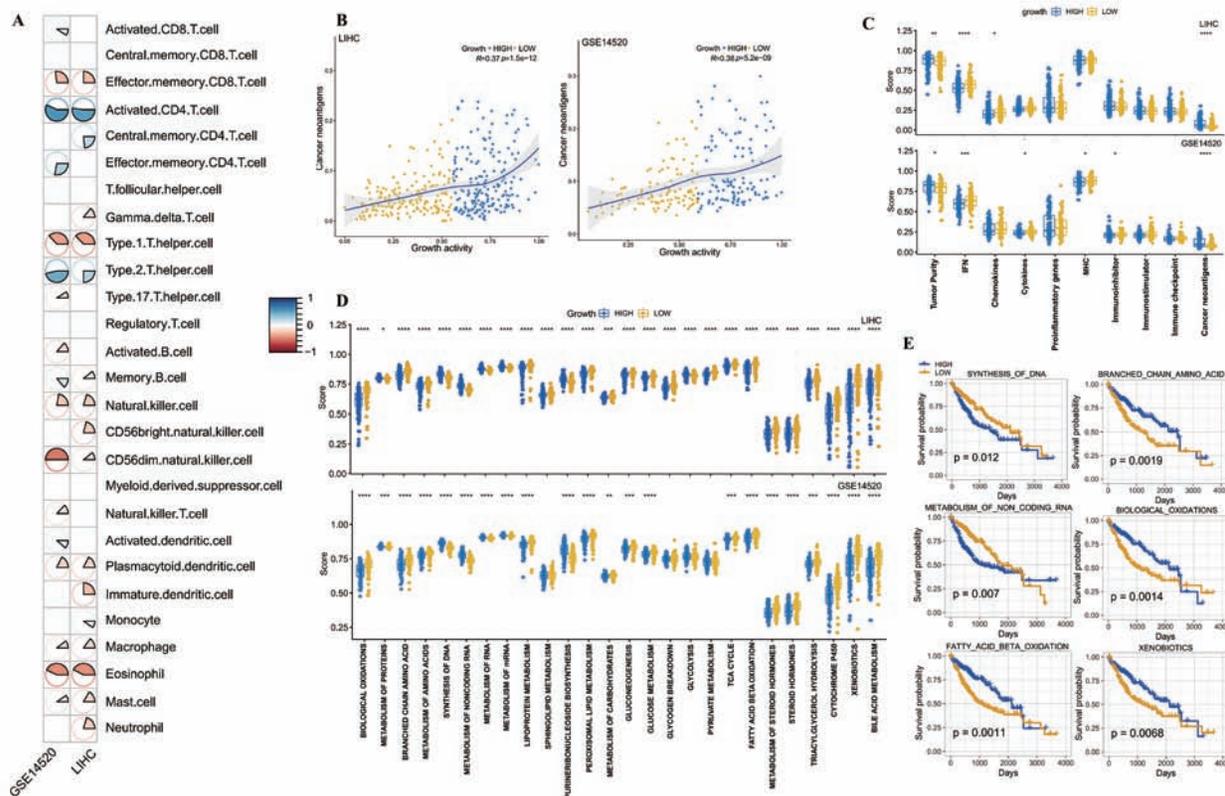


Fig. 5. Tumor rapid growth is associated with immune-metabolism. (A) Correlation between growth activity and tumor-infiltrating lymphocytes' infiltration. (B) Tumor growth was highly correlated with neoantigen production both in the LIHC and GSE14520 cohort. (C) Dysregulation of immune signatures between growth^{high} and growth^{low} groups in LIHC and GSE14520 respectively. (D–E) Metabolic reconfiguration between the growth^{high} and growth^{low} groups in LIHC and GSE14520 respectively, which have prognosis effect.

might contribute to gene expression in HCC related to tumor growth speed. Taking a genetic-centric view, we specified the set of gene expression changes that correlate with the alteration of the signature. The number of target genes whose expression are differentially affected varies widely across the different genetic change of growth signature. For example, *BIRC5*, playing dual roles in mitosis and cell survival of HCC,⁴⁴ was independently correlated with expression levels of 920 genes. These data supported the notion that genetic change of these hub gene results in a rapid tumor growth.

Moreover, the growth signature exhibited a significant correlation with certain genomic features, such as tumor purity and neoantigens. Further, no obvious association between rapid growth and active antitumor immune signatures was found. In addition, a significant negative correlation was observed between growth activity and energy metabolism integration, biological oxidation, vitamins, fatty acid, and glucose metabolism, *etc.* (Supplementary Table 5). The above results supported the idea of a link between tumor growth, metabolic landscape reconfiguration, and clinical progression of cancer. Accordingly, aggressive malignant phenotypes of cancer cells obtained by accumulated mutations change metabolic phenotypes for proliferation represented by nucleotide and amino acid metabolism. Indeed, this hypothesis of highly coordinated growth program warrants further study.

Finally, candidate drugs have been inferred based on the growth signature. Numerous cell cycle inhibitors have been designed over the past decades.⁴⁵ The current findings of growth-specific drugs for HCC would have potential implica-

tions in warranting future studies toward developing targeted and combinatorial therapeutics for HCC.

Conclusions

Given the inherently modular profile of tumor growth, the present study revealed a unifying portrait of the growth signature of HCC, and could be extended to pan-cancer. Next, the study offered information to better define how specific organizations of genes are able to orchestrate rapid growth. Further, the growth signature has potential prognostic and therapeutic intervention value for HCC, lightening the way toward tailoring the targeted therapeutics for HCC.

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Conflict of interest

The authors have no conflict of interests related to this publication.

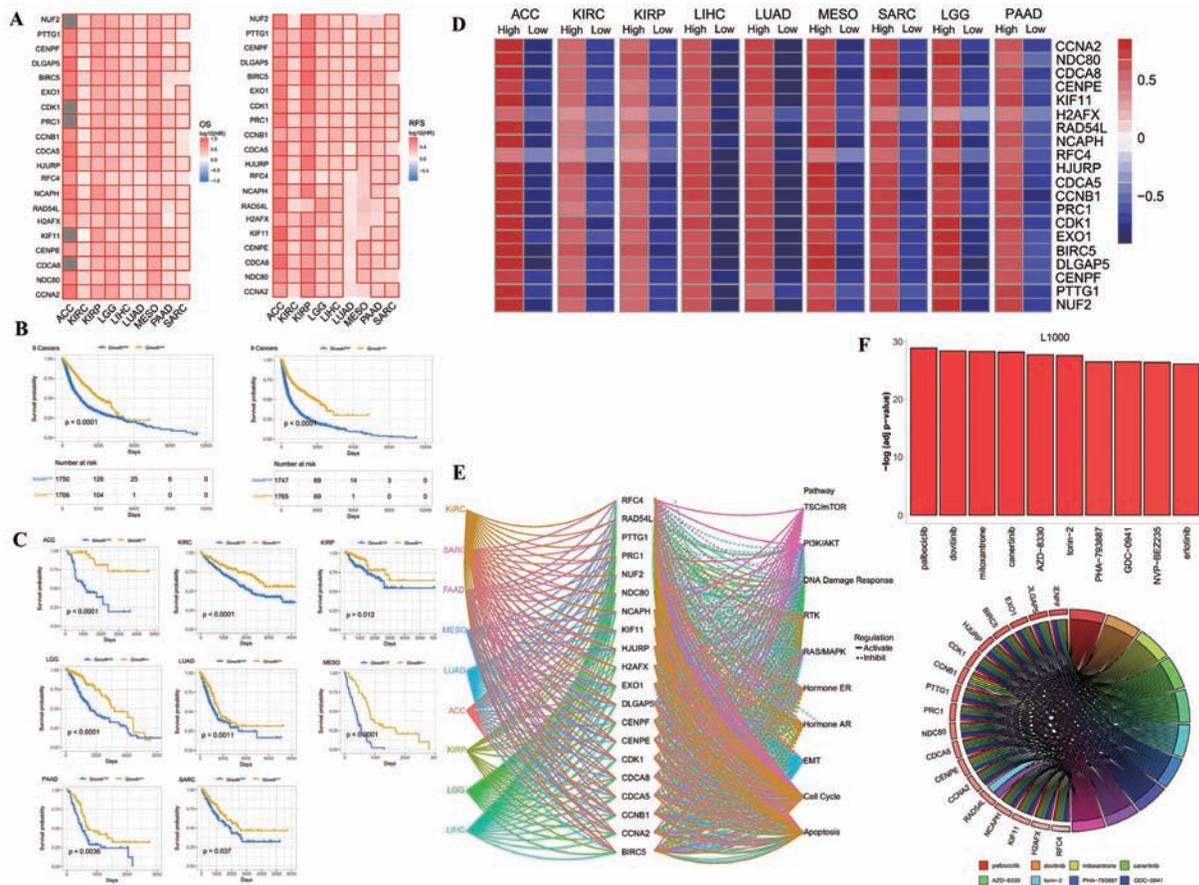


Fig. 6. Growth signature in diverse cancers and harbor clinically actionable genes. (A) Survival map of the hub genes within the signature for overall survival or recurrence-free survival in nine cancer types. (B) Survival plot of growth^{high} vs. growth^{low} in nine cancers, using median cut-off. (C) Survival plot of rapid growth activity in nine cancers. (D) The average mRNA expression in growth^{high} and growth^{low} samples across nine cancers. Red indicates relatively high expression, while blue indicates relatively low expression. (E) The network showing the relationship between genes and pathways in different cancer types. The solid line indicates activation, and the dashed line indicates inhibition. (F) The top 10 drugs (upper panel) enriched for the growth signature by L1000 and their targeted genes (lower panel).

Author contributions

Contributed to study concept and design (XZ and JY), acquisition of the data (JmL and YxL), assay performance and data analysis (JW, YL, AqL, AnC, and JtF), drafting of the manuscript (JW and YL), critical revision of the manuscript (JW and XZ), supervision (JY).

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Bacterial Infections in Cirrhotic Patients in a Tertiary Care Hospital

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Abstract

Background and Aims: Patients with cirrhosis are immunocompromised and at higher risk of developing infections compared to the general population. The aim of this study was to assess the incidence of infections in cirrhotic patients in a large academic liver center and investigate potential associations between infections, bacteria isolated, therapeutic regimens used, and mortality. **Methods:** This was a retrospective chart review study, including 192 patients. All patients had a diagnosis of cirrhosis and were admitted to University Hospital. Information collected included demographics, etiology of cirrhosis, identification of bacteria from cultures, multidrug-resistant (MDR) status, antibiotics administered, intensive care unit (ICU) admission, and patient mortality. **Results:** Infections were present in 105 (54.6%) patients, and 60 (31.2%) patients had multiple infections during a hospitalization(s) for infections. A total of 201 infections were identified. Urinary tract infections (UTIs) were the most common infection (37.8%), followed by bacteremia (20.4%), pneumonia (12.9%), spontaneous bacterial peritonitis (SBP) (11.9%), abscess/cellulitis (6.0%), infectious diarrhea (6.0%), and other (5.0%). *Escherichia coli* was the most common bacteria isolated (13.4%), both among sensitive and MDR infections. MDR bacteria were the cause for 41.3% of all infections isolated. Fungi accounted for 9.5% of infections. 21.9% of patients had decompensation from their infection(s) that required ICU care, and 14.6% of patients died during hospitalization or soon after discharge. **Conclusions:** The incidence of infections in cirrhotic patients is much higher than in their non-cirrhotic counterparts (54.6%), even higher than prior studies suggest. As many of these infections are caused by MDR bacteria and fungal organisms, stronger empiric antibiotics and antifungals should be considered when initially treating this

immunocompromised population. However, once organism sensitivities are discovered, narrowing of antibiotic regimens must occur to maintain good antibiotic stewardship.

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Introduction

Patients with cirrhosis are known to be immunocompromised and therefore more susceptible to infection.¹ Among cirrhotic patients, 32–34% are diagnosed with bacterial infections on hospital admission or during their hospital course, significantly higher than the 5–7% overall infection rates for hospitalized patients in general.² The incidence of infection rises to 45% in cirrhotic patients who are admitted for gastrointestinal hemorrhage.² The mechanism behind the decrease in immune function has been reported to be multifactorial.

The liver plays a key role in immune defense as the largest organ of the reticuloendothelial system (RES).¹ The liver comprises 90% of the RES, with its combined Kupffer and sinusoidal endothelial cell masses. In the setting of cirrhosis, the number of these specialized cells are decreased, leading to concurrent RES functional impairment and increased rates of bacteremia.¹ This effect is compounded by the formation of portosystemic shunts which decrease blood flow through the liver and thereby avert the RES, allowing less bacteria and endotoxins to be removed from the circulation by the liver.¹ It has also been shown that patients with cirrhosis have reduced neutrophil mobilization and phagocytic activity; this deficit correlates with the degree of liver disease.² In addition, the aforementioned decreased phagocytic activity is compounded by decreased bactericidal and opsonization capacity.² Moreover, cirrhotic patients have lower levels of protective proteins, including immunoglobulins (Igs)M, IgG, and IgA in ascitic fluid, as well as decreased concentrations of C3, C4, and CH50 in both the blood and ascitic fluid in combination, leading to further decreased bactericidal activity.²

In addition to an impaired immune system, cirrhotic patients are at higher risk for developing pathological bacterial translocation. In cirrhosis, the sympathetic nervous system is up-regulated, leading to slower gut motility and allowing bacterial stasis and overgrowth. Normally, there is a 100–1,000:1 ratio of anaerobes to Gram-negative bacilli (GNB) in the gut, with anaerobes rarely translocating across gut mucosa. But, due to the slower gut motility, the flora changes and GNB overpopulate. Portal hypertension

Keywords: Cirrhosis; Bacterial infections; Multidrug resistance; Fungal infections.

Abbreviations: AIDS, acquired immune deficiency syndrome; CI, confidence interval; CRE, carbapenem-resistant Enterobacteriaceae; EASL, European Association for the Study of the Liver; ESBL, extended spectrum beta lactamase; GNB, Gram-negative bacilli; HIV, human immunodeficiency virus; ICU, intensive care unit; Ig, immunoglobulin; MDR, multidrug-resistant; MELD, model for end-stage liver disease; MRSA, methicillin-resistant *Staphylococcus aureus*; NAC-SELD, North American Consortium for the Study of End-Stage Liver Disease; OR, odds ratio; RES, reticuloendothelial system; RR, relative risk; SBP, spontaneous bacterial peritonitis; SIBO, small intestinal bacterial overgrowth; SIRS, systemic inflammatory response syndrome; SOFA, sequential organ failure assessment; VRE, vancomycin-resistant enterococci; UTI, urinary tract infection. Received: 16 August 2020; Revised: 15 November 2020; Accepted: 5 December 2020

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causes intestinal submucosal edema, which disrupts the mucosal barrier, leading to increased permeability of these GNB across the intestinal walls into lymph nodes, the blood stream, and ultimately the initially sterile peritoneal fluid.¹ Small intestinal bacterial overgrowth (SIBO) has also been associated with bacterial translocation and infection.³

The systemic inflammatory response syndrome (SIRS) is a process that cirrhotic patients are at higher risk for due to their elevated levels of endotoxins, proinflammatory cytokines (*e.g.*, TNF- α and IL-6), as well as nitric oxide. SIRS contributes to immune dysregulation in cirrhotics, as well as other complications. Endotoxins like lipopolysaccharides from Gram-negative bacteria and peptidoglycans from Gram-positive bacteria also result in a considerable increase in pro-inflammatory cytokines that lead to significant inflammation.⁴ Cirrhotics have been shown to form higher levels of pro-inflammatory cytokines compared to non-cirrhotics when exposed to endotoxins.⁵ Increased nitric oxide production leads to oxidative stress and further vasodilation. SIRS can lead to portal hypertension-related complications like variceal bleeding, hepatic encephalopathy, and renal failure.⁴ SIRS has been linked with sepsis in the past, when associated with infection, though the new Sepsis-3 criteria now focus on organ dysfunction due to abnormal host response to infection, as measured by the sequential organ failure assessment (commonly known as SOFA) and qSOFA scores.^{6,7}

The gut microbiota has also been shown to be altered in cirrhosis. Studies have shown that the amount of beneficial autochthonous bacteria (*e.g.*, *Lachnospiraceae*, *Ruminococcaceae*, and *Clostridiales*) are reduced in cirrhosis, with a subsequent increase in potentially pathogenic taxa (like *Staphylococcaceae*, *Enterobacteriaceae*, and *Enterococcaceae*). A reduction in autochthonous taxa can be deleterious, as they produce short chain fatty acids that decrease colonic inflammation, contend with pathogenic bacteria for nutrients, create antibacterial peptides, and possibly improve the intestinal barrier.⁸

The most common bacterial infections in cirrhotic patients have been reported to be SBP (25–31%), urinary tracts infections (20–25%), pneumonia (15–21%), bacteremia (12%), and soft tissue infections (11%).⁹ It has been reported that nearly 75% of bacterial infections in cirrhotic patients are caused by Gram-negative bacteria (like *E. coli*, *Klebsiella*, *Pseudomonas*, *Vibrio*, *etc.*), with Gram-positive bacteria comprising only 20.2% and anaerobes only 3.2%.⁹ However, with increased use of quinolone prophylaxis, frequent hospitalizations, and the high number of invasive procedures cirrhotic patients undergo, there has been a shift to increasing numbers of infections caused by Gram-positive organisms (38–70%).⁹ It has been reported that infections in cirrhotic patients encompass a 4-times higher risk of mortality compared to those in non-cirrhotics.¹⁰ In fact, cirrhotic patients who developed infections were shown to have a 1-month mortality rate of 30% and a 1-year mortality rate of 60%.¹⁰

Given the high mortality rates associated with infections in cirrhotic patients and the changing types of infections, we decided to study the distribution of infections in one large academic center. The purpose of this retrospective chart review study was to assess the prevalence of infections in cirrhotic patients in a large urban academic liver center and investigate potential associations between infections, bacteria isolated, therapeutic regimens used, and mortality.

Methods

We performed a retrospective chart review of 263 patients who were admitted to the hepatology inpatient service at a

tertiary care hospital. The protocol was reviewed and approved by the Rutgers New Jersey Medical School Institutional Review Board. Inclusion criteria were 1) adult patients between the ages of 18 and 80 years, and 2) diagnosis of cirrhosis. The diagnosis of cirrhosis was made radiographically or by histology. Patients were excluded from the study if they were 1) diagnosed with human immunodeficiency virus (commonly known as HIV) or acquired immune deficiency syndrome (commonly known as AIDS), 2) were on immunosuppressive medications after transplantation, or 3) had evidence of additional immunodeficiency syndromes or were on immunosuppressive agents for other disorders (*i.e.* patients with rheumatoid arthritis on steroids).

Infections were defined in the following ways. SBP was defined by a polymorphonuclear cell count in the ascitic fluid of ≥ 250 , with secondary bacterial peritonitis diagnosed by the same ascitic fluid findings after an abdominal procedure recently performed. Bacteremia was diagnosed by positive blood cultures and UTIs by urinalyses with 10 leukocytes or more and/or positive urine cultures. Respiratory tract infections were defined by chest x-rays with evidence of consolidation and/or positive sputum cultures, and cellulitis/other infections were noted by consistent physical exam findings. Diagnosis of other infections were made based on conventional criteria.

Blood and urine samples were collected as per University Hospital protocol. Blood cultures were collected peripherally in two sets of aerobic/anaerobic bottles from different sites, with cultures drawn from central lines only if there was high suspicion for line infections. Urine samples were drawn from patients via clean catch if the patient did not have a catheter placed, otherwise were drawn from urinary catheters if present. Straight catheterization was utilized if the patient was unable to void and did not have a catheter placed.

The electronic medical records were reviewed and data were extracted as follows: patient age, sex, etiology of cirrhosis, identification of bacteria isolated from cultures, multidrug-resistant (MDR) status, antibiotics administered, admission to intensive care unit (ICU), and patient mortality during or soon after hospitalization. Infections that were characterized as MDR included extended spectrum beta lactamase (ESBL) producing *E. coli* and *Klebsiella* species, methicillin-resistance *Staphylococcus aureus* (MRSA), *E. faecium* and *E. faecalis* resistant to vancomycin (VRE), and all bacteria resistant to at least three different antibiotics.

For statistical analysis, continuous variables were summarized as median (interquartile range) and categorical variables were summarized as percentages. Distributions of continuous variables were tested for normality by the Kolmogorov-Smirnov test, and, if skewed, were in-transformed. For continuous variables, the linear trend across infection variable (no infection, infection, MDR infection) was determined by analysis of variance; categorical variables were tested by the χ^2 trend test. Binary logistic regression analysis was applied to assess the association between patients' clinical data and in-hospital mortality. Covariates that were univariably associated with in-hospital mortality (exploratory $p < 0.10$) were entered into a multivariable model using the stepwise backward likelihood ratio method. All analyses were performed with a complete dataset using SPSS software (SPSS 27.0; IBM Corp., Armonk, NY, USA). All tests were two-tailed, and p values < 0.05 were considered statistically significant.

Results

A total of 263 patient charts were reviewed from patients admitted to the hepatology inpatient service from 2008–

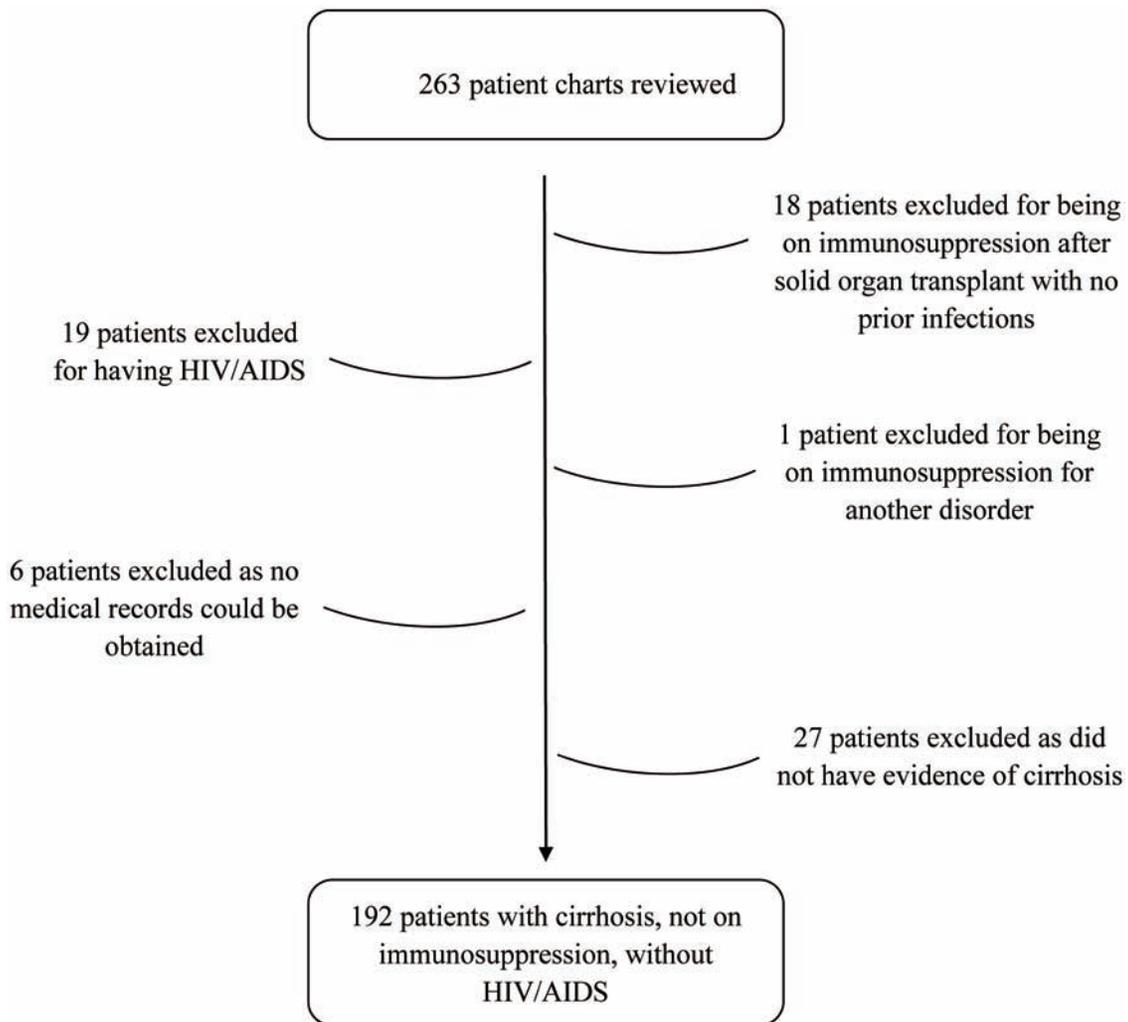


Fig. 1. Patient selection process.

2014. Eighteen patients were excluded for being on immunosuppressive medications after solid organ transplant, with no pre-transplant infections. An additional nineteen patients were excluded for carrying a diagnosis of HIV or AIDS. One patient was excluded for being on immunosuppressive medications for another disorder, and six patients were excluded as there was no information that could be found in the medical records for them. Twenty-seven patients could not be included as they did not have evidence of cirrhosis. This left 192 patients who met the criteria to be a part of the study (Fig. 1).

Men were more common than women, making up 62.6% of the study population. The mean age among patients diagnosed with infection was 55.8 years and 57.3 years among patients without infection. Hepatitis C was the most common etiology of cirrhosis, both in patients with infection (31.5%) and patients without infection (29.7%), closely followed by alcohol (30.6% in those with infections and 27.0% in those without infections). Other baseline data are mentioned in Table 1. The model for end-stage liver disease (MELD) scores were noted to be significantly higher in infected patients (whether MDR or non-MDR) compared to non-infected patients (22 and 20 vs. 13 respectively, $p < 0.001$). Ascites also was significantly more likely to be present in infected patients (whether MDR or non-MDR)

compared to non-infected patients (89% and 73% vs. 53% respectively, $p < 0.001$).

Infections were present in 105 (54.6%) patients, and 60 (31.2%) patients were noted to have multiple infections during a hospitalization or multiple hospitalizations for infections. A total of 201 infections were identified in these patients. UTIs were the most common infection identified (37.8%), followed by bacteremia (20.4%), pneumonia (12.9%), SBP (11.9%), abscess/cellulitis (6.0%), infectious diarrhea (6.0%), and other (5.0%) (Fig. 2).

A total of 174 organisms were isolated from these infections, while 31 infections were culture-negative. *E. coli* was the most common bacteria isolated (13.4%), both among sensitive and MDR infections (20.5% in MDR infections and 11% in sensitive infections). Other prevalent bacteria isolated included *Klebsiella* (9%), *S. epidermidis* (6.5%), *S. aureus* (6%), and VRE (6%) (Fig. 3). Other frequent sensitive bacterial isolates included *Klebsiella* (8.8%), *S. aureus* (7.7%), and *Clostridium difficile* (7.7%). Other prevalent MDR bacteria included VRE (14.5%), *Klebsiella* (12%), and *S. epidermidis* (10.8%). MDR bacteria were the cause for 41.3% of all infections isolated. Fungi (*Candida* species and *Aspergillus*) were isolated in 19 cases, accounting for 9.5% of infections (Fig. 4). Fungemia, in particular, was present in five cases, or 2.5% of infections. Thirty-one infections

Table 1. Distribution of patients' clinical data and trends across different infection categories

Clinical data	No infection	Infection	MDR infection	<i>p</i> -value*
Age, median (IQR)	58 (50–63)	56 (53–63)	56 (51–61)	0.49
Male sex, %	69	58	62	0.36
Diabetes mellitus, %	29	24	31	0.87
MELD score, median (IQR)	13 (9–23)	20 (16–27)	22 (16–29)	<0.001*
Ascites, %	53	73	89	<0.001*
Hepatic encephalopathy, %	32	49	50	0.051
Rifaximin prior to admission, %	39	38	37	0.82

**p*-value for trend; *p*-value <0.05 indicates statistical significance.

were noted to be culture-negative, with either pneumonia based on imaging findings ($n=10$), SBP based on body fluid analysis ($n=7$), cellulitis ($n=9$), or UTI based on positive urinalysis ($n=5$).

Vancomycin and piperacillin-tazobactam were the most commonly prescribed antibiotics (vancomycin in 40.8% of infections and Pip-Tazo in 39.8%). Ceftriaxone and levofloxacin were used in 21.9% and 17.4% of infections respectively. Among the patients, 21.9% had decompensation from their infection(s) that required ICU care and 14.6% of patients died during hospitalization or soon after discharge. This is in comparison to the 29.7% of patients who had infections but did not require ICU stay or succumb to their infection. When reviewing factors that were associated with increased risk for mortality, MELD score, presence of ascites, and presence of infection (MDR or non-MDR) were found on univariate analysis to have a significant association. On multivariate analysis, MELD score [odds ratio (OR): 7.9, 95% confidence interval (CI): 2.3–27.3, $p=0.001$] and presence of infection (MDR or non-MDR) (MDR OR: 16.9, 95% CI: 2.0–139.2, $p=0.009$; non-MDR OR: 12.5, 95% CI: 1.5–105.6, $p=0.02$) were found to be significantly associated with mortality (Table 2).

Discussion

Patients with cirrhosis are known to be at a higher risk of developing infections. This study showed that in our population of cirrhotic patients, slightly less than 55% had an infection on admission or developed one during hospitalization. This is a higher percentage than even prior papers suggest, emphasizing the delicate status of cirrhotic patients and that they are at high risk for developing infections and other forms of decompensation. These results may also have to do with our location as a tertiary care center in a large city. The fact that some of our patients had multiple hospitalizations with infections diagnosed may have also contributed to this higher percentage. These infections are made all the more critical given their significant association to patient death in this study. High priority should be given to prevention/early treatment of infections in cirrhotic patients, with appropriate, early broad antibiotic coverage in those suspected to have/with infections, given their associated worse outcomes.

Of interest was the large amount of fungal infections that were diagnosed. While often dismissed in immunocompetent

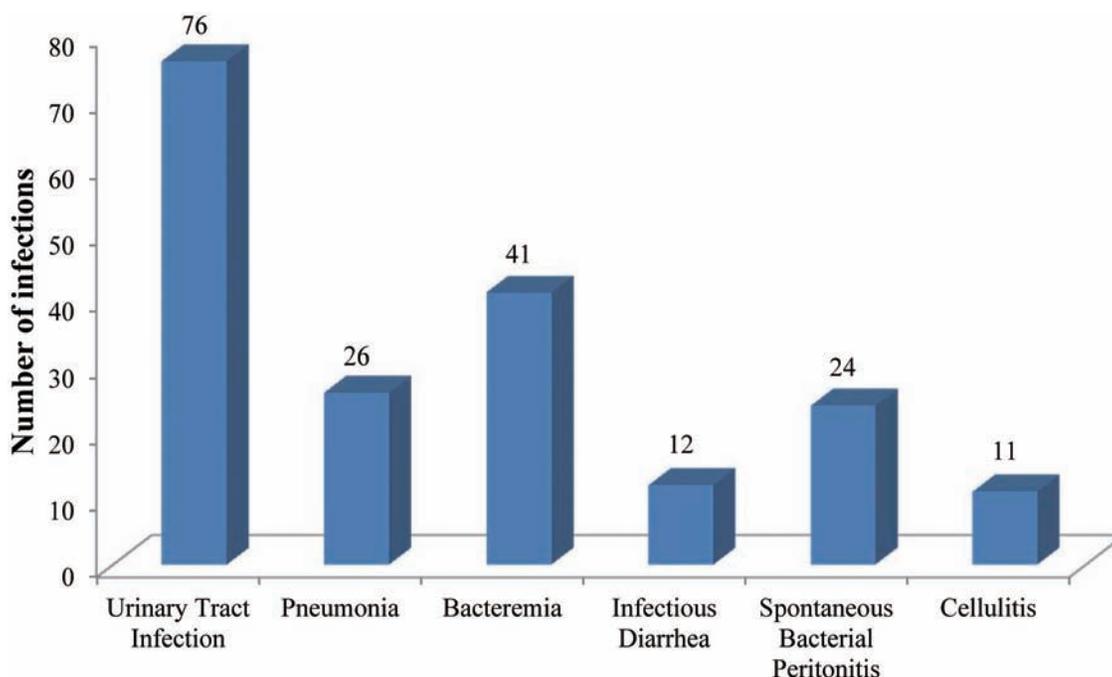


Fig. 2. Top six types of infections in cirrhotic patients.

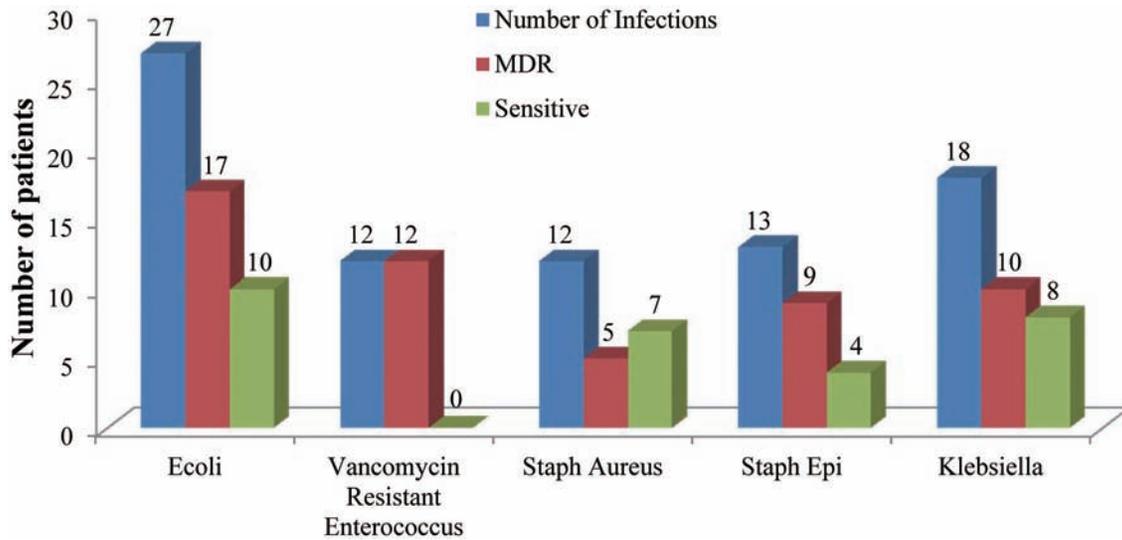


Fig. 3. Top five bacteria isolated from cirrhotic patients.

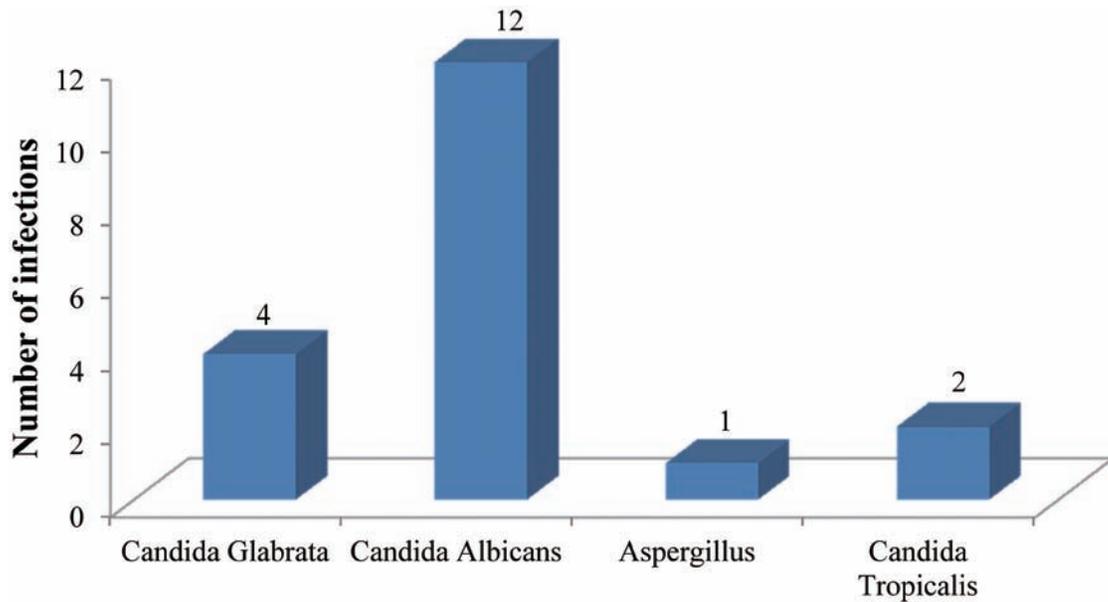


Fig. 4. Types of fungal infections in cirrhotic patients.

Table 2. Associations between patient’s clinical data and in-hospital mortality

	Univariable model		Multivariable model ^a	
	OR (95% CI)	p-value*	OR (95% CI)	p-value*
MELD score ^b	11.3 (3.7–34.2)	<0.001*	7.9 (2.3–27.3)	0.001*
Ascites	15.8 (2.1–119.4)	0.008*	7.3 (0.9–61.1)	0.07
Hepatic encephalopathy	1.9 (0.8–4.3)	0.10	x	
Rifaximin prior to admission	2.2 (1.0–4.8)	0.06	x	
Infection	17 (2.1–135.8)	0.007*	12.5 (1.5–105.6)	0.020*
MDR infection	29.7 (3.8–232.3)	0.001*	16.9 (2.0–139.2)	0.009*

OR indicates odds ratio for dying during index hospitalization; 95% CI indicates 95% confidence interval for the corresponding OR. ^aOnly clinical parameters with $p < 0.10$ in univariate analysis are reported in the table. ^bPer 1-Ln unit change in MELD score. *p-value < 0.05 indicates statistical significance. x excluded in backward selection (*i.e.* not significant for prediction).

patients as colonizers that are not responsible for sepsis/decompensation, we diagnosed numerous fungal infections, in particular many cases of fungemia, that were responsible for significant decompensation in our patients. These findings point to a gap in our management strategies for cirrhotic patients with suspected infections. While invasive fungal infections have been generally agreed to have serious complications in immunocompromised states, less focus has been given to infections regarded as fungal colonization.^{11,12} Lahmer *et al.*,¹² however, showed that even patients with fungal infections that did not meet criteria for invasive fungal infection (*i.e.* fungemia, biopsies of affected areas with fungal involvement, or chest x-ray/CT scan findings of lung or other organ) were still independently associated with higher mortality rates. Other studies have noted that the incidence of invasive fungal disease in the ICU was higher in patients with liver disease compared to other high-risk groups, though the mortality rates were not shown to differ.¹³

Although the above results make a compelling case for early and aggressive antibiotic use, it is clear that changes in the gut microbiome are occurring as a result of increasing antibiotic use. Bajaj *et al.*¹⁴ evaluated the difference in fungal and bacterial stool profiles of different populations. When comparing healthy controls to outpatient cirrhotic patients on/off antibiotics to hospitalized uninfected/infected culture-negative/infected culture-positive cirrhotic patients, there was a decrease in fungal diversity when comparing inpatients to outpatients, cirrhotics to healthy controls, and culture-positive infected inpatients to culture-negative infected patients or outpatients on antibiotics, with higher concentrations of *Ascomycota* species noted. As the more common type of fungal infections in cirrhosis (peritonitis, fungemia, and esophagitis) are often due to *Ascomycota* and portend a poor prognosis, antibiotics may exacerbate the incidence/risk of these infections.

A recent multicenter, prospective study from the North American Consortium for the Study of End-Stage Liver Disease (NACSELD) cohort evaluated inpatient cirrhotic patients to determine risk factors contributing to the development of fungal infections and their significance on 30-day survival. Among hospitalized and infected patients, 12.7% were found to have fungal infections, all of which were nosocomial. Of the 134 fungal infections, 104 were noted after a prior bacterial infection and antibiotic use; however, the location of bacterial infection or type of bacteria isolated was not shown to significantly determine the development or location of fungal infections. This suggests antibiotic use for treatment of bacterial infections as a possible risk factor for the development of fungal infection. Other independent risk factors for fungal infections included diabetes, ICU admission, acute kidney injury, and bacterial infections diagnosed at admission. Fungal infections were also associated with worse 30-day survival. Most fungal infections had a 30% 30-day mortality rate, though fungemia and fungal peritonitis (forms of invasive fungal infections), had >50% 30-day mortality rates.¹⁵

Close surveillance for fungal infections versus empiric antifungal coverage for patients with suspected infection and evidence of clinical deterioration or clinical deterioration despite antibiotic treatment of bacterial infections may help improve outcomes in this regard. While more is being learned at this time, further research into the topic of fungal infections in cirrhotic patients and surveillance, and potential prophylaxis is needed.^{16,17}

Multidrug-resistant bacteria were isolated in over 41% of infections in our study. This correlates with the increasing rates of MDR infections being isolated in general and a change in the epidemiology of infections being isolated in cirrhotic patients. Studies have documented the increase in MDR infections, ranging from 23–47%.^{18–20} Prior studies have shown that nosocomial infections, long-term nor-

floxacin prophylaxis, infection by MDR bacteria in the last 6 months, and recent use of beta-lactam antibiotics have all been independently associated as risk factors for the development of MDR infections in cirrhotic patients.¹⁸ More recently, Piano *et al.*²¹ performed a multicenter, intercontinental, prospective study evaluating the prevalence of bacterial and fungal infections in patients with cirrhosis. Their study showed a global prevalence of MDR bacteria of 34%. However, there were significant differences in MDR bacteria depending on geographic areas, with Asia having the highest prevalence. India had the highest prevalence, with 73% of bacteria isolated being MDR. This was attributed to over-the-counter access of antibiotics in the community and an increased presence of antibiotics in the environment. Independent risk factors for MDR bacterial infections were infections occurring in Asia (specifically India), antibiotic utilization 3 months prior to hospital admission, and site of infection. MDR infections were associated with decreased resolution rate of infection, higher incidence of shock and *de novo* organ failures, and higher in-hospital mortality compared to non-MDR infections. Interestingly, there was a lack of an association between quinolone prophylaxis and MDR infections. This may be due to the fact that only 10% patients in the study were on SBP prophylaxis, but bears further analysis. One of the most important findings was that giving appropriate empiric antibiotic coverage was independently associated with better in-hospital and 28-day survival rates. The efficacy of empiric antibiotics was the only modifiable predictor of mortality.

This increase in MDR infections has larger implications in regards to our antibiotic choices in this population of patients, namely, that patients' infections must be classified appropriately as community acquired, healthcare-associated, or nosocomial, so that the appropriate strength antibiotics can be started at admission. This is a critical time in the course of therapy, as empiric antibiotic regimens that are not effective in the initial 24–48 hour period while cultures are being processed have been associated with increased mortality.²²

In the past, third-generation cephalosporins had been the first choice of empiric treatment for SBP and several other infections, including UTIs. Studies have shown, however, evidence of bacterial resistance to third generation cephalosporins in 21.5% to 57% of SBP cases in Europe, with only 67% efficiency of empirical treatment with these agents.¹⁹ As a result, the European Association for the Study of the Liver (known as EASL) established guidelines advocating for stronger empiric antibiotics in nosocomial infections (piperacillin-tazobactam or meropenem +/- glycopeptide), with mention that these choices should be tailored to the prevalent MDR bacteria of the local area.²³ The most recent guidelines set by EASL recommend that healthcare-associated infections should be treated with carbapenems like nosocomial infections if the prevalence of MDR organisms is high in the area. They have also added piperacillin-tazobactam as a treatment option in community acquired infections.²⁴ The efficacy of these guidelines has been shown in practice. Piano *et al.*²⁵ looked at broader-spectrum antibiotic regimens in comparison to third generation cephalosporins in the treatment of nosocomial SBP, and found that meropenem with daptomycin was more effective than ceftazidime as empirical antibiotic treatment (86.7% vs. 25% response to treatment, with 90% of ceftazidime non-responders responding to meropenem/daptomycin). The effectiveness of the initial empiric antibiotic treatment was noted to be a strong predictor of 90-day transplant-free survival in this patient population. The fact that 22% of the patients in our study required ICU care and nearly 15% succumbed to their illnesses is further testament to the need for broader empiric antibiotics for cirrhotic patients on presentation to the hospital with likelihood of sepsis.

Moreover, new types of MDR infections are becoming more prevalent. Carbapenem-resistant Enterobacteriaceae (referred to as CRE) were uncommon prior to the year 2000 but have doubled in prevalence in the last decade among healthcare-associated infections.²⁶ And, while healthcare-associated infections and nosocomial infections are where the majority of these cases have been noted in the past, studies are showing increasing rates of community acquired CRE infections, ranging from 7.7–29.5% globally and from 5.6–10.8% in the USA.^{26,27} These findings bring to light the conundrum of antibiotic use in cirrhotic patients. Antibiotic prophylaxis and empiric broad spectrum antibiotic use has an important function, as it can prevent bacterial infections and positively impact liver decompensation and overall survival. However, it can lead to antibiotic resistance, potentially exacerbating the situation in the longer run.²⁸ Understanding local antibiotic resistance patterns is important to tailoring empiric regimens that will improve survival outcomes without unnecessarily increasing risks for antibiotic resistance.

Given the increasing rates of MDR infections being isolated, with their associated mortality, it is important to consider new and innovative treatment and/or preventative options for bacterial infections in cirrhotic patients. Rifaximin has been evaluated in the recent past as a new option for prevention of SBP. Elfert *et al.*²⁹ looked at 262 patients with cirrhosis and a prior episode of SBP, and randomly assigned them to receive either 1,200 mg of rifaximin or 400 mg of norfloxacin daily for 6 months. The rifaximin prophylaxis group was shown to have significantly lower rates of recurrent SBP when compared to the norfloxacin group (3.9% vs. 14.1%, $p=0.04$). Additionally, there were 3-times less encephalopathy-related deaths in the rifaximin group compared to the norfloxacin group. Menshawey *et al.*³⁰ performed a meta-analysis of six studies including 973 patients to study the role of rifaximin in prevention of SBP. They found that rifaximin in addition to norfloxacin resulted in statistically lower rates of SBP [relative risk (RR): 0.58, 95% CI: 0.37–0.92, $p=0.02$] and hepatic encephalopathy (RR: 0.38, 95% CI: 0.17–0.84, $p=0.02$), compared to the norfloxacin monotherapy group. They also found no significant differences between rifaximin and norfloxacin in regards to frequency of SBP (RR: 0.49, 95% CI: 0.24–1.01, $p=0.05$). Our own study did not see much of an impact of rifaximin on infection rates, with 37.4% of those with infections on rifaximin compared to 38.5% of those without infections.

This study has some limitations. It is a retrospective study, and so is at risk of selection and researcher bias. UTIs were noted to be the most common infection isolated, though a subset of these cases may have been related to asymptomatic bacteriuria, rather than actual infection. Our data also revealed a number of cases of *S. epidermidis* infections. While appropriate techniques were utilized in obtaining cultures, it is possible that contamination may have contributed to the number of these cases. Additionally, our patients included those of geriatric age group, whose immunosenescence may have put them at higher risk for the development of infections. It is also a single-center study in an urban tertiary care hospital, receiving referrals from neighboring hospitals of decompensated cirrhotic patients, and may not be fully generalizable to different demographic centers. Further prospective, multicenter trials can help to corroborate these findings in a more substantial way.

In short, our study shows that the incidence of infections in cirrhotic patients is much higher than that of their non-cirrhotic counterparts, and even higher than prior studies of cirrhotic patients suggest. As a large component of these infections are caused by MDR bacteria and fungal organisms, stronger empiric antibiotics and antifungals need to be considered when initially treating this immunocompromised population who may not mount appropriate im-

munologic responses to these pathogens. However, once organism sensitivities have been discovered, narrowing of antibiotic regimens needs to occur to maintain good antibiotic stewardship.

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Conflict of interest

Pyrpoulos NT is a recipient of research grants from Mallinckrodt, Valeant, Gilead, Bayer, Beigene, Genfit, Prometheus, Grifols, Intercept, Zydus and Eisai, which are outside the submitted work. Lingiah VA has no conflict of interests related to this publication.

Author contributions

Reviewed the literature, performed the majority of data acquisition and analysis, manuscript preparation, and preparation of figures and tables (VAL), designed, co-authored and revised the manuscript (NTP).

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Drugs for Non-alcoholic Steatohepatitis (NASH): Quest for the Holy Grail

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is a global epidemic that is likely to become the most common cause of chronic liver disease in the next decade, worldwide. Though numerous drugs have been evaluated in clinical trials, most of them have returned inconclusive results and shown poorly-tolerated adverse effects. None of the drugs have been approved by the Food and Drug Administration for treating biopsy-proven non-alcoholic steatohepatitis (NASH). Vitamin E and pioglitazone have been extensively used in treatment of biopsy-proven nondiabetic NASH patients. Although some amelioration of inflammation has been seen, these drugs did not improve the fibrosis component of NASH. Therefore, dietary modification and weight reduction have remained the cornerstone of treatment of NASH; moreover, they have shown to improve histological activity as well as fibrosis. The search for an ideal drug or 'Holy Grail' within this landscape of possible agents continues, as weight reduction is achieved only in less than 10% of patients. In this current review, we summarize the drugs for NASH which are under investigation, and we provide a critical analysis of their up-to-date results and outcomes.

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Introduction

Nonalcoholic fatty liver disease (NAFLD) is an emerging global epidemic. It is the most common cause of chronic liver disease in the western world.¹ The pooled prevalence of NAFLD in Asia is 30% (95% confidence interval [CI]: 28.13–31.15).² The term nonalcoholic steatohepatitis (NASH) was coined almost 4 decades ago.³ Recently, NAFLD was renamed as

Keywords: Fatty liver; NAFLD; NASH; Obeticholic acid; Saroglitazar.

Abbreviations: CI, confidence interval; FDA, Food and Drug Administration; FXR, farnesoid X receptor; NAFLD, nonalcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; OCA, obeticholic acid; PIVENS, pioglitazone versus vitamin E versus placebo for the treatment of nondiabetic patients with nonalcoholic steatohepatitis; PPAR, peroxisome proliferator-activated receptor; TONIC, treatment of NAFLD in children; UDCA, ursodeoxycholic acid.

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“metabolic (dysfunction)-associated fatty liver disease”, or “MAFLD”.⁴ However, the change in terminology is unlikely to alter the management of these patients and hence will not be referred to in this manuscript. In this review, instead, we will focus on the drugs used for NASH. There have been several trials to find an ideal drug for the treatment of NASH.^{5–10} Despite several advances in the understanding of the pathophysiology underlying this common disease, to date, there are no drugs approved by the Food and Drug Administration (FDA) for the treatment of either simple steatosis or NASH. Recently, FDA has accepted a new drug application (not approved) for OCA, a farnesoid X receptor (FXR) agonist, which was shown to improve fibrosis in NASH.¹¹ In this review, we outline the mechanisms and targets of such, and discuss some of the major drug trials in NASH.

Journey of drugs in NASH

For the approval of a drug for a disease, it needs to undergo preclinical trials, followed by Phase 1 safety studies, which are supported by Phase 2 studies. Phase 2 studies are dose-finding trials and aid in evaluating efficacy of a drug. Once a drug is found to be effective, it undergoes Phase 3 studies that compare the safety and efficacy of the drug with existing therapies. Then, the drug is placed for review before an appropriate drug review committee that may/may not approve the drug.¹² Post-approval, there are phase 4 trials, a strategy which is also known collectively as ‘post-marketing surveillance’. The objective of the phase IV studies is to check the drug’s performance in real-life scenarios, to study the long-term risks and benefits of the drug, and to discover any rare side effects. Interestingly, for each phase of the trial, there are recommended endpoints to be achieved before proceeding to the next stage of a trial in adult patients, such as those with NASH.¹³ However, there are no clear endpoints for pediatric NASH.¹³ For NASH, the endpoint for a phase 3 trial is NASH resolution (defined as disappearance of ballooning and disappearance or persistence of minimal, lobular inflammation that does not qualify for the diagnosis of NASH), with or without a reduction in fibrosis stage by one point.¹³

There are certain major hindrances to conducting a clinically relevant trial in NASH.^{14,15}

Some of the limiting factors are:

1. Biopsies are required to define participants and are needed to establish efficacy, as well.
2. Many trials have high screen fail rate, due to stricter inclusion criteria.
3. There is often a high placebo response rate in the control group. The reasons for this are unclear but may be due to behavioral changes in the control group, resulting in weight loss.

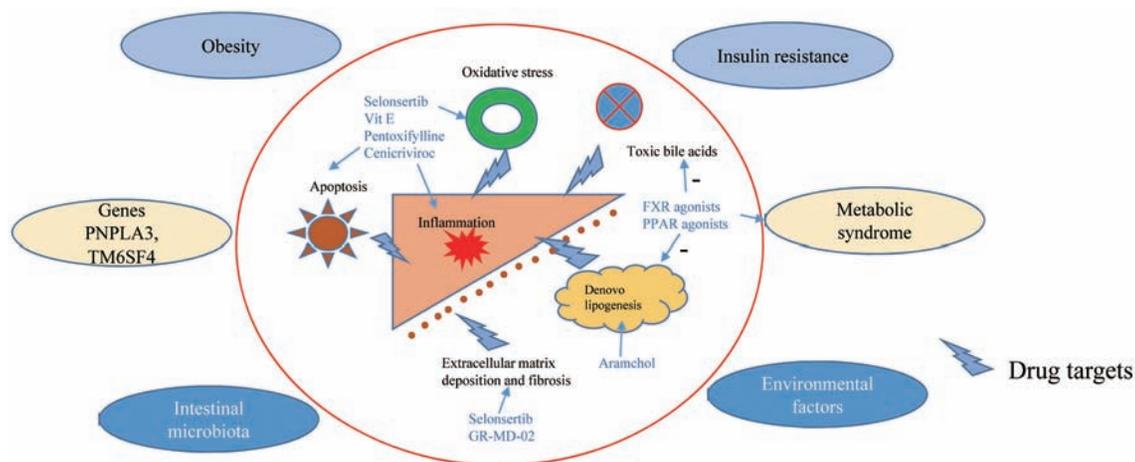


Fig. 1. Factors implicated in the pathogenesis of NASH and possible drug targets. The factors which lead to NAFLD are depicted in the outer circle, while the changes that occur inside the liver are shown within the red circle. The drug targets are shown in blue lightning arrows. Abbreviations: FXR, farnesoid X receptor; PNPLA, patatin-like phospholipase domain-containing protein A; PPAR, peroxisome proliferator-activated receptors; TM6SF4, transmembrane 6 superfamily 2 human gene; Vit E, vitamin E.

4. Most of the studies use two primary endpoints.
 - 4a. Improvement in fibrosis, with no worsening of NASH
 - 4b. NASH resolution without worsening of fibrosis
5. Challenges in developing clinical endpoints, due to the lack of specific symptoms of NASH.
6. Difficulties in managing confounders, such as recording alcohol intake before and during the study, and the lack of uniformity in diet and physical activity.

Histopathology and grading of NASH

There are two major grading systems of NASH. One which is frequently used in clinical trials is NAFLD Activity Score-Clinical Research Network (NAS-CRN) and has been validated to compare the biopsies. NAS-CRN consists of three components of activity, viz. steatosis (score 0–3), lobular inflammation (score 0–3), and ballooning (score 0–2), with a maximum score of 8. A score ≥ 5 suggests definite NASH. Fibrosis is graded from 0–4 (0: no fibrosis to 4: cirrhosis).¹⁶ NAS-CRN has been validated in both children and the adult population. The Steatosis activity fibrosis (SAF) scoring system aids in the diagnosis of NASH and can be used in clinical trials.¹⁷ The SAF score is semi-quantitative and differs slightly from the NAS-CRN, with lobular inflammation scored from 0 to 2 (instead of 3) and ballooning from 0–2 (1: clusters, reticulated cytoplasm; 2: enlarged hepatocytes, as opposed to few and many in NAS-CRN scoring). As the final score is meant to represent a diagnosis, steatosis must be >0 , wherein activity (ballooning plus lobular inflammation) must be ≥ 2 , in which ballooning is at least 1. Fibrosis is based on the NAS-CRN scale and is reported as “F”.

Pathogenesis and targets

To find an ideal drug for NASH, it is essential to understand its pathogenesis and identify a single ideal target. The target may exist at multiple levels and may also be outside the liver itself. Whether NAFLD is the ‘hepatic manifestation’ of metabolic syndrome or a pathogenic determinant of metabolic syndrome is still unknown.^{18,19} However, there is a growing body of evidence strongly supporting the notion that NAFLD precedes the development of type 2 diabetes mellitus (T2DM) and metabolic syndrome.^{18,20} It is also a

paradox to find out if the drugs that is appropriate for lean and obese NASH patients would be similar. An ideal drug for NASH would be a drug that targets fat deposition, has anti-inflammatory and antifibrotic properties, and reduces cardiovascular risk, which is the most frequent cause of mortality in NASH.^{21,22} The pathogenesis of NAFLD is complex, involving extrinsic predisposing factors and intrinsic genetic factors.²³ Insulin resistance, however, remains central to the development of NAFLD. The complex interplay of different factors in pathogenesis is shown in Fig. 1.

Currently, drug treatment is indicated for patients with progressive NASH (*i.e.* NASH activity with bridging fibrosis/cirrhosis), early-stage NASH (at high risk for disease progression; age >50 years, metabolic syndrome, diabetes mellitus or increased alanine transaminase), or NASH with high necroinflammatory activity.^{24,25} Most of the pharmacotherapy trials have been carried out in biopsy-proven NASH patients (biopsy is must to prove NASH/inflammation). Several drugs have shown initial promise but failed to meet the critical endpoint of improvement in fibrosis scores. The drugs and modalities of treatment that have been tried in patients with NASH are discussed below.

Lifestyle changes for the treatment of NASH

The most effective and proven therapy for NASH is weight loss. Analysis of data from eight randomized control trials has shown that $>5\%$ weight loss leads to resolution hepatic steatosis, and $\geq 7\%$ improves the inflammatory score of NAS (NAFLD activity score).²⁶ But, reportedly, only 50% of patients can achieve a weight loss of 7%.²⁷ Lastly, a weight loss of $\geq 10\%$ results in the resolution of early fibrosis, in approximately 45% of subjects.²⁷

Physical activity of ≥ 150 m/week is associated with improvement in liver enzymes, irrespective of the weight loss. More than 8,000 steps per day is associated with a reduction in all-cause mortality; however, the number of steps required for reduction in NAS score/liver enzymes has not been evaluated.²⁸

Dietary interventions

A triple hit behavioral phenotype exists, which involves i)

sedentary behavior, ii) low physical activity, and iii) poor diet, all of which are well known to be associated with poor cardio-metabolic health, NAFLD, and overall mortality.²⁹ Along with physical activity, a healthy diet is recommended for NAFLD patients.²⁴ Refined sugar and sugar-sweetened beverages are a common source of empty calories.²⁹ These fructose-rich diets increase the hepatic synthesis of triglycerides. Hence, sugar-sweetened beverages should be avoided. Mediterranean diet is primarily plant-based (whole grains, legumes, fruit, vegetables), low in carbohydrates (limited simple sugars and refined carbohydrates), and rich in monounsaturated (mostly olive oil) and omega-3 fats, and which incorporates limited red meat, and low-fat dairy products, which has been shown to improve the hepatic steatosis and insulin resistance and is the recommended diet for NAFLD.^{30,31} Recent studies have shown that 1 year of a hypocaloric (750 Kcal/day) diet combined with 200 m of exercise per week has a dose relationship with weight loss and leads to improvement in NASH, including fibrosis.²⁷ The combination of a hypocaloric diet (reduction by 500–1,000 kcal/day) and moderate-intensity exercise would provide the best likelihood of sustaining weight loss over time.²⁴ Currently, the time-tested diet and exercise therapy remains the most effective and cost saving intervention for management of NAFLD.

Drugs

Antioxidants

Vitamin E: Vitamin E has been studied in the landmark PIVENS (adult patients) and TONIC trials (pediatric population), where the antioxidative and free radical scavenging property of vitamin E has been hypothesized to improve NASH.^{6,8} Indeed, there was a significant improvement in steatosis and inflammation in patients treated with vitamin E for 96 weeks compared to placebo.⁶ However, there was no improvement in fibrosis.⁶ There have been conflicting reports on the rise in all-cause mortality in patients receiving vitamin E.^{32,33} However, a small increase in prostatic cancer due to long term administration of vitamin E is known.³⁴ Though the drug is well tolerated, the current guidelines recommend vitamin E (rrr- α -tocopherol) at a daily dose of 800 IU/day in non-diabetic adults with biopsy-proven NASH, weighing the risk-benefit ratio before initiation of treatment.²⁴ The long-term safety of administration of vitamin E for more than 6 months is unclear.

Pioglitazone: Pioglitazone, an agonist of peroxisome proliferator-activated receptor (PPAR) γ , was evaluated in the same PIVENS study. Pioglitazone has been hypothesized to increase the adipocyte uptake of fatty acids, thereby potentially drawing fat away from the hepatocytes.⁶ The use of 30 mg of pioglitazone for 96 weeks in non-diabetic patients showed a significant improvement in NASH compared to placebo. However, there was no improvement in fibrosis.⁶ The major drawback of vitamin E and pioglitazone is the lack of improvement in fibrosis, which is an important determinant of outcomes in NASH.³⁵ Thus, the clinical relevance of the results from the PIVENS study is limited. Further, weight gain, bladder cancer, and bone loss are major concerns associated with pioglitazone.²⁴ Of note, pioglitazone may be used for both diabetic and non-diabetic patients with biopsy-proven NASH only after explaining the risks and benefits in detail.^{24,31}

Ursodeoxycholic acid (UDCA): UDCA at a dose of 13–15 mg/kg body weight in patients with biopsy-proven NASH has not shown any benefit when compared with placebo and is not recommended for NASH.⁷ Animal studies on a side-chain-shortened homologue of UDCA, nor- (n)UDCA, can

attenuate the progression of NASH.³⁶ A recent human study (phase 2 trial) reported significant improvement in serum ALT levels at 12 weeks with the use of nUDCA at 1,500 mg per day compared to placebo.³⁷ Further phase III studies are required to confirm if the drug can meet the recommended endpoints.

Omega-3 fatty acids: Omega-3 fatty acids can reduce oxidative stress, lipotoxicity, and inflammation in patients with NASH.³⁸ There have been conflicting reports about efficacy of omega-3 fatty acids in NAFLD.^{10,39} The optimum dose has not yet been determined. However, the benefits of omega-3 fatty acid supplementation have been noted with a dose of ≥ 0.83 g/day.⁴⁰ Currently, they can be used to treat hypertriglyceridemia in NASH but not for the treatment of NAFLD or NASH.²⁴

Metformin: Early studies with metformin showed improvement of insulin resistance, liver chemistries, and a modest reduction in hepatic steatosis.⁴¹ Subsequently, two meta-analyses with the use of metformin in NASH showed no benefit and are currently not recommended for the treatment of NASH.^{42,43}

Pentoxifylline (PTX): PTX inhibits several pro-inflammatory cytokines, including tumor necrosis factor (TNF)- α .⁴⁴ PTX increases hepatic glutathione levels in mice with steatohepatitis induced by a methionine choline-deficient diet and reduces the production of oxygen radicals induced by prolonged ischemia time in rat livers.^{44,45} PTX down-regulates profibrogenic cytokines and procollagen I expression in a rat model of biliary duct occlusion.⁴⁶ Although preclinical studies have demonstrated the efficacy of PTX in NASH, there are conflicting reports in human trials of PTX.^{45,47} PTX is currently not recommended for NASH, due to insufficient evidence.

Drugs in the pipeline for NASH

The increasing burden of NASH worldwide has kept researchers astute to discover a new drug. NASH is associated with a high lifetime economic burden. In the absence of treatment, the total direct cost of illness for these patients will continue to grow.⁴⁸ Several drugs are in the pipeline for the treatment of NASH. Although none of these drugs appear as ideal, many of them seem promising. Some of these drugs are detailed in Table 1.

Obeticholic acid (OCA): Bile acid signal receptors are abundant in the liver, kidney, adipose tissue, small intestine, and immune cells.⁴⁹ OCA, 6 α -ethyl chenodeoxycholic acid (INT 747), is a semi-synthetic derivative of chenodeoxycholic acid. It is a 100-times more potent agonist of FXR than chenodeoxycholic acid.^{50,51} OCA is rapidly absorbed orally and reaches a peak plasma concentration in approximately 1.5 hours after intake and has a steady-state half-life of 4 days. The drug is not affected by food intake. The mean volume distribution of OCA is 618 L and is about 99% protein bound. The liver extensively metabolizes it into glycine and taurine conjugates. OCA undergoes extensive enterohepatic circulation, and >85% of metabolites are excreted in feces. FXR activation is mediated by binding of OCA to FXR receptors, which leads to increased secretion of FGF19 from the ileum. This results in formation of the β -klotho-FGF4-FGF19 complex, which inhibits CYP7A1 expression and bile acid synthesis. Besides, there is an increase in bile salt exporter protein (known as BSEP) and multidrug resistance 3 (known as MDR3) protein, promoting efflux of bile from hepatocytes. Further, OCA has a mild suppressive effect on the transforming growth factor-beta gene and extracellular matrix reorganization and stellate cell activation. The mechanism of action of OCA is summarized in Fig. 2.

The phase 2b study of OCA in NASH, called the 'FLINT'

Table 1. Mechanism of novel therapies for NASH

Name of drug	Mechanism of action	Phase of study	Comments
OCA	FXR agonist	3	Review with FDA for approval likely in June 2020
Elafibranor	PPAR α agonist	3	Missed primary endpoints in 2015 and 2020
Aramchol	Stearoyl Co-A desaturase 1 inhibitor and prevents <i>de novo</i> lipogenesis	3	Results of the ARMOR study are awaited
Saroglitazar	Dual PPAR α agonist	Phase 2 studies in USA and phase 3 studies in India	Currently approved by the DCGI. Abstracts have shown more effect on steatohepatitis.
Geniviroc	CCR2 and CCR5 chemokine receptor antagonist	CENTAUR study has shown improvement	Data from AURORA study are expected by September 2020
Emricasan	Pan-caspase inhibitor	Phase 2	Did not meet the primary endpoint
Belapectin	Galectin-3 inhibitor	Phase 2 study on effect in NASH and fibrosis and reduction of portal pressure	No improvement in fibrosis or portal hypertension
GLP-1 agonists: liraglutide and semaglutide	GLP-1 hormone	3	Studies with semaglutide hold promise and have shown fibrosis improvement

Abbreviations: CCR, CC chemokine receptor; DCGI, Drugs Controller General of India; FDA, Food and Drug Administration; FXR, farnesoid X receptor; GLP, glucagon-like peptide, NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; OCA, obeticholic acid; PPAR, peroxisome proliferator-activated receptors.

study, showed a significant improvement of NAS score by ≥ 2 points, without worsening of fibrosis, in 45% of patients receiving OCA at 25 mg, as compared with 21% in placebo ($p=0.0002$). Thirty-five percent in the OCA group also showed improvement in fibrosis compared to 19% in the placebo group ($p=0.004$).⁵² However, this study was terminated early due to administrative reasons. This was followed by a subsequent phase 3 study (the REGENERATE trial) involving 2,400 NASH patients with F2-3 fibrosis. They were randomized in 1:1:1 ratio to receive either placebo or OCA at 10 mg or OCA at 25 mg per day for 48 weeks.⁵³ An interim analysis at 18 months on 931 patients showed improvement in fibrosis by ≥ 1 stage, with no worsening of NASH in 23% of patients receiving OCA at 25 mg, when compared with 12% in the placebo arm ($p=0.0002$) and 18% in the OCA 10 mg group ($p=0.04$ for placebo).¹¹ A dose-dependent decrease in liver chemistries was observed from 3 months to 18 months. Similarly, improvement in NAS score by 2 points without worsening of fibrosis was significantly higher in the OCA 25 mg group when compared to placebo (36% vs. 24%; $p=0.0012$), with no such difference between the OCA 10 mg and placebo groups (30% vs. 24%; $p=0.11$).¹¹ Considering the vast number of NASH patients worldwide, this promising data would help to avoid many of the liver transplants attributable to NASH. Recent studies have shown that underlying genetic abnormalities may identify a cohort of patients who would respond to OCA.⁵⁴

Pruritus and a rise in low-density lipoprotein cholesterol (LDL) are the two major concerns with the use of OCA. The most common side effect reported with OCA is pruritus. Pruritus on treatment was reported in 19% of patients on placebo compared to 28% with OCA at 10 mg and 51% with OCA at 25 mg.¹¹ Nearly 10% had to discontinue OCA (25 mg) due to pruritus compared to 1% each in the OCA 10 mg and placebo arms.^{11,55} The general concern for a physician is introducing pruritus in an asymptomatic disease. However, in a recent abstract, the interim analysis of the REGENERATE trial showed lower patient-reported outcomes than the general population on a specific questionnaire (the chronic liver disease questionnaire-NASH), and the patient-reported out-

comes improved with OCA treatment.⁵⁶ The authors further argued that pruritus is present in 21% of individuals at baseline, which is also unclear. Female gender, gastrointestinal comorbidity, and psychiatric comorbidity were associated with clinically important low itch score. However, data suggested that OCA-related pruritus occurs early in the treatment, without any subsequent worsening or negative impact on patient-reported outcomes. Importantly, thus far, OCA is the only drug to have met the endpoint of fibrosis improvement.

Besides, OCA therapy increases small very low-density lipoprotein (VLDL) particles, large and small LDL particles, and reduces high-density lipoprotein particles at 12 weeks, which reverses after drug discontinuation.⁵⁷ Whether this leads to an increased risk of cardiovascular mortality in NASH patients is not yet known. Concurrent use of statins (in the CONTROL trial) lowered the LDL cholesterol to below baseline as early as 4 weeks after initiation and has an acceptable tolerability profile.⁵⁷ The FDA approved OCA in 2016 for use in primary biliary cholangitis at a dose of 5–10 mg/day for non-responders in Child A cirrhotics and non-cirrhotics. While the recommended dose for Child B/C cirrhotics is 5 mg/weekly, to a maximum dose of 10 mg twice weekly.⁵⁸

PPAR agonists

These are a group of nuclear receptor proteins that act as modulators of gene expression by functioning as transcription factors.⁵⁹ They have a role in lipid, protein and carbohydrate metabolism, as well as in cellular differentiation. Fibrates consist of fenofibrate, clofibrate, gemfibrozil, are PPAR α agonists. They help in breakdown and transport of fatty acid and are found in abundance in the liver, skeletal muscle, and endothelial cells.⁶⁰ Studies with fibrates in NASH did not show any difference in the improvement in either steatosis or fibrosis.^{61,62} A possible reason for their ineffectiveness in humans is because of the lower expression of PPAR α in humans when compared to mouse models.

Thiazolidinediones (TZD), pioglitazone, and rosiglitazone are PPAR γ agonists. PPAR γ , which is mainly located in the ad-

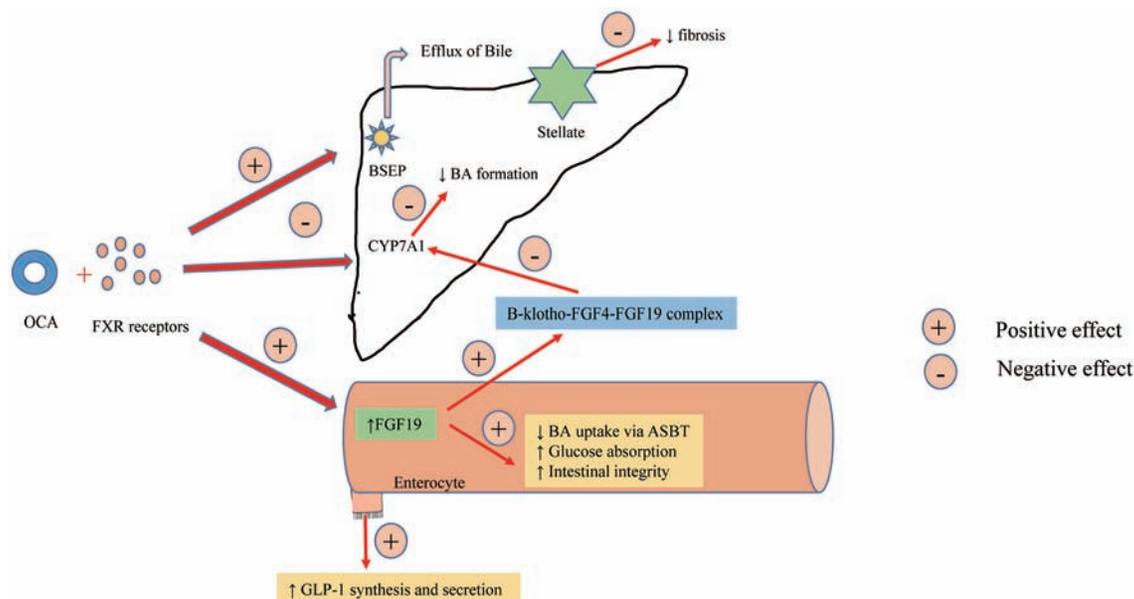


Fig. 2. Proposed mechanism of action of OCA in NASH. In the small intestine (orange cylinder) OCA binds to FXR receptors and through formation of the β -klotho-FGF4-FGF19 complex inhibits CYP7A1 and decreases bile acid synthesis. OCA increases the expression of the bile salt exporter protein BSEP and the multidrug resistance 3 protein MDR3, promoting efflux of bile from liver. OCA also decreases stellate cell (green star) activation, leading to decrease in fibrosis. OCA within the enterocytes leads to increased GLP-1 formation and improves insulin sensitivity. Abbreviations: ASBT, apical sodium-dependent bile acid transporter; BA, bile acid; CYP7A1, cytochrome P450 family 7 subfamily A member 1; FGF, fibroblast growth factor; GLP-1, glucagon-like peptide-1.

ipose tissue, plays a vital role in the regulation of adipocyte differentiation, adipogenesis, and lipid metabolism.⁶³ They have shown to improve glucose uptake and increase fatty acid oxidation, and insulin secretion, leading to improvement in insulin sensitivity.⁶⁴ Pioglitazone, a weak PPAR γ agonist, showed a significant decrease in serum ALT and total hepatic lipid content and an increase in adiponectin expression in mouse models.⁶⁵ In a trial of 55 patients with impaired glucose tolerance (or T2DM), pioglitazone administered with a hypocaloric diet improved steatosis and inflammation but not fibrosis.⁶⁶ Even in the PIVENS trial, there was no improvement in fibrosis.⁶ (Pioglitazone has been discussed in the previous paragraph). Rosiglitazone, a potent PPAR γ agonist, on the other hand, has shown some beneficial effects in rodent models.⁶⁷ Rosiglitazone improved steatosis and transaminase levels, despite significant weight gain.⁶⁴ Even prolonged therapy of rosiglitazone had no substantial improvement in NAS score.⁶⁸ A meta-analysis of four trials did not show improvement in fibrosis with the use of TZD therapy; although, there was a significant improvement in steatosis and inflammation.⁶⁹ Weight gain remains the major concern associated with the use of TZD.^{6,64,70}

PPAR β/δ agonists, are universally present in all cells of the body and are involved in regulating mitochondrial metabolism and fatty acid beta-oxidation.⁷¹ The data regarding the effectiveness of PPAR δ agonists for the treatment of NAFLD are limited, precluding formulation of any conclusions.⁷²

Dual agonists: Glitazars are a group of drugs that have PPAR α/γ agonism and can improve dyslipidemia and insulin resistance.⁷³ Muraglitazar and aleglitazar posed severe safety concerns for cardiovascular events and weight gain and were withdrawn.^{74,75} Saroglitazar, a dual PPAR α/γ agonist, was recently approved by the Drugs Controller General of India (known as the DCGI) for the treatment of NASH. Saroglitazar has promising results in the treatment of NASH. Elafibranor, a PPAR α/δ agonist, also had favorable results in preclinical trials. Bezafibrate is also a dual PPAR α/δ agonist and has some *in vitro* studies supporting its use in NASH, but there are no clinical studies on its efficacy in NASH.⁷⁶

Saroglitazar: PPAR α agonism is thought to affect fatty acid catabolism/dyslipidemia, while PPAR γ has an impact on glycemic control and insulin sensitization. A combination of fenofibrate (PPAR α) and rosiglitazone (PPAR γ) improved diabetic dyslipidemia and glycemic control.⁷⁷ Saroglitazar is a dominant PPAR α agonist and is effective in improving insulin sensitivity.^{78,79} Saroglitazar in mice has shown to ameliorate NASH through down-regulation of the hepatic lipopolysaccharide/toll-like receptor-4 pathway and inhibition of adipocyte dysfunction.⁸⁰ Saroglitazar prevents weight gain, normalizes liver enzymes, improves insulin resistance, dyslipidemia, and hepatic inflammation in NASH mice.⁸⁰ Saroglitazar also led to a significant change in adipokine levels, resulting in a substantial decrease in serum leptin and TNF- α level.⁸⁰ In NASH models, saroglitazar reduced hepatic steatosis, inflammation, ballooning, and fibrosis.⁸¹ It also reduced liver enzymes and the expression of inflammatory and fibrosis biomarkers. Saroglitazar led to a significant reduction in the NAS score, better than that achieved with pioglitazone and fenofibrate.^{81,82} The mechanism of action of saroglitazar is shown in Fig. 3. A recent randomized multicenter placebo-controlled trial using different doses of saroglitazar (the EVIDENCE IV trial) was presented as an abstract at the annual meeting of the American Association for the Study of Liver Diseases (AASLD). Saroglitazar at 4 mg improved dyslipidemia, hepatic steatosis, and insulin resistance, when compared to placebo (LO10 AASLD 2019).⁸³ Another phase 3 multicenter, double-blind, randomized study concluded that saroglitazar at 4 mg for 52 weeks improved NAS score, transaminitis, and lipid profile without fibrosis worsening [Abstract 1427, APASL liver meet 2020 Hepatol Int (2020) 14 (Suppl 1): S326].

Elafibranor: Elafibranor (GFT505) is a PPAR α/δ agonist under evaluation for the treatment of NASH. It has been shown in *in vivo* studies on Western diet-fed, human apolipoprotein E2 transgenic mice to improve steatosis, inflammation, and fibrosis.⁸⁴ The study also demonstrated that the drug decreased hepatic lipid accumulation and inhibited pro-inflammatory and profibrotic gene expression. In the initial multicenter, double-blind placebo-controlled randomized tri-

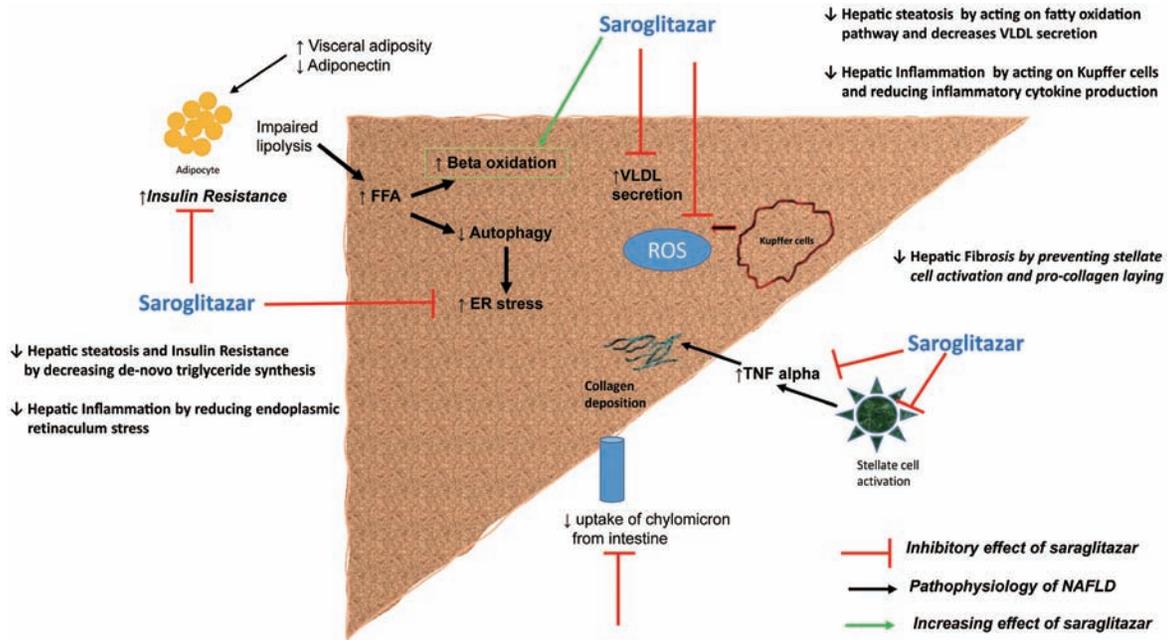


Fig. 3. Mechanism of action of saroglitazar in NASH. The pathophysiology of NASH is depicted in the pathway with black arrows. Saroglitazar increases beta-oxidation (green arrows) and decreases lipolysis (red arrows), leading to decrease in free fatty acids. In addition, it reduces ER stress and stellate cell activation (red arrows). This leads to decreased hepatic steatosis and inflammation, reduction in hepatic fibrosis, and improvement in insulin resistance. Abbreviations: ER, endoplasmic reticulum; FFA, free fatty acid IL-6, interleukin-6; ROS, reactive oxygen species; TG, triglyceride; TNF, tumor necrosis factor; VLDL, very low-density lipoprotein.

al (the GOLDEN-505 trial), elafibranor at 80 mg and 120 mg were compared against placebo for treatment of NASH for 52 weeks.⁸⁵ In the intention-to-treat (ITT) analysis, no difference was noted in the protocol-defined primary outcome, which was NASH resolution without fibrosis worsening. However, in a post ad hoc modified endpoint, NASH resolution without fibrosis worsening was higher with elafibranor at 120 mg compared to placebo (19% vs. 12%; $p=0.045$). Improvement in NAS score was seen in 20% of patients with elafibranor at 120 mg compared to only 11% in the placebo group ($p=0.018$).⁸⁵ Elafibranor was associated with a mild reversible rise in serum creatinine but had no adverse effects on cardiac profile or body weight. Recently, the disappointing results of the phase 3 trial on elafibranor (the RESOLVE-IT trial) were announced. In the ITT analysis of 1,070 patients, the response rate (NASH resolution without fibrosis worsening) was 19.2% for patients who received elafibranor at 120 mg compared to 14.7% for the placebo arm. Twenty-five percent of patients who received elafibranor at 120 mg achieved fibrosis improvement compared to 22.4% in the placebo arm. There was also no significant improvement in other biochemical parameters.

Lanifibranor (IVA337) is a pan-PPAR agonist, which has been shown to improve all the histological factors of NASH, including fibrosis, in experimental mouse models.⁸⁶ A phase 2 randomized placebo-controlled trial is under evaluation for assessing the safety and efficacy of lanifibranor in patients with T2DM and NAFLD (NCT03459079).

Arachidyl-amido cholanolic acid (aramchol): Aramchol is an inhibitor of stearoyl-Co-A desaturase 1 (known as SCD1), which is an enzyme located in the endoplasmic reticulum and catalyzes the rate-limiting step of monounsaturated fatty acid formation and prevents *de novo* lipogenesis.⁸⁷ Initial studies on the methionine and choline-deficient diet model of NASH showed down-regulation of SCD1, along with increasing flux through the trans-sulphuration pathway, thereby maintaining cellular redox homeostasis.⁸⁸

A double-blind, multicenter placebo-controlled trial on

biopsy-proven NASH comparing aramchol at 100 mg or 300 mg against placebo for 3 months concluded that there was significant reduction in hepatic fat content with aramchol at 300 mg.⁸⁹ An open-labeled safety study was conducted on 16 healthy volunteers (Abstract #2326 Liver meeting AASLD 2019). Twice-daily dosing with aramchol at 300 mg resulted in significantly higher exposures than once-daily dosing of aramchol at 600 mg. Both dosing regimens were safe and tolerable, without any adverse effects. Currently, a double-blind, placebo-controlled randomized phase 3 study with aramchol at 300 mg in subjects with NASH and F2-3 who are overweight (or obese) and have prediabetes or adequately controlled T2DM (the ARMOR study) is underway (NCT04104321).

Cenicriviroc (CVC): CVC is a dual human C-C motif chemokine receptor type 2 and 5 (CCR2/CCR5 chemokine) antagonist. CVC-mediated antagonism of CCR2 reduces the recruitment, migration, and infiltration of pro-inflammatory monocytes and macrophages at the site of liver injury.^{90,91} CCR5 antagonism by CVC is expected to additionally impair the migration, activation, and proliferation of collagen-producing activated hepatic stellate cells/myofibroblasts.^{91,92} The CENTAUR phase 2b study included patients with NAS score ≥ 4 and NASH-CRN fibrosis stage 1–3. The study concluded that CVC improved fibrosis in patients with NASH, and most of these improvements occur at year 1 and are maintained until the end of the 2nd year.^{93,94} Phase 3 (the AURORA study) trial is designed to strengthen the findings of this drug further.⁹⁵ In this multicenter, randomized, double-blind, placebo-controlled study (NCT030328740) of approximately 2,000 adults with histological evidence of NASH and F2-3 fibrosis will be randomized in 2:1 ratio to oral CVC 150 mg or placebo once daily. The primary efficacy endpoint includes the proportion of subjects with ≥ 1 -stage improvement in liver fibrosis and no worsening of steatohepatitis at 1 year. The results are expected by September 2020.

Glucagon-like peptide-1 (GLP-1) inhibitors: Liraglutide is a human incretin (GLP-1) agonist. Liraglutide allevi-

ated the features of metabolic syndrome in rats fed with a high-fat diet.⁹⁶ Liraglutide improved glucose tolerance, reduced weight gain, triglyceride levels, and liver fat accumulation.⁹⁶ The initial randomized study compared liraglutide at 1.8 mg against placebo for 12 weeks. Liraglutide reduced the body mass index, improved hepatic and adipose tissue insulin sensitivity, and also improved glycemic control, all of which form the major component in NASH pathogenesis.⁹⁷ A subsequent multicenter randomized phase 2 placebo-controlled trial (the LEAN trial) found that liraglutide is safe, well-tolerated, and leads to histological resolution of NASH (39% vs. 9% in placebo; $p=0.019$).⁹⁸ The progression of fibrosis was also more significant in the placebo arm (36% vs. 9%; $p=0.04$). Common side effects with liraglutide were gastrointestinal in 81% of patients, and the most common were nausea and diarrhea.⁹⁸ There are conflicting reports of increased incidence of pancreatic cancer and acute pancreatitis with this incretin analogue.^{99,100} However, further studies are ongoing comparing liraglutide and bariatric surgery in obese Asian NASH patients, and results are awaited (NCT02654665).

Semaglutide is another GLP-1 agonist discovered in 2012 and approved for the treatment of T2DM since 2017. It is currently being investigated for NASH. A double-blind placebo-controlled trial for 52 weeks with semaglutide and lifestyle modification has shown significant weight loss compared to liraglutide and placebo.¹⁰¹ The cardiovascular outcomes of 104 subjects with dose of semaglutide at 0.5 or 1.0 mg/week in T2DM (the SUSTAIN-6 trial; NCT01720446) and a 52-week weight management trial with a dose of semaglutide at 0.05-0.4 mg/day (NCT02453711) were analyzed. Semaglutide had cardioprotective effects in T2DM patients. Among subjects treated with semaglutide (especially at 0.4 mg/day), the proportion of patients with metabolic syndrome approximately halved during the trial compared with the baseline.¹⁰² Semaglutide also reduced inflammatory markers and aminotransferases.¹⁰² The investigation of semaglutide at 0.1, 0.2 and 0.4 mg/day for NASH resolution without fibrosis worsening after 72 weeks of therapy has recently been completed, and the results are expected (NCT02970942).

Galectin-3 inhibitors (GT-MD-02): Galectins are conserved proteins with the ability to bind β -galactosides through carbohydrate-recognition domains.¹⁰³ Galectin-3 contains a C-terminal carbohydrate-recognition domain linked to an N-terminal protein-binding domain and is a unique chimeric galectin.¹⁰³ In the cytoplasm, galectin-3 is vital for cell survival due to its interaction with specific survival-associated proteins. In the nucleus, galectin-3 promotes pre-mRNA splicing and regulates gene transcription, whereas extracellular galectin-3 modulates cell-cell interactions. Thus, it is involved in cell differentiation, inflammation, fibrogenesis, and the host defense.¹⁰⁴

Galactoarabino-rhamnogalacturonan, belapectin (GR-MD-02), binds mostly to galectin-3 receptors and has been hypothesized to manipulate the upstream events in the pathogenesis of NASH, which leads to substrate overload.¹⁰⁵ The sequential dose-ranging, placebo-controlled, double-blinded safety study in biopsy-proven NASH patients with advanced fibrosis (Brunt stage 3) revealed no difference in adverse effects when GR-MD-02 single or three weekly repeated doses of 2, 4 or 8 mg/kg was used.¹⁰⁶ Galectin-3 ablation protects from diet-induced NASH by decreasing hepatic advanced lipoxidation end products' accumulation, with attenuation of inflammation, hepatocyte injury, and fibrosis.¹⁰⁷ A multicenter phase 2b, randomized trial has enrolled patients with NASH, cirrhosis, and portal hypertension to randomly receive biweekly infusions of belapectin at a dose of 2 mg/kg or 8 mg/kg or placebo for 52 weeks. Although belapectin was safe, it was not associated with a significant reduction in hepatic venous pressure gradient

(HVPG) or fibrosis compared to placebo. In the subgroup analysis of patients without esophageal varices, 2 mg/kg belapectin did reduce HVPG and development of varices.¹⁰⁸

Emricasan: Emricasan is a pan-caspase inhibitor that acts on the final apoptotic pathway involved in the pathogenesis of NASH. In a murine model of NASH, hepatocyte apoptosis was attenuated by emricasan, which led to an improvement in fibrosis, bringing forth the use of emricasan as an antifibrotic agent in NASH.¹⁰⁹ A subsequent multicenter study involving cirrhotic patients (etiology: alcohol, hepatitis C virus, and NASH) demonstrated a significant reduction in model for end-stage liver disease (MELD score), Child-Pugh scores, international normalized ratio, and total bilirubin in patients with MELD scores ≥ 15 following emricasan 25 mg.¹¹⁰ A multicenter, double-blind, randomized trial recruited 263 patients with NASH-related cirrhosis and baseline HVPG of ≥ 12 mmHg. These patients were treated with twice-daily with oral emricasan at 5 mg, 25 mg or 50 mg, or placebo in a 1:1:1:1 ratio for up to 48 weeks. The primary endpoint was change in HVPG (Δ HVPG) at week 24. Secondary endpoints were changes in biomarkers (aminotransferases, caspases, cytokeratins) and development of liver-related outcomes. Although emricasan was safe, there was no reduction in HVPG or biomarkers.¹¹¹ Similarly, another randomized placebo-controlled trial with NASH patients with F1-F3 fibrosis with emricasan (5 mg or 50 mg) for 72 weeks did not improve liver histology in patients with NASH fibrosis and showed a trend towards worsening of fibrosis and ballooning.¹¹² This drug is unlikely to hold promise.

Selonsertib: Selonsertib is an inhibitor of apoptosis signal-regulating kinase 1 (ASK1), with potential anti-inflammatory and antifibrotic properties. A preliminary study of NASH was conducted with F2-3 fibrosis patients treated with selonsertib at 6 mg or 18 mg orally alone or in combination with simtuzumab (125 mg subcutaneously weekly) or simtuzumab alone for 24 weeks. The study demonstrated a decrease in hepatic collagen with the use of selonsertib.¹¹³ A follow-up study that used magnetic resonance imaging (MRI)-based evaluation of fibrosis assessment in patients receiving selonsertib showed a positive trend warranting further investigations.¹¹⁴ Two subsequent randomized, double-blind, placebo-controlled, phase 3 trials of selonsertib in patients with NASH and bridging fibrosis F3 (STELLAR-3 trial) or compensated cirrhosis (STELLAR-4 trial) were conducted, wherein patients were randomized 2:2:1 to receive selonsertib at 18 mg, selonsertib at 6 mg, or placebo once daily for 48 weeks.¹¹⁵ Neither of these trials met the primary endpoint, and no improvement in fibrosis was noted.

Tipelukast: MN-001 is an antifibrotic and anti-inflammatory molecule that acts by antagonizing leukotriene receptors (referred to here as LT), phosphodiesterases, and 5-lipoxygenase (referred to here as 5-LO).¹¹⁶ The inhibitory effect of tipelukast on the 5-LO/LT pathway may contribute to its antifibrotic effects. In an interim report of nine NASH/NAFLD patients with hypertriglyceridemia who completed tipelukast (MN-001) at 250 mg qd for the first 4 weeks, tipelukast significantly reduced triglycerides.¹¹⁷ The complete results are awaited (NCT02681055).

Volixibat: Volixibat (SHP626) is a potent inhibitor of the apical sodium-dependent bile acid transporter (ASBT). In the initial phase 1 study, the absorption of the drug was found to be very low after oral ingestion.¹¹⁸ The drug was reviewed based on the hypothesis that ASBT inhibition in the terminal ileum would facilitate the removal of free cholesterol in the liver by reducing the recirculation of bile acids to the liver and promoting new bile acid synthesis. A randomized controlled trial comparing volixibat at 0.5 mg, 1 mg, 5 mg, or 10 mg against placebo for 28 days in healthy volunteers and T2DM patients showed increased fecal excretion of bile acids with an elevation of serum C4 levels in both groups.¹¹⁹ A recent randomized, double-blind trial

recruited steatosis and non-cirrhotic NASH patients treated with volixibat at 5 mg, 10 mg, or 20 mg or placebo once daily for 48 weeks.¹²⁰ The study failed to reach the primary endpoint, which was defined as a $\geq 5\%$ reduction in MRI-proton density fat fraction (PDFF) and $\geq 20\%$ reduction in serum ALT level at interim analysis at 24 weeks, and the study was terminated.

NS-0200: The 5' adenosine monophosphate-activated protein kinase (AMPK)/Sirtuin 1 (Sirt1) pathway is a crucial regulator of mitochondrial biogenesis, energy, and lipid metabolism.¹²¹ Activation of this pathway may reverse or at least prevent excess hepatic lipid accumulation and inflammation. L-leucine is an activator of the SIRT1/AMPK pathway, serving as a partial mimetic of calorie restriction in preclinical models, and thereby modulates lipid and energy metabolism and increases insulin sensitivity.¹²² The combination of leucine with low-dose metformin reversed hepatic steatosis in preclinical studies in murine models of T2DM and NASH. Adding low-dose sildenafil enhances this effect by stimulating endothelial nitric oxide synthase activity, leading to exacerbated stimulation of Sirt1 and thereby attenuating inflammation and fibrosis.^{123,124} NS-0200 is a leucine-metformin-sildenafil fixed-dose combination that may reduce hepatic steatosis and improve metabolic parameters. The study revealed that high-dose NS-0200 relatively reduced hepatic fat by 15.7% from baseline in the high ALT group ($p < 0.005$), while no such benefit was seen at lower doses.¹²⁵ Further studies are underway for this combination. The effect of high- and low-dose NS-0200 on the fat content (MRI-PDFF) is being assessed in a randomized, 16-week, placebo-controlled, double-blind study in NAFLD patients (NCT02546609).

Conclusions

Although many drugs fared well in animal models, the clinical utility of these drugs is limited in humans. The animal models are usually controlled for various confounders, and hence they may be an accurate representation of disease. However, no animal models can completely replicate the heterogeneous nature and physiological condition of human beings. The response to an injury is different in each individual. Hence, very few drugs have managed to progress into use in clinical practice. Despite many drugs being under evaluation, the only established treatment of NASH at this point is weight loss. Medications are currently used with no FDA approval as an adjunct to lifestyle changes in patients with biopsy-proven NASH. The two drugs ahead in the pipeline are OCA, which is awaiting FDA approval and saroglitazar, which has been approved only in India for use in NASH, although long-term data on fibrosis improvement are still awaited.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Conceptualization and study design (MS, AVK), data acqui-

sition (MS, MP), initial drafting of the manuscript (MS, AVK, MP), figure generation (MS, PK), critical assessment of the manuscript, and provision of intellectual input (DNR, PNR), article guarantor (AVK). All authors approved the final draft.

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New Drugs on the Block—Emerging Treatments for Nonalcoholic Steatohepatitis

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Abstract

Patients with nonalcoholic steatohepatitis (NASH) are at higher risk of progression to advanced stages of fibrosis, cirrhosis, hepatocellular carcinoma and other end-stage liver disease complications. When addressing treatment of NASH, we have limited approved options, and the mainstay of therapy is lifestyle intervention. Extensive research and revelation in the field of pathogenesis of NASH has offered new possibilities of treatment and emerging new drugs that are being tested currently in numerous preclinical and clinical trials. These drugs target almost all steps in the pathogenesis of NASH to improve insulin sensitivity, glucose and lipid metabolism, to inhibit *de novo* lipogenesis and delivery of lipids to the liver, and to influence apoptosis, inflammation and fibrogenesis. Although NASH is a multifactorial disease, in the future we could identify the predominating pathological mechanism and, by choosing the most appropriate specific medication, tailor the treatment for every patient individually.

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Introduction

Nonalcoholic steatohepatitis (NASH) and nonalcoholic fatty liver are histologically two distinguishable subtypes of nonalcoholic fatty liver disease (NAFLD). Nonalcoholic fatty liver requires more than 5% fat infiltration of the liver and NASH, alongside fat infiltration, is characterized by inflammation

and hepatocyte injury.¹ Although hepatic steatosis within NAFLD is a widespread disease, with prevalence in some parts of the world up to 40%,² NASH is present in only 10% to 20% of individuals with NAFLD, but when accompanied by significant fibrosis is associated with increased overall mortality, primarily from cardiovascular diseases.¹ Population projection models estimate that 3% to 6% of adults have NASH, and according to current trends, the prevalence of NASH is expected to rise by 15% to 56% until 2030.^{3,4}

Patients with NASH have a higher risk of progression to advanced stages of fibrosis, cirrhosis, hepatocellular carcinoma and other end-stage liver disease complications.⁵ At the time of NASH diagnosis about 25% of patients have a moderate to severe stage of fibrosis (F>2), and in around 40% of NASH patients fibrosis will progress at a rate of 1 stage per 10 years.⁶ Although often clinically silent, more than 20% of patients with NASH will develop end-stage liver disease over their lifetime.^{7,8} A meta-analysis of 86 studies and more than 8 million patients from 22 countries has shown that in comparison with NAFLD, NASH has greater overall mortality (11.77 to 0.77 per 1,000 person-years) and liver related mortality (25.56 to 15.44 per 1,000 person-years).⁶

When addressing treatment of NASH, we have limited approved options, and the mainstay of therapy is lifestyle intervention, including changes in diet and exercise regimes, with an emphasis on weight reduction of more than 7%.^{1,9,10}

Several drugs have been proposed for treating NASH, but according to the guidelines of European and American societies, metformin (an insulin sensitizer) is not recommended because it showed no effect on liver histology although it has a beneficial effect on insulin resistance and alanine aminotransferase (ALT) levels.^{1,11} Pioglitazone, a thiazolidinedione and intra-nuclear peroxisome proliferator-activated receptor (PPAR) γ agonist that is in use for diabetes mellitus treatment, was evaluated in several trials for the treatment of NASH. In the PIVENS randomized controlled trial, patients with biopsy-proven NASH without diabetes mellitus received vitamin E (800 IU/day) and pioglitazone (30 mg/day) for 96 weeks and were compared with patients who received placebo.¹² The therapy with vitamin E was associated with amelioration of NASH (43% vs. 19%); however, when compared with the placebo, the pioglitazone therapy was not that successful (34% and 19%).¹² Both therapies were associated with reduction of hepatic steatosis, inflammation and liver laboratory tests but without an improvement in fibrosis scores.¹² Pioglitazone is, however, associated with substantial side-effects, such as weight gain, fluid retention, heart failure and bone loss, so the American Association for the Study of Liver Diseases 2018 Guidelines state that pioglitazone may be used in biopsy-proven NASH

Keywords: Fatty liver; Steatohepatitis; Nonalcoholic fatty liver disease; Treatment.

Abbreviations: ALT, alanine aminotransferase; ASK-1, apoptosis signal-regulating kinase 1; BMI, body mass index; CCR, C-C chemokine receptor type; DPP4, dipeptidyl peptidase 4; FGF, fibroblast growth factor; FXR, farnesoid X receptor; GLP-1, glucagon-like peptide-1; MAFLD, metabolic-associated fatty liver disease; MR, magnetic resonance; NAFLD, nonalcoholic fatty liver disease; NAS, NAFLD activity score; NASH, nonalcoholic steatohepatitis; NF κ B, nuclear factor kappa B; PPAR, peroxisome proliferator-activated receptor; SGLT, sodium-glucose co-transporter.

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Table 1. New drugs in phase 2 and phase 3 clinical trials

Agent	Mechanism of action	Treatment/intervention	Patients	Phase	ClinicalTrials.gov identifier
Elafibranor	Dual PPAR α and PPAR δ agonist	Elafibranor (120 mg) vs. placebo	NASH (NAS>4) and F2/F3	3	NCT02704403
Saroglitazar	Dual PPAR α and PPAR γ agonist	Saroglitazar (1,2 or 4 mg) vs. placebo	NASH and/or NAFLD (biopsy or noninvasive)	2	NCT03061721
Lanifibranor	pan-PPAR agonist	Lanifibranor (800 mg and 1,200 mg) vs. placebo	NASH (biopsy)	2	NCT03008070
Liraglutide	GLP-1 receptor agonist	Liraglutide (0,6–3 mg) vs. exercise + diet vs. bariatric surgery	NASH (biopsy or noninvasive)	3	NCT02654665
Semaglutide	GLP-1 receptor agonist	Semaglutide (0.1, 0.2, 0.4 mg) vs. placebo	NASH (biopsy)	2	NCT02970942
Dapagliflozin	SGLT inhibitor	Dapagliflozin (10 mg) vs. placebo	NASH and DM (biopsy)	3	NCT03723252
Pegbelfermin	FGF21 analog	Pegbelfermin (3 doses) vs. placebo	NASH and F3 (biopsy)	2	NCT03486899
Cenicriviroc	CCR2/CCR5 antagonist	Cenicriviroc (150 mg) vs. placebo	NASH and F2/F3 (biopsy)	3	NCT03028740
Tropifexor	FXR agonist	Tropifexor monotherapy vs. combination with cenicriviroc	NASH with F2/F3 (biopsy)	2	NCT03517540
Resmetirom	Thyroid hormone receptor β agonist	Resmetirom (80 mg, 100 mg) vs. placebo	NASH (NAS>4)	3	NCT03900429
Firsocostat	Acetyl-CoA carboxylase inhibitor	Firsocostat vs. fenofibrate, cilofexor, selonsertib	NASH (F2/F3 and F4/cirrhosis)	2	NCT02781584, NCT03449446

Abbreviations: CCR2/CCR5, C-C chemokine ligand types 2 and 5; DM, diabetes mellitus; FGF21, fibroblast growth factor-21; FXR, farnesoid X receptor; GLP-1, glucagon-like peptide-1; NAFLD, nonalcoholic fatty liver disease; NAS, NAFLD activity score; NASH, nonalcoholic steatohepatitis; PPAR, peroxisome proliferator-activated receptor; SGLT, sodium-glucose co-transporter.

patients after discussing the risks and benefits.¹ Vitamin E has been associated with increased incidence of intracranial bleeding and prostate cancer; however, NASH patients who could have benefited from vitamin E were not included in the study nor did the study take into account other confounding factors (smoking, supplements).¹³ Orlistat, as well as lipid lowering agents used in treating hyperlipidemia, have been used in patients with NASH, and although their use is safe, the outcomes of their effect on treating NASH are inconclusive.^{1,11}

As a result of better understanding the underlying processes in the development of NAFLD and NASH, as well as a long withstanding opinion that the nomenclature for NAFLD and NASH doesn't suffice, an international working group represented by Eslam *et al.*¹⁴ proposed a change in nomenclature to metabolic-associated fatty liver disease or "MAFLD".¹⁴ This change would, in their opinion, better depict the character of this disease and patients that suffer from it, and alienate the labeling alcoholic burden. This could also propose a change in the study design of pre-clinical and clinical trials that would take into consideration the specific heterogeneity of the pathological pathways and specific characteristics of the patients and thus give more reliable results for the studied drugs.¹⁴

Extensive research and revelation in the field of pathogenesis of NASH has offered new possibilities of treatment and emerging new drugs that are being tested now in numerous preclinical and clinical trials (Table 1). These drugs target almost all steps in the pathogenesis of NAFLD and NASH, aiming to improve insulin resistance, glucose and lipid metabolism, to inhibit lipogenesis and delivery of lipids to the liver, and to influence apoptosis, inflammation and fibrogenesis.¹⁵ Figure 1 depicts the proposed mechanisms of action of these agents in the pathogenesis of NAFLD and NASH.

Intestinal microbiome and associated dysbiosis has

emerged as an important factor that indirectly, through intestinal permeability and bacterial metabolites, influences several mechanisms responsible for NASH progression. The therapeutic options that could influence these steps are depicted in Figure 2.

Drugs improving insulin sensitivity and modulating glucose and lipid metabolism

PPARs

PPARs are nuclear receptors that regulate metabolic homeostasis, cell differentiation and immune-inflammation.¹⁶ We distinguish several PPAR intracellular receptor subtypes, as they exert different distribution and actions in different tissues.¹⁶ The first extensively studied PPAR γ agonist, pioglitazone, already mentioned in the introduction, modulates glucose uptake, insulin signaling, fatty acid uptake, triglyceride synthesis and hydrolysis, as well as inflammation and maturation of macrophages.¹⁶

PPAR α is most extensively pronounced in the liver and exerts its regulatory roles through fatty acid transport and β -oxidation to deliver lipids in the liver, and has been shown to decrease triglycerides and increase high-density lipoprotein cholesterol in serum.¹⁷ It also influences inflammation through nuclear factor-kappa B (NF κ B) action modulation and reduces the expression of acute-phase genes.¹⁷ PPAR γ is mostly expressed in the adipose tissue and controls lipogenesis, adipocyte differentiation, and glucose metabolism.¹⁸ By promoting the storage of fatty acids, such as triglycerides, PPAR γ acts as an insulin sensitizer and prevents ectopic fat accumulation.^{18,19} Maeda *et al.*²⁰ showed that PPAR γ agonists caused a significant rise in plasma adiponectin concentrations. Adiponectin is a protein derived

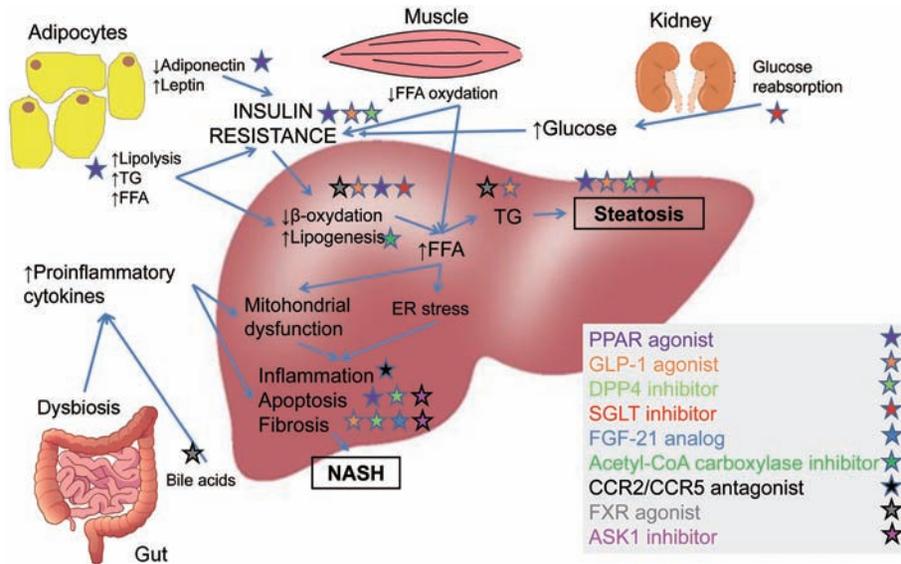


Fig. 1. Proposed mechanisms of action of new agents for treatment of NASH. Abbreviations: ASK-1, apoptosis signal-regulating kinase 1; CCR2/CCR5, C-C chemokine ligand types 2 and 5; DPP4, dipeptidyl peptidase 4; ER, endoplasmic reticulum; FFA, free fatty acids; FGF21, fibroblast growth factor-21; FXR, farnesoid X receptor; GLP-1, glucagon-like peptide-1; NASH, nonalcoholic steatohepatitis; PPAR, peroxisome proliferator-activated receptor; SGLT, sodium-glucose co-transporter; TG, triglycerides.

from fat tissue that possesses anti-atherogenic properties and suppresses cytokine production from macrophages and expression of adhesion molecules in vascular endothelial cells. PPAR δ agonists produce similar effects as PPAR α on liver lipid metabolism, exhibit a positive influence on insulin sensitivity, and promote an alternative activation effect on macrophages and Kupffer cells that leads to attenuation of tissue inflammation.^{21,22}

Elafibranor is a dual PPAR α and PPAR δ agonist, that was investigated in a phase II multicenter, randomized placebo-controlled study at two dose regimes (80 mg and 120 mg once a day versus placebo) over 52 weeks in biopsy-proven NASH patients.²³ Treatment with 120 mg of elafibranor reduced liver enzymes, lipid values, glucose profiles,

and markers of systemic inflammation in comparison with placebo, and there was a statistically significant amelioration of NASH activity without aggravation of fibrosis.²³ Furthermore, the patients that experienced NASH regression had a significant decline in fibrosis stage compared to those without NASH regression.²³ Elafibranor, although, was well-tolerated and caused an increase in serum creatinine level that was reversible.²³ A phase III multicenter study (RESOLVE-IT) on elafibranor (120 mg per day versus placebo) is ongoing and planned to enroll 2,000 patients with liver biopsy-proven NASH (NAFLD activity score (NAS) of >4) and F2-3 fibrosis (NCT02704403).²⁴

Saroglitazar is a dual PPAR α and PPAR γ agonist that exhibits a predominant PPAR α effect with a moderate PPAR γ

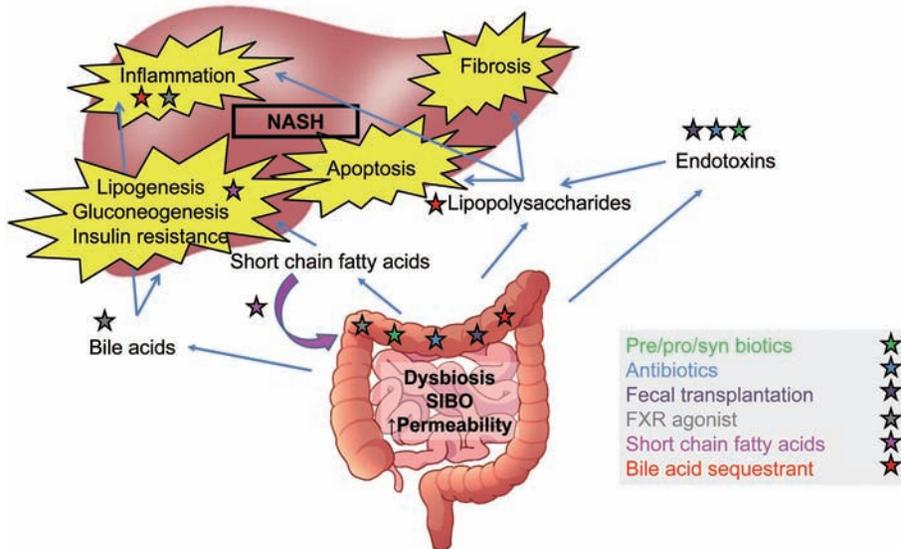


Fig. 2. Proposed mechanism of action of treatments for NASH targeting microbiome changes. Abbreviations: NASH, nonalcoholic steatohepatitis; FXR, farnesoid X receptor; SIBO, small intestinal bacterial overgrowth.

effect; as such, it provides a positive effect on lipid metabolism and insulin sensitivity without the side effects caused by PPAR γ activation.²⁵ Since it has a nonrenal route of elimination, it has been shown to be safe in patients with deteriorated renal function.²⁵ A review of 18 selected studies on patients with diabetic dyslipidemia that were treated with saroglitazar 4 mg once daily for at least 12 weeks showed a consistent mean regression in lipid levels and glycosylated hemoglobin levels with an increase in mean high-density lipoprotein cholesterol levels from baseline as well as an incline in ALT levels and fatty liver (evaluated by FibroScan™) in NAFLD patients with diabetic dyslipidemia.²⁶ There is an ongoing phase II study on saroglitazar on NASH/NAFLD patients that will investigate the safety of treatment and impact on serum ALT levels (NCT03061721).²⁷

Lanifibranor is a next-generation pan-PPAR agonist that in a NAFLD mouse model improved insulin resistance and steatohepatitis (biopsy-assessed hepatic steatosis, inflammation, ballooning and fibrosis), that combines and exceeds specific effects of the single PPAR agonists.¹⁸ Currently, efficacy and the safety of two doses (800 mg and 1,200 mg) of lanifibranor per day for 24 weeks is being evaluated in a phase II study versus placebo in adult NASH patients with moderate to severe necroinflammation without cirrhosis (NCT03008070).

Glucagon-like peptide receptor agonists and dipeptidyl peptidase 4 inhibitors

Glucagon-like peptide-1 (GLP-1), recognized as physiologic incretin, is a hormone secreted from the distal ileum and colon that increases insulin synthesis and secretion, decreases glucagon secretion, decreases hepatic gluconeogenesis, suppresses appetite, and delays gastric emptying.²⁸ It has been shown that hepatocytes express GLP-1 receptors and that GLP-1 agonists reduce steatosis and influence lipid metabolism by decreasing lipogenesis and increasing oxidation of fatty acids.²⁹

Liraglutide, a first class GLP-1 receptor agonist, was studied on a hepatic stellate cell (rat and human) model, and it was found that liraglutide markedly improved the stellate cell phenotype and diminished cell proliferation.³⁰ Rats with cirrhosis treated with liraglutide had lower portal pressure, lower intrahepatic vascular resistance, and significant improvement in fibrosis and endothelial function.³⁰ These antifibrotic effects of liraglutide therapy were also recorded in human liver and the proposed mechanism is an GLP1-R-independent and NF κ B-Sox9-dependent one.³⁰ Furthermore, metabolic and hepatic beneficiary effects of liraglutide were studied in an obese NASH mouse model, and found reduced body weight, reduced hepatic steatosis, and reduced collagen 1a1 and galectin-3 content.³¹ In a randomized, phase 2 study, liraglutide was compared to placebo in obese, biopsy-confirmed NASH patients.³¹ After the treatment with liraglutide, 40% of patients had NASH resolution compared with 9% in the placebo group. Only 9% of patients on liraglutide versus 36% of patients on placebo had progression of fibrosis.³² Results also showed improvement in the metabolic risk factors such as weight, glucose and high-density lipoprotein cholesterol levels; the main reported adverse events were mild to moderate, and included diarrhea, constipation and loss of appetite.³² Currently ongoing is a phase 3 study that is comparing effects of liraglutide and bariatric surgery on anthropometric measures, liver function, insulin resistance, endothelial function and biomarkers of NASH (CGH-LiNASH, NCT02654665). A recently published study on 30 obese NAFLD patients showed that liraglutide administered at 3 mg daily for 26 weeks, followed by 26 weeks of only weight gain prevention, compared with diet and exercise

modifications only, had significant reductions in weight, liver fat fraction (measured by magnetic resonance imaging), serum ALT and caspase-cleaved cytokeratin-18 at 26 weeks.³³ However, those benefits were not sustained after discontinuation of treatment, in contrast with effects of lifestyle modification.³³

Exenatide, also a GLP-1 receptor agonist, in a NASH mouse model showed an improvement of mitochondrial tricarboxylic acid cycle flux after a 8-week treatment, a significant decrease in insulin resistance, steatosis, hepatocyte lipotoxicity and hepatic triglyceride content as well as lower expression of hepatic lipogenic genes (Srebp1C, Cd36) and genes involved in inflammation and fibrosis.³⁴ Exenatide treatment for 12 weeks (5 mg twice daily for 4 weeks then 10 mg twice daily for 8 weeks) combined with insulin glargine in diabetic, obese, NAFLD patients was associated with a greater reduction of body weight, waist circumference, liver fat (appraised by ultrasound) and liver enzymes than with intensive insulin therapy (93% vs. 67%); at the end of treatment, up to 43% of patients had no liver steatosis.³⁵ The most common side effects were similar to liraglutide, and were found in up to 40% of patients.³⁵ A relatively small open-label study on eight patients with diabetes mellitus and biopsy-proven NASH found that 28 weeks of exenatide treatment made no significant difference in liver histology, and only three of the eight subjects did meet the primary end point of improved histopathology, with 1 to 2 point fibrosis improvement seen in four subjects and fibrosis worsening by 1 point in one subject and staying the same in three subjects.³⁶ More studies on exenatide are needed to draw firmer conclusions on its role in treating NASH patients.

Semaglutide, a novel GLP-1 receptor agonist, was investigated in a recently completed phase 2 placebo-controlled trial comparing the efficacy and safety of different doses in 320 NASH patients and the results are awaited (NCT02970942). Since NASH patients have a greater risk of cardiovascular mortality, semaglutide could have potential benefit compared to other GLP-1 receptor agonists, since it was shown to be able to prevent cardiovascular events as well as reduce body mass and ALT level.^{37,38}

Dipeptidyl peptidase 4 (DPP4) inhibitors exert their effect by blocking the enzyme DPP4, which is involved in the degradation of GLP-1 and other incretins.²⁸ Serum DPP4 levels are elevated in NASH patients and correlate well with the histopathological severity of NASH. DPP4 levels are also positively associated with liver fibrosis and hepatocyte apoptosis.²⁸ Sitagliptine, an DPP4 inhibitor, prevented infiltration of adipose tissue by CD8(+) T-cells and M1 macrophages, decreased PAI-1 expression, and had a positive effect on liver lipid metabolism and liver fat infiltration.³⁹ It was also shown in a mouse model that sitagliptine could prevent the progression of hepatocellular carcinoma related to NASH.⁴⁰ However, a relatively small study on biopsy-proven NASH patients after 24 weeks of sitagliptine (100 mg) showed no improvement of fibrosis or NAS versus placebo.⁴¹ Conflicting results were found in larger studies on biopsy-confirmed NASH patients, where the same dose of sitagliptine (100 mg) given for 1 year improved NAS by ameliorating steatosis and ballooning, regardless of diabetic state,⁴² and in another 24-week administration trial showed no superiority compared to placebo in reducing liver fat infiltration in prediabetic patients with NAFLD or those with diabetes mellitus and NAFLD.⁴³ Linagliptine, another DPP4 inhibitor, was shown to have both anti-inflammatory and antisteatotic activity in NASH.⁴⁴ It was evaluated in a NASH mouse model as well, in combination with empagliflozine, a sodium-glucose co-transporter (SGLT)-2 inhibitor. A combination of linagliptine and empagliflozine ameliorated NASH with a stronger anti-fibrotic effect.⁴⁵ There are still no human studies being conducted

to verify the safety and utility of linagliptine in the treatment of NASH.

SGLT inhibitors

SGLT inhibitors act by reducing glucose reabsorption in the proximal tubule in the kidney, leading to glucosuria and plasma glucose reduction.⁴⁶ They have been used in treating diabetes mellitus, but as several mouse model studies showed beneficial effect on liver function and prevention of fibrosis associated with NASH, it has become an interesting option for treating NASH in humans.^{47,48} Ipragliflozin, a SGLT2 inhibitor was investigated in a NASH mouse model, where ipragliflozin had a positive effect on free fatty acid serum concentration, liver lipid metabolism, reduced apoptosis and fibrosis.⁴⁶ The same results were confirmed in a similar study using a mouse NASH model and 4-week therapy, in which ipragliflozin improved glucose metabolism, reduced insulin resistance, and improved liver steatosis and fibrosis by reducing inflammation and oxidative stress in the liver.⁴⁸ In humans, in patients with diabetes mellitus type 2 and NAFLD, ipragliflozin reduced liver fat (as estimated indirectly by calculating liver fat index).⁴⁹ A Japanese study retrospectively included 130 diabetes mellitus patients with proven NASH, and selected patients with altered liver enzymes when adding ipragliflozin to their DPP-4 inhibitor or GLP-1 receptor antagonist therapy, and found after treatment significantly decreased ALT and the Fibrosis-4 score.⁵⁰

In a randomized, active-controlled, open-label trial on 57 patients with diabetes mellitus type 2 and NAFLD who were treated with dapagliflozin (5 mg/d) for 24 weeks or placebo, hepatic steatosis and stiffness were assessed noninvasively (by transient elastography and controlled attenuation parameter).⁵¹ Based on their findings, dapagliflozin improved liver steatosis in diabetes mellitus type 2 and NAFLD patients, and ameliorated liver fibrosis only in patients with significant liver fibrosis assessed non-invasively by transient elastography.⁵¹ In a randomized placebo-controlled double-blind multicenter study on participants with diabetes mellitus type 2 and NAFLD, dapagliflozin monotherapy reduced liver serum markers, cytokeratin (CK) 18-M30 and CK 18-M65, and plasma fibroblast growth factor (FGF)-21.⁵² A phase 3 study on the histological efficacy and safety of dapagliflozin in NASH patients is ongoing (NCT03723252).

The effect of empagliflozin on liver steatosis in type 2 diabetes mellitus patients and NAFLD was the focus of an investigator-initiated, prospective, open-label, randomized clinical study to examine the effect of 10 mg of empagliflozin per day when included in the treatment of type 2 diabetes mellitus versus standard treatment without empagliflozin.⁵³ Hepatic liver fat was measured by magnetic resonance (MR) imaging proton density fat fraction, and found that the empagliflozin group had a significant reduction of liver fat and ALT levels.⁵³ A multicenter study on patients with diabetes mellitus type 2 to evaluate the impact of empagliflozin (25 mg daily) or placebo for 24 weeks on lipid content, liver energy metabolism and body composition evaluated by 1H magnetic resonance (MR) spectroscopy was recently completed, and results are awaited (NCT02637973).

FGF21 analog (pegbelfermin)

FGF21 is a regulator of energy metabolism and in a study on patients with NAFLD (defined by MR proton spectroscopy) and NASH (defined by biopsy), plasma FGF21 levels were higher in patients with NASH compared to those without NASH or NAFLD. Plasma FGF21 levels correlated positively

with the stage of necroinflammation ($p=0.02$) and fibrosis ($p<0.001$) but not with steatosis ($p=0.60$).⁵⁴ Endogenous FGF21 has a short half-life of 1 to 2 hours, but various modification strategies have been used to create longer acting FGF21 analogues.^{55,56} Pegbelfermin is a recombinant analog of human FGF21 that was evaluated in a multicenter randomized, double-blind, placebo-controlled study in biopsy-proven NASH in overweight adults (body mass index of >25 kg/m²) in subcutaneous two dose group administration (10 mg daily and 20 mg weekly).⁵⁶ After 16 weeks of treatment, patients in both groups showed a significant amelioration of steatosis (assessed by MR spectroscopy), levels of noninvasive fibrosis biomarker (N-terminal type III propeptide), and amelioration of liver stiffness and transaminase levels.⁵⁶ A phase 2b randomized, double-blind, placebo-controlled study evaluating the safety and efficacy of pegbelfermin in patients with NASH and stage 3 fibrosis is active but not recruiting any new patients. The primary outcome was determined as achievement of ≥ 1 stage amelioration in fibrosis without progression of NASH or NASH improvement with no progression of fibrosis (as determined by liver biopsy) (NCT03486899). Results are awaited.

Statins

Although statins have been extensively used in treating cardiovascular diseases, their use has been widely underestimated in treating NASH, probably due the common misinterpretation that statins damage the liver, as seen in elevation of liver enzymes during treatment. In a multicenter cohort of 1,201 European individuals who underwent liver biopsy for suspected NASH, statin use was recorded in 107 subjects and was associated with an improvement in liver steatosis, NASH and fibrosis stage F2–F4 development; however, this effect was limited in patients with the I148M PNPLA3 variant.⁵⁷

Resmetirom (thyroid hormone receptor β -agonist)

Resmetirom, a thyroid hormone receptor β -agonist has been shown in a mouse model to reduce liver steatosis by targeting dyslipidemia.⁵⁸ In a 36-week randomized, double-blind, placebo-controlled multicenter study on adults with biopsy-confirmed NASH (fibrosis stages 1–3) and hepatic fat fraction of at least 10% (assessed by MR proton density fat fraction) resmetirom treatment resulted in improvement in steatosis in NASH patients.⁵⁹ Adverse events were mostly mild or moderate, with a higher incidence of transient diarrhea and nausea caused by the resmetirom.⁵⁹ A phase 3, multinational study (MAESTRO-NASH) on resmetirom at 80 mg or 100 mg compared to placebo to achieve NASH resolution on liver histology in non-cirrhotic NASH patients with stage 2 or 3 fibrosis is recruiting and expected to end in 2024 (NCT03900429).

Firsocostat (acetyl-CoA carboxylase inhibitor)

Firsocostat is an acetyl-CoA carboxylase inhibitor that targets *de novo* lipogenesis through inhibition of acetyl-CoA to malonyl-CoA conversion. In a phase 2 trial that included 126 patients with NASH and fibrosis, 20 mg daily of firsocostat for 12 weeks showed significant reduction in liver fat by 29%; however, during treatment, an increase in plasma triglyceride levels was recorded, with 16 patients having levels of more than 500 mg/dL.⁶⁰ Two phase 2 clinical trials on firsocostat in monotherapy or in combination with other therapies for NASH regarding safety, efficacy and tolerabil-

ity, one on patients with stage 2 and 3 fibrosis and another in participants with bridging fibrosis or compensated cirrhosis are expected to finish soon, and results of these studies are awaited (NCT02781584, NCT03449446).

Drugs modulating hepatocyte injury, inflammation, apoptosis and fibrosis

Inflammation is a crucial step in NASH pathogenesis, and it has been found that Kupffer cells secrete C-C chemokine ligand types 2 (CCL2) in response to hepatocyte injury which, downstream, leads to monocyte recruitment and influx to the liver, where they mature into proinflammatory macrophages.^{61–63} These activated macrophages express proinflammatory cytokines, which in turn activate hepatic stellate cells, promote their survival, and stimulate fibrogenesis.⁶³ C-C chemokine receptor types 2 (CCR2) and 5 (CCR5), and their ligands, C-C chemokine ligand types 2 (CCL2) and type 5 (CCL5) were found to be up-regulated in NASH.⁶⁴

Cenicriviroc, a dual CCR2/CCR5 antagonist with a long plasma half-life (30–40 h in humans), was studied in a phase 2b study (CENTAUR) in the treatment of NASH and liver fibrosis.⁶³ The study evaluated efficacy and safety of 150 mg per day of cenicriviroc over 2 years for obtaining improvement in NAS at the first year relative to screening biopsy, without progression of fibrosis.⁶³ After 1 year of cenicriviroc, two times more patients achieved regression of fibrosis without worsening of steatohepatitis compared with placebo (20% vs. 10%; $p=0.02$).⁶³ A phase III study to evaluate the efficacy and safety of 150 mg per day of cenicriviroc versus placebo for the treatment of liver fibrosis in adult subjects with NASH and stage 2 or 3 liver fibrosis (AURORA trial) is still recruiting patients and results are awaited (NCT03028740).

Farnesoid X receptor agonists

Farnesoid X receptor (FXR) is a nuclear receptor expressed in the liver, gallbladder, and intestines. It is a modulator of bile acid, glucose and lipid metabolism.^{65–67} In the intestine, FXR modulates FGF15 and FGF19 synthesis and delivery to the liver through the portal circulation.⁶⁵ In the liver, FGF15 and FGF19 stimulate glycogen synthesis and suppress gluconeogenesis but also decrease triglyceride accumulation.⁶⁶ FXR activation reduces activity of SREBP-1c, a key transcription factor in regulation of triglyceride synthesis.⁶⁷ Obeticholic acid, which acts as an FXR agonist, has been evaluated in a phase 2 study on NASH patients without cirrhosis (FLINT trial), which randomized 283 patients to 25 mg of obeticholic acid or placebo for 72 weeks.⁶⁸ Although obeticholic acid was successful in the FLINT trial in reducing NAS by 2 points without worsening of fibrosis, a large number of patients developed significant pruritus and experienced a rise in total serum cholesterol and low-density lipoprotein.⁶⁸ Since these changes were attributed to suppression of *de novo* bile acid synthesis and an escalation in reverse cholesterol transport, future research was focused on development of FXR agonists that would have better efficacy, safety and tolerability profiles.⁶⁹ Tropifexor, a novel FXR agonist, that modulates gene expression in the liver and intestines in low doses with low systemic exposures was found to have a good tolerability profile in healthy volunteers and is now being evaluated in patients with NASH.⁶⁹ A combination therapy of tropifexor and cenicriviroc is also being evaluated in a phase 2 study (TANDEM trial) in patients with NASH and liver fibrosis (NCT03517540).

Selonsertib (apoptosis signal-regulating kinase 1 inhibitor)

Selonsertib is an apoptosis signal-regulating kinase 1 (known as ASK1) inhibitor that is intended to target the p38/JNK pathway, which is activated by TNF α and intracellular oxidative stress, and results in apoptosis and fibrosis.⁷⁰ A phase 2 multicenter study of selonsertib, given for 24 weeks once daily in doses of 6 mg and 18 mg, with or without simtuzumab, was undertaken in NASH patients with liver fibrosis (stage 2 or 3).⁷¹ Paired pretreatment and post-treatment liver biopsies and noninvasive diagnostic methods were used to evaluate the efficacy of treatment. After the treatment, a significant number of patients in the 18 mg selonsertib group achieved one or more stage amelioration in fibrosis compared to the 6 mg selonsertib group and simtuzumab alone (43% vs. 30% vs. 20%, respectively).⁷¹ Reduction in fibrosis was associated with reduction in liver stiffness and apoptosis markers measured by noninvasive methods.⁷¹ However, further trials of selonsertib on NASH patients but with stage 3 fibrosis (STELLAR-3) and stage 4 fibrosis/compensated cirrhosis (STELLAR-4) found that 48 week treatment with selonsertib (6 or 18 mg daily dose) had no significant effect on liver serum tests or fibrosis progression evaluated noninvasively.⁷²

Drugs targeting gut microbiome changes

Although the composition of gut microbiome varies among individuals, the prevailing bacterial phyla are *Firmicutes* and *Bacteroidetes*, that make up around 90% of the microbiome, and *Actinobacteria* and *Proteobacteria*. The prevailing bacterial phyla is responsible for a specific metabolite profile that can influence liver and overall metabolism, specifically metabolites such as bile acids, lipopolysaccharides and short-chain fatty acids.⁷³ It has been shown that the changes in gut microbiome and concentrations of the aforementioned metabolites is important in NAFLD pathogenesis and progression, but a specific microbiome composition in NAFLD has not yet been identified.^{74,75} A prospective study on fecal microbiome in adult NAFLD patients, carried out by Loomba *et al.*⁷⁶ on the association between microbiome composition and advanced stages of fibrosis in NAFLD, identified 37 bacterial species that vary depending on the disease stage. However, the authors state that these results could reflect changes within the microbiome that occur with age and that further studies are needed to see if specific microbial species are responsible for the gut-liver crosstalk and progression of NAFLD.⁷⁶ Prebiotics, probiotics, and antibiotics have been investigated in animal and human studies, in attempts to modify the microbiome and influence NAFLD. In animal studies, it has been shown that prebiotics and synbiotics can influence gene expression to modify β -oxidation and lipogenesis, thus effecting liver fat infiltration, inflammation, and insulin resistance.^{77,78} Results in human studies regarding their use in NAFLD were modest; although, most of these studies were not accompanied by a histological confirmation and had a small population sample.^{79,80} A recent meta-analysis on the use of prebiotics, probiotics and synbiotics for NAFLD concluded that prebiotic and probiotic use was associated with a reduction in body mass index (BMI) and modest influence on serum aminotransferase levels and lipid profile, without ameliorating inflammation.⁷⁹

Antibiotics

Antibiotics have been relatively successfully used in treating

NAFLD. An example is a trial with rifaximin, where a 28-day treatment induced a decrease in BMI, serum aminotransferases, and gamma-glutamyl transferase.⁸⁰ Since then, there have been many trials with opposing results, but the antibiotic treatment for NAFLD and obesity is still intriguing, based on the high number of ongoing trials.⁸¹ However, although short-term antibiotic treatment could prove beneficial, long-term and frequent use of antibiotics could cause a much greater problem of antibiotic resistance.

Fecal transplantation

Fecal transplantation, successful in treatment of *Clostridium difficile* infection, has been a promising treatment for microbial dysbiosis in NAFLD. However, recently published results from a randomized controlled trial on 21 patients with NAFLD that underwent autologous and allogenic fecal transplantation, and were followed up until 6 months from the procedure, found that although allogenic transplantation reduced small intestinal permeability it did not influence insulin resistance (measured by HOMA-IR) or hepatic steatosis measured by MR proton density fat fraction.⁸²

Modification of bacterial metabolites

A specific microbiome composition is also responsible for various points in bile acid metabolism and circulation in the body. Enterohepatic circulation of bile acids is regulated by the microbiome since it affects synthesis of amino acids necessary for bile acid liver conjugation as well as the expression of terminal ileum transporters that reabsorb around 95% of intestinal bile acids.^{83,84} As mentioned in the previous section, the FXR receptor is a nuclear receptor expressed in the liver, gallbladder and intestines, and a modulator of bile acid metabolism.^{65–67} Obeticholic acid is a synthetic derivative of chenodeoxycholic acid that as well as cholic acid is a primary bile acid synthesized in the liver. In study by Friedman *et al.*,⁸⁵ obeticholic acid induced suppression of bile acid synthesis (measured by reduced levels of 7 α -hydroxy-4-cholesten-3-one) and caused an increase in Gram-positive bacteria species (*S. thermophilus*, *L. casei*, and *L. lactis*). The FLINT trial on obeticholic acid in NASH patients, as elaborated earlier, emphasized the need for FXR agonists with better efficacy, safety and tolerability profiles.⁶⁸

Specific short chain fatty acids have been found to indirectly influence NASH progression, as well as previously described bile acids, in interplay with microbiome composition.⁸⁶ Short chain fatty acids are products of complex carbohydrate fermentation that cannot be digested by the organism, and the most prevalent are acetate, butyrate, and propionate.⁸⁷ They exert their effects by binding to specific receptors in the colon to increase GLP-1 and other insulin sensitizing peptides.⁸⁸ Their overall effects on liver, skeletal muscle and adipose tissue *in vitro* promote *de novo* lipid synthesis, fat oxidation, anabolism, and insulin sensitivity.⁸⁹ Moreover, studies have shown that they strengthen the intestinal barrier and reduce gut permeability and, by that, exhibit anti-inflammatory effects that are an important component in NASH development.^{90,91} In humans, the interplay between specific short fatty acids and the residing microbiome is more complex, and studies that addressed specific therapeutic procedures influencing short fatty acid intestine composition are inconsistent in their conclusions, so further research is necessary.⁸⁹

Lipopolysaccharides are a structural component of Gram-negative bacteria and of endotoxins that in healthy microbiota and intact intestinal barrier enter the hepatic circulation in only small amount and are thereby eliminated by Kupffer

cells after recognition by Toll-like receptors.⁹² In patients with NASH, there is an up-regulation of the pro-inflammatory response in liver that results from a major influx of lipopolysaccharides and other bacteria metabolites due to impaired intestinal barrier and altered microbiome.⁹³ Agents that could affect this pathway are extensively researched, and a recently published study on sevelamer (that acts as a hydrophilic bile acid sequestrant) showed great potential in affecting liver fibrosis in a diet-induced NASH animal model.⁹⁴ Sevelamer improved the composition of the gut microbiome, improved the intestinal barrier, promoted fecal excretion of lipopolysaccharides and, by that, reduced the concentration of lipopolysaccharides in liver and suppressed the proinflammatory Toll-like receptor pathway.⁹⁴ Studies on sevelamer in human NASH patients have not yet been conducted.

Conclusions

NASH represents an important global health burden with significant morbidity and the available treatment options are still unsatisfactory. However, new treatments for NASH are emerging. There is a large number of new drugs that are being tested in the preclinical setting and understanding NASH pathogenesis has a crucial role in their development. The microbiome composition has been shown to be related to changes in gut permeability, leaking lipopolysaccharides, and metabolism of short-chain fatty acids, all indirectly influencing proinflammatory and profibrotic pathways in the liver. Fecal microbiota transplantation and influencing the microbiome composition through bile acids and agents affecting other microbial metabolites have, in that way, been recognized as possible mechanisms to influence the development of NASH, potentially to reverse the changes preceding NASH and even influence NASH stage regression.

After years of not being able to actively treat NASH other than with diet and exercise modification, with only limited pharmacological possibilities, we are now expecting drugs which target specific points in NASH pathogenesis. Although NASH is a multifactorial disease, in the future, we could identify the predominating pathological mechanism and, by choosing specific medications, tailor the treatment for every patient individually.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Study concept and design (MD, SSS), acquisition of data (SSS, LVJ, DH), analysis and interpretation of data (MD, SSS, LVJ, DH), drafting of the manuscript (SSS, LVJ), critical revision of the manuscript for important intellectual content (MD, LVJ, DH), administrative, technical, or material support, study supervision (LSD, MD).

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An Update on Efficacy and Safety of Emerging Hepatic Antifibrotic Agents

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Abstract

Liver fibrosis represents a response to chronic liver injury. Metabolic dysfunction-associated fatty liver disease and metabolic dysfunction-associated steatohepatitis are the most common chronic liver diseases, both with increasing incidence. Therefore, there is a great impetus for development of agents targeting these conditions. Accumulating data on possible treatment options for liver fibrosis are emerging in the literature. However, despite extensive research and much effort in the field, approved agents for liver fibrosis are still lacking. In this critical review, we have summarized the main data about specific treatment options for liver fibrosis gained from ongoing clinical trials, with an emphasis on efficacy and safety of these agents.

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Keywords: Liver fibrosis; MAFLD; MASH; Clinical trials; Antifibrotic agents; Efficacy; Safety.

Abbreviations: γGT, gamma-glutamyl transferase; ACC, acetyl-coenzyme A carboxylase; ALT, alanine aminotransferase; ARB, angiotensin receptor blocker; ASK1, apoptosis signal-regulating kinase; AST, aspartate aminotransferase; CCR, C-C motif chemokine receptor; DAA, direct-acting antiviral; FGF, fibroblast growth factor; FXR, farnesoid X receptor; GLP, glucagon-like peptide; HCV, hepatitis C virus; HIV, human immunodeficiency virus; IgA, Immunoglobulin A; IL, interleukin; LOXL2, lysyl oxidase-like homolog 2; MAFLD, metabolic dysfunction-associated fatty liver disease; MASH, metabolic dysfunction-associated steatohepatitis; MC, mast cell; MR, magnetic resonance; OCA, obeticholic acid; PBC, primary biliary cholangitis; PPARs, peroxisome proliferator-activated receptors; PSC, primary sclerosing cholangitis; SCD1, stearoyl CoA desaturase 1; T2DM, type 2 diabetes mellitus; THR, thyroid hormone receptor; UDCA, ursodeoxycholic acid; VAP-1, vascular adhesion protein-1.

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Introduction

Liver fibrosis represents a pathogenic response to chronic liver injury.¹ Among the etiologies, viral hepatitis B and C infections have been historically cited as the most common causes. In the last 30 years, certain changes in prevalence have occurred as a consequence of the spread of Western lifestyle and therapeutic advances in the treatment of some liver diseases. Intriguingly, while the prevalence of chronic hepatitis B and alcoholic liver disease has remained stable, the prevalence of chronic hepatitis C has decreased. Along with the increasing prevalence of type 2 diabetes mellitus (T2DM), obesity and metabolic syndrome, prevalence of metabolic dysfunction-associated fatty liver disease (MAFLD) has notably increased.² MAFLD,³ in particular, has become the most common form of chronic liver disease, with global prevalence of 25.4%,⁴ and a major cause of liver cirrhosis and hepatocellular carcinoma.³ Other, less common etiologies include cholestatic disease (primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC)), autoimmune hepatitis and such genetic diseases as hemochromatosis, alpha antitrypsin deficiency, and cystic fibrosis.⁴

However, regardless of the cause, a common and the most prominent feature of all advanced chronic liver diseases is liver fibrosis.² At the histopathological level, liver fibrosis represents qualitative and quantitative changes in extracellular matrix and deposition of type I collagen, primarily, which ultimately results in disorganization of the liver parenchyma architecture.⁴ Fibrosis stage is the strongest predictor of prognosis in patients with chronic liver diseases, linked not only to liver-related but also to extrahepatic morbidity and mortality.⁵

During the last decades, basic science studies have investigated the underlying molecular and pathophysiological mechanisms of liver fibrosis in detail, aiming to explore potential new drug targets. Currently, at least six groups of fundamental pathophysiological mechanisms of liver fibrosis development could be distinguished (Table 1) and consequently treatment strategies can be differentiated as treatments of the underlying disease(s), targeting cell death, liver metabolism, gut-liver axis, fibrogenesis (in the

Table 1. A comprehensive list of hepatic antifibrotic therapeutic agents with their proposed mechanisms of action, pharmaceutical target of interest, indication and affiliation to ongoing or recently completed phase of clinical trial

Mechanism of action	Therapeutic agent	Target	Clinical trial phase	Indication	Trial name and id	Results and safety	Expectations
Underlying disease	DAA	HCV	N/A	HCV	N/A	N/A	N/A
Cell death	Vitamin E	Antioxidants	III	MASH	PIVENS-NCT00063622	Improvements in ALT, AST and hepatic steatosis reduction; generally safe	+
	Emricasan (IDN-6556)	Pan-caspase inhibition	II	MASH	ENCHORE NF-NCT 02686762	No effect; generally safe	-
	Selonsertib (GS-4997)	ASK1 inhibition	II	MASH	GS-US-384-1497-NCT02466516	Amelioration of liver fibrosis; generally safe	+
			III	MASH	STELLAR 3-NCT03053050	No effect; generally safe	-
			III	MASH cirrhosis	STELLAR 4-NCT03053063	No effect; generally safe	-
			II	MASH	ATLAS-NCT03449446	Selonsertib monotherapy group discontinued following termination of STELLAR trials	--
Liver metabolism	UDCA	Secondary bile acid; inhibition of MCs and decrease in histamine production	N/A	MASH; PSC; PBC	N/A	N/A	N/A
	Obeticholic acid	FXR agonism	III	MASH	REGENERATE-NCT02548351	Improved liver fibrosis and liver biochemistry; increased risk of liver injury in patients with mild liver impairment	++
	Cilofexor (GS-9674)	FXR agonism	II	MASH	REVERSE-NCT03439254	Results are expected	0
	Tropifexor (TXR, LJM452)	FXR agonism	III	MASH	GS-US-402-1852-NCT02854605	Improvements in liver biochemistry and markers of cholestasis; safe	+
	EDP-305	FXR agonism	IIa	MASH	PRIMIS-NCT03890120	Results are expected	0
	Fenofibrate	PPARA agonism	Pilot study	PBC	FLIGHT-FXR-NCT02855164	Improvements in ALT and markers of cholestasis; safe	++
			II	PBC	ARGON 1-NCT03421431	Decrease in AST; safe	++
			II	PBC	INTREPID-NCT03394924	Decrease in ALT, AST and markers of cholestasis; safe	+
			II	PBC	NCT00575042	Improvements in liver biochemistry; safe	+

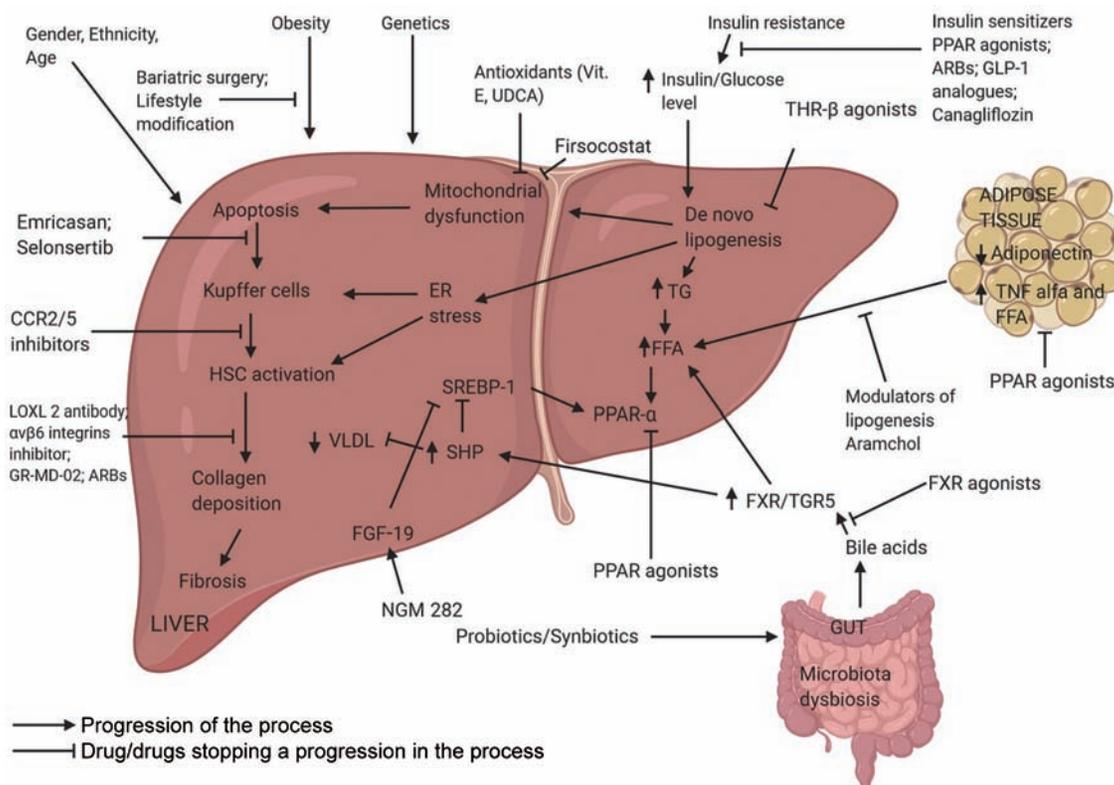


Fig. 1. Treatments for metabolic dysfunction-associated fatty liver disease (MAFLD) and metabolic dysfunction-associated steatohepatitis (MASH) according to mechanism of action. Abbreviations: ARB, angiotensin receptor blocker; CCR2/5, C-C motif chemokine receptor; ER, endoplasmic reticulum; FFA, free fatty acids; FXR, farnesoid X receptor; GLP, glucagon-like peptide; HSC, hepatic stellate cell; LOXL 2, lysyl oxidase-like homolog 2; PPAR, peroxisome proliferator-activated receptor; SHP, small heterodimer partner; SREBP, sterol regulatory element binding protein; TG, triglycerides; TGR5, Takeda G protein-coupled receptor 5; THR, thyroid hormone receptor; TNF, tumor necrosis factor; UDCA, ursodeoxycholic acid; VLDL, very low-density lipoprotein.

narrow sense of the word), and inflammation.

In this critical review, we will present the latest findings on underlying molecular and pathophysiological mechanisms in the development of liver fibrosis and their utilization as potential therapeutic targets, as shown in Fig. 1. The boundaries between aforementioned pathways are arbitrary and *in vivo* these pathways overlap continuously. Although clinical trials have examined the effectiveness of lifestyle changes, bariatric surgery and hepatitis C and B suppression, however, the same is out of the scope of this review. This review focuses on pharmaceuticals; particularly, we will highlight data about efficacy and safety of these potential drugs gained from the currently ongoing or recently completed phases 2 and 3 clinical trials.

Treatment options for different pathophysiological mechanisms of liver fibrosis development

Treatments of the underlying disease

Although cirrhosis is considered to be a definitive and irreversible stage of hepatic fibrosis, several studies have shown that the cure or suppression of hepatitis C and B can result in regression of hepatic fibrosis and even cirrhosis.^{6,7} Specific etiologic treatments for chronic hepatitis B and C that have resulted in sustained virologic response have also been associated with decreased fibrosis and cirrhosis regression. Similarly, lifestyle changes or bariatric surgery achieving weight loss in patients with metabolic dysfunction-associated stea-

tohepatitis (MASH) resulted in reduction of the histological features of MASH and fibrosis reversal.^{8,9}

Therefore, any treatment for liver fibrosis would ideally include treatment of the underlying disease as well. However, this is not always possible, and also, on the other hand, in some cases of known and cured-cause of chronic liver disease, the persistence of fibrosis or even its progression can still be present.^{9,10} One possible explanation for that paradox might be that beyond a certain limit, the fibrogenetic process gains a certain relative autonomy.¹¹ In such cases, etiologic treatment cannot eliminate life-threatening complications. Therefore, regardless of the etiological factor, novel antifibrotic drugs represent an utmost need for patients with cirrhosis.

Treatments targeting cell death

In chronic liver disease, hepatocyte death is a profibrotic trigger.¹² Moreover, an association between the extent of hepatocyte death and the progression of fibrosis in MAFLD was confirmed.¹³ Thus, it can be assumed that inhibition of cell death pathways constitutes a promising target for future antifibrotic treatments. However, different cell types contribute to profibrotic or antifibrotic actions, respectively. Hepatic stellate cells are a main collagen-producing cells in liver fibrosis and, therefore, their apoptosis in terms of regulated death could be crucial in stopping and reversing fibrosis.¹⁴ Therefore, the current concept of antifibrotic therapy is cell-type specific.^{12,14}

Vitamin E is a long-known antioxidant agent which could

act protectively in the liver.¹⁵ Since it inhibits apoptosis and oxidative stress, it could have a positive and maybe even a curative effect. According to some studies, a therapy with vitamin E could enhance biochemical and histological parameters in MAFLD.^{16,17} These results are promising but sparse due to a short-lasting duration of these trials. Also, safety and efficacy of vitamin E has yet to be evaluated. In the PIVENS (pioglitazone, vitamin E or placebo for nonalcoholic steatohepatitis) trial, vitamin E was investigated as a therapy for MASH. The results were preferable in patients treated with high-dose vitamin E (800 IU/day) for 96 weeks compared to placebo. There was an improvement in some histopathology findings recorded. Improved steatosis and decreased alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were found; however, effect on fibrosis was not detected.^{17,18} Although a lot of vitamin E benefits have been confirmed, some studies have indicated that it could raise all-cause mortality, prostate cancer in males older than 50, and hemorrhagic stroke risk.¹⁶ Meta-analysis by Xu *et al.*¹⁹ confirmed that vitamin E supplementation is promising, with significant improvements in some histological parameters in MASH patients, except fibrosis. Since, the results cannot be generalized, as trials were limited, with a short follow-up duration and a small number of participants, additional large-scale high-quality studies are needed to obtain more comprehensive information on vitamin E supplementation for clinical use.

Apoptosis and production of inflammatory cytokines (interleukin (IL)-1 β and IL-18), among others factors, are regulated by caspases and enzymes activated by lipotoxicity. Thus, a more specific therapeutic option could be the inhibition of a pan-caspase enzyme by the use of emricasan, a pan-caspase inhibitor (IDN-6556, PF-03491390, Conatus Pharmaceuticals). Effective blockage of liver injury and fibrosis by emricasan thoroughly inhibiting apoptosis of hepatocytes has been demonstrated in a murine model of MASH.²⁰ These data supported the hypothesis that emricasan may be a prospective antifibrotic drug in treatment of MASH.²⁰ One week of emricasan treatment ameliorated liver fibrosis, portal hypertension and improved liver function in rats with advanced cirrhosis in a preclinical study in 2019. Since beneficial effects, without any apparent hepatic or systemic adverse effects, were demonstrated with emricasan, further clinical evaluation in the treatment of advanced chronic liver disease was encouraged.²¹ However, in a double-blind, placebo-controlled phase 2 clinical trial (ENCORE-NF, NCT02686762) involving 318 MASH patients with fibrosis (stages F1–F3) after 72 weeks, emricasan did not improve histopathological findings of liver fibrosis in the majority of patients. On the contrary, it may have even worsened it. ALT levels were lowered for short-term; however, it seems that emricasan could have redirected liver cells to a different mechanism of cell death and thus resulted in progression of fibrosis.²² Persistent normalization of ALT in serum was observed during 72 weeks in a small subset of patients. Therefore, only in a minor subgroup of patients, emricasan may have improved fibrosis, as determined by liver biopsy. Other caspase inhibitors under clinical development, VX-166 (Vertex) and GS-9450 (Gilead) have been reviewed recently.²

The third promising pathophysiological pathway is inhibition of the apoptosis signal-regulating kinase 1 (ASK1) preventing activation of the aberrant intracellular cascade pathway, ending in cell death. It has been shown that inhibition of ASK1 resulted in the alleviation of hepatic steatosis in diabetic obese mice (over a decade ago).²³ Selonsertib (GS-4997), an ASK1 inhibitor, was investigated in rodent model of MASH, with promising results published in 2016. Improvements in cholesterol, bile acid and lipid metabolism, and a reduction of liver steatosis and fibrosis were shown.²⁴ Subsequently, a “pilot trail” with selonsertib was

conducted in 72 MASH patients with grades 2 or 3 fibrosis in 2018. This phase 2 trial (NCT02466516) confirmed fibrosis improvement, as determined by liver biopsy and magnetic resonance (MR) elastography. Decrease in liver collagen content as well as less inflammation, and a decreased liver stiffness were found.²⁵ Recently selonsertib was investigated in two large phase 3 trials in MASH patients with either advanced fibrosis (STELLAR-3, NCT03053050) or cirrhosis (STELLAR-4, NCT03053063), respectively. However, the primary endpoint after 48 weeks was not met in either study and selonsertib actually exhibited worse results than placebo.^{26,27} Nevertheless, selonsertib was included in the phase 2b of multicenter, randomized, double-blind, placebo-controlled ATLAS trial (NCT03449446), with acetyl-coenzyme A carboxylase (ACC) inhibitor firsocostat and the farnesoid X receptor (FXR) agonist cilofexor in 395 MASH patients with fibrosis (stage F3) or compensated cirrhosis (F4).²⁸ However, the selonsertib monotherapy group was discontinued after the STELLAR trials termination.²⁹

Treatments targeting liver metabolism

Most of the new pharmacological approaches are aiming to diminish altered hepatic metabolism using targets such as the FXR, the family of peroxisome proliferator-activated receptors (PPARs), ACC, fibroblast growth factor (FGF) 21, or glucagon-like peptide (GLP) 1 agonists.³⁰

One of the medications often used in treatment of cholestatic liver disease is ursodeoxycholic acid (UDCA), a secondary bile acid. The proposed mechanism of action of UDCA is decrease of histamine production by mast cells (MCs). MCs enter liver tissue and initiate fibrosis. Thus, inhibition of MCs infiltrating liver might be a potential efficient therapy for PSC. UDCA treatment significantly reduced levels of gamma-glutamyl transferase (γ -GT) and total bile acids according to a study from 2017.³¹ Effectiveness of vitamin E and UDCA in MAFLD and in patients without diabetes were compared recently. UDCA was as effective as vitamin E when combined with a specific dietary guidance and lifestyle modification. UDCA can be used as an alternative to vitamin E in the treatment of MASH, as it has minimal side effects and equal tolerability.³² Side effects of UDCA are diarrhea, elevated creatinine, elevated blood glucose, leukopenia, peptic ulcer, skin rash, and thrombocytopenia.³²

FXR is expressed in liver cells and intestine. Actually, it belongs to a nuclear receptor family of transcription factors and as such it regulates bile acid flow. Bile is a toxic substance, but the toxic effect of bile on hepatocytes and bile duct epithelial cells can be minimized physiologically through various mechanisms, such as high apical membrane cholesterol and sphingomyelin content, bile hydration and alkalinization, micellar binding of bile acids, mucin formation, and particularly bile flow.³³ FXR is being explored as a prospective drug for cholestasis, since it could potentially protect liver cells by binding bile acids. When bile acids are bound to FXR, bile acid circulation is diminished and lipids and glucose metabolism are regulated. FXR has an important role in lipid synthesis, and in very low density lipoprotein and triglyceride metabolisms also. Recently, FXR expression was compared in specimens from fibrotic patients with MASH and MAFLD and it was shown that FXR had lower expression in MASH specimens compared to MAFLD, respectively, so it could be hypothesized that FXR might slow liver fibrosis.³⁴

Obeticholic acid (OCA) is a synthetic bile acid and a potent activator of the FXR. In rodent models of MASH, it improved insulin resistance and altered lipid metabolism, showing positive effects on liver fibrosis.³⁵ A preclinical study in 2014 found that cirrhosis complications in the form of decreased portal hypertension may be affected in the

cirrhotic rat model.³⁶ Currently, there is an ongoing randomized global phase 3 study to evaluate the impact of OCA on MASH with fibrosis (REGENERATE, NCT02548351) and on MASH with cirrhosis (REVERSE, NCT03439254), expected to be completed in 2022. After 72 weeks of treatment with 25 mg of OCA, the REGENERATE study showed improved histology and biochemistry of liver fibrosis.³⁷ Pruritus was found in 51% of patients as the most common side effects, and in addition an increase in serum cholesterol levels was found in 17% of patients (compared to 7% of patients on placebo). This low-density lipoprotein increase could be resolved by administration of statins but its clinical significance on cardiovascular mortality is still unclear. Pruritus as side effect of OCA was registered earlier in 23% of patients in the FLINT study in 2015.³⁸ OCA improved the histological features of MASH but its long-term benefits and safety have not been clarified yet.³⁸ Malaise, severe pruritus, lowered high-density lipoprotein cholesterol, abdominal pain and discomfort were documented as adverse effects of OCA. According to the Food and Drug Administration, OCA increases the risk of serious and fatal liver injury in patients with moderate to severe liver impairment when dosed more frequently than recommended. It may also increase the risk of liver injury in patients with mild liver impairment receiving the recommended dose. However, the drug is approved as a second-line therapy for PBC.³⁹

Cilofexor (GS-9674) is a nonsteroidal FXR ligand. It was tested in 2019 in a phase 2 double-blind, placebo-controlled study (NCT02854605) in patients with MASH. Improvements were seen in biochemistry liver function tests and markers of cholestasis. However, the most common side effect recorded was pruritus. Other side effects also reported were rib fracture, diarrhea/dehydration, and acute kidney injury.⁴⁰ There is also an ongoing phase 3 trial (NCT-03890120, PRIMIS) on 400 patients with PSC. Results are expected in February 2023.⁴¹

Tropifexor (LJN-452) is another promising nonsteroidal FXR agonist. According to preclinical research in two distinctive rodent models of MASH, it demonstrated impressive effects in 2019. After 4 weeks of tropifexor therapy, an amelioration of MASH histopathology and reduction in liver triglycerides were observed. Mice that received tropifexor showed dose-dependent reversal of liver fibrosis.⁴² Of course, these promising findings need further evaluation in clinical trials. An active phase 2 trial (NCT02855164) by Novartis Pharmaceuticals (FLIGHT) on 351 patient with MASH was completed.⁴³ First results showed amelioration of ALT levels and markers of cholestasis.⁴⁴ According to one study from 2019, which included 95 healthy volunteers, the tolerance was good at a single dose up to 3,000 µg and in multiple doses up to 60 µg without significant alterations of plasma lipids in healthy volunteers.⁴⁵

EDP-305 is also an FXR ligand. In animal models it improved liver fibrosis.⁴⁶ It was evaluated for nonalcoholic steatohepatitis in a phase 2a study (ARGON-1) by ENANTA Pharmaceuticals. EDP-305 efficiently reduced AST level after 12 weeks of therapy. Its adverse events were pruritus, nausea, vomiting, diarrhea, headache, and dizziness. One more trial with this agent is expected, a 2b trial (Argon-2) for nonalcoholic steatohepatitis.⁴⁷ Also a phase 2 study (INTREPID, NCT03394924) was completed in January 2020 on 68 participants with PBC. Results were improvements in ALT, AST, and markers of cholestasis. However, the primary endpoint of at least 20% reduction in ALT was not met.⁴⁸

Another interesting group of drugs are the PPAR agonists. PPARs have an important role in the metabolism of glucose and lipids and thus they have a great potential in therapy of MAFLD. PPAR α appears to play a role in the development of MAFLD. PPARs also have a crucial role in beta-oxidation of lipids and play an important role in hepatic fibrosis.¹¹ An agent from this group is fenofibrate. A study conducted

in rats from 2012 showed amelioration of portal pressure and hepatic fibrosis.⁴⁹ A pilot trial in 2010 (NCT00575042), including 20 PBC patients with an incomplete response to UDCA, published its results. Patients were treated with a combination of fenofibrate and UDCA. Results were significant improvements in liver biochemistry. The most prominent side effect was heartburn.⁵⁰ Another study which included 90 patients with MAFLD published in 2015 did not show significant improvements in fibrosis.⁵¹

Thiazolidinediones, as PPAR γ ligands, are a group of insulin-sensitizing medications, including rosiglitazone and pioglitazone. The FLIRT study from 2008 included 63 patients with MASH receiving rosiglitazone. Only half of the patients were responders and had improvements in liver biochemistry, but histopathological amelioration of fibrosis was absent.⁵² A FLIRT 2 trial (NCT00492700) was conducted in 2010, including 53 patients with MASH, among which 18 of them were treated with rosiglitazone. It improved liver biochemistry in the short term but in the long term it was not as efficient.⁵³ Also, thiazolidinediones have some side effects, such as weight gain, edema, bone loss, increased risk of bladder cancer, and cardiovascular complications, which limit their use.⁵⁴ However, in a meta-analysis by Musso *et al.*,¹⁸ which included eight randomized clinical trials with 516 biopsy-proven MASH patients, 24 months pioglitazone therapy was associated with reversal of advanced fibrosis, improved overall fibrosis stages, and resolution of MASH, regardless of the presence of diabetes.⁵⁵ The already mentioned PIVENS study (NCT0063622), which included 247 patients with MASH without diabetes, showed improvements in ALT and AST levels, and hepatic steatosis reduction. However, improvements in liver fibrosis were not detected. Data from a trial (NCT00994682) regarding pioglitazone were published in 2018. Participants were MASH patients, among which 52 had T2DM and 49 were prediabetic. Improvements were seen in histopathological findings of fibrosis and in insulin tissue sensitivity.⁵⁶

A PPAR δ agonist seladelpar (MBX-8025) was tested in obese, dyslipidemic, and diabetic mouse model, where amelioration was confirmed by lipid levels, liver biochemistry results and hepatic cell ballooning.⁵⁷ Consequently, it is being evaluated for promising potential as a novel therapy for PSC in a phase 2 trial (NCT04024813)⁵⁸ and for MASH (NCT03551522) and the results are expected in December 2020.⁵⁹ Currently, there is also an ongoing phase 3 study (NCT03602560) including seladelpar (ENHANCE) in 240 participants with PBC. The results are expected in December 2021.⁶⁰

Elafibranor (GFT-505) is a combined PPAR α and PPAR δ agonist. A phase 2b trial GOLDEN-505 (NCT01694849) was published in 2016 in patients with MASH, showing improvements in hepatic enzymes, levels of lipids and glucose. Elafibranor modestly resolved MASH histology, but it has not proven histological resolution of fibrosis.⁶¹ Currently, there is an ongoing clinical trial (RESOLVE-IT) for elafibranor in phase 3 investigation (NCT02704403). Final data are expected to become publicly available in 2021,⁶² when, based on confirmed efficacy and safety of elafibranor itself or in combinations with other developmental drugs, its possible role in the future treatment of MAFLD/MASH will be elucidated.^{62,63} However, an interim analysis published in May 2020 has shown disappointing results, without reaching neither the primary nor secondary endpoint of the study.⁶⁴

Saroglitazar is another representative from the PPAR dual (α and γ) ligands group. It has been shown in preclinical studies that it targets all the segments of MASH.⁶⁵ An analysis from 2019 has concluded that it ameliorates ALT levels and MAFLD.⁶⁶ There are two active phase 2 clinical trials assessing saroglitazar in patients with MASH and assessing its safety in PBC. A phase 2 trial (NCT03061721, EVIDENCES IV) was completed in 106 MASH patients, resulting in

amelioration of ALT levels, hepatic steatosis, dyslipidemia, and insulin resistance.⁶⁷ EVIDENCES VI (NCT03863574) will be finished in December 2020 and EVIDENCES VII in July 2021 (NCT03617263).^{68,69} Results of a phase 2 trial (EPICS, NCT03112681) assessing saroglitazar in PBC are expected to become publicly available in July 2020.⁷⁰ However, the drug is already registered in India for diabetes treatment.⁴

There is one available pan-PPAR agonist, lanifibranor. Lanifibranor (IVA 337), a moderately potent and well-balanced pan-PPAR agonist⁷¹ is being evaluated as a potential drug candidate for MASH treatment in an ongoing 2b trial (NCT03008070-NATIVE) by INVENTIVA. The results are expected to be published in 2020.⁷² Lanifibranor is also being investigated as a potential drug treatment of MAFLD and T2DM in another phase 2 trial (NCT03459079), and these results are expected in March 2021.⁷³

Bezafibrate is a pan-PPAR agonist that was evaluated in patients with PBC who had an inadequate response to UDCA alone in a 24-month placebo-controlled phase 3 trial (NCT01654731-BEZURSO). Co-administration of bezafibrate to UDCA resulted in a complete biochemical response in 31% of patients, which was significantly higher than in the placebo and UDCA-treated group. Amelioration of pruritus, fatigue, and noninvasive measures of liver fibrosis were persistent, with observed enhancement of biochemical parameters.⁷⁴

Telmisartan is a unique angiotensin receptor blocker (ARB) that modulates PPAR- γ activity and thereby increases sensitivity to insulin, which decreases hepatic fat accumulation. It also blocks the angiotensin II receptor, which inhibits hepatic stellate cell activation and suppresses hepatic fibrogenesis.⁷⁵ One study (FANTASY) was conducted in a period from 2012 to 2014, comparing the effect of two ARBs (telmisartan and losartan) and aiming to support a theory that telmisartan might be more effective than losartan in MAFLD and in insulin resistance. Participants in this randomized controlled trial were patients with MAFLD, T2DM and hypertension treated with telmisartan and losartan. There was no remarkable enhancement in these two groups. Free fatty acids were decreased in the group telmisartan group. Also, one thing worth mentioning is that the liver-to-spleen ratio was better in the telmisartan group.⁷⁵ Another prospective randomized controlled trial was conducted in 2016 on 35 MASH patients, in whom telmisartan plus life style modification were introduced, and 15 MASH patients, in whom only life style modification was introduced. It revealed that telmisartan significantly improved the histology of MASH patients. One more trial was published in 2018 which included two groups of patients with MAFLD treated with placebo and telmisartan (dose of 20 mg per day). Telmisartan decreased the level of IL-1 β and IL-1 and thus had an antifibrotic effect. Neither telmisartan nor placebo improved histopathology findings in MAFLD.⁷⁶ Telmisartan side effects included sinus pain and congestion, back pain, diarrhea, sore throat, flu-like symptoms, upset stomach, muscle pain, headache, dizziness, fatigue, and nausea.⁷⁷ Despite their potential benefit, ARBs are not used for non-cardiovascular indications because of dose-limiting hypotension.⁷⁸

The thyroid hormone receptor β is a main liver receptor for thyroxine. There are two available selective ligands for MASH in this group, MGL-3196 (resmetirom), which initially targeted dyslipidemia, and VK2809. Results from a phase 2 trial (NCT02912260) including 348 patients with MASH that evaluated the use of resmetirom was published in 2019. Resmetirom-treated patients showed reduction in liver fat as assessed by MR imaging-proton density fat fraction. Reported side effects were mostly mild or moderate and balanced between groups, except for diarrhea and nausea.⁷⁹ Resmetirom (MGL-3196) is also being investigated in an ongoing phase 3 trial (MAESTRO-NASH) in MASH and cirrhosis (NCT03900429) patients. The first results are expected to

be published in March 2024.⁸⁰ VK2809 showed improvements in liver lipids in a 3-month placebo-controlled phase 2 trial.⁴ VK2809 is currently in an ongoing phase 2b trial for nonalcoholic steatohepatitis (NCT04173065-VOYAGE). The results are expected in November 2021.⁸¹ In 2019, a phase 2 study (NCT02927184) regarding VK2809 use in patients with MAFLD and hypercholesterolemia was completed.⁸² It showed amelioration in hepatic fat content in a 12-week period, with no serious side effects, as compared to placebo. Both ALT and AST levels were reduced.⁸³

ACC1 and ACC2 have a crucial role in fatty acid metabolism. Inhibition of ACC1 and ACC2 represents an interesting pharmacological approach for another group of agents targeting lipid metabolism through simultaneous inhibition of fatty acid synthesis and stimulation of fatty acid oxidation. An agent from this group is firsocostat. Recently, in a study on diet-induced rodent models of MAFLD, an improvement in liver steatosis was shown; however, a significant increase in plasma triglyceride levels was also observed.⁸⁴ Therefore, based on the results of this study, the potential therapeutic utility of liver-directed ACC inhibition in the treatment of MAFLD remains to be further investigated.⁸⁴ It is worth mentioning that this drug induced hypertriglyceridemia that has shown a good response to statin treatment.⁴ Also, a phase 2 trial (NCT02856555) evaluating safety and efficacy of GS-0976, an ACC inhibitor in liver, was recently completed and decrease in hepatic steatosis and selected markers of fibrosis, and liver biochemistry was confirmed.⁸⁵ However, reported side effects included pruritus, headache, diarrhea, and nausea.²⁹

Aramchol (arachidyl amido cholanoic acid) is a conjugate of fatty bile acids. It inhibits stearoyl coenzyme A desaturase 1, which is crucial in lipogenesis. A phase 2 trial (ARREST) completed in 2018 showed improvements in hepatic fibrosis.⁸⁶ Aramchol is currently in a phase 3 ongoing trial (ARMOR) for MASH (NCT04104321).⁸⁷

There are also newly emerging indication opportunities for agents already used to treat diabetes, as numerous patients with MASH are diabetics as well. GLP-1 agonists exert various beneficial effects in T2DM, such as enhancement of glucose-dependent insulin secretion, acceleration of β -cell proliferation, inhibition of β -cell apoptosis, inhibition of motility and gastric emptying, and stimulation of the sensations of satiety and fullness by direct action on the central nervous system, with reduction in body weight.⁸⁸ Two agents that might be beneficial in MASH treatment are liraglutide and semaglutide. A phase 2 trial (NCT01237119, LEAN) investigating efficacy and safety of liraglutide, a long-acting GLP-1 analogue in nonalcoholic steatohepatitis patients, was published relatively recently. Amelioration of histopathology findings was confirmed; however, side effects including diarrhea, loss of appetite, and constipation were also observed.⁸⁹ A phase 2 study (NCT 03987451) exploring efficacy and safety of semaglutide in MAFLD is ongoing and the results are expected to become publicly available in June 2021.⁹⁰

A study was published in 2018 regarding a sodium-glucose cotransporter 2 inhibitor, canagliflozin. The results were amelioration of markers for hepatic fibrosis among included patients with T2DM and MASH. There were no serious side effects. As authors of the study stated, the limitation of this trial is a small number of participants.⁹¹ A phase 3 study (TCTR20190118008) evaluating canagliflozin in MASH was completed in March 2020 and the results are still expected.⁹²

Treatments targeting gut-liver axis

The term "gut-liver axis" represents an anatomical and func-

tional unit formed of the small intestine and liver. A number of signals, including hormones, chemokines and growth factors are released from the small intestine and enter the liver via the portal vein. Also, on the other hand, bile acids and other signals (IgA immunoglobulins) reach the small intestine via the bile.⁹³ In a critical analysis of clinical trials in 2017, it was vividly described as “Whatever comes from the gut enters the liver and the portal circulation is the afferent and the biliary tree the efferent of the gut-liver-axis”.⁹⁴ In patients with liver fibrosis and cirrhosis, certain changes within the gut-liver axis have been demonstrated, including changes in the intestinal microbiota, as a potential target for future antifibrotic drugs. In a prospective study in 2017, it was demonstrated that MAFLD patients had a specific metagenomic-derived signature of fecal microbiota which enabled distinguishing patients with mild/moderate fibrosis from advanced fibrosis.⁹⁵ In several experimental animal models the influence gut-derived signals, (e.g., intestinal mucus layer and the content of the intestinal microbiota on the fibrotic response in the liver) was determined.⁹⁶

Future perspectives of antifibrotic drugs include modulating the gut microbiome. Twenty-one randomized clinical trials on the effect of probiotics/synbiotics in MAFLD patients were evaluated in a recent meta-analysis. The authors reported reduction in ALT, improvement in liver stiffness measurement by elastography, and amelioration of steatosis as determined by ultrasound imaging, primarily as a probiotics/synbiotics effect on microbiome composition.⁹⁷ However, given the heterogeneity of the analyzed data and since neither of randomized clinical trials examined sequential histological findings, as determined by liver biopsy, the authors concluded that additional well-designed studies are needed to determine the true value of probiotics/synbiotics for treatment of liver fibrosis. It is worth mentioning that currently there is an ongoing randomized clinical trial (PRO-BILIVER TRIAL, NCT03467282) assessing 46 participants with MASH, receiving 1 g of probiotic mix in comparison to placebo. Results are expected in December 2021.⁹⁸

Within the gut-liver axis field, promotion of useful gut signals could have promising results. In humans, FGF19 is a gut-derived hormone which regulates glucose homeostasis and bile acid synthesis. Its beneficial metabolic effects were tested in a multicenter, randomized, double-blind, placebo-controlled, phase 2 clinical trial (NCT02443116) involving 82 biopsy-confirmed MASH patients with fibrosis stages 1–3. An engineered FGF19 analogue (NGM282, aldafermin) rapidly (after 12 weeks) and significantly diminished liver fat content, as measured by MR imaging. The vast majority of patients experienced at least one side effect, mostly the mildest (injection site reactions, diarrhea, abdominal pain, and nausea), while only a small percentage of patients experienced more severe side effects.⁹⁹ Since the drug has an acceptable safety profile, further studies are approved. Currently, there is ongoing 2b phase of multicenter, randomized, double-blind, placebo-controlled clinical trial (ALPINE 2/3, NCT03912532). The aim is to evaluate histologic response and also safety and tolerability of subcutaneously administered NGM282 for 24 weeks. The study included 152 histologically-confirmed MASH patients with fibrosis stage 2 or 3. Still, there is no result data available and it is estimated to be completed in December 2020.¹⁰⁰

Treatments targeting fibrogenesis (myofibroblast activation and extracellular protein deposition)

Studies on xenograft liver models determined 10 years ago that the key enzyme involved in extracellular collagen accumulation is lysyl oxidase-like homolog 2 (LOXL2). It significantly contributes to collagen stabilization catalyzing cross-

linkage of collagen, and by such action has a profibrotic effect. Inhibition of LOXL2 using monoclonal antibodies had optimistic and promising preclinical results.¹⁰¹ Also, in animal models, irreversible inhibition of LOXL2 has resulted in decreased fibrosis in mice.¹⁰²

However, the results from the 2b phase of a clinical trials in MASH patients investigating the efficacy of simtuzumab as monoclonal antibody against LOXL2 were negative (NCT01672866, NCT01672879). Indeed, the study was terminated prematurely (after 96 weeks) for inefficiency of simtuzumab, which had not shown any beneficial effect when compared to placebo.¹⁰³ Similar negative results were registered in a phase 2 study for PSC (NCT01672853) without significant reductions in fibrosis.¹⁰⁴ Also, in a study including hepatitis C virus (commonly known as HCV) patients, human immunodeficiency virus (commonly known as HIV) patients, or patients with HCV-HIV co-infection and advanced liver disease, simtuzumab showed no improvement in liver fibrosis after 22 weeks.¹⁰⁵

Inhibition of integrins, which are the receptors functioning in interactions between extracellular matrix and cells and also activators of pure profibrotic cytokine-transforming growth factor β -1, could have promising results in the treatment of hepatic fibrosis. Inhibitor of the α v β 6 integrin (GSK3008348) was investigated in phase 1 clinical trial (NCT02612051) for the first time in humans for the treatment of idiopathic pulmonary fibrosis. The drug was well tolerated, with an acceptable safety profile; consequently, further studies are warranted.¹⁰⁶

Treatments targeting inflammation

The boundaries between aforementioned pathways are arbitrary and, *in vivo*, these pathways overlap continuously. This is especially true for inflammatory pathomechanisms.

In liver fibrosis, one of the functionally important inflammatory but also fibrogenetic pathways for targeted therapeutic action is the expression of the carbohydrate molecule galectin 3 on inflammatory macrophages. Therefore, inhibition of this pathway by GR-MD-02 (galactoarabinorhamnogalacturonate, belaepectin; Galectin Therapeutics) was tested in several rodent models, resulting in effective reduction of liver fibrosis.¹⁰⁷ After 3 years of the first human phase 1 GT-020 study, the safety and efficacy of GR-MD-02 has been demonstrated.¹⁰⁸ Though, in two phase 2 clinical trials, the efficacy of GR-MD-02 in MASH patients did not produce the expected results. In a phase 2 clinical trial involving 30 MASH patients with advanced fibrosis (NCT02421094), GR-MD-02 had no significant effect on non-invasive biomarkers of liver inflammation or fibrosis after 16 weeks, as measured by magnetic resonance elastography and shear-wave ultrasonic elastography, since histopathology monitoring was not planned for this study.¹⁰⁹ In another phase 2b, randomized clinical trial of the safety and efficacy of GR-MD-02 involving 162 patients with MASH, cirrhosis, and portal hypertension (NCT02462967), GR-MD-02 showed no significant effect on liver fibrosis and liver-related outcomes. However, in a subgroup of patients without esophageal varices, GR-MD-02 has shown beneficial effects in alleviating portal hypertension and development of varices.¹¹⁰ According to a review from December 2019, a phase 3 clinical trial on GR-MD-02 in cirrhotic MASH patients began in the last quarter of 2019.¹¹

The chemokine-receptor axis plays a pivotal role in liver inflammation pathways. In chronic liver disease, chemokines are released by Kupffer cells, stressed hepatocytes, endothelial and hepatic stellate cells. Liver inflammation is regulated by proinflammatory chemokines, promoting recruitment, migration and infiltration of inflammatory

cells (lymphocytes, monocytes, neutrophils) to the injury site. However, different chemokines and their receptors are characterized as the basis of various chronic liver diseases. Even more so, recent research has shown that certain chemokines and immune cells could have anti-inflammatory and antifibrotic impacts.¹¹¹ One of the fundamental profibrotic pathways in chronic liver disease is the infiltration of inflammatory monocytes via the CC chemokine receptor (CCR)2.¹¹¹ Accordingly, inhibition of CCR2 in an experimental murine model of MAFLD has confirmed reduction of hepatic inflammation and fibrosis.¹¹² Another chemokine receptor, CCR5, had also been determined to exert profibrogenic effects in murine models, predominantly via impact on hepatic stellate cell activation.¹¹³ An inhibitor of dual chemokine receptors type 2 and 5 (CCR2/CCR5) was developed by Allergan, based on previous successful preclinical research, and it was called cenicriviroc. In a 2-year randomized, double-blind, multinational phase 2b clinical trial involving 289 subjects with noncirrhotic MASH and liver fibrosis (stages 1–3; CENTAUR (NCT02217475)), cenicriviroc has been evaluated as a promising antifibrotic therapeutic agent.¹¹⁴ Although the primary outcome of steatohepatitis alleviation has not been reached, the secondary endpoint of liver fibrosis improvement by ≥ 1 stage was achieved after 1 year of therapy. Exploratory analyses after to 2 years of follow-up showed sustained treatment benefit, with greater effects in patients with advanced fibrosis.¹¹⁵ At a dose of 150 mg daily, cenicriviroc showed a satisfactory safety profile in patients with mild-to-moderate hepatic impairment, with only a few drug-related side effects (*i.e.* fatigue with a frequency of 2.8%, and diarrhea with a frequency of 2.1%).¹¹⁵ A phase 3 multicenter, randomized, double-blind trial on efficacy and safety of cenicriviroc at a dose of 150 mg, known as the AURORA study (NCT03028740), is currently underway, with enrollment of 2,000 histologically-proven MASH subjects with fibrosis stage F2 or F3.¹¹⁶

The third inflammatory target is the vascular adhesion protein-1 (VAP-1), also termed in the literature as amine oxidase copper-containing 3. It is a membrane-bound amine oxidase, which has a dual effect in initiating lymphocyte migration to the site of hepatic injury and also promoting oxidative stress.¹¹⁷ In four murine models of hepatic injury, inhibition of VAP-1 resulted in reduced inflammatory cell recruitment to the liver and ameliorated liver fibrosis.¹¹⁷ Boehringer Ingelheim has been investigating BI 1467335 in a phase 2a trial (NCT03166735), as a pharmacological inhibitor of VAP-1 in 150 MASH patients.¹¹⁸ However, the company decided to discontinue the study for MASH due to risk of drug interactions.¹¹⁹

Conclusions

Liver fibrosis remains without approved pharmacotherapy. The treatment of the underlying disease, if possible, is the sole approach, with liver transplantation being the ultimate therapeutic option. For cases of MAFLD, representing the leading cause of liver fibrosis in the world, only a few specific therapeutic recommendations are available.¹¹⁸ Since fibrosis, as a consequence of chronic liver injury, is considered a key factor in the prognosis and overall mortality, its resolution is the main goal in new antifibrotic therapeutic approaches. Basic research and clinical trials have reinforced the complexity of underlying signaling pathways in liver fibrosis development. In this context, novel antifibrotic drugs are targeting cell death, abnormal liver metabolism, the gut-liver axis, myofibroblast activation, extracellular protein deposition, and inflammation. Promising new antifibrotic compounds currently in phase 3 clinical trials are obeticholic acid (FXR agonism) in REVERSE trials, elafibranor (dual

PPAR α/δ agonist) in the RESOLVE-IT study, and cenicriviroc (CCR2/CCR5 inhibitor) in the AURORA study. A key issue in the future will be potential combination therapy with a synergistic effect, which could target multiple underlying pathophysiological mechanisms of fibrosis. The modest effect sizes of different antifibrotic drugs will likely lead to pursuit of drug combinations personalized to each stage of the MAFLD disease spectrum.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Conceived of and designed the article, and critically revised the manuscript (RS, MS), performed critical revision of the manuscript for important intellectual content, obtained funding, and provided administrative, technical and material support (RS), performed literature searches and wrote the manuscript (VRR, KB), updated the text of the manuscript (MS, RS), performed literature searches and table and diagram drawing (JR), critical revision of the manuscript for important intellectual content (AT).

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Diversity in NAFLD: A Review of Manifestations of Nonalcoholic Fatty Liver Disease in Different Ethnicities Globally

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Abstract

Globally, the rise in prevalence of obesity and metabolic syndrome as a whole has been linked to increased access to processed foods, such as refined sugars and saturated fats. Consequently, nonalcoholic fatty liver disease (NAFLD) is on the rise in both developed and developing nations. However, much is still unknown on the NAFLD phenotype with regards to the effect of ethnic diversity. Despite similarities in dietary habits, it appears that certain ethnicities are more protected against NAFLD than others. However, manifestations of the same genetic polymorphisms in different groups of people increase those individuals' predisposition to NAFLD. Diets from different regions have been associated with a lower prevalence of NAFLD and have even been linked to regression of hepatic steatosis. Socioeconomic variations amongst different regions of the world also contribute to NAFLD prevalence and associated complications. Thus, a thorough understanding of ethnic variability in NAFLD is essential to tailoring treatment recommendations to patients of different backgrounds.

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Introduction

The current landscape of liver disease has evolved over the last few decades. With the advent of highly effective therapy for viral hepatitis, nonalcoholic fatty liver disease (NAFLD) has become one of the most common etiologies of chronic liver disease.¹ NAFLD is defined by detection of hepatic steatosis,

or the presence of macrovesicular fat in >5% of hepatocytes, either by imaging or histology, after exclusion of secondary causes and alcoholic fatty liver disease.² Currently, international experts consensus have proposed the more comprehensive and practical nomenclature of “metabolic dysfunction-associated fatty liver disease (MAFLD)”, which is defined by evidence of hepatic steatosis plus one of the following three criteria: overweight/obesity, presence of type 2 diabetes mellitus (DM), or evidence of metabolic dysregulation.^{3,4}

The increased prevalence of NAFLD is driven in large part by the increasing presence of metabolic syndrome, obesity, sedentary lifestyle, and improved access to food supplies globally.⁵ The effect is seen not only in adults but also in children and adolescents.⁶ This review will focus primarily on ethnic differences in NAFLD prevalence and its socioeconomic effect, in addition to risk factors, manifestations, diagnosis, and outcomes.

Diagnosis and manifestations

NAFLD is a spectrum of disease continuity, ranging from simple steatosis, steatohepatitis (NASH) with or without different stages of fibrosis, cirrhosis, and hepatocellular carcinoma (HCC).⁷ Even though most cases of HCC develop in cirrhotic patients, there is increasing evidence of development of HCC in non-cirrhotic NASH.⁸ The majority of individuals with NAFLD are asymptomatic, and the diagnosis is often discovered incidentally during workup for elevated liver function tests or via imaging for another purpose. Compared to the non-NAFLD population, a higher rate of fatigue impairing physical function has been found in NAFLD/NASH patients. Energy level is found to be lower in patients with significant hepatic fibrosis compared to a normal/mild hepatic fibrosis group.^{9,10} Pruritus has also been reported among NASH patients.¹¹ Other symptoms include abdominal bloating/swelling, abdominal discomfort, sleep disturbance, or apnea.¹² There are no data on difference in presentations amongst various ethnicities.

NAFLD is most often diagnosed non-invasively with abdominal imaging; although, a liver biopsy is the gold standard to determine the presence steatohepatitis and stage of fibrosis. It is worth noting that ultrasound sensitivity to NAFLD is poor when steatosis occupies less than 30% of the liver, making it an inferior modality to diagnose the condition at the 5% liver fat reference.¹³ However, specific biomarker panels such as the fatty liver index (FLI) as validated in Italy (AUROC 0.84)¹⁴ and the hepatitis steatosis index (HIS) as validated in Korea (AUROC 0.81)¹⁵ can augment ultrasonography and assist in better defining the risk of steatosis in patients of different ethnicities.

Due to the higher cost and/or health risks associated with other modalities, such as computed tomography or liver biopsy, ultrasound is primarily used to diagnose patients in

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Abbreviations: APRI, aspartate aminotransferase/platelet ratio index; ARFI, acoustic radiation force impulses; BMI, body mass index; CAP, controlled attenuation parameter; DM, diabetes mellitus; FIB-4, fibrosis-4 index; FLI, fatty liver index; GWAS, genome-wide association studies; HCC, hepatocellular carcinoma; HIS, hepatitis steatosis index; MAFLD, metabolic dysfunction-associated fatty liver disease; MRE, magnetic resonance elastography; MS, metabolic syndrome; NAFLD, nonalcoholic fatty liver; NASH, nonalcoholic steatohepatitis; NHANES, National Health and Nutrition Examination Survey; PNPLA3, patatin-like phospholipase domain-containing protein 3; TE, transient elastography.

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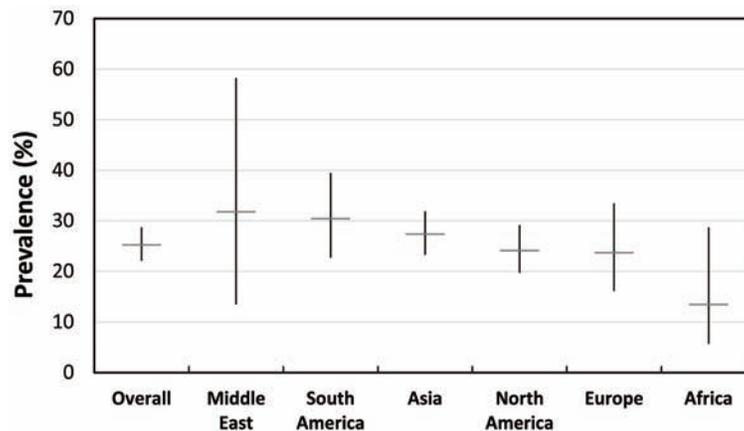


Fig. 1. Prevalence of NAFLD by geographical regions.

developing countries.¹⁶ Controlled attenuation parameter (CAP) is an ancillary tool to conventional ultrasonography and measures how hepatic fat attenuates ultrasound waves while liver stiffness is measured by transient elastography. It can assist in detecting and grading hepatic fat, although it has not been shown to reliably estimate the quantity of liver fat.¹⁷ Magnetic resonance imaging, *i.e.* spectroscopy or proton density fat fraction, is increasingly being used in NAFLD clinical trials to detect hepatic steatosis in both Western and Eastern countries.¹⁸ Factors that prevent magnetic resonance imaging from consideration as a first-line diagnostic tool include accessibility due to specialized equipment parts and expertise of the operator and radiologist.¹⁹ A noninvasive algorithm developed in Finland, known as the NAFLD liver fat score, used ¹H-MRS as a reference and had an AUROC of 0.86–0.87, but used serum insulin level as a part of its panel, which limited its accessibility to a wider patient population.²⁰

Assessing the stage of hepatic fibrosis is important in NAFLD as it can predict the risk of disease progression and mortality.²¹ Non-invasive tests including serum biomarkers and imaging tests have been developed to detect hepatic fibrosis in risk stratification. Non-invasive serum biomarkers include simple tests, such as aspartate aminotransferase/alanine aminotransferase ratio, NAFLD fibrosis score, fibrosis-4 (commonly known as FIB-4) index, and aspartate aminotransferase/platelet ratio index (commonly known as APRI), and complex tests, such as FibroTest/FibroSure, FibroSpect, enhanced liver fibrosis panel, Pro-C3 based predictive fibrosis score and NIS4, which are not commercially available yet.²² However, the simple non-invasive biomarkers are less sensitive in identifying advanced fibrosis in South Asians compared to Caucasians.²³ The imaging tests including transient elastography, acoustic radiation force impulses (commonly known as ARFI), 2D shear wave elastography and magnetic resonance elastography (commonly known as MRE) have been shown to accurately detect advanced fibrosis.²⁴ ARFI and 2D shear wave elastography imaging can reliably detect cirrhosis, although the data for transient elastography and MRE are more robust.^{25,26} However, studies investigating these different modalities that were performed in patients from Asia, Europe and the USA have shown no obvious differences in performance amongst different patient ethnicities.

Epidemiology

Prevalence

The global prevalence of NAFLD varies across different re-

gions. It has been estimated to be around 25.24% of the population based on data from 2016. It is highest in the Middle East followed by South America and Asia. The prevalence of NAFLD in North America and Europe is estimated around 24%, while it was lowest in Africa (Fig. 1).²⁷ NAFLD is known to occur between the 4th and 6th decades of life,² although older age is related to higher rates of developing the disease. Traditionally, it was thought that NAFLD occurred more in men, although some studies dispute this sex bias. The median age of women who develop NAFLD is also higher than that of men.²⁸ While studies from Thailand and Sri Lanka have found increased prevalence of NAFLD in females, in the USA, China, and Spain the prevalence was higher in men.²⁹ Many factors, including diagnostic modalities used and inter-country variation in obesity prevalence by sex, could contribute to the sex discrepancies found among studies.

The Dallas Heart Study is a key study that explored NAFLD distribution amongst different ethnicities in the USA.³⁰ Using magnetic resonance spectroscopy, the investigators observed that there was a significant difference amongst patients of Hispanic, Caucasian, and African American ethnicity with respect to the prevalence of NAFLD, with those of Hispanic origin being more predisposed. Similar data was also derived from the Multiethnic Cohort Study in Hawaii, although the authors reported a significantly increased prevalence in Japanese Americans.³¹ Individuals of Alaskan-Native ethnicity have been reported to have a NAFLD prevalence under 5%, although this should be confirmed in future studies.³²

Studies from both the USA and South America have established that not all patients of Hispanic origin have the same predisposition to NAFLD. While the prevalence among the overall Hispanic population has been reported as high as 29%, within the USA, individuals of Hispanic ethnicity from Mexico had a significantly higher prevalence of NAFLD than patients from Puerto Rico or the Dominican Republic.³³ Moreover, patients from Central America and South America also had higher a prevalence of NAFLD that was not explained by differences in rates of metabolic syndrome, physical activity, or diet.³⁴ For example, studies from Brazil estimate the prevalence of NAFLD to be around 30%.³⁵ In a study of patients who underwent bariatric surgery, advanced stage of disease was likely to occur more in Hispanic (43%) and non-Hispanic white (46%) patients compared to non-Hispanic Black (21%) patients.³⁶

In Europe, the prevalence of NAFLD varies greatly among the different countries; although similar to patients around the globe, those with metabolic syndrome had a NAFLD prevalence of around 50%.³⁷ It is worth noting that Eastern

European countries such as Romania reported lower prevalence at around 20% in the same group of individuals.³⁸ Within European countries, prevalence also has proven to vary among different ethnicities. In Greece, prevalence of NAFLD is reported to be about 41% and in Spain was found to be 33% in males and 20% in woman.²⁹ In the UK, the highest incidence of abnormal liver tests were among patients of Bangladeshi, Pakistani, and Indian heritage.³⁹

Interestingly, although patients of Asian ethnicity have a lower body mass index (BMI) on average than other racial groups, they continue to have a higher prevalence of NAFLD, reported as high as 29.62%, which has increased significantly over the past two decades. The reported prevalence rates from Korea and Taiwan are 24–40% and 15–27% respectively, compared to Japan at 9–18%. Indonesia has the highest prevalence (51.04%).⁵

Although literature from Africa is limited, the reported prevalence was among the lowest worldwide, with Nigeria reporting 8.7% prevalence.⁴⁰ In fact, the Dallas Heart Study noted African Americans to have the lowest prevalence of NAFLD in the USA; although, they do have a higher chance of in-hospital mortality, longer hospital stay, and poorer discharge destination compared to non-Hispanic Caucasians.⁴¹ Moreover, a South African study found that progression of fibrosis in patients with NAFLD seems to occur less frequently in those of Black African ethnicity.⁴²

A meta-analysis of the literature published on NAFLD from around the world noted that the highest prevalence of NAFLD is in South America and the Middle East.⁴³ In the Middle East, it is estimated to be 20–30%; although, wide variation appears to exist between various countries. For example, Iran reports the prevalence to be 4.1%, while Saudi Arabia cites 16.6%.²⁹ Unfortunately, the majority of Middle Eastern countries have not published extensive studies on NAFLD and inconsistencies in regulation, practice, and reporting have affected the quality of data in certain national registries.⁴⁴

Complications/outcomes

Complications and comorbidities of NAFLD are diverse among different ethnicities. The most common cardiovascular complications or comorbidities in China are carotid and coronary artery disease, followed by hypertension.⁴⁵ In the USA, African Americans with NAFLD are more likely to have abdominal aorta calcification than non-Hispanic Caucasians, Chinese Americans and Hispanics, with a 41% higher prevalence.⁴⁶ In a recent meta-analysis of NAFLD patients comparing patients of different ethnicities, there was no statistically significant difference in severity of fibrosis among Hispanics, non-Hispanic Caucasians, and Blacks. Regarding outcomes of NAFLD, including progression to cirrhosis, development of HCC, liver-related mortality and all-cause mortality, the data are heterogeneous.⁴⁷ The annual incidence of HCC in Asian countries is higher than the global incidence rate for HCC (1.8 cases per 1,000 person-years vs. 0.44 case per 1,000 person-years), which may be related to a lack of public awareness of the disease. Consequently, this may lead to delays in proper intervention, monitoring and screening for HCC in these countries.^{29,48}

Some studies suggest that Hispanics have higher rates of disease progression and fibrosis.³⁰ Data analysis from the Scientific Registry of Transplant Recipients between 2002 and 2019 demonstrated that the rate of NASH is lower in Black patients and the majority of liver transplant waitlist candidates for NASH are of Hispanic ethnicity. Moreover, NASH-HCC is the leading indication for transplantation in Hispanic patients.⁴⁹

Socioeconomic effect

There is a significant socioeconomic effect on NAFLD. Shifting the pattern of lifestyle and diet have contributed to the prevalence of obesity and NAFLD in Asia. The urbanization of countries in Asia promotes the risks of NAFLD in those populations with increasing prevalence of obesity and DM. The lowest prevalence of disease is in Japan, while the highest rates of NAFLD associated-cirrhosis is in Japanese-Americans,⁵⁰ suggesting the implication of lifestyle and socioeconomic conditions in the prevalence of NAFLD.⁴⁸ Similarly, differences in prevalence of NAFLD between Africans in Nigeria and African Americans indicates the implication socioeconomic effect on the epidemiology.⁵¹ The prevalence among the younger Chinese population (<60 years-old) was also higher compared to similarly aged patients in the West that is due to rapidly increasing in prevalence of NAFLD from 2008–2010 to 2015–2018 along with rapid change in socioeconomic status.⁴⁵ A population-based study in southwest Iran suggested that patients with a high socioeconomic level were likely to have NAFLD.⁵² On the other hand, socioeconomic conditions impact the outcomes of NAFLD. The proportion of deaths from NASH was lowest in the high-income Asia Pacific region.⁵¹ Poor awareness of NAFLD and less health care utilization in China may contribute to the higher incidence of HCC.^{45,53,54}

Risk factors

Weight

The World Health Organization estimated in 2016 that there are more than 1.9 billion adults who are considered overweight or obese.²⁹ The USA has the highest overall number of obese adults and Indonesia is reported to have the lowest. In terms of prevalence, Oceania has the highest prevalence, while the Middle East is second in prevalence.²⁹ A recent study demonstrated lower rates of overweight and obese individuals in China, with a higher prevalence and incidence of NAFLD than Western countries. Genetic factors with an involvement in high frequency of NAFLD promotion genes and low frequency of NAFLD risk reduction genes might contribute to the predisposition of NAFLD and metabolic disease in the Chinese population.^{45,48} The World Health Organization has classified Asians with BMIs of 23–27.5 kg/m² to be at increased risk of obesity-related conditions.⁵⁵

NAFLD in lean individuals, which is commonly defined as NAFLD in patients with a BMI <25 kg/m², has been studied among different ethnicities and nationalities, including Asians, Indians, and Caucasians. The overall prevalence of lean NAFLD is 5–26% in Asian populations, and 7–20% in Western populations. These data should be interpreted with caution, given the different BMI cut-offs among different regions. Among Asian countries, the prevalence ranges from approximately 7.27–8% in China, 4.2% in Taiwan, 19.3% in Hong Kong, 12.6–22.4% in Korea, 15.2% in Japan, and 6.4% in India. Compared to Asia, the data in Caucasians are sparse, with smaller sample sizes. A meta-analysis of 93 international studies estimates that 40% of patients with NAFLD globally are non-obese and an estimated 20% were classified as lean NAFLD. The prevalence of non-obese NAFLD notably varied quite significantly amongst different countries, with a reported prevalence as low as 25% in Pakistan to more than half of the general Austrian and Swedish population.⁵⁶ Other country specific studies have reported lean NAFLD rates of 16% in Italy, 17% in the Dallas Heart Study, and 7.4% in the largest epidemiological study in USA. Lean NAFLD is associated with proinflammatory

visceral adipose tissue, central obesity, higher triglyceride level and early adulthood weight gain in Asian populations, whereas it is associated with insulin resistance, younger age, female sex, and hypercholesterolemia in the Caucasian population.⁵⁷ Another study demonstrated 2- to 3-fold higher risk of insulin resistance in Asian Indians compared to matched Caucasians, Africans, and Eastern Asians.⁵⁸ A multiethnic study in the USA also showed that non-obese Hispanics, Chinese Americans, and South Asians were at higher risk of being metabolically ill health compared to non-Hispanic Caucasians due to insulin resistance.⁵⁹

In longitudinal studies, development of advanced liver disease in lean NAFLD is associated with hypertriglyceridemia and higher creatinine in Asians. Moreover, lean NAFLD patients in one study were more likely have disease progression compared to obese NAFLD patients amongst Caucasians.⁵⁷ In fact, global estimates of NASH and stage 2 or greater fibrosis in non-obese and lean NAFLD patients are 39.0% and 29.2%, respectively. Consequently, this has been linked to all-cause, liver-related, and cardiovascular-related mortality rates of 12.1, 4.1, and 4.0 per 1,000 person-years in this particular demographic.⁵⁶ A multicenter study from Italy further classified the risk of disease progression and comorbidities in lean vs. obese patients with NAFLD. In this patient cohort, individuals with lean NAFLD were less likely to have metabolic syndrome and its associated complications, including hypertension, diabetes and carotid artery disease, and less likely to have NASH or advanced fibrosis compared to overweight and obese NAFLD patients. Interestingly, waist circumference appeared to be an important factor in determining the risk of associated comorbidities and disease progression. In fact, lean NAFLD patients with a medium waist circumference (men: 94–102 cm; women: 80–88 cm) were more likely to be diabetic than overweight and obese patients with a similar waist circumference. In the overall study population, individuals in the highest waist circumference category were at the highest risk for disease progression, regardless of BMI.⁶⁰ Factors that might contribute to lean NAFLD encompasses concomitant alcohol intake, endocrine disorders (polycystic ovarian syndrome, hypothyroidism, growth hormone deficiency), congenital and acquired lipodystrophy from human immunodeficiency virus medications, association with certain drugs (such as steroids, amiodarone, methotrexate and tamoxifen), inborn errors of metabolism (lysosomal acid lipase deficiency), genetic factors [polymorphisms in the gene that encodes for patatin-like phospholipase domain-containing protein 3 (*PNPLA3*)], nutritional factors (starvation, total parental nutrition), and gastrointestinal surgery (jejunioileal bypass).⁶¹

Metabolic syndrome

Metabolic syndrome (MS) includes abdominal obesity, DM, hypertension, and dyslipidemia.⁶² Both obesity and DM are increasing globally and are correlated with increased prevalence of NAFLD. Type 2 DM is a major risk factor for the development of NAFLD and accelerates progression to advanced liver disease and increase risk for mortality. According to the World Health Organization, there has been an increase in DM from 108 million in 1980 to 422 million in 2014, with a notable increase amongst patients in low and middle income countries.⁶³ The prevalence of NAFLD/NASH among type 2 DM is over 60%.⁶⁴ Similarly, the prevalence is 52.55% in Asians.⁴⁸ In the USA, Hispanics have a 12.8%, non-Hispanic Blacks have 13.2% and Asian Americans have 9% higher risk of type 2 DM, respectively, when compared to non-Hispanic Caucasians.⁶⁵ Native American adults in Southern Arizona have one of the highest preva-

lence rates of DM in the world, estimated at 33%. According to the 2019 International Diabetes Atlas, the prevalence of diabetes in adults was 8.5% in South and Central America, 12.2% in the Middle East and North Africa, 4.7% in Africa, 11.5% in South-East Asia, 11.4% in the Western Pacific, and 6.3% in Europe.⁶⁵

Hypertension is another risk factor for NAFLD and has a bidirectional relationship with NAFLD. The severity and progression of NAFLD have been associated with hypertension. On the other hand, NAFLD is also a possible risk factor for development of hypertension.⁶⁶ In the USA, the prevalence of hypertension is higher in non-Hispanic Blacks compared to non-Hispanic White and Hispanic populations.⁶⁷ When the 2017 American College of Cardiology/ American Heart Association hypertension guideline was applied globally, the prevalence of hypertension increased in Canada (32% to 46%),⁶⁸ India (29% to 43%),⁶⁹ China (25% to 50%),⁷⁰ and South Korea (28% to 48%),⁷¹ respectively.

Dyslipidemia induces inflammation and increases cytokine production and oxidative stress, triggering pathogenesis of NAFLD. Moreover, dyslipidemia is responsible for cardiovascular risk in NAFLD patients.⁷² Hypertriglyceridemia was found to be highest in East Asians and non-Hispanic Whites and lowest in the African American population.⁷³ The prevalence of high low-density lipoprotein cholesterol was higher in Asian Indians, Filipinos, Japanese, and Vietnamese compared to non-Hispanic Whites.⁷⁴ MS was found to be present in 34%, 62%, 31%, 33%, and 37% of patients with NAFLD in Asia, Europe, Middle East, North America and South America, respectively. The incidence of MS is higher among NAFLD patients compared to controls. In Asia, the incidence increases by 14% if patients have at least three components of MS compared to patients with fewer MS.⁵ NAFLD patients with multiple components of MS are at higher risk for advanced fibrosis and all-cause mortality. Each additional MS condition worsens the risk of liver-related and all-cause mortality based on The Third National Health and Nutrition Examination Survey (commonly known as NHANES).⁷⁵

Genetics

With the advent of genome-wide association studies (commonly referred to as GWAS), many genes have been brought to the forefront of NAFLD research. A select few of these genes have been further explored with respect to their role in NAFLD development in different ethnicities, and many more are under investigation.

One of the most notable genes in the field of NAFLD codes for *PNPLA3*. In particular, the genetic variant rs738409, which results in substitution of methionine for isoleucine, resulting in a loss of function in the *PNPLA3* protein, contributes to increased accumulation of triglycerides in lipid droplets within hepatocytes as compared to cells with functional *PNPLA3*.⁷⁶ This variant has been associated with increased susceptibility to NAFLD in a variety of different ethnicities, including Hispanics, African Americans, East Asians, and South Asians. Particularly in studies from Asia, a “lean NAFLD” phenotype, as previously mentioned, is well described. These patients have a BMI that is lower than the commonly accepted obese range, and yet still develop NAFLD at notable rates.⁷⁷ A study from Hong Kong noted that the rs738409 allele is more common in patients with “lean NAFLD” than in obese patients.⁷⁸ Another gene variant of *PNPLA3*, rs6006460, results in a phenotype characterized by lower than average hepatic fat. This variant, interestingly, occurs in 10% of African Americans as opposed to <1% in either Caucasians or Hispanics,⁷⁹ and may in part explain the lower observed incidence of NAFLD amongst this

patient demographic.

Another gene linked to the development of NAFLD that has also been studied within the context of ethnicity codes for the membrane-bound *o*-acyltransferase domain-containing 7 protein. The rs641738 variant has been associated with increased NAFLD risk in Europeans⁸⁰ but has not been studied in other populations as extensively. Two other genes with variants associated with NAFLD, *TM6SF2* and *GCKR*, have been well described. *TM6SF2* codes for a protein of unknown function, although different variants have been linked to increased hepatic lipid content, as well as increased levels of aspartate aminotransferase/alanine aminotransferase.⁸¹ *GCKR*, which codes for a glucokinase regulator, similarly has variants that are associated with hepatic fat accumulation.⁸² A population-based study among Hispanic/Latino adults in the USA found a high frequency of *PNPLA3* G (41%) and a low frequency of *TM6SF2* T (5%) in Hispanics/Latinos. Among Hispanics, the *PNPLA3* G frequency was the highest in Mexicans (52%) and the lowest in Dominicans (23%).⁸³ In another study that compared genetic predisposition between Chinese and Caucasians, the frequency of *PNPLA3* polymorphism was higher and that of *TM6SF2* was lower in Chinese compared to Caucasians.⁴⁵

GWAS studies have also yielded a number of different genes associated with NAFLD, including but not limited to *LPIN1*, *Tribble-1*, *FDFT1*, *ERLIN1*, *etc.* Genes involved in the pathophysiology of other liver conditions have also been noted to contribute to risk of NAFLD development; for example, mutations in *HFE*, most commonly noted in hereditary hemochromatosis, have been cited as risk factors for NAFLD.⁸⁴ It is also worth mentioning that ongoing investigations into epigenetics, microRNA, and mitochondrial RNA may improve our understanding of how NAFLD presents in different ethnicities in the future.

Lifestyle

Diet

Although it seems intuitive that certain elements of diet and weight control would predispose specific populations to NAFLD, the literature that explores these factors is more recent. Overall, increased intake of saturated fats, fructose, and cholesterol-rich sources predisposes individuals to NAFLD.⁸⁵ Although evidence currently is limited, sedentary behavior has been increasingly accepted as an independent risk factor for NAFLD.⁸⁶ As an example to the beneficial effect of physical activity, aerobic exercise that contributes to progressive increase in fat-free, lean mass can provide protection against NAFLD. Dietary elements typical of the Western Diet in addition to Western eating habits, such as snacking, have been shown to independently contribute to hepatic steatosis.⁸⁷ Soft-drink consumption, rampant in the West, has been shown to increase liver fat by 140% over a period of 6 months in otherwise healthy individuals.⁸⁸ Relatedly, it was found that a diet of 3 g fructose/kg increases the amount of hepatic fat in adult men.⁸⁹

Limiting consumption of carbohydrates has generally been noted to improve NAFLD;⁹⁰ however, if carbohydrates are examined overall, a focus on ethnic diet variations presents a more complicated picture. For example, the traditional Chinese diet is high in carbohydrates but is also vegetable-rich and has proven to be low-risk for NAFLD. Consistent with earlier mentioned data, a more recent study noted that "Westernization" of South Korean food, via refined grains, processed meats, fried foods, *etc.*, correlated with increased incidence of NAFLD diagnosed by ultrasound.⁹¹ Such regions have also noted that weight gain in general, irrespective of diet, seems to predispose people

to NAFLD in certain regional studies. Korean studies have noted that weight gain as low as 2 kg can contribute to NAFLD development.⁹² More specifically, data from Hong Kong reported that the presence of central obesity predisposes patients to NAFLD.⁹³

Variations and similarities in the standard diet within a geographic region or amongst individuals of a particular ethnic background has allowed researchers to investigate the different nutritional patterns that promote hepatic steatosis and the progression of NAFLD, as well as whether a specific diet affects disease progression differently in patients of various backgrounds. Ultimately, this informs healthcare providers when counseling patients with NAFLD on ideal eating habits, and whether recommendations can be generalizable to individuals of various ethnicities.

The Western dietary pattern, containing large amounts of red meat, processed meat, and fried foods, has a well-established link to the development of MS.⁹⁴ Unfortunately, this diet has established itself and its associated adverse health consequences globally. A study of 170 Iranians with NAFLD evaluated the effects of an Iranian, Western and "healthy dietary patterns" on liver fibrosis. The Western dietary pattern was strongly associated with fibrosis, with an odds ratio of 4.21. The investigators noted that higher consumption of red meat, hydrogenated fats, and soda drinks increased the odds of fibrosis measured by elastography, while a diet rich in low-fat dairy, nuts, fruit, and coffee or tea was protective.⁹⁵ Interestingly, the positive association between a Western diet and NAFLD was not replicated in a prospective cross-sectional study of 1,190 Korean patients with and without NAFLD. Four dietary patterns and their association with a diagnosis of hepatic steatosis were analyzed, including a traditional Korean diet, Western and high-carbohydrate diets, and a simple meal pattern diet. As previously mentioned, this study revealed no association between a Western or carbohydrate-rich diet and the presence of NAFLD, while a traditional Korean diet was positively correlated with presence of the disease.⁹⁶ While the absence of an association between increased carbohydrate intake and NAFLD is somewhat surprising, data from other studies have been inconsistent with regards to the effect of carbohydrate intake. Patients who were on a 2-week carbohydrate-restricted diet had similar weight loss but more hepatic fat reduction than patients who were on a reduced calorie-only diet.⁹⁰ However, another study demonstrated similar improvements in hepatic steatosis between patients on a high-carbohydrate diet and low-carbohydrate diet when weight loss was comparable,⁹⁷ suggesting that the true benefit of carbohydrate restriction on NAFLD arises primarily when it is linked to weight reduction. However, a low carbohydrate diet in patients with NAFLD has been associated with a decrease in alanine aminotransferase, although the diet was primarily soy-based.⁹⁸

In contrast to the Korean dietary study and similar to findings of the Iranian investigators, a study out of Greece confirmed the increased odds of NAFLD with a fast-food type dietary pattern, while also confirming that when unsaturated fatty acid intake was divided into quartiles, those in the second quartile had an over 50% reduced odds of NAFLD compared to individuals within the first quartile of dietary intake.⁹⁹ With respect to fats, a potential therapeutic strategy includes increased consumption of both mono-unsaturated fatty acids and poly-unsaturated fatty acids. Increased intake of poly-unsaturated fatty acids results in greater reduction in hepatic steatosis when used in combination with a heart healthy diet compared to dieting alone.¹⁰⁰ In fact, a meta-analysis has found that omega-3 fatty acids derived from seafood sources have a positive effect on hepatic steatosis.¹⁰¹

Given the increased popularity and presence globally of the Western style diet, the above data generally seem to

suggest a benefit to avoiding this type of nutritional behavior. In fact, a study assessing patients (Framingham Heart Study), consisting primarily of Caucasian patients in the USA reinforced the benefits of focusing on alternative diets for liver fat accumulation. The study investigated how changes in the Mediterranean-style diet score and Alternative Healthy Eating Index affected liver fat and new-onset fatty liver. An increase in either dietary score was inversely associated with liver fat accumulation and incident NAFLD, with a reduction in the odds of fatty liver by 26% for every 1-standard deviation increase in Mediterranean-style diet score. Moreover, individuals with a higher genetic predisposition to NAFLD as determined by single nucleotide polymorphisms and decreased Mediterranean-style diet score or Alternative Healthy Eating Index scores had higher liver fat compared to patients with improved or stable scores.¹⁰² Thus, adopting the Mediterranean diet, typically characterized by high intake of olive oil, nuts, fruits, vegetables, legumes, and fish, with wine in moderation, can be suggested to patients with NAFLD, especially given the broad health benefits related to a variety of different health conditions related to MS. Despite heterogeneity in the way the Mediterranean Diet is defined in different studies, it has consistently shown favorable health outcomes.¹⁰³ Studies from multiple regions of the world have reported a marked regression of NAFLD when patients switched to the Mediterranean Diet,¹⁰⁴ reinforcing its broad appeal regardless of patient ethnicity. A large randomized control trial to evaluate the benefit of this diet, independent of weight loss, is currently underway in Australia.¹⁰⁵

With respect to protein intake, it seems that a diet consisting of a larger proportion of protein does not necessarily aid in improvement of NAFLD. However, a moderate protein diet encompassing 25% of total caloric intake has been shown to be optimal, and higher percentages do not necessarily reduce body fat content any better.¹⁰⁶

Ultimately, the guidance provided by a patient's health-care provider is key to successful changes in dietary habits that can ultimately improve or reverse hepatic steatosis. Studies show that patients will make better nutritional choices after having expressed better understanding of what NAFLD is, reinforcing the importance of patient education. Moreover, as a preventative measure, recommending dietary patterns that reflect an adherence to a healthy diet can reduce NAFLD risk in the general population. An analysis of the multiethnic cohort consisting of patients of African American, Japanese American, Latino, Native Hawaiian, and Caucasian descent revealed that high Healthy Eating Index and Dietary Approaches to Stop Hypertension scores were associated with lower risk of fatty liver, with no observed differences by race or ethnicity.¹⁰⁷ However, a more recent analysis of the same cohort focused on the specific components of enrollees' diets at baseline and association with NAFLD. Overall, when comparing 2,974 patients with NAFLD and 29,474 matched controls, intake of poultry, cholesterol, processed red meat and red meat in general was associated with NAFLD. When stratified by race and ethnicity, poultry intake and cholesterol intake was only significantly associated with NAFLD in Whites and Native Hawaiians. Processed red meat correlated significantly with NAFLD in Latinos and Whites, and increased fiber intake was protective in these two ethnic groups.¹⁰⁸ The damaging effects of red meat consumption and the associated risk of NAFLD was further demonstrated in a cross-sectional study of 789 adults. After controlling for BMI, smoking, alcohol intake, physical activity, energy and saturated fat and cholesterol intake, the consumption of meat in general and red or processed meat was associated with increased odds for insulin resistance and NAFLD.¹⁰⁹ Thus, while an overall healthy diet is recommended for all patients, with the ultimate goal of achieving meaningful weight loss, a specific list of foods to avoid may

be tailored to a patient based on race and ethnicity.

Intermittent fasting has recently gained popularity as an alternative to more traditional diets, aimed at reducing weight and improving obesity-related comorbidities. It includes diets that focus on a particular eating pattern where caloric intake is eliminated during a predefined period of time, and has consistently proven beneficial for both weight loss and reduction in obesity-related comorbidities.¹¹⁰ A randomized controlled trial assessing a modified alternative-day calorie restriction diet, another form of intermittent fasting, found that adherence to this diet amongst patients with NAFLD was excellent (75–83%) with a significant reduction in liver steatosis and fibrosis (measured by shear wave elastography) amongst individuals randomized to 8 weeks of the intervention diet arm.¹¹¹

Physical activity

Exercise as an intervention in NAFLD has also been studied, although the data concerning its benefits independent of the weight loss requires further clarification. Studies have noted a 20–30% decrease in hepatic lipid content with general exercise. Interestingly, even when these patients regain lost weight, there seems to be a persistent long lasting beneficial effect on liver fat and insulin resistance.¹¹² Aerobic exercise has been shown to reduce hepatic triglycerides in sedentary and obese patients.¹¹³ Weight resistance exercise has also been linked to a reduction in hepatic steatosis without weight loss.¹¹⁴ Although evidence on aerobic vs. resistance training is mixed, combination therapy seems to be superior to either. However, weight loss was a confounder when comparing aerobic with resistance exercise and a combination of the two,¹¹⁵ and thus these recommendations require further investigation prior to unlinking their benefits to weight loss alone.

Non-obese patients with underlying NAFLD or NASH can be more challenging to manage in the absence of approved pharmacotherapy. As mentioned previously, the strongest evidence for the management of NAFLD and NASH comes from studies focused on interventions that achieve weight loss and increased physical activity. While implementing these interventions intuitively makes sense in obese individuals or those with other components of MS, it is less obvious for those considered within normal BMI range, which has been more commonly seen in patients of east Asian background.¹¹⁶ However, a study examining the effect of diet modification and exercise on hepatic steatosis in 1,365 potential living donors with NAFLD on initial biopsy revealed that although only 5% of patients were obese at the start of the study, histological improvement on repeat biopsy was observed in 85.8% of participants.¹¹⁷ Some of these findings may, in part, be related to the response to weight loss seen with variants of the *PNPLA3* alleles. More specifically, the GG genotype that is more commonly observed in Asian patients with "lean NAFLD" has been linked to a favorable histologic response to diet modification leading to weight loss. Thus, despite the challenges associated with recommending weight loss to patients with a normal BMI, interventions that reduce weight are likely to be beneficial in this patient population. Moreover, although "lean NAFLD" is well recognized in patients of Asian background, with prevalence as high as 19% in Asia compared to 7% in the USA, this phenotype is now increasingly recognized in other races. In fact, a large Swedish cohort with 646 patients with biopsy-proven NAFLD, the "lean NAFLD" prevalence was reported as 19%,¹¹⁸ identical to estimates from Asia, making these recommendations more generalizable than previously thought.

Studies assessing the efficacy of caffeinated beverages

have shown consistent results. Overall, while the majority of studies do not show any significant improvement in steatosis with increased caffeine intake, coffee and other caffeinated beverages may have a protective effect against the development or presence of fibrosis.¹¹⁹ An analysis of the multiethnic cohort evaluating the association between coffee intake and chronic liver disease and HCC revealed an inverse association between increased coffee consumption and the incidence of HCC or chronic liver disease. In fact, consuming ≥ 4 cups of coffee a day was associated with a 41% reduction in HCC and a 71% reduction in chronic liver disease, when compared to non-coffee drinkers. This association did not differ based on patient race or ethnicity. Given the risk of HCC associated with NAFLD, including in the absence of cirrhosis, a balanced increase in coffee intake may be beneficial regardless of race.¹²⁰

Conclusions

The prevalence of NAFLD varies globally. Different ethnicities carry distinct risks for NAFLD. The shifting paradigm of socioeconomic status, lifestyle, and dietary habits plays an important role in raising the incidence of NAFLD irrespective of genetic and geographic background. Those changes are diverse among different ethnicities and/or countries. The disparity in availability and accessibility to certain diagnostic tests and healthcare utilization in different countries also impacts the rates of outcomes or complications. We have briefly summarized the characteristics of NAFLD in different ethnic populations in Table 1. While it is important to develop universal guidelines that can be used for patients of any ethnic background, it is also imperative that diagnosis and management of NAFLD is tailored differently amongst various populations. We need more effort in educating patients on NAFLD and improvement in the utilization of non-invasive tests to risk-stratify these patients. Obtainment of more data on socioeconomic effects of NAFLD is necessary, as such plays a major role in development and complications of NAFLD. Finally, it is important for nations to work together to implement education and prevention programs to decrease the growing burden from this disease.

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Conflict of interest

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Author contributions

Drafting of manuscript (QY), critical revision of the manuscript for the important intellectual content (MATH, ZT, NP), administrative support (NP), supervision (NP).

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Table 1. Summary characteristics of NAFLD in different ethnicities

Ethnicity	Characteristic
Caucasian	More obese NAFLD than lean NAFLD
Hispanic	Leading cause of NASH-HCC transplant in the USA
Asian	More lean NAFLD than other ethnicities Urbanization and change in pattern of lifestyle and diet contributed to NAFLD Poor awareness of NAFLD and less health care utilization
Non-Hispanic Black or African American	Higher chance of in-hospital mortality, longer hospital stay and poorer discharge destination Need more data on prevalence in Africa

- disease: An international expert consensus statement. *J Hepatol* 2020; 73: 202–209. doi:10.1016/j.jhep.2020.03.039.
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Noncanonical NF- κ B Signaling Pathway in Liver Diseases

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Abstract

The noncanonical NF- κ B signaling pathway is an important branch of NF- κ B signaling. It is involved in regulating multiple important biological processes, including inflammation and host immune response. A central adaptor protein of the noncanonical NF- κ B pathway is NF- κ B-inducing kinase (NIK), which activates the downstream kinase IKK α to process p100 to p52, thereby forming the RelB/p52 heterodimer to initiate the expression of target genes. Currently, many specific inhibitors and monoclonal antibodies targeting or triggering this pathway are being developed and tested for various diseases, including cancers, autoimmune diseases, and virus infection. Given that aberrant activation of the noncanonical NF- κ B pathway is frequently observed in various liver diseases, targeting this pathway may be a promising therapeutic strategy to alleviate liver inflammation. Moreover, activation of this pathway may contribute to the antiviral immune response and promote the clearance of persistent hepatotropic virus infection. Here, we review the role of the noncanonical NF- κ B pathway in the occurrence and development of different liver diseases, and discuss the potency and application of modulating the noncanonical NF- κ B pathway for treatment of these liver diseases.

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Abbreviations: I κ B, κ B inhibitory factor; NIK, NF- κ B-inducing kinase; TNFR, tumor necrosis factor receptor; LTBR, lymphotoxin beta receptor; BAFFR, B-cell-activating factor belonging to TNF family receptor; RANK, receptor activator for nuclear factor κ B; Fn14, fibroblast growth factor-inducible 14; TRAF, tumor necrosis factor receptor-associated factor; MCSFR, macrophage colony stimulating factor receptor; IKK, I κ B kinase; cIAP, cellular inhibitor of apoptosis protein; TBK1, TANK binding kinase 1; TNAP, TRAF- and NIK-associated protein; miRNA, microRNA; NAFLD, nonalcoholic fatty liver disease; ALD, alcoholic liver disease; NASH, nonalcoholic steatohepatitis; BAFF, B-cell-activating factor belonging to TNF family; KO, knockout; mTECs, mouse thymic medullary epithelial cells; PSC, primary sclerosing cholangitis; PBC, primary biliary cirrhosis; Treg, regulatory T cell; HBV, hepatitis B virus; HCV, hepatitis C virus; HCC, hepatocellular carcinoma; PHX, partial hepatectomy; TWEAK, TNF-like weak inducer of apoptosis.

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Introduction

The NF- κ B family of transcription factors, including NF- κ B1 p50, NF- κ B2 p52, RELA (p65), c-Rel and RelB,¹ are involved in diverse biological processes, such as inflammation, apoptosis, proliferation, and development.^{2,3} These NF- κ B subunits form various homodimers or heterodimers that bind to κ B enhancers of target genes and regulate their transcription.³ In resting cells, the NF- κ B dimer is inactive and is sequestered in the cytoplasm by binding to members of the κ B inhibitory factor (I κ B) family. In activating cells, NF- κ B signaling is activated through a series of signaling cascades, following the ligation of various cell surface receptors with paired ligands.³

NF- κ B signaling transduction can be divided into canonical or noncanonical NF- κ B signaling pathways (Fig. 1). The canonical pathway has been well studied, and is known to rely on the degradation of I κ B α .⁴ This pathway is also known to be rapid and transient. In contrast, activation of the noncanonical NF- κ B signaling pathway has been shown to rely on the processing of p100. This process is characteristically slow and persistent, and is regulated in a strict and complex manner through the activity of a variety of proteins.^{5,6} NF- κ B-inducing kinase (NIK) is a central and specific signal component in the noncanonical NF- κ B signaling pathway,^{6,7} while inducible p100 processing is a central step in noncanonical NF- κ B signaling transduction.⁵

Recent studies have suggested that the noncanonical NF- κ B signaling pathway is involved in regulating multiple important biological processes, such as immune inflammation, development of lymphoid organs, and B and T cell survival and maturation.⁴ In addition, the well-characterized functions of the noncanonical NF- κ B pathway are also found to be dysregulated in the pathogenesis of various liver diseases, including metabolic liver disease, autoimmune liver disease, and viral hepatitis. Here, we review the expression and function of the noncanonical NF- κ B signaling molecules in various liver diseases. In particular, we also discuss the therapeutic potency of modulating noncanonical NF- κ B signaling for treatment of liver diseases.

Signaling molecules of noncanonical NF- κ B signaling pathway

Important signaling molecules of the noncanonical NF- κ B signaling pathway include receptors and adaptor proteins. The best known noncanonical NF- κ B receptors belong to the tumor necrosis factor receptor (TNFR) superfamily and include lymphotoxin beta receptor (LTBR),⁸ B-cell-activating factor belonging to TNF family receptor (BAFFR),^{9,10} CD40,^{9,10} receptor activator for nuclear factor κ B (RANK),¹¹

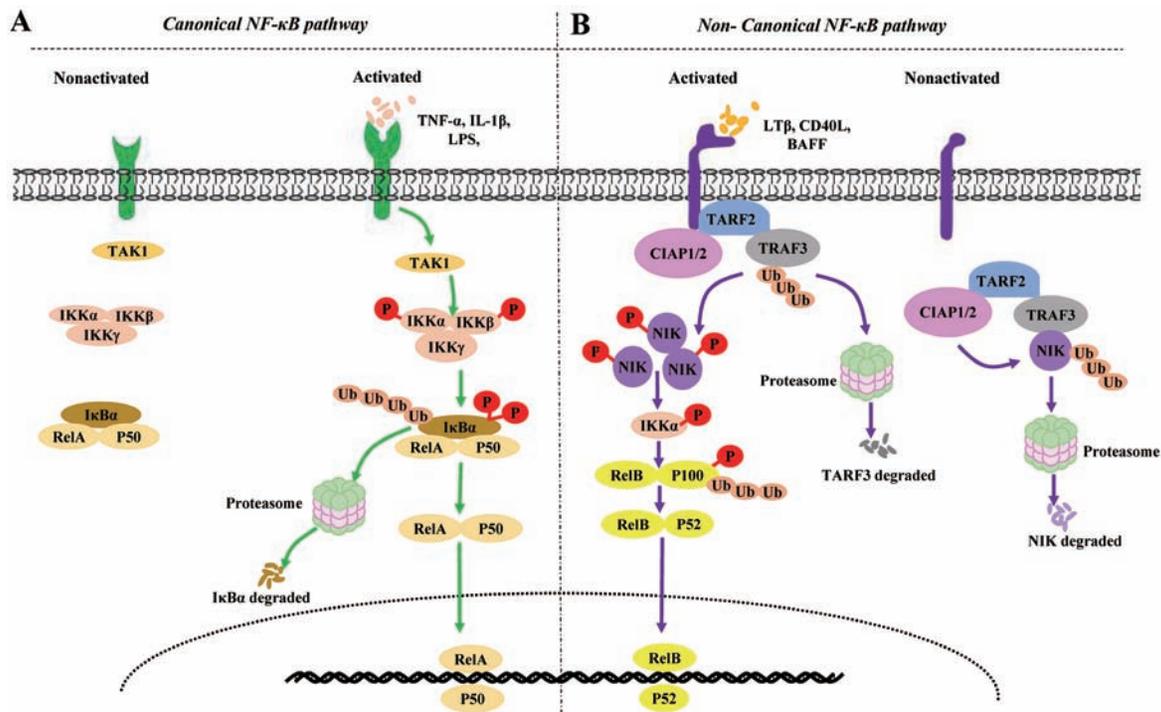


Fig. 1. Canonical and noncanonical NF- κ B signaling pathways in resting vs. receptor-stimulated cells. (A) Canonical NF- κ B signaling pathways. In resting cells, the binding of members of the I κ B family, such as the prototypical I κ B member I κ B α , to classical NF- κ B complexes, particularly NF- κ B1 p50-RelA and NF- κ B1 p50-c-Rel dimers, inhibit the nuclear translocation of NF- κ B complexes. The canonical pathway is activated by signals from various immune-related receptors. In various proinflammatory cytokine-stimulated cells, ligand-receptor interactions initially activate TAK1. Subsequently, TAK1 activates the IKK complex and mediates I κ B α phosphorylation and ubiquitin (Ub)-dependent proteasomal degradation, leading to the rapid and transient nuclear translocation of the classical NF- κ B dimers. (B) Noncanonical NF- κ B signaling pathways. In resting cells, the cIAP1/2-TRAF2-TRAF3 multisubunit ubiquitin ligase complex is composed of TRAF2, TRAF3, and cIAP1/2, in which TRAF2 acts as the adaptor protein, linking TRAF3 and cIAP1/2. The binding of NIK to TRAF3 leads to the constitutive degradation of NIK in a Ub- and proteasome-dependent manner. Following ligand (such as LTB β , CD40L or BAFF) stimulation, the cIAP1/2-TRAF2-TRAF3 complex is recruited to the receptors. The focus of cIAP1/2 mediated K48 ubiquitination and proteasome-dependent protein degradation shifts from NIK to TRAF3, which induces TRAF3 degradation and NIK stabilization. Subsequently, phosphorylated NIK phosphorylates IKK α , which, in turn, phosphorylates NF- κ B2 p100, resulting in the processing of p100 into p52 and the nuclear translocation of RelB-p52 heterodimers. In addition, stabilized NIK is regulated via a negative feedback loop, involving IKK α -mediated phosphorylation.

fibroblast growth factor-inducible 14 (Fn14),¹² and OX40 (also called CD134).¹³ A common feature of these receptors is the presence of a TNFR-associated factor (TRAF)-binding motif, which recruits different TRAF molecules, particularly TRAF2 and TRAF3, to the receptor complex during ligand binding. This is a critical step, leading to degradation of TRAF molecules and activation of the noncanonical NF- κ B pathway by downstream signals.^{14,15} Several non-TNFR receptors can also mediate noncanonical NF- κ B pathway activation, such as macrophage colony-stimulating factor receptor (MCSFR), a growth factor receptor that regulates macrophage differentiation and proliferation.^{14,15}

The essential adaptor proteins belong to TRAF2, TRAF3, NIK (also known as MAP3K14), I κ B kinase α (IKK α) complex, precursor p100, RelB, and the p100 processed product, p52. In resting cells, TRAF2 acts as an adaptor protein connecting TRAF3 with cellular inhibitor of apoptosis protein (cIAP)1/2 to form a TRAF3-TRAF2-cIAP1/2 multisubunit ubiquitin ligase complex.¹⁶ TRAF3 is not only an NIK binding protein but also a key adaptor protein that regulates the level of NIK.¹⁷ Although TRAF3 cannot catalyze the formation of K48-linked ubiquitin chains, it acts as a bridge between cIAP1/2 and NIK, thereby allowing cIAP1/2 to mediate K48 ubiquitylation and proteasome-dependent NIK degradation, which maintains NIK at a low level.^{5,18} When the receptor is stimulated, the target of cIAP1/2-mediated K48 ubiquitination and proteasome-dependent protein degradation changes from NIK to TRAF3 through raising the TRAF3-TRAF2-cIAP1/2 complex to receptors, which results

in TRAF3 degradation and stabilizes NIK expression.^{5,16} However, some stimulatory signals can also stabilize NIK expression by inducing the degradation of TRAF2 or cIAP.^{19–21} The accumulation of NIK induces phosphorylation and polyubiquitination of p100, leading to the release of p52. Subsequently, the RelB/p52 heterodimer translocates into the nucleus and initiates the expression of target genes^{19–21} (Fig. 1). The targets regulated by this pathway include several cytokine and chemokine coding genes, such as CCL19 (also called ELC), CCL21 (also known as SLC), CXCL13 (also named BLC), VCAM1, ICAM1, and MADCAM1.^{19–21} Inducible p100 processing by activating NIK is the centerpiece in the activation of the noncanonical NF- κ B signaling pathway and involves the strict regulation of multiple processes, such as phosphorylation, ubiquitination, and ubiquitin-like modification.⁵ Notably, although IKK α is thought to be a key regulator of p100 phosphorylation, activating IKK α alone is not enough to activate the noncanonical NF- κ B pathway, which additionally requires the activity of NIK.^{6,22}

The noncanonical NF- κ B signaling pathway is regulated at multiple levels. NIK, as the first central regulatory component of this pathway, is itself regulated by various factors. TRAF2, TRAF3, and cIAP are negative regulators of NIK^{19–21} and are essential for maintaining a low NIK level in resting cells. NIK is also regulated through a feedback mechanism involving its downstream kinase, IKK α .²³ In mouse B cells and fibroblasts, TRAF3 degradation following receptor activation does not lead to a sustained increase in NIK levels but instead maintains NIK at a steady level. Furthermore,

compared with wild-type cells, IKK α -deficient cells express higher basal levels of NIK.²³ However, the negative feedback regulation of NIK by IKK α cannot replace the function of the TRAF3-TRAF2-cIAP1/2 complex, which controls the basal level of NIK.

Several additional factors have recently been implicated in the regulation of NIK. In BAFFR- and CD40-activated B cells, TANK binding kinase 1 (TBK1) phosphorylates NIK and initiates NIK degradation.²⁴ Monarch-1 (also known as NLRP12) interacts with NIK to induce NIK ubiquitination and subsequent proteasome-dependent degradation in myeloid cells. Moreover, monarch-1 may indirectly regulate the level of NIK by stabilizing the TRAF3 level.^{25,26} Another potential negative regulator of NIK is TRAF- and NIK-associated protein (TNAP). In TNAP-transfected cells, the kinase activity of NIK is suppressed, which inhibits p100 processing.²⁷ Notably, most studies investigating the role of these factors in NIK regulation have used specific cell lines, and whether their function has tissue-cell specificity merits further investigation. IKK α is also regulated by several factors. A recent study identified microRNA (miR)-223, miR-15a, and miR-16 as specific negative regulators of IKK α that regulate the activation of the noncanonical NF- κ B signaling pathway during macrophage differentiation.²⁸

Role of noncanonical NF- κ B signaling pathways in different liver diseases

The liver is an important metabolic organ of the body, and is involved in biosynthesis, biotransformation, and detoxification. *In vivo*, the liver is associated with the metabolism of glucose, lipids, and proteins, as well as that of alcohol, drugs, and poisons. Consequently, the liver is often affected by a wide variety of pathogenic factors. Liver damage is always closely associated with the occurrence of intrahepatic inflammation and an impaired immune response. The non-canonical NF- κ B signaling pathway plays significant roles in the development and regulation of the immune system, and also has a role in multiple immune inflammatory diseases. Recent studies have demonstrated that dysregulation of the noncanonical NF- κ B signaling pathway is related to the pathophysiological mechanisms associated with several liver diseases, including nonalcoholic fatty liver disease (NAFLD), alcoholic liver disease (ALD), autoimmune liver disease, and viral hepatitis (Table 1³⁰⁻⁵⁹). Furthermore, aberrant activation of the noncanonical NF- κ B pathway in different cell types (such as hepatocytes and thymic epithelial cells) can elicit different effects on the pathogenesis of liver diseases.²⁹⁻³¹

NAFLD/nonalcoholic steatohepatitis/ALD

NAFLD is an acquired, metabolic stress-induced liver injury, and is closely related to insulin resistance and genetic susceptibility. The most significant pathological feature of NAFLD is the existence of vesicular steatosis in the liver in the absence of alcohol and other well-defined liver injury-related factors. NAFLD is divided into two categories: non-alcoholic fatty liver and nonalcoholic steatohepatitis (NASH); importantly, NASH patients are prone to progression to liver fibrosis and cirrhosis. NAFLD/NASH is associated with multiple metabolic characteristics including obesity, diabetes, dyslipidemia, and hypertension. It has also been shown that B-cell-activating factor belonging to TNF family (BAFF) is linked to the histological severity of NASH.⁶⁰ Mechanistically, the BAFF/BAFFR signal was also found to exert a protective role in hepatic steatosis via the down-regulation of the expression of steatogenesis-related genes

in hepatocytes.³² RANK plasma levels and peripheral blood mononuclear cell mRNA levels were also found to be decreased in NAFLD patients.³³ Similarly, the plasma OX40 level was also positively correlated with disease severity in NASH patients. OX40, a key regulator of innate and adaptive immunity in the liver, promoted NASH initiation and development by increasing the functions of proinflammatory monocytes, macrophages, and T cells.³³ This suggests that the noncanonical NF- κ B signaling pathway may have a role in the formation and development of NAFLD/NASH.

The activity of NIK and the expression of P52 (the activated form of NF- κ B2) are increased in the liver, while NIK has been shown to promote glucagon responses in obese mice. Obese mice with liver-specific inhibition of NIK expression exhibited reduced glucagon responses and hepatic glucose production as well as resistance to hyperglycemia and glucose intolerance.³⁵ In line with that study, NIK deletion in hepatocyte or immune cells suppressed liver inflammation and lipogenic programs, thereby protecting against high fat diet-induced liver steatosis.³⁶ Conversely, NIK overexpression in mouse hepatocytes triggered liver injury, extensive liver inflammation, oxidative stress, and liver fibrosis, leading to weight loss and premature death. However, these effects were independent of noncanonical NF- κ B signaling pathway activation. Instead, the mechanism was related to the overexpression of NIK in hepatocytes, which induced activated bone marrow-derived macrophages to secrete proapoptotic molecules, which stimulated hepatocyte apoptosis.³¹ Other studies have demonstrated that liver-specific expression of the carboxyl terminus of HSC70-interacting protein inhibited the activation of the noncanonical NF- κ B signaling pathway and reversed the liver injury caused by hepatocyte-specific overexpression of NIK through the promotion of NIK degradation.³⁷ Moreover, B022, a small-molecule NIK inhibitor, also suppressed NIK-induced liver inflammation and liver injury.³⁸ The results of those studies suggest that NIK-mediated activation of noncanonical NF- κ B signaling in the liver or immune cells may be a potential pathogenic factor for the occurrence of NAFLD and related metabolic disorders; however, the underlying mechanisms require further investigation.

ALD includes four pathological stages: alcoholic fatty liver, alcoholic hepatitis, alcoholic liver fibrosis, and alcoholic cirrhosis. The mRNA level of Fn14 was associated with acute mortality in alcoholic steatohepatitis.³⁹ One study found that mice after alcohol intervention and ALD patients both showed abnormally high levels of NIK mRNA and p52 protein in the liver.⁴⁰ Hepatocyte-specific NIK deletion was shown to protect mice from alcoholic steatosis by sustaining hepatic fatty acid oxidation, whereas NIK overexpression contributed to hepatic lipid accumulation with disrupted fatty acid oxidation, which promoted the occurrence of ALD.⁴⁰ This indicates that the hepatocyte-specific activation of NIK is associated with ALD. Although the noncanonical NF- κ B signaling pathway is activated in the liver of ALD patients, whether it is necessary for ALD development remains to be verified.

Drug-induced liver disease

Drug metabolism and clearance are mainly accomplished in the liver through biotransformation and the bile secretion pathway. However, drugs can be transformed into toxic metabolites through the action of cytochrome p450 in the liver. These metabolites can induce lipid peroxidation, dyslipidemia, and ion pump inactivation, resulting in liver injury, liver failure, liver fibrosis, and cirrhosis. Innate and adaptive immunity both play important roles in drug-induced liver injury. A recent study demonstrated that the

Table 1. Expression and function of signaling molecules of the noncanonical signaling pathway in liver diseases

Liver diseases	Signaling molecules	Expression and function	Ref
NAFLD/ NASH/ALD	BAFFR and BAFF	Severe obesity and insulin resistance are found in BAFFR ^{-/-} mice fed a high-fat diet; BAFF down-regulates the expression of steatogenesis-related genes	32
	RANK	Decreased RANK plasma level and peripheral blood mononuclear cell mRNA level in NAFLD patients	33
	OX40	Plasma OX40 level is positively correlated with disease severity in NASH patients; OX40 deficiency decreases liver fat accumulation, lobular inflammation, and focal necrosis in mice with NASH by regulating both innate and adaptive immunity	34
	Fn14	Fn14 mRNA expression is correlated with acute mortality in ALD	39
	NIK	Liver-specific inhibition of NIK expression shows resistance to hyperglycemia and glucose intolerance in obese mice; NIK deletion in hepatocytes or immune cells suppress liver inflammation and lipogenic programs in mice on high-fat diet; Hepatocyte-specific NIK deletion protects mice from alcoholic steatosis by sustaining hepatic fatty acid oxidation	31,35–37,40
Drug-induced liver disease	OX40	OX40 exacerbates paracetamol-induced liver injury by promoting the function of proinflammatory macrophage and CD4 ⁺ T cells	41
	NIK	Inhibiting NIK signaling ameliorates inflammation, oxidative stress and damage in carbon tetrachloride-induced liver injury	38,42
Autoimmune liver disease	LT β	Increased level of LT β in the bile duct cells of PSC patients	43
	BAFF	Increased level of BAFF in peripheral blood of PBC patients; BAFF promotes Treg apoptosis and inhibits cytokine production by activating B cells in PBC patients	44
	NIK	NIK ^{-/-} mice or mice null for <i>Nik</i> in the thymus develop autoimmune hepatitis, liver injury, and liver fibrosis spontaneously	44
	IKK α	Specific ablation of IKK α in mTECs results in severe T cell-mediated autoimmune hepatitis, liver injury, and liver fibrosis	30
	RelB	Deletion of RELB from liver parenchymal cells protects mice from 5-diethoxycarbonyl-1,4-dihydrocollidine-induced cholestatic liver fibrosis	43
Viral hepatitis	LTBR	LTBR activation induces APOBEC3B expression, which can degrade covalently closed circular DNA (the template of HBV replication) in hepatocytes	48
	BAFF	Increased plasma level of BAFF in patients with chronic HBV infection	46
	OX40 and OX40L	Tregs may suppress natural killer cell-mediated hepatocytotoxicity directly through the interaction between OX40 and OX40L, which may be one of the mechanisms underlying the chronic hepatitis B-associated liver disease; Treatment with OX40 agonists improves HBV antigen clearance in young mice	45,46
	NIK	Overexpression of NIK stimulating by interferon- α inhibits HCV replication in infected hepatocytes; Activation of NF- κ B in hepatocytes is occasioned by the up-regulation of NIK during the initial stage of HBV infection; NIK deletion can cause increased resistance to HCV infection in hepatocytes	47,49,50
	IKK α	IKK α deletion can lead to an increase in resistance to HCV infection in hepatocytes	50
Virus-associated HCC	LTBR and LT β	Increased levels of LT α β and LTBR in HBV- or HCV-induced hepatitis and HCC patients; Liver-specific LT α β expression in mice induces liver inflammation and HCC	51
	BAFFR	Decreased BAFFR expression in B cells is associated with tumor size and clinical stage	52
	NIK	Increased activation of NIK-dependent NF- κ B signal promote growth and resistance to the chemotherapeutic drug 5-FU in HBV-related HCC	53–55

Table 1. Expression and function of signaling molecules of the noncanonical signaling pathway in liver diseases - (continued)

Liver diseases	Signaling molecules	Expression and function	Ref
Liver regeneration	LTBR	Mice deficient for LTBR display liver injury, low survival rates, and reduction in hepatocyte proliferative capacity after PHx	56
	BAFF	The increased level of BAFF in the liver of C57/B6 mice after PHx; Mice treated with anti-BAFF neutralizing antibodies show impaired liver regeneration within 72 h of 70% PHx	57
	Fn14 and TWEAK	Fn14 and TWEAK KO mice, as well as wild-type mice treated with anti-TWEAK antibodies, show reduction in hepatocyte and cholangiocyte proliferation after PHx	58
	NIK	Hepatocyte-specific NIK KO mice display the greater ability of hepatocyte proliferation and liver regeneration after the PHx	59
	IKK α	Hepatocyte-specific IKK α KO mice display the greater ability of hepatocyte proliferation and liver regeneration after the PHx	59

plasma level of OX40 was significantly increased in mice treated with paracetamol or carbon tetrachloride as well as in patients presenting with drug-induced liver injury.⁴¹ Furthermore, OX40 played a key role in promoting the function of proinflammatory macrophages and CD4⁺ T cells, thereby exacerbating paracetamol-induced liver injury.⁴¹ These observations indicate that OX40/OX40L-mediated activation of the noncanonical NF- κ B signaling pathway may be involved in the progression of drug-induced liver injury. Moreover, the hepatic levels of NIK mRNA and p52 protein were significantly increased in mice with carbon tetrachloride-induced liver injury.^{31,38} The small-molecule NIK inhibitor, B022, can inhibit NIK signaling, including noncanonical NF- κ B signaling pathway activation and expression of CCL2, CCL5, CXCL5, TNF- α , IL-6, and other inflammation-related genes, thereby reducing acute liver inflammation, oxidative stress, and liver injury induced by carbon tetrachloride.³⁸ Similarly, apigenin, a flavonoid found in many plants, can mitigate liver injury by ameliorating inflammation and oxidative stress through suppressing the noncanonical NF- κ B pathway.⁴² These results suggest that activation of the noncanonical NF- κ B signaling pathway may promote the development and progression of drug-induced liver diseases. At the same time, selective small-molecule NIK inhibitor 46 (XT2) or B022 are effective at inhibiting drug-induced liver injury and liver inflammation, which shows that NIK is an attractive drug target in drug-induced liver diseases.^{38,61}

Autoimmune liver disease

Autoimmune hepatitis is a type of liver inflammatory injury disease resulting from autoimmune abnormalities. Genetically susceptible individuals develop autoimmune hepatitis mostly due to environmental factors. Autoimmune hepatitis is characterized by the accumulation of immune cells that can recognize autoantigens and self-attack in the liver. Elevated serum transaminase and immunoglobulin G levels, positive serum autoantibodies, and moderate-to-severe interfacial hepatitis are also commonly observed in patients with autoimmune hepatitis. The noncanonical NF- κ B signaling pathway, an indispensable immunoregulatory factor, plays important roles in the development and regulation of B cell and T cell-mediated immune responses.⁵ Abnormal T cell development is an important risk factor for autoimmune diseases. NIK and its downstream signal, IKK α , are central components of the noncanonical NF- κ B signaling pathway. Studies have reported that *Nik* knockout (KO) mice developed autoimmune hepatitis, liver damage, and liver fibrosis, and displayed growth retardation and premature

death (most KO mice died before 13 weeks of age).²⁹ Other studies have demonstrated that mice null for *Nik* in the thymus, but not in the liver or bone marrow, developed fatal autoimmune liver disease, intrahepatic inflammation, and liver fibrosis mediated by CD4⁺ T cells.²⁹ Similarly, specific ablation of either NIK or IKK α in mouse thymic medullary epithelial cells (mTECs) resulted in severe T cell-mediated autoimmune hepatitis, injury, and fibrosis in the liver, leading to premature death.³⁰ The reason for this phenomenon may be related to the dysregulation of the noncanonical NF- κ B pathway mediated by NIK/IKK α in mTECs, which abrogates mTEC development and leads to the breakdown of central T cell immune tolerance. These observations indicate that dysregulation of the noncanonical NF- κ B signaling pathway in the thymus has a role in autoimmune liver disease development, and even causes death in some severe cases, which may be related to the failure of central T cell immune tolerance.

Primary sclerosing cholangitis (PSC), an autoimmune disease of the liver, is a chronic cholestasis syndrome characterized by extensive inflammation and fibrosis in the intrahepatic and extrahepatic biliary tract systems. The levels of LT β and RelB are up-regulated in the bile duct cells of patients with various chronic liver diseases, including PSC.⁴³ Moreover, results in mice showed that RelB activation (non-canonical NF- κ B subunit) induced ductular reaction, oval cell activation and the progression of biliary fibrosis. Additionally, the transformation of the secretory and proliferative phenotype in cholangiocytes was shown to be dependent on lymphotoxin β and RelB,⁴³ suggesting that activation of the noncanonical NF- κ B pathway in cholangiocarcinoma cells may promote PSC progression. The etiology of primary biliary cirrhosis (PBC) is also related to autoimmunity. The concentration of BAFF was increased in peripheral blood of PBC patients. In addition, BAFF-activated B cells could induce Treg cell apoptosis and reduce the expression of IL-10 and TGF- β , resulting in the loss of self-tolerance.⁴⁴ Therefore, BAFF-mediated noncanonical NF- κ B pathway activation in B cells may be involved in the occurrence of PBC.

Viral hepatitis

In chronic viral hepatitis, continuous viral replication and dysregulated host immune function are the main causes of the progressive development of the disease. The noncanonical NF- κ B pathway is closely related to the differentiation, proliferation, and maturation of various immune cells. Hence, abnormal immune function in patients with chronic viral hepatitis may be associated with dysregulation of in-

tracellular noncanonical NF- κ B pathway activation.

OX40 is one of the receptors that can activate the non-canonical NF- κ B signaling pathways, maintaining T cell excitation, promoting the proliferation of effector T cells and memory T cells, inducing T cell phenotype transformation, and inhibiting regulatory T cell (Treg) differentiation and activity. Interestingly, effector T cell depletion and Treg accumulation have been observed in chronic virus-related hepatitis. Therefore, OX40/OX40L-induced noncanonical NF- κ B pathway activation may be related to the development of chronic viral hepatitis. One study reported that an increased number of hepatic Tregs accumulated along with the recovery of liver injury in natural killer cell-mediated hepatitis B virus (HBV) transgenic mice with the oversensitive liver injury phenotype, triggered by a low dose of concanavalin A.⁴⁵ Further findings indicated that Tregs may directly suppress natural killer cell-mediated hepatocytotoxicity through OX40/OX40L interaction in a cell-cell contact-dependent manner,⁴⁵ which may be one of the mechanisms underlying the chronic hepatitis B-associated liver disease. Publicover *et al.*⁴⁶ also showed that the expression of OX40L in liver innate immune cells is essential in HBV immunization. Treatment with OX40 agonists led to improved HBV antigen clearance in young mice, and also enhanced the strength of T cell responses in both young mice and adult mice that had been exposed to HBV when they were young and had subsequently developed a chronic HBV infection serological profile.⁴⁶ However, additional studies are needed to determine whether OX40/OX40L interaction is involved in the activation of the noncanonical NF- κ B pathway in these processes. BAFF is necessary for the activation of B lymphocytes. Although one study demonstrated that the serum BAFF level was higher in patients with chronic HBV infection than in the normal population,⁶² the role of BAFF in the disease remains unclear.

Surprisingly, during the initial stage of HBV infection, cDNA microarray and western blot analysis showed that the mRNA and protein levels of TRAF2 and NIK were up-regulated in primary normal human hepatocytes.⁴⁷ In addition, LTBR, a member of the TNFR superfamily, can activate NIK-mediated noncanonical NF- κ B signaling. The activation of LTBR induced APOBEC3B expression, which can degrade covalently closed circular DNA (the template of HBV replication) in hepatocytes.⁴⁸ These findings suggest that the activation of NIK exerts antiviral effects in hepatocytes. Although NIK is an indispensable component of noncanonical NF- κ B signaling pathway, NIK activity is not specific to this pathway. Thus, it is not clear whether elevated intrahepatic NIK expression exerts antiviral effects by activating the noncanonical NF- κ B signaling pathway or through another as yet unidentified mechanism. Notably, HBV polymerase (pol) can inhibit the activation of the noncanonical NF- κ B pathway by suppressing the nuclear translocation of NF- κ B subunits in hepatoma cells, thereby antagonizing host innate immune responses.⁶³ The above results indicate that noncanonical NF- κ B pathway activation is inhibited in the liver of patients with chronic HBV infection. Recently, it was found that the Smac mimetic Birinapant, which might activate the noncanonical NF- κ B pathway by targeting cIAP1 and cIAP2, was able to promote the apoptosis of virus-infected hepatocytes to clear persistent HBV infection.^{64,65}

Previous studies have shown that hepatitis C virus (HCV) replication can be inhibited in infected hepatocytes following interferon- α -stimulated overexpression of NIK.⁴⁹ However, NIK and IKK α deletion can lead to an increase in resistance to HCV infection in hepatocytes.⁵⁰ HCV infection promoted the expression of NIK in hepatocytes at the post-transcriptional level, and then NIK directly activated IKK α to promote HCV replication, lipogenesis and lipid droplet formation.⁵⁰ These results suggest that activation of the noncanonical NF- κ B pathway is closely related to hepatocyte susceptibil-

ity to HCV. These results suggest that excessive intrahepatic NIK expression not only plays an antiviral role but also increases susceptibility to hepatitis virus infection, thereby promoting disease progression. A possible explanation for this phenomenon may be that the NIK-mediated functions of the noncanonical NF- κ B pathway may differ according to the disease stage. Alternatively, NIK and IKK α may induce additional signaling molecular pathways that counteract the noncanonical NF- κ B pathway, thereby leading to different effects.

Viral associated hepatocellular carcinoma

The pathogenesis of hepatocellular carcinoma (HCC) is associated with a variety of etiologies, including chronic HBV and HCV infections. The development of HCC is inseparable from the immunosuppressive microenvironment. The non-canonical NF- κ B pathway plays important roles in the development and regulation of the immune system and may be involved in HCC pathogenesis. One study found that the expression of LTBR and that of its ligands (LT α and LT β) was up-regulated in HBV- or HCV-associated HCC, while the liver-specific expression of LT α / β can induce liver inflammation and tumor formation in mice.⁵¹ The expression of BAFFR, which is closely related to B cell survival and maturation, was decreased in B cells in the peripheral blood of HCC patients with chronic HBV infection, and this decreased BAFFR expression was significantly correlated with tumor size and clinical stage.⁵² All the above-mentioned receptors and corresponding ligands can activate the noncanonical NF- κ B signaling pathway, which suggests that the activation of this signaling pathway may play an important role in the initiation and development of HCC.

In addition, the overexpression of miR-98-5p markedly inhibited the proliferation, migration, and invasive ability of tumor cells and promoted cell apoptosis in HBV-related HCC via decreasing the protein expression of NIK. Furthermore, overexpression of miR-98-5p also significantly inhibited tumor growth and decreased the expression of NIK in a mouse xenograft tumor model.⁵³ HBV infection promoted an increase in NIK-dependent activation of NF- κ B, which not only promoted the occurrence of HBV-related HCC but was also correlated with HCC resistance to the chemotherapeutic drug fluorouracil (commonly known as 5-FU).^{54,55} Small interfering RNA-mediated inhibition of the NIK-dependent activation of NF- κ B reduced resistance to 5-FU in HBV-related HCC.⁵⁵ This suggests that HBV may promote the development of HCC and its drug resistance through noncanonical pathways mediated by NIK.

Role of noncanonical NF- κ B signaling pathways in liver regeneration

The liver has a powerful regenerative capacity. Following 70% partial hepatectomy (PHx), rodents can regain normal liver mass within a week via reparative hepatocyte replication. However, when impaired, this process can exacerbate the pathogenesis of acute or chronic liver diseases. LTBR is one of the receptors that can activate the noncanonical NF- κ B signaling pathway. Mice deficient for LTBR signaling displayed clear liver injury, low survival rates, and reduced hepatocyte proliferative capacity after PHx. Similar phenomena were also observed in mice deficient for the LTBR ligand LT α after PHx, suggesting that the LT β /LT α signaling pathway enhances hepatocyte regeneration.⁵⁶ The expression of BAFF was up-regulated in the liver of C57/B6 mice after PHx. Conversely, within 72 h of undergoing 70% PHx, mice treated with anti-BAFF neutralizing antibodies died, showing

reduced microvascular density in their remaining liver tissue as well as impaired liver regeneration, suggesting that BAFF-mediated signaling improved hepatocyte regeneration.⁵⁷ Moreover, compared with control mice, both Fn14 and TNF-like weak inducer of apoptosis (TWEAK) KO mice showed reduced hepatocyte and cholangiocyte proliferation after PHx. A similar phenomenon was also observed in wild-type mice treated with anti-TWEAK antibodies,⁵⁸ which indicated that the TWEAK/Fn14 signal is essential for mouse liver regeneration after PHx. All of the above-mentioned receptor-ligand interactions can activate the noncanonical NF- κ B signaling pathway. Consequently, these results imply that activation of the non-canonical NF- κ B signaling pathway may be associated with liver regeneration; however, the underlying mechanism needs to be further determined.

Compared with NIK^{flox/flox} mice, hepatocyte-specific NIK KO and hepatocyte-specific IKK α KO mice displayed a greater ability for hepatocyte proliferation and liver regeneration after the PHx due to the stable deletion of NIK or IKK α in hepatocytes.⁵⁹ Hepatotoxin treatment or high-fat diet feeding inhibited PHx-stimulated hepatocyte replication in mice. Under the same condition, inactivation of hepatic NIK markedly enhanced hepatocyte proliferation.⁵⁹ The above findings suggest that activation of the noncanonical NF- κ B signaling pathway plays important roles in hepatocyte regeneration. Mechanistically, to some extent, activated hepatic NIK/IKK α inhibited liver regeneration by suppressing the cell mitotic pathway-JAK2/STAT3 pathway.⁵⁹ Therefore, targeting the NIK may also accelerate liver regeneration, which would also be of concern in acute liver failure.

Therapeutic targeting of the noncanonical NF- κ B signaling pathway in liver diseases

Given that aberrant activation of the noncanonical NF- κ B pathway is observed in various liver diseases, targeting this pathway may be a promising therapeutic option. For example, the anti-BAFF monoclonal antibody belimumab has been approved to be clinically administered to systemic lupus erythematosus patients.⁶⁶ Increased level of BAFF in peripheral blood can also be observed in patients with autoimmune liver disease, and a study has found that BAFF promotes Treg cell apoptosis and inhibits cytokine production by activating B cells in PBC patients.⁴⁴ Therefore, BAFF antagonists might be applicable to the autoimmune liver diseases. In addition, the results showing that selective small-molecule NIK inhibitor 46 (XT2) or B022 are effective at inhibiting drug-induced liver injury and liver inflammation indicate that NIK is an attractive target in drug-induced liver diseases.^{38,61} It will be interesting to test these NIK inhibitors with different scaffolds for treatment in clinical trials. Furthermore, IKK α deletion can lead to increased resistance to HCV infection and ability for hepatocyte proliferation and liver regeneration.^{50,59} Teglarinad chloride, one of the IKK α /IKK β inhibitors which has passed phase 1 clinical trials for malignant melanoma, lymphoma and solid tumors,⁶⁷ may be used in treatment of HCV infection and acute liver failure. Although some specific inhibitors and/or monoclonal antibodies are currently being applied to a broad range of diseases in which aberrant activation of noncanonical NF- κ B signaling occurs, further studies are needed to determine whether they can be used in the treatment of liver diseases.

Conclusions

Extensive investigation has led to a deeper understanding of the signal transduction mechanisms and biological functions associated with the noncanonical NF- κ B signaling

pathway. Its functions are indispensable, especially for the production of a normal immune response and in inflammatory diseases. Numerous studies have also shown that abnormalities in noncanonical NF- κ B signaling are closely related to the occurrence of various liver diseases, which provides a theoretical basis for the discovery of drugs targeting the noncanonical NF- κ B pathway.

Despite these advances, several questions remain to be addressed. First, most studies that have investigated the noncanonical NF- κ B pathway have focused on the cytoplasmic regulation of upstream kinases, such as NIK, IKK α , and NF- κ B heterodimers, while little attention has been paid to the nuclear regulation of NF- κ B, especially that related to RelB-p52 heterodimers. Second, although receptor binding to specific ligands can activate the noncanonical NF- κ B pathway, the target genes that are regulated by different receptor-activated signaling pathways in specific cell types remains unclear. Third, although activation of the noncanonical NF- κ B pathway is completely dependent on NIK, this is not the only function of this protein. Therefore, it remains to be determined whether NIK mediates the activation of additional signaling pathways that may interact with the noncanonical NF- κ B pathway. Fourth, although several studies have shown that NIK-induced noncanonical NF- κ B signaling is closely related to various liver diseases, it is unclear how this pathway regulates the expression of target genes involved in the development of these diseases. Moreover, although the canonical and noncanonical NF- κ B pathways are known to interact, whether this interaction is associated with the promotion of liver disease, and how the two pathways regulate each other, requires further investigation. Solving these problems will contribute to exploring more specific and effective methods for treating liver diseases based on the noncanonical NF- κ B pathway.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Collected the data and drafted the manuscript (XZ, CQ, LX), made critical revision of the manuscript for important intellectual content (XZ).

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Role of ALDH2 in Hepatic Disorders: Gene Polymorphism and Disease Pathogenesis

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Abstract

Aldehyde dehydrogenase 2 (ALDH2) is a key enzyme of alcohol metabolism and it is involved in the cellular mechanism of alcohol liver disease. *ALDH2* gene mutations exist in about 8% of the world's population, with the incidence reaching 45% in East Asia. The mutations will result in impairment of enzyme activity and accumulation of acetaldehyde, facilitating the progression of other liver diseases, including non-alcoholic fatty liver diseases, viral hepatitis and hepatocellular carcinoma, through adduct formation and inflammatory responses. In this review, we seek to summarize recent research progress on the correlation between *ALDH2* gene polymorphism and multiple liver diseases, with an attempt to provide clues for better understanding of the disease mechanism and for strategy making.

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Introduction of gene polymorphisms in aldehyde dehydrogenase 2

Function of aldehyde dehydrogenase 2 in human beings

The aldehyde dehydrogenases play a key role in the metabolism of toxic aldehydes. Some are produced in human

bodies, such as 4-hydroxy-2-nonenal (4-HNE) and malondialdehyde (MDA), while others were obtained from the environment, like formaldehyde, acrolein, and ethyl.^{1,2} As a member of the ALDH superfamily, ALDH2 is the most sensitive isoform to irreversible inactivation and is also the most sensitive to inactivation by toxics, such as 4-HNE.¹ This enzyme could metabolize acetaldehyde (ACH) to acetate irreversibly in a redox reaction (Fig. 1).³ Disturbances in the expression of *ALDH2* will dampen its metabolic capacity and result in accumulation of ACH consequently. Based on its electrophilic feature, ACH could bind with biomolecules such as proteins or DNA and destroy cell integrity, which contributes to the development of various human diseases,⁴ such as endocrine disorders, cardiovascular diseases, pulmonary diseases, oral cancers, gastrointestinal cancers, Fanconi anemia, and dermatitis.^{5–7}

ALDH2 gene and polymorphisms

ALDH2 is a polypeptide consisting of 517 amino acids, principally expressed in the liver but also in other organs, such as heart, kidney, muscle, and brain.⁸ Its coding gene is located on chromosome 12 (12q24.2), which is 44 kilobases in length and comprises 13 exons.⁹ After translation, the expressed protein is transported to the mitochondrial matrix to participate in dehydrogenase, esterase and reductase reactions in liver and fat tissues particularly. Studies of the human genome have shown 19 kinds of functional *ALDH* genes in total, with a wide range of expression and substrate specificities, among which the *ALDH2* gene has the highest expression and exclusively harbors existence of genetic polymorphisms.¹

As it encodes a key enzyme for alcohol metabolism, *ALDH2* also has an important functional single nucleotide polymorphism (SNP), the *rs671-Glu504Lys* variant, which has significantly reduced activity compared to the wild type.¹⁰ The *rs671* variant exists in 30–45% of East Asians (Chinese, Japanese, and Korean) and 8% of the world's population.^{11,12} The incidence of this mutation in China is as high as 37–59%.^{13–16} Nowadays, the *rs671*SNP locus at exon 12 is of special concern in worldwide research. According to sequencing detection,¹⁷ a G→A point mutation is prone to occur at exon 12, causing the original glutamic acid (Glu) to be replaced by lysine (Lys), whose mutation is named *ALDH2Glu504Lys* (SNP*rs671*).

ALDH2rs671 SNPs are composed of three genotypes: GA, AA and GG. GA is a heterozygous mutation, also named as *ALDH2*1/*2* (Glu/Lys). AA is a homozygous mutation, also known as *ALDH2*2/*2* (Lys/Lys). GG is the normal allele, without mutation (Fig. 2). The majority of studies on these genotypes have confirmed that the GA genotype has 10–20% of the enzyme activity compared wild type, while the

Keywords: Aldehyde dehydrogenase 2; Gene polymorphisms; Alcoholic liver disease; Non-alcoholic fatty liver disease; Viral hepatitis; Hepatocellular carcinoma.
Abbreviations: 4-HNE, 4-hydroxy-2-nonenal; ACH, acetaldehyde; ALD, alcoholic liver disease; ALDH2, aldehyde dehydrogenase 2; ALT, alanine aminotransferase; AMPK, AMP-activated protein kinase; CHB, chronic hepatitis B; CHC, chronic hepatitis C; CYP2E1, cytochrome P4502E1; GGT, γ -glutamyl transpeptidase; Glu, glutamic acid; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HSC, hepatic stellate cell; Lys, lysine; HDL-C, high-density lipoprotein cholesterol; HIF-1 α , hypoxia-inducible factor-1 alpha; ISGs, interferon-stimulated genes; MDA, malondialdehyde; NAFLD, non-alcoholic fatty liver disease; NF- κ B, nuclear factor-kappa B; PP2A, protein phosphatase 2A; ROS, reactive oxygen species; SNP, single nucleotide polymorphism; SREB-1, sterol regulatory element binding protein-1 pathway; STAT, signal transducer and activator of transcription; TG, triglyceride.

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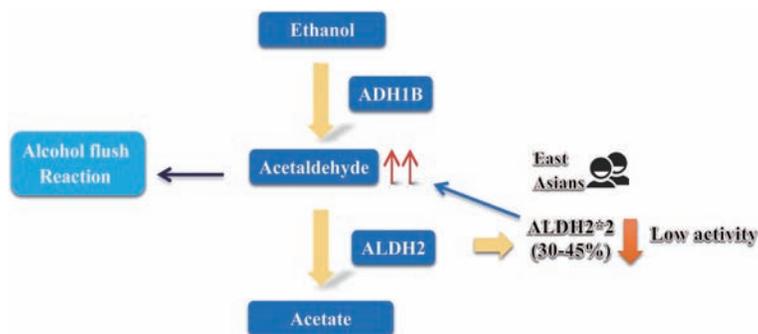


Fig. 1. Alcohol metabolism and enzymes that strongly impact alcohol consumption. The *ALDH2*2* variant exists in 30–45% of East Asians (Chinese, Japanese, and Korean), which has low activity.

AA genotype loses more than 96% of the enzymatic activity. As a result, individuals with GA or AA mutations could show up to 6 or 19 times greater ACH concentrations, respectively, as compared with wild type after alcohol intake.¹⁸

Distribution of *ALDH2* alleles in different populations

The genotype frequencies of the *ALDH2* gene polymorphisms vary among different races. The rare *ALDH2*2* allele has been observed in Caucasians, Africans and Southeast Asians but it is widely present in East Asians.^{19,20} There is a report of this mutation being found in about 560 million people of East Asian descent and reducing enzymatic activity by approximately 60% to 80% in *ALDH2*1/*2* heterozygotes.¹⁹ Among East Asians, the *ALDH2* allele frequencies are diverse among Japanese, Korean, and Chinese. In China, the *ALDH2*2* gene frequency in some Chinese aboriginal populations (e.g., Korean, Uighur, Zhuang and Olunchun) is lower compared to the Chinese Han population. In the Chinese Han population, the *ALDH2*2* allele frequency is 17% to 29%, the proportion of individuals with *ALDH2*1/*2* heterozygotes is 36% to 44%, and the proportion of individuals with *ALDH2*2/*2* homozygotes is 7% to 8%.^{21,22}

Related liver diseases

Alcoholic liver disease

Alcoholic liver disease (ALD) is a direct outcome of chronic ethanol consumption and is considered as an important health problem worldwide. ALD encompasses a broad spectrum of liver injuries, including steatosis, fibrosis, cirrhosis, and alcoholic hepatitis.²³ The incidence of ALD has been increasing yearly because of the rapid boom in alcohol con-

sumption in many developing countries over the past decade.²⁴ The prevalence of ALD in China, the USA, Europe, and Japan is 4.5%, 6.2%, 6%, and 1.56–2.34%, respectively.^{24–27} There are about 260 million people occasionally, habitually and excessively drinking, and appropriately 2.5 million people die from ALD each year.²⁸ Hence, ALD pathogenesis and therapy have always been the focus of national researchers.

The *ALDH2* Glu504lys polymorphism is tied closely to occurrence and development of ALD in related individuals,²⁹ though its polymorphism does not contribute to alcohol dependence in the Turkish population.³⁰ Regardless of homozygous AA or heterozygous GA status, both guarantee elevated ACH level after alcohol drinking. A single-center study from the Fifth Medical Centre of the General Hospital of the Chinese People's Liberation Army reported that only 2.3% of ALD patients have the *ALDH2*2* allele, compared with 14.5% of the proportion of healthy controls (281 and 535 controls; odds ratio [OR] of 0.13 and 95% confidence interval [CI] of 0.07–0.24).³¹ In Korea, Lee *et al.*³² found that the *ALDH2*1* allele is associated with a higher frequency of alcoholic cirrhosis ($p=0.001$). Likewise, a meta-analysis of 12 studies found that people with the *ALDH2*1* allele are more likely to go on to develop alcoholic liver cirrhosis compared with those with either the *ALDH2*1/*2* or *ALDH2*2/*2* genotype.³³ Based on the activity of the enzyme after gene mutation, *ALDH2*2/*2* should have produced a poor protective effect of ethanol; however, it brings some body information, such as facial flushing, reminding those with *ALDH2*2/*2* to be alert to alcohol intake and usually leading to little excessive ethanol consumption.³⁴ On the contrary, without the gene reminder, those with *ALDH2*1* are not aware of consuming excessive alcohol.

The protection from the *ALDH2* Glu504lys polymorphism has also been verified by Liu's team,³⁵ whose result demonstrated that individuals carrying this polymorphism are protected from alcohol drinking, with a 4-fold decrease in risk. Ma *et al.*³⁶ and Li *et al.*¹⁶ also provided further evidence that

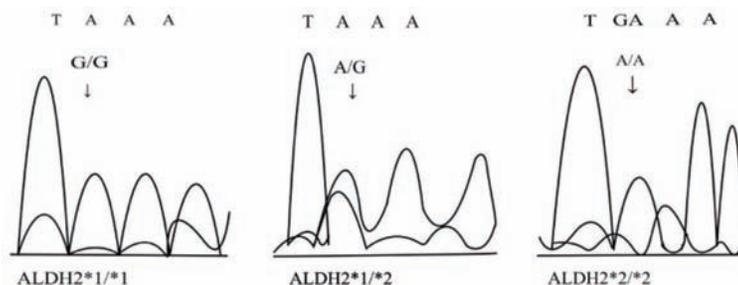


Fig. 2. Genotypes of *ALDH2rs671*.

Table 1. Recent clinical studies on the relationship between the ALDH2 polymorphism and ALD

Year	Conclusion	Reference
2001	The <i>ALDH2</i> *2 gene protects against the development of alcoholism.	32
2012	Strong protective effect of the <i>ALDH2 504lys</i> (*2) allele against alcoholism and alcohol-induced medical diseases in Asians.	16
2015	The absence of the <i>ALDH2</i> *2 allele in both alcoholics and controls suggests that this polymorphism does not contribute to alcohol dependence in the Turkish population.	30
2015	ALDH2 plays a beneficial role in ameliorating chronic alcohol intake-induced hepatic steatosis and inflammation, through regulation of autophagy.	37
2016	People with the <i>ALDH2</i> *1 allele are more likely to go on to develop alcoholic liver cirrhosis compared with those with the <i>ALDH2</i> *1/*2 or <i>ALDH2</i> *2/*2 genotype.	33
2016	Polymorphisms in <i>ALDH2</i> exerted significant indirect effects on hepatocellular carcinoma risk, mediated through alcohol drinking.	35
2016	The <i>ALDH2 Glu504Lys</i> polymorphism and 'alcohol flush' are not harmless in the study's Asian population.	36
2017	Individuals who carry at least one copy drink typically less and are protected against heavy alcohol use and alcohol use disorders.	34
2018	Patients with the <i>ALDH2 504lys</i> variant were less associated with ALD compared to those with <i>ALDH2 504glu</i> .	31
2019	ALDH2 deficiency is associated with elevated acetaldehyde and glucocorticoids post-alcohol consumption, thereby inhibiting T cell activation and hepatitis.	37

Abbreviations: ALD, alcohol liver disease; ALDH2, aldehyde dehydrogenase 2; Glu, glutamic acid; Lys, lysine.

the mutation and "alcohol flush" are not harmless in this Asian population. In other words, the *ALDH2* gene mutation is a protective factor in the alcohol-drinking population in East Asia, while it is weaker in European and African populations.³⁴ In fact, the Eastern culture encourages or challenges people to drink more alcohol in social activities, and sometimes people with flushing may not be able to escape or reject such alcoholism.

Aerobic glycolysis is involved in alcohol metabolism, which could be inhibited by a known factor: corticosterone. As is shown in the animal experiment of Gao's team,³⁷ a higher level of serum corticosterone is detected in ethanol-fed *Aldh2*(-/-) mice, compared to the wild type mice. Gao's team³⁷ also found that acute alcohol drinking in humans was related to elevated plasma glucocorticoid levels in human subjects, with higher levels in those with inactive ALDH2 than active ALDH2. To conclude, the progress of aerobic glycolysis is impaired by ethanol, especially in those with *ALDH2**2. Meanwhile, they succeeded in restored con-canavalin A-mediated hepatitis via blockade of corticosterone. Therefore, aerobic glycolysis-related signaling pathways may be a key factor. Interestingly, the authors found that glucose metabolism in T cells could be disrupted by ACH through inhibition of the aerobic glycolysis-related signaling pathways. In addition, weakened autophagy is involved and compromised lysosomal activity will lead to abnormal stacking of ethanol or acetaldehyde by-product including protein or DNA adducts. Guo *et al.*³⁸ reported that observations both *in vivo* and *in vitro* are in favor of a beneficial role of ALDH2 in alcohol intake-facilitated fatty liver and inflammation through autophagy regulation (Table 1^{16,30-37}).

The traditional hypothesized pathway is through oxidative stress. ALDH2 dramatically attenuates hepatic oxidative stress induced by chronic alcohol intake and favors a role of oxidative stress in ethanol- and ALDH2-elicited hepatic responses, by restoring autophagy and reopening autophagy flux. Additional ethanol consumption will increase the production of NADH/NAD⁺, and reactive oxygen species (ROS) in the mitochondrial electron transport chain. Then, ROS is able to activate nuclear factor-kappa B (NF-κB) and

its downstream proinflammatory signal, and correspondingly aggravate inflammation and hepatocyte damage.³⁹⁻⁴¹ Moreover, Zhong *et al.*⁴² selected mitochondrial ALDH2 as a promising therapeutic target for ALD. They said that it accelerates aldehyde clearance and reverses hepatic steatosis and apoptosis in mice. Therefore, artificial modulation of *ALDH2* expression may be a potential therapeutic intervention for alcoholism and ALD in the future.^{43,44}

As mentioned above, variants in ALDH2 decrease the rate of ACH conversion to acetate because it blocks its ability to remove ACH and results in a strong aversive reaction. Therefore, if we can find a medium to intervene this mechanism and develop a blocker, we will alleviate this effect. It is also suggested that physicians should pay attention to explore the potential immunosuppressive therapy in alcoholics.

Non-alcoholic fatty liver disease

It has become more and more accepted that non-alcoholic fatty liver disease (NAFLD) stands for not just a single type of liver disease but the hepatic manifestation of complicated metabolic dysfunctions. NAFLD covers a wide range of liver pathologies, including steatosis, steatohepatitis, fibrosis/cirrhosis and liver failure.^{14,45} Nowadays, NAFLD has become the leading cause of chronic liver diseases on earth and its global prevalence is appropriately 25%.⁴⁶⁻⁴⁹ Prevalence rates of NAFLD were estimated to be 22.4%, 24.13%, 23.71%, 25%, 31% and 32% in China, the USA, Europe, Japan, the Middle East, and South America, respectively.^{14,24,50,51} In the USA, NAFLD is estimated to be the most common cause of chronic liver disease, affecting between 80 and 100 million individuals, among whom nearly 25% progress to non-alcoholic steatohepatitis.¹⁴ A recent report of data from the National Health and Nutrition Examination Survey ranging from 1988 to 2010 indicated that modest alcohol consumption (7-21 g/day) is associated with decreased mortality among patients with NAFLD.⁵² In contrast to the studies of 58,927 patients with NAFLD in Korea, even moderate drink-

ers (10–29.9 g/day) exhibited an increased tendency to progress to fibrosis compared with non-drinkers.⁵³ Whether consuming moderate alcohol could be a lifestyle intervention in the treatment requires further investigation.

In China and East Asia, genome-wide association studies found that *ALDH2 rs671* is a susceptible gene locus for obesity and significantly associated with increased body mass index and visceral fat deposition.^{10,11} A control study reported that a significant accumulation of the 4-HNE protein adduct and a significant up-regulation of *ALDH2* protein expression are found in the two groups of non-alcoholic steatohepatitis patients, suggesting that *ALDH2* plays a role in combating non-alcoholic steatohepatitis oxidative stress. We have reason to speculate that *ALDH2* gene mutation will reduce the oxidative stress protection response of liver recovery in non-alcoholic steatohepatitis patients. On the contrary, Alda-1, as an activator of ALDH2, suppresses PINK1/PARKIN-mediated mitophagy and its usage in apoE-mice has led to improvement in the degree of arteriosclerosis and hepatic steatosis, indicating that activation of ALDH2 can improve NAFLD on the condition of mitochondrial injury caused by vinyl chloride.⁵⁴ Alda-1 protects against liver damage under these conditions via increasing clearance of aldehydes and preserving mitochondrial respiratory function.

In the field of biomedicine, researchers have found that inhibition of *ALDH2* enhances the ACH-mediated hepatocyte sterol regulatory element binding protein-1 (SREB-1) pathway activity and promotes triglyceride (TG) deposition in the liver. A cohort study⁵⁵ conducted by Japanese researchers followed 341 patients who never or seldom drank or drank less for 4–6 years and found that the incidence of NAFLD is higher in carriers of the mutant gene *ALDH2*2* than in non-carriers. Carriers of *ALDH2*2* with elevated γ -glutamyl transpeptidase (GGT) levels (>25.5 IU/L) have a significantly increased risk of NAFLD. A recent Japanese study investigated the association of *ALDH2rs671* genotype with liver disease in 1,768 alcohol-dependent Japanese men. They found that the ineffective *ALDH2* Glu/Lys genotype increases the ratio or regression coefficient of age- and alcohol-adjusted fatty liver, ketonuria and serum high-density lipoprotein cholesterol (HDL-C) level, and reduces liver cirrhosis and serum TG level. Through logistic regression analysis, Chinese scientists also found that the GGT level in carriers of the GA/AA type is significantly higher than that of the GG type, suggesting that carriers of the GA/AA type are more prone to suffer liver cell damage and more severe fatty changes than carriers of the GG type. Nevertheless, another animal study suggested that *ALDH2*-deficient individuals may be resistant to steatosis and blood alanine aminotransferase (ALT) elevation but be more prone to liver inflammation and fibrosis following alcohol consumption.¹⁵ Furthermore, a human experiment showed that the prevalence of elevated ALT level increases with the accumulation of components of metabolic syndrome and that the correlation between active ALDH2 and elevated ALT level is sensitive.⁵⁶ Their logistic regression analysis also revealed that body mass index, TG level, and *ALDH2* genotype are associated with ALT elevation. This result coincides with the findings of the genome-wide association studies.

To conclude, the relationship between ALDH2 and the NAFLD disease spectrum has begun to enter the laboratory stage but it needs a large number of research studies to clarify the connection.

Viral hepatitis

Although the global incidence of viral hepatitis, hepatitis B virus (HBV) infection mainly, is going down, it continues

to play an important role in developing countries.⁵⁷ There are approximately 257 million people with chronic HBV infection globally, including 68% in Africa and the Western Pacific, according to a World Health Organization report.⁵⁸ In China, chronic hepatitis B (CHB) and chronic hepatitis C (CHC) affect 90 million and 10 million people, respectively. In developed countries such as the USA, Japan, and the European Union, the prevalence of HBV is much lower (0.71–1.17%), but the prevalence of hepatitis C virus (HCV) (1.10–1.56%) is higher than in China (HBV: 6.52%; HCV: 0.72%). In 2016, the Global Health Sector Strategy on viral hepatitis called for elimination of viral hepatitis as a major public health threat by 2030.⁵⁹ However, unlike other liver diseases, the relationship between viral hepatitis and *ALDH2* remains unclear.

HCV infection is an important cause of chronic liver disease, with nearly 71 million chronically infected people worldwide.⁵⁷ HCV and alcohol intake are both risk factors for accelerated fibrosis progression,⁶⁰ and alcohol use in the setting of HCV infection is correlated with increased rates of fibrosis progression.⁶¹ Based on previous studies,^{62,63} the correlation between *ALDH2* and HCV could be explained by the two following aspects: enhanced virus replication and immunity suppression.

For one thing, the metabolite ACH could help to activate the expression of miR-122 and miR-34a, both able to stimulate HCV replication.⁶⁴ Correspondingly, a large number of virus products brought about by strong virus replication will promote hepatocellular apoptosis. Apoptosis has a secondary amplification effect on the viral lethality in the liver, which not only delays virus clearance but also aggravates liver cell damage. And, then, Kupffer cells and hepatic stellate cells (HSC) are driven by interleukins to aggregate and participate in the phagocytosis and clearance of apoptotic bodies.⁶² This process will accelerate the inflammatory responses and fibrogenesis in the liver. Meanwhile, ACH could increase the activity of protein phosphatase 2A (PP2A).⁶⁵ PP2A could reduce methylation of signal transducer and activator of transcription (STAT)-1 and formation of the protein inhibitor of the activated STAT-1 PIAS-1-STAT-1 complex.⁶⁴ Ultimately, the damage will enhance destruction of STAT-1 caused by HCV, thereby increasing the apoptosis (Fig. 3).

For another, some scientists have claimed that impairment of immunity is a probable cause. Ethanol exposure enhances the inhibitory effect of HCV on innate immunity, thereby activating the spread of the virus in the liver and eventually leading to impaired adaptive immunity.⁶³ The expression of interferon-stimulated genes (commonly referred to as ISGs) compromises over 300 antiviral molecules that synergistically exert innate immunity and are under control of the catalysis of retinol and retinoic acid biogenesis.⁶⁵ Interestingly, the toxicity of these two substances can be suppressed by ALDH metabolism. It means that inhibition of ALDH will hinder the body's antiviral ability through the ISGs pathway. Therefore, one of the molecular mechanisms for the synergism between HCV and alcohol abuse in liver disease progression is hepatocyte metabolism involving ethanol-retinol metabolic competition.⁶⁶

In addition, activated T cells can be combined with other immune cells to form a positive feedback effect, being aroused by various cellular factors in turn, in a bid to stir up inflammation and inhibiting further liver damage. Gao *et al.*³⁷ discovered the phenomena that alcohol-fed *Aldh2*^{-/-} mice were less sensitive to concanavalin A-induced T cell hepatitis than wild type mice. Their further study suggested that ACH directly restrained cytokine production in T cells by means of the inhibition of aerobic glycolysis or stimulation of corticosterone release, leading to the occurrence of suppressed T cell hepatitis in ethanol-fed *Aldh2*^{-/-} mice. What is more, there is a certain correlation between the HBV epi-

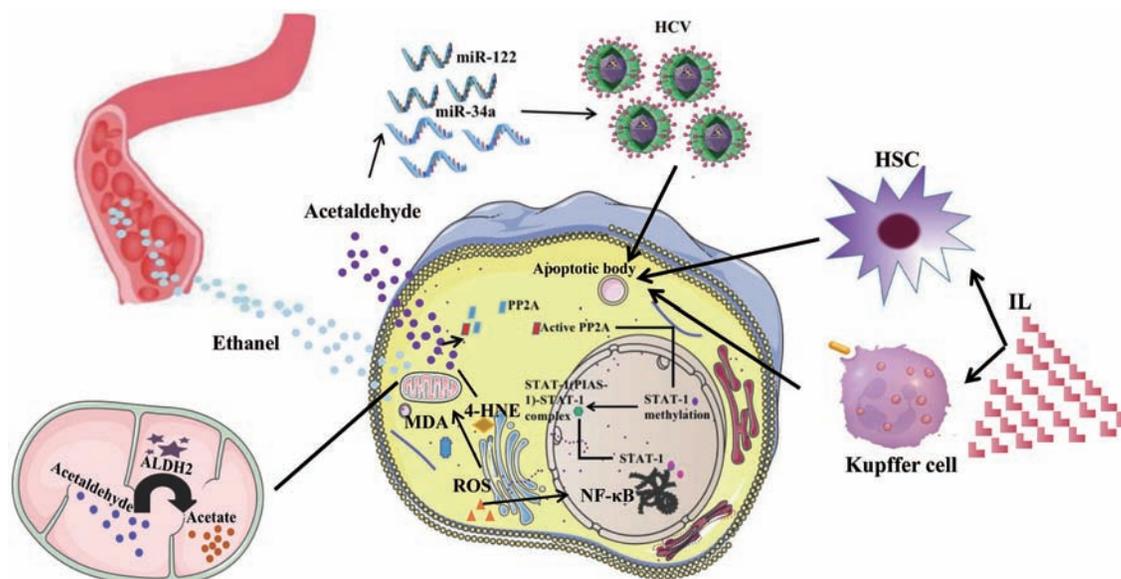


Fig. 3. Effect of ALDH2 in HCV infection and toxic aldehydes. Ethanol is converted to acetaldehyde by the cytosolic enzyme ADH. Then acetaldehyde is converted to acetate by ALDH2. ACH could help to activate the expression of miR-122 and miR-34a, both of which are able to stimulate hepatitis C virus replication, leading to apoptosis. Kupffer cells and hepatic stellate cells (HSC) are driven by interleukins and aggregate to participate in the phagocytosis and clearance of apoptotic bodies. ACH also increases the activity of PP2A. PP2A could reduce methylation of signal transducer and activator of transcription (STAT)-1 and formation of the protein inhibitor of activated STAT-1 PIAS-1-STAT-1 complex. 4-hydroxy-2-nonenal (4-HNE) and malondialdehyde (MDA) are toxic aldehydes in human bodies, which are produced by reactive oxygen species (ROS). Meanwhile, ROS could activate nuclear factor-kappa B (NF-κB) and its downstream targets.

demographic area and the distribution of *ALDH2* gene-defect areas in East Asia.⁶⁷ Almost all patients with *ALDH2* mutation genotypes are linked with HBV infection.⁶⁸ Among patients with chronic HBV, homozygous carriers of the *ALDH2**2 mutant gene have a significantly increased risk of developing liver cirrhosis.

Generally speaking, there is an optimistic link between *ALDH2* and HCV, and alcohol is undoubtedly a factor that aggravates the development of HCV disease and may also be a break-through point in the design of research experiments.

Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is the sixth most common cancer and the third leading cause of cancer mortality in the world.⁶⁹ In China, HCC has emerged as one of the top three malignant tumors, according to rankings by prevalence and mortality.^{70,71} The prevalence rates of HCC were reportedly 0.03%, 0.01%, <0.01%, and <0.01% among the general population in China, the USA, Europe, and Japan, respectively.²⁵ Meanwhile, HCC cases are increasing rapidly in China,⁶⁹ which accounts for approximately 90% of all cases of primary liver cancer.⁷² Therefore, scientists have been endeavoring to explore the relationship between *ALDH2* gene mutation and HCC (Table 2^{35,73-79}).

Generally, HBV infection and ALD are two major liver diseases with HCC developing tendency.⁶⁰ Hou *et al.*⁷³ inhibited aggressive behavior *in vitro* and in mice by forcing the expression of *ALDH2* in HCC cells. Recently, Liu *et al.*³⁵ explored the association between *ALDH2* polymorphisms and the risk of HCC among CHB patients, and their result showed that *ALDH2* polymorphisms has nothing to do with HCC but does protect against developing HCC through habitual alcohol drinking, which was reported in another research study as well.⁷⁴ Similarly, based on an analysis of 4,155 hepatitis B surface antigen seropositive participants,

there is a distinct relationship between an increased risk of HCC in the HBV-positive cirrhosis population and *ALDH2* gene polymorphisms.⁷⁵ Recently, a study involving both mice and human patients showed that *ALDH2* gene deficiency correlates well with a higher risk for advancement of alcohol related-fibrosis to HCC.⁷⁶

A meta-analysis conducted by Chen *et al.*⁷⁷ found that the *ALDH2 rs671* polymorphism is not associated with HCC susceptibility in East Asians, and this is similar to the conclusion from Liu *et al.*³⁵ Interestingly, Huang *et al.*⁷⁸ found that the *ALDH2* polymorphisms had a certain impact on resolution of HCC in patients. The result showed that HCC patients with a defective allele of *ALDH2* have a promising postoperative outcome, after Kaplan-Meier analysis and univariate followed by multivariate Cox proportional hazard analysis indicated that the GG genotype is an independent clinical predictor for shorter time-to-distant metastasis (adjusted $p=0.019$) and shorter overall survival (adjusted $p=0.001$). Although the *ALDH2**2 mutation itself does not lead to liver cancer directly, it will reduce *ALDH2* protein levels and liver enzyme, which eventually is related to the accumulation of ACH in the blood and carcinogenic mutations. Likely, the results of animal experiments show that the mouse *ALDH2 (E487K)* mutation significantly promotes the occurrence and development of mouse liver cancer.⁷⁹

Unfortunately, despite a series of strong evidence supporting ethanol as an environmental risk factor for HCC, the exact pathways by which alcohol causes HCC are still under exploration. ACH has been shown to affect DNA replication and repair mechanisms. After chronic alcohol exposure, *Aldh2*-deficient animals produce a large amount of harmful oxidized mitochondrial DNA via extracellular vesicles, which can be delivered into neighbor HCC cells and subsequently activate multiple oncogenic pathways, to promote HCC development (Fig. 4).⁷⁶ What is more, consuming a large amount of ethanol induces microsomal ethanol metabolism by cytochrome P4502E1 (known as CYP2E1) and leads to additional production of acetaldehyde, as well as an in-

Table 2. Recent clinical studies on the relationship between the *ALDH2* polymorphism and HCC

Author	Year	Conclusion	Reference
Liu <i>et al.</i>	2016	Polymorphisms in <i>ALDH2</i> had significant indirect effects on HCC risk, mediated through alcohol drinking.	35
Hou <i>et al.</i>	2017	Inhibiting aggressive behavior both <i>in vitro</i> and in mice by forcing the expression of <i>ALDH2</i> in HCC cells.	73
Ye <i>et al.</i>	2018	The mutant genotypes of <i>ALDH2</i> may be protective factors for HCC susceptibility in Guangxi Province, China.	74
Chien <i>et al.</i>	2016	GG genotype of <i>ALDH2 rs671</i> was an HCC risk predictor in cirrhotic chronic hepatitis B patients.	75
Seo <i>et al.</i>	2019	<i>ALDH2</i> deficiency is associated with an increased risk of alcohol-related HCC development from fibrosis in human patients and in mice.	76
Chen <i>et al.</i>	2020	<i>ALDH2 rs671</i> polymorphisms are not associated with HCC susceptibility in East Asians.	77
Huang <i>et al.</i>	2019	HCC patients carrying a defective allele of <i>ALDH2</i> had a favorable postoperative outcome.	78
Jin <i>et al.</i>	2015	<i>ALDH 2</i> plays a role of tumor suppressor by maintaining the stability of genome in the liver, and the common human <i>ALDH 2</i> variant would become an important risk factor for hepatocarcinogenesis.	79

Abbreviations: ALDH2, aldehyde dehydrogenase 2; CYP2E1, cytochrome P4502E1; HCC, hepatocellular carcinoma.

crease in free radicals that can result in cell death, DNA damage, and even production of other carcinogenic substances.^{80,81} Other hypothesized pathways have included the transactivator protein X that is encoded by HBV and re-molded to the extracellular matrix through hypoxia-inducible factor-1α (HIF-1α) target genes and the lysyl oxidase (HIF-1α/LOX) pathway to promote HCC metastasis.⁸² The ALDH2-acetaldehyde-redox-AMP-activated protein kinase (AMPK) axis participates in the regulation of ACH levels, which is activated by ALDH2. Therefore, identifying ALDH2 expression levels in HCC might be a useful biomarker for determining prognosis and developing targeted therapies that are urgently needed to treat patients with HCC.

In addition, human liver cancer tissue test results show

that *ALDH2*2* protein is extremely unstable in human liver, and the low expression of *ALDH2* protein has a certain correlation with the formation of liver cancer. The *Journal of Hepatology* also reports that a deficiency in the *ALDH2* gene expression is associated with an increased risk of HCC in patients with hepatitis B cirrhosis who overtake alcohol. Both *in vivo* and *in vitro* studies have found that liver cells from *ALDH2*-deficient mice can produce a large amount of harmful oxidized mitochondrial DNA,³² which is transferred to adjacent liver cells through extracellular vesicles and can activate multiple carcinogenic pathways involving ACH (JNK, STAT3, BCL-2, and TAZ) to promote the occurrence of alcohol-related HCC.⁷³ ALDH2 could also affect metabolism by regulating the ALDH2-acetaldehyde-redox-AMPK

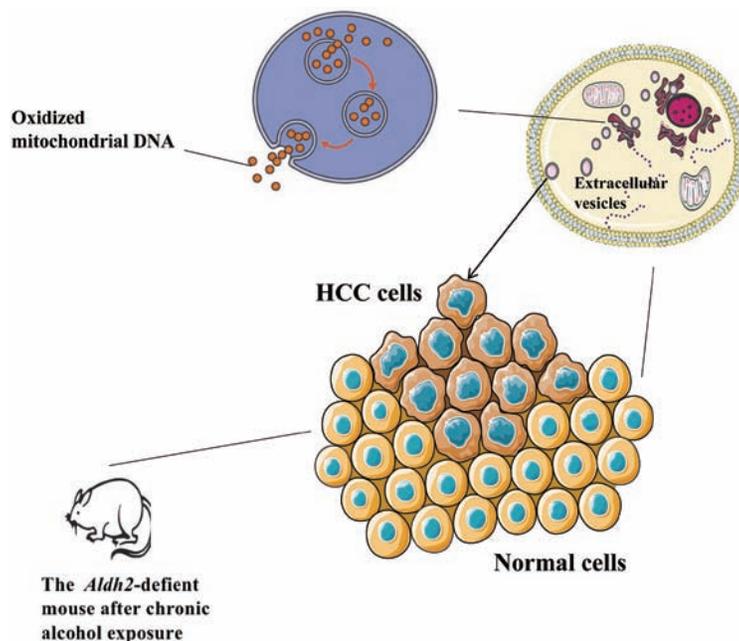


Fig. 4. Effect of ALDH2 on HCC cells. After chronic alcohol exposure, the *Aldh2*-deficient mice produce a large amount of harmful oxidized mitochondrial DNA which are delivered into neighboring hepatocellular carcinoma (HCC) cells via extracellular vesicles.

axis,⁸³ because AMPK can direct lipid metabolism to regulate tumor cell growth and survival. Actually, in proteomics studies, among the proteins related to metabolism and liver function, ALDH2 does not have the highest expression level of enzymes, compared to ALDH1, ALDH4 and ALDH9A, according to the characteristics of the metabolic subgroup;⁸⁴ however, we cannot look down upon the role of *ALDH2* in HCC. Zahid *et al.*⁸⁵ reported transcriptional suppression of alcohol metabolism regulators, and demonstrated that *ALDH2*, downstream of the mTOR signal, is partly responsible for triggering oncogenic transformation of hepatocytes, resulting in disease onset and progression in HCC *in silico*.

In summary, *ALDH2* is a potential risk factor for HCC. However, the clinical correlation between *ALDH2* gene polymorphism and the occurrence and development of liver cancer remains to be further studied.

Conclusions

ALDH2 is a key enzyme in alcohol metabolism, and its genetic mutations are mainly clustered in East Asia. The genetic mutations of *ALDH2* will depress ALDH2 enzyme activity and provoke accumulation of ACH, which will lead to the destruction of liver cells. Importantly, *ALDH2* gene mutation and the potential impact of ACH on T cell response may become one of the factors affecting the progression of liver disease and outcomes of global liver disease. In conclusion, understanding the impact of disease progression related to the *ALDH2* gene may be helpful for the improvement of future liver disease prevention strategies.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Review design (BC), drafting of the manuscript and figure design (QW), language and grammar perfection (XL), revising the manuscript for important intellectual content (QW, BC, ZZ), critical revision (ZZ).

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An Ecological Evaluation of Vinyl Chloride Exposure and Liver Cancer Incidence and Mortality in Texas

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Abstract

The goal of this analysis was to evaluate the association between county-level ambient vinyl chloride (VC) and county-level liver cancer incidence and mortality rates in Texas. Modeled county-level ambient VC data were obtained from the National Air Toxics Assessment. Age-adjusted county-level liver cancer incidence rates were abstracted from the Texas Cancer Registry and age-standardized county-level liver cancer mortality rates were obtained from the peer-reviewed literature. Multivariable imputation was utilized to impute incidence rates in counties with suppressed liver cancer incidence rates. Negative binomial and Poisson regression models were utilized to evaluate the association between county-level ambient VC and county-level liver cancer incidence and mortality rates, respectively, adjusted for county-level heavy drinking prevalence, hepatitis mortality rates, median income, and race (percent Hispanic). County-level ambient VC was not associated with county-level liver cancer incidence or mortality rates. Specifically, when compared to the lowest tertile of ambient VC, the middle (relative risk [RR]: 1.06, 95% confidence interval [CI]: 0.95–1.19) and highest (RR: 1.03, 95% CI: 0.90–1.17) tertiles of ambient VC were not associated with liver cancer incidence. Similarly, county-level ambient VC in the middle (RR: 0.95, 95% CI: 0.85–1.05) and highest (RR: 0.93, 95% CI: 0.82–1.05) tertiles were not associated with liver cancer mortality. This analysis suggests that county-level ambient VC is not associated with liver cancer incidence or mortality in Texas. Our study provides novel results regarding liver cancer risk from low-level non-occupational exposure to ambient VC.

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Abbreviations: ASL, angiosarcoma of the liver; ASPEN, Assessment System for Population Exposure Nationwide; ATSDR, Agency for Toxic Substances and Disease Registry; BRFSS, Behavioral Risk Factor Surveillance System; CDC, Centers for Disease Control and Prevention; EPA, Environmental Protection Agency; HCC, hepatocellular carcinoma; IARC, International Agency on Cancer Research; NAACCR, North American Association of Central Cancer Registries; NATA, National Air Toxics Assessment; NCHS, National Center for Health Statistics; NCI, National Cancer Institute; NPCR, National Program of Central Cancer Registries; PEL, Permissible Exposure Limit; PVC, polyvinyl chloride; RR, relative risk; SEER, Surveillance and Epidemiology End Results; VC, vinyl chloride.

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Introduction

Vinyl chloride (VC), also called vinyl chloride monomer, is a compound used in the manufacture of the polymer polyvinyl chloride (PVC). Due to its versatility and technical properties, PVC is used in various industrial and consumer plastic and vinyl products, including pipes, building supplies, automotive parts, clothing, and medical devices.¹ The PVC global market is projected to increase approximately 3% per year until 2021.²

In 2012, the International Agency on Cancer Research (IARC) classified VC as a group 1 carcinogen based on evidence from animal and occupational epidemiology studies.³ Specifically, IARC concluded that there was sufficient evidence in humans that VC causes angiosarcoma of the liver (ASL) and hepatocellular carcinoma (HCC) due to findings from two large, multicenter cohort studies (one in the USA and one in Europe) of workers at VC and PVC production or processing plants.^{3–5} IARC also discussed a meta-analysis on occupational exposure to VC that concluded that VC workers may experience an increased risk of HCC.^{3,6} The meta-analysis authors noted that findings may have been influenced by the under diagnosis of true ASL and that there was likely variability in the probability and level of exposure among production workers, as direct measurements of VC exposure were not reported in the underlying studies.⁶

The 2012 IARC evaluation was specific to workers occupationally exposed to VC who were potentially simultaneously exposed to other industrial chemicals and agents. It is important to note that IARC did not perform an evaluation of the association between non-occupational VC exposure and cancer risk. The Agency for Toxic Substances and Disease Registry (ATSDR) reported that inhalation of air containing VC is the most likely non-occupational exposure route for the general population.⁷ According to the Environmental Protection Agency (EPA), VC is present in ambient air due to discharge of exhaust gases from VC manufacturing or processing facilities or evaporation from areas where chemical wastes are stored.⁸ Ambient VC concentrations are typically around 1 µg/m³ or less, with concentrations near emission sources ranging from trace levels to over 2,600 µg/m³.^{7,9} For comparison, the Occupational Safety and Health Administration's Permissible Exposure Limit (PEL) is 1 ppm (2,500 µg/m³).¹⁰ While there is information on low-dose exposure among occupational cohorts, there is limited evidence available on the potential risk of liver cancer among individuals non-occupationally exposed to ambient VC.¹¹

VC and PVC plants tend to be clustered primarily along the Texas and Louisiana Gulf coast, which may result in potentially elevated ambient VC concentrations in these areas due to plant emissions. In 2017, Cicalese *et al.*¹² published an ecological study on the association between air pollution (arsenic, benzene, 1,3-butadiene, and VC) and liver cancer incidence in Texas, concluding that their findings suggest that VC is a "significant contributor" to the incidence

of liver cancer in Texas. As noted in our recent Letter to the Editor, this analysis had severe methodological limitations related to insufficient latency period, missing data due to data suppression, lack of adjustment for confounders, and inappropriate model selection.¹³ Due to these limitations and the data gap concerning the potential health effects of non-occupational ambient VC exposure, the objective of the current study was to perform a more rigorous and comprehensive ecological evaluation of the association between exposure to county-level ambient VC and both liver cancer incidence and mortality in Texas. Our analysis has the additional benefits of allowing for adjustment for important liver cancer risk factors, imputation of incidence rates for suppressed counties, consideration of both liver cancer incidence and mortality, and utilization of more conventional Poisson and spatial regression modeling.

Methods

Exposure data

Modeled county-level VC data were obtained from the 1996 National Air Toxics Assessment (NATA). NATA is the EPA's review of air pollutants in the USA, where emissions data from stationary sources (*e.g.*, large waste incinerators and factories), area and other sources (*e.g.*, small manufacturers, wildfires, dry cleaners) and mobile outdoor sources (*e.g.*, vehicles) are used as inputs in air quality models to estimate the county-level and census tract-level concentrations of various chemicals.¹⁴ The EPA uses the Assessment System for Population Exposure Nationwide (ASPEN) simulation model to combine emissions data with meteorological data to estimate concentrations of air pollutants, accounting for rate/location of release, wind speeds and direction, breakdown and transformation, and deposition.¹⁵ The 1996 assessment was selected to allow for sufficient disease latency, as this was the earliest dataset available. Specifically, we obtained the county-level median estimated annual average ambient VC ($\mu\text{g}/\text{m}^3$) for every county in Texas. Additionally, we performed a sensitivity analysis using the county-level 95th percentile ambient VC instead of the county-level median ambient VC.

NATA provides data on both ambient VC (concentration of VC in the open air) and exposure VC (concentration of VC that a person may breathe over time, accounting for time that a person spends indoors and outdoors).¹⁶ Due to the study objectives, we selected ambient VC for the final model; however, we also performed a sensitivity analysis using county-level median exposure VC.

Liver cancer data

Incidence: Liver cancer incidence rates were obtained from the Texas Cancer Registry (TCR). The TCR collects data through passive and active surveillance and meets the high quality data standards put forth by the Centers for Disease Control and Prevention (CDC) and the North American Association of Central Cancer Registries.¹⁷ Specifically, county-level liver cancer incidence rates (per 100,000 population), age-adjusted to the 2000 USA Standard Population, were queried for each county in Texas from 2006 to 2015.

Mortality: Liver cancer mortality rates were abstracted from the Mokdad *et al.*¹⁸ (2017) analysis of cancer mortality patterns across USA counties. Briefly, Mokdad *et al.*¹⁸ used death records from the National Center for Health Statistics (NCHS), population counts from the Census Bureau, the NCHS, and the Human Mortality Database, and validated

small area estimation models to estimate county-level mortality rates for various cancer endpoints. Age-standardized 2010 liver cancer mortality rates (per 100,000 population) were calculated using the USA Census population.¹⁸

Latency period: Our liver cancer incidence (2006–2015) analysis allowed for a 10–19 year latency period (ambient VC data from NATA 1996). Similarly, our liver cancer mortality (2010) analysis provided a 14 year latency period.

Potential confounding variables

Known risk factors of liver cancer were considered as potential confounding variables. County-level sex (percent male), race (percent Hispanic), and income (median household income in the past 12 months, inflation-adjusted dollars) were abstracted from the 2010 American Community Survey (5-year averages).^{19–21} Data from 2010 were selected from among the available years (2005–2017) to correspond to the midpoint of the outcome data. County-level age-standardized total cigarette smoking prevalence (non-daily and daily smoking; 2010) and age-standardized prevalence of heavy drinking (consumption, on average, of more than one drink per day for women and two drinks per day for men in the past 30 days; 2010) were reported in the peer-reviewed literature based on calculations using validated small area estimation methods and data from the Behavioral Risk Factor Surveillance System (BRFSS).^{22,23} County-level age-adjusted obesity (body mass index ≥ 30) prevalence data were collected from the CDC.²⁴ Lastly, age-standardized hepatitis mortality rates (per 100,000 population) for 2010 were obtained from the USA's Infectious Disease Mortality Rates dataset from the Institute for Health Metrics and Evaluation.²⁵

Incidence rate imputation for suppressed counties

The TCR suppresses incidence rate and cancer count data when there are fewer than 16 reported cancer-specific cases in a county, based on the threshold used by the National Program of Central Cancer Registries (NPCR) at the CDC, the North American Association of Central Cancer Registries (NAACCR), and the Surveillance and Epidemiology End Results (SEER) Program at the National Cancer Institute (NCI). Additionally, the TCR does not provide incidence rate, population size, or cancer count data for counties with a risk population of <1,000 persons. Of the 254 counties in Texas, 102 had fewer than 16 cases of liver cancer reported from 2006–2015 and 8 had risk populations <1,000 persons. Overall, 110 counties (43%) had missing liver cancer incidence data due to suppression or an insufficient at-risk population. Therefore, we used a step-wise selection ($p=0.10$) negative binomial regression model (accounting for over-dispersion in the reported incidence data) to perform multivariable imputation to estimate liver cancer incidence rates for counties with suppressed rate data in the TCR. Multiple imputation is a technique for handling missing values, where the distribution of the observed data is used to estimate multiple datasets (to reflect uncertainty around the true value) that are then pooled for statistical analysis.²⁶ Variables selected for the multivariable imputation model were county-level race (percent Hispanic), smoking prevalence, heavy drinking prevalence, hepatitis mortality rate, and median income. Three counties had imputed liver cancer incidence rates that were negative, which were set to zero for purposes of the statistical analyses. Imputed incidence rates for the remaining counties were rounded to the nearest tenth to match the data format of rates reported by the TCR.

Exploratory spatial data analyses

Exploratory spatial data analyses were performed to qualitatively evaluate the association between county-level ambient VC, liver cancer incidence and mortality rates, and potential confounders to inform statistical analyses and modeling. Additionally, the NATA 1996 dataset provided exposure VC data at both the county- and census tract-level. Therefore, we explored exposure VC at the census tract-level to assess the distribution of exposure VC within counties.

Statistical analyses

County-level ambient VC data were log (base 10) transformed (due to positive skewness), and then both ambient VC and covariate data were categorized into tertiles (low, medium, and high) (Table 1). Ambient VC and covariate data were modeled as categorized tertiles, with the lowest tertile serving as the reference group. A sensitivity analyses was performed using scored tertiles (1, 2, 3), instead of categorized tertiles (2 vs. 1 and 3 vs. 1).

For liver cancer incidence analyses, negative binomial regression models were utilized due to over-dispersed incidence data. For liver cancer mortality analyses, Poisson regression models were used, as the mortality data were not over-dispersed. For both models, unadjusted univariate analyses were performed for ambient VC and each covariate variable. Statistically significant predictors of liver cancer (county-level heavy drinking prevalence, hepatitis mortality rates, median income, and race [percent Hispanic]) were included in the adjusted multivariable liver cancer incidence and mortality models. These covariates were also statistically significantly associated with ambient VC using univariate linear regression. County-level smoking prevalence, obesity prevalence, and sex (percent male) were not statistically significant predictors of liver cancer incidence or mortality and were excluded from the final models; however, we performed a sensitivity analysis with all of these variables included. The calculated risk estimates for all models were incidence/mortality rate ratios (relative risk [RR]; exponentiated beta-coefficients from the models) with 95% confidence intervals (CIs). All analyses were performed using STATA 14.2.

Sensitivity analyses

In addition to tests previously described, various sensitivity analyses were performed to further assess the consistency of findings. Different alternative imputation methods were evaluated, including assuming that each suppressed county had: 1) 8 cases of liver cancer (median of range of 0–15 cases), 2) 15 cases of liver cancer (maximum number of cases that would still be suppressed), and 3) a liver cancer incidence rate similar to the Texas average (9.5 cases per 100,000 population, as reported by the TCR). For the assumed 8 and 15 cases models, we were unable to calculate incidence rates for the counties with a risk population of <1,000 persons ($n=8$), as specific population sizes were not reported by the TCR. Therefore, we utilized the imputed incidence rates from the multivariable imputation method for these counties.

To examine spatial correlation, a geospatial regression was performed using GeoDa 1.12.1.131. It is likely that area-level factors influence exposure observations, where individuals living near each other experience exposures that are more similar than exposures experienced by individuals living further away. Spatial regression can account for this

Table 1. Descriptive results for county-level VC and covariates

Variable	Tertile 1			Tertile 2			Tertile 3		
	n ^a	Mean	Range	n ^a	Mean	Range	n ^a	Mean	Range
Median ambient VC, $\mu\text{g}/\text{m}^3$	85	7.87×10^{-6}	4.12×10^{-8} – 1.78×10^{-5}	85	4.45×10^{-5}	1.86×10^{-5} – 8.99×10^{-5}	84	2.19×10^{-3}	9.10×10^{-5} – 1.02×10^{-1}
Median ambient VC – Log (base 10) transformed, $\mu\text{g}/\text{m}^3$	85	-5.24	-7.39 – -4.75	85	-4.40	-4.73 – -4.05	84	-3.41	-4.04 – -0.99
Heavy drinking prevalence, %	91	4.76	2.7–5.7	88	6.24	5.8–6.7	75	7.65	6.8–16.5
Hepatitis mortality rate, per 100,000	98	0.26	0.15–0.32	72	0.36	0.33–0.39	84	0.53	0.40–2.2
Median household income, \$	85	33,679	19,959–38,702	85	42,018	38,832–45,929	84	54,632	46,128–83,889
Race, percent Hispanic	85	10.7	0–17.0	85	24.3	17.1–35.5	84	58.1	35.8–98.3
Sex, percent male	85	48.4	44.9–49.1	85	49.8	49.1–50.6	84	54.2	50.7–68.4
Smoking prevalence, %	87	18.9	14.0–21.1	85	22.5	21.2–23.8	82	25.9	23.9–29.6
Obesity prevalence, %	91	28.0	23.3–29.1	79	29.7	29.2–30.3	84	32.0	30.4–36.8

^aIdentical values were assigned to the same tertile, which may result in an unequal distribution of the number of counties across the tertiles.

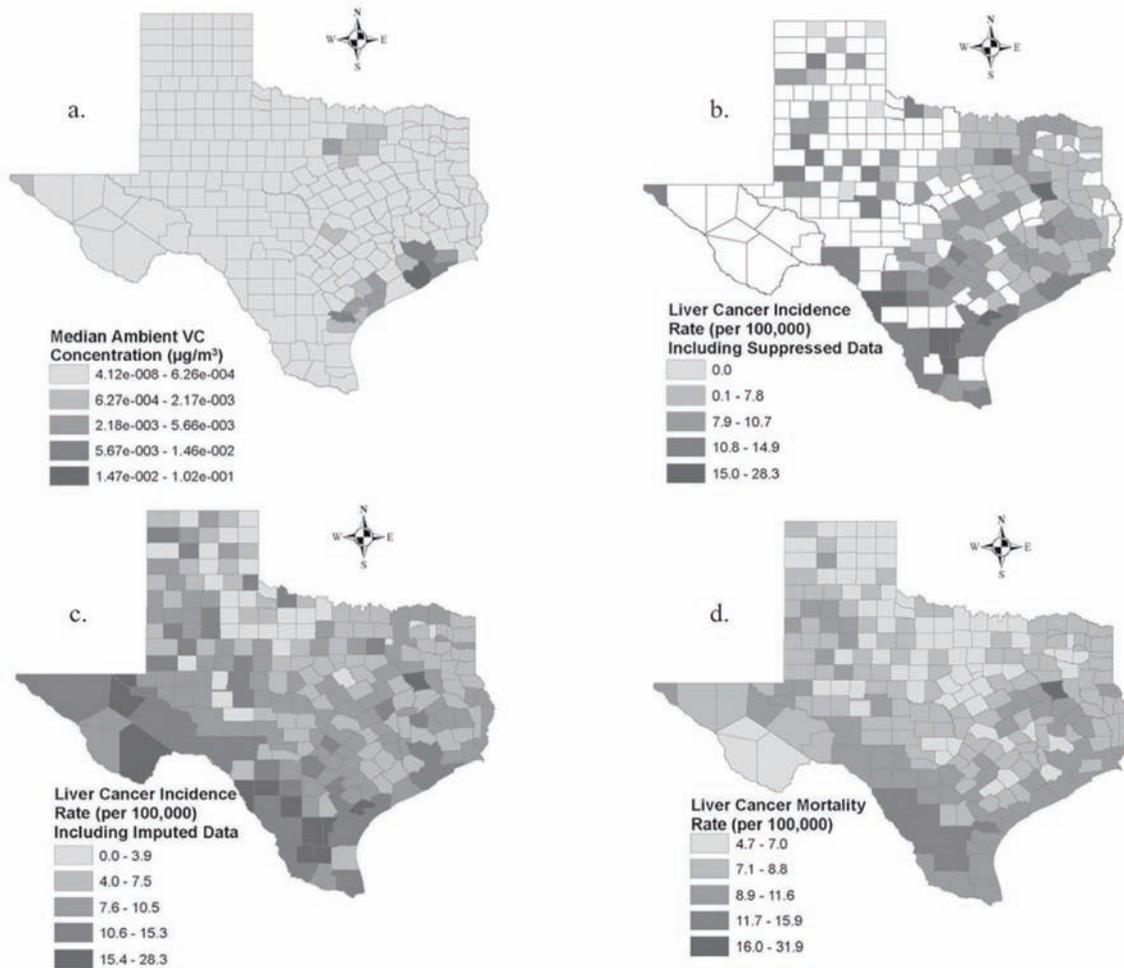


Fig. 1. Spatial distribution of county-level data. (A) Median ambient VC ($\mu\text{g}/\text{m}^3$). (B) Liver cancer incidence rate (per 100,000), including suppressed data. (C) Liver cancer incidence rate (per 100,000), including imputed data. (D) Liver cancer mortality (per 100,000).

spatial correlation, which is not possible with a negative binomial or Poisson regression models. Weights were created using rook contiguity (neighbor defined as a county sharing a common border), and scored tertiles were used for both exposure and covariate data.^{27,28} A spatial lag regression model was selected based on diagnostics from the ordinary least-squares regression. A spatial error regression model was also ran as an additional sensitivity analysis.

Results

VC exposure

Modeled county-level median ambient VC was reported for all 254 counties in Texas, ranging from $4.12 \times 10^{-8} \mu\text{g}/\text{m}^3$ (McMullen County) to $0.0102 \mu\text{g}/\text{m}^3$ (Brazoria County). The median ambient VC in Texas was $5.85 \times 10^{-4} \mu\text{g}/\text{m}^3$. Counties with imputed liver cancer incidence rates (suppressed counties) had a lower average county-level median ambient VC in comparison to counties with reported liver cancer incidence rates in the TCR (not suppressed counties) (Supplementary Table 1). The spatial distribution of median ambient VC by Texas county is shown in Fig. 1.

Exposure VC data were available at both the county-level

and census-tract level in the NATA 1996 dataset, and exploratory spatial data analyses revealed that within the same county, exposure VC varied across the census tract-level for some counties. For example, the largest difference was in Brazoria County, where the county-level median exposure VC was $0.073 \mu\text{g}/\text{m}^3$, while the census tract-level exposure VC within Brazoria County ranged from 0– $0.74 \mu\text{g}/\text{m}^3$. Out of the 254 counties, 27 (11%) had an order of magnitude or larger difference between the maximum census tract-level VC exposure and the median county-level exposure VC (e.g., similar to Brazoria County) (Supplementary Table 2). Only 6% of counties had an order of magnitude or larger difference between the census-tract level 95th percentile exposure VC and the county-level 95th percentile exposure VC values (Supplementary Table 2).

Liver cancer incidence

Liver cancer incidence rates (per 100,000 population) ranged from 0 (Cottle, Sterling, Lipscomb, and Sherman Counties) to 28.3 (Brooks County) in the dataset from the TCR (not suppressed counties), and ranged from 0 (Armstrong, Franklin, and Throckmorton Counties) to 18.6 (Jim Hogg County) in the imputed dataset (for suppressed counties). The average liver cancer incidence rate in the data-

Table 2. Liver cancer incidence and mortality rate ratios

Variable ^a	Category, tertile	Liver cancer incidence		Liver cancer mortality	
		Unadjusted RR (95% CI)	Adjusted RR (95% CI) ^b	Unadjusted RR (95% CI)	Adjusted RR (95% CI) ^b
Median ambient VC	Low (ref)	NA	NA	NA	NA
	Medium	1.01 (0.87–1.17)	1.06 (0.95–1.19)	0.94 (0.84–1.04)	0.95 (0.85–1.05)
	High	1.11 (0.95–1.28)	1.03 (0.90–1.17)	0.97 (0.87–1.07)	0.93 (0.82–1.05)
Heavy drinking	Low (ref)	NA	NA	NA	NA
	Medium	1.39 (1.21–1.59)*	1.38 (1.23–1.56)*	1.08 (0.97–1.20)	1.07 (0.95–1.20)
	High	1.65 (1.43–1.89)*	1.66 (1.46–1.88)*	1.21 (1.09–1.35)*	1.22 (1.08–1.37)*
Hepatitis	Low (ref)	NA	NA	NA	NA
	Medium	1.27 (1.10–1.47)*	1.14 (1.01–1.28)*	1.12 (1.00–1.25)*	1.08 (0.97–1.22)
	High	1.38 (1.21–1.59)*	1.16 (1.03–1.30)*	1.26 (1.14–1.40)*	1.19 (1.06–1.33)*
Race, percent Hispanic	Low (ref)	NA	NA	NA	NA
	Medium	1.21 (1.06–1.39)*	1.23 (1.09–1.39)*	1.00 (0.89–1.11)	1.00 (0.89–1.11)
	High	1.69 (1.48–1.93)*	1.68 (1.50–1.88)*	1.26 (1.14–1.40)*	1.22 (1.10–1.36)*
Income	Low (ref)	NA	NA	NA	NA
	Medium	0.90 (0.78–1.04)	0.87 (0.78–0.97)*	0.94 (0.85–1.04)	0.94 (0.84–1.04)
	High	0.75 (0.65–0.87)*	0.76 (0.67–0.85)*	0.80 (0.72–0.89)*	0.83 (0.74–0.93)*

^aVC data were log transformed, and then both VC and covariate data were divided into tertiles (low, medium, and high). ^bAdjusted for heavy drinking prevalence, hepatitis mortality rates, income, and race (percent Hispanic). **p*-value <0.05. Abbreviations: CI, confidence interval; RR, rate ratio; VC, vinyl chloride.

set from the TCR was 9.0, while the average liver cancer incidence rate in the imputed dataset was 7.3. The spatial distribution of liver cancer incidence rates by Texas county is shown in Fig. 1 (for both suppressed and imputed counties). Based on visual inspection, there was no evidence of a spatial association between county-level ambient VC and liver cancer incidence rates (Fig. 1).

County-level ambient VC was not associated with county-level liver cancer incidence in either the univariate or multivariable negative binomial regression models. Specifically, in the unadjusted model, the middle and highest tertiles of county-level ambient VC were associated with liver cancer incidence RRs of 1.01 (95% CI: 0.87–1.17) and 1.11 (95% CI: 0.95–1.28), respectively, when compared to the lowest tertile of ambient VC. The absence of association between county-level ambient VC tertile and liver cancer incidence persisted (RR: 1.06, 95% CI: 0.95–1.19 and RR: 1.03, 95% CI: 0.90–1.17 for the middle and highest tertiles, respectively) upon adjustment for county-level heavy drinking prevalence, hepatitis mortality rate, race (percent Hispanic) and median income. Increasing tertiles of county-level heavy drinking prevalence, hepatitis mortality rate, and race (percent Hispanic) were associated with a statistically significant increased rate of liver cancer incidence, while increasing tertiles of county-level median income were associated with a statistically significant decreased rate of liver cancer incidence (Table 2).

Liver cancer mortality

Liver cancer mortality rates (per 100,000 population) ranged from 4.72 (Hartley County) to 31.9 (Anderson County). The average liver cancer mortality rate was 8.1. The spatial distribution of liver cancer mortality rates by Texas county is shown in Fig. 1. Based on visual inspection, there was no evidence of a spatial association between county-level am-

bient VC and liver cancer mortality rates (Fig. 1).

County-level ambient VC was not associated with county-level liver cancer mortality in either the univariate or multivariable Poisson regression models. In the unadjusted model, the middle and highest tertiles of county-level ambient VC had liver cancer mortality RRs of 0.94 (95% CI: 0.84–1.04) and 0.97 (95% CI: 0.87–1.07), respectively, when compared to the lowest tertile of county-level ambient VC. In the adjusted model, the middle (RR: 0.95, 95% CI: 0.85–1.05) and highest (RR: 0.93, 95% CI: 0.82–1.05) tertiles of ambient VC were not associated with liver cancer mortality, when compared to counties with the lowest tertile of ambient VC (Table 2). The highest tertile of county-level heavy drinking prevalence, hepatitis mortality rate, and race (percent Hispanic) were associated with a statistically significant increased rate of liver cancer mortality, when compared to counties with the lowest tertile. In contrast, counties with the highest tertile of county-level median income were associated with a statistically significant decreased rate of liver cancer mortality (Table 2).

Sensitivity analyses

County-level ambient VC was consistently not associated with county-level liver cancer incidence or mortality rates across various sensitivity analyses (Supplementary Table 3). For example, both county-level 95th percentile ambient VC and median exposure VC were not associated with liver cancer incidence or mortality rates. Additionally, no association was observed between county-level median ambient VC and liver cancer incidence or mortality rates when the final multivariable model also included the covariates of county-level smoking prevalence, obesity prevalence, and sex (percent male). Similarly, no association was observed when each suppressed county was assigned the Texas average liver cancer incidence rate. When each suppressed county was

assigned 8 or 15 cases of liver cancer, the highest tertile of county-level ambient VC was associated with a statistically significant decreased rate of liver cancer incidence, when compared to the lowest tertile of ambient VC. Further, county-level ambient VC was not associated with county-level liver cancer incidence in both the spatial lag and spatial error regression models, while county-level ambient VC was associated with a statistically significant decreased rate of liver cancer mortality in both the spatial lag and spatial error regression models (Supplementary Table 3).

Discussion

This ecological analysis provides no evidence that Texas county-level ambient VC is associated with either county-level liver cancer incidence or mortality rates in Texas. This study has the strength of evaluating ambient VC levels that the general population could experience. There is evidence that high cumulative exposure to VC in occupational settings (*i.e.* $\geq 2,271$ ppm-years or $>2,500$ ppm-years) is associated with an increased risk of HCC.^{11,29} However, the potential health effects of lower exposure to ambient levels (*e.g.*, emissions from manufacturing and processing facilities) have not been well studied. In our analysis, low-level county-level ambient VC (maximum concentration of $0.0102 \mu\text{g}/\text{m}^3$ [approximately 4 ppt]) was not associated with county-level liver cancer incidence or mortality rates in Texas. Our findings contrast with the conclusions of the Cicalese *et al.*¹² (2017) analysis, which reported that VC is a significant contributor to the incidence of liver cancer in Texas (Cicalese and colleagues did not specify if they used ambient or exposure VC concentrations).

This analysis considered multiple well-known risk factors of liver cancer as potential confounders, including sex, race, heavy drinking prevalence, hepatitis mortality rates, smoking prevalence, income, and obesity. Our analysis suggests that county-level heavy drinking prevalence, hepatitis mortality rates, median income, and race (percent Hispanic) were all significant predictors of liver cancer incidence and mortality. These results are in agreement with other studies that have reported that heavy drinking and hepatitis virus infections are associated with an increased risk of liver cancer.^{30–32} It has also been reported that income is inversely associated with liver cancer incidence, likely due to differences in risk factors across income levels.³³ Additionally, the American Cancer Society reports that race is a risk factor of liver cancer, with higher rates of liver cancer occurring among Asian Americans, Pacific Islanders, and Hispanics/Latinos compared to other races and ethnicities among the USA's population.³⁴ The consideration of potential confounding factors is an important strength of our study that builds upon the only other published study on this topic. While the Cicalese *et al.*¹² (2017) study did adjust for county-level hepatitis C infection prevalence, the number of prisons in each county (as a proxy of hepatitis infection), and obesity prevalence, they did not consider several other important risk factors, suggesting that their results may be susceptible to bias.

A limitation of the underlying data in our analysis was the high prevalence of missing liver cancer incidence rate data (43% of counties). Rather than simply excluding the missing data, this analysis has the strength of using various methodologies to impute liver cancer incidence rates for the suppressed counties. Counties with suppressed liver cancer incidence data had a lower average population size than counties with reported incidence rates (Supplementary Table 1). This is logical, as the TCR suppresses counties with less than 16 cases of cancer and counties with an insufficient at-risk population size. Additionally, counties with imputed incidence rates (suppressed counties) had a lower

average and lower bound median ambient VC than counties with reported incidence rates (not suppressed counties), by an order of two magnitudes (Supplementary Table 1). Therefore, this study increased the county sample size and representativeness by including a wider range of exposure across populations in Texas. Additionally, exclusion of the suppressed counties may result in bias if the excluded counties (due to low liver cancer counts) were also counties with low ambient VC. In this scenario, exclusion of the suppressed counties could result in a biased overestimate of the association.

We used NATA data from 1996, TCR data from 2006–2015, and mortality data from 2010, which allows for a minimum of 10 years between exposure and disease diagnosis. Studies that do not allow for sufficient induction and latency periods for liver cancer result in biased risk estimates, as the short latency time periods are potentially biologically irrelevant, as any impact on disease would not yet have been clinically diagnosed or detected. Liver cancer latency (not specific to a certain exposure) has been estimated to be 10.8 years, defining latency as the time from cancer initiation to diagnosis.³⁵ It has also been reported that the median latency for angiosarcoma of the liver and HCC deaths among workers at VC or PVC plants are 36 and 48 years, respectively.¹¹ Another study reported that the latency for malignant hepatoma (predominantly ASL, but HCC and cholangiocellular carcinoma were also noted) among PVC production workers ranged from 12 to 34 years.³⁶ It should be noted that these latency estimates are specific to disease mortality rather than disease diagnosis. We included the earliest NATA data available but it is possible that the latency periods in this study may not be sufficient for some disease diagnosis or mortality.

As an ecological study, this analysis is limited to examining ambient VC, potential confounders, and liver cancer incidence/mortality rates at the county-level rather than the individual level. Ecological studies are inherently limited by specification bias, aggregation bias, and temporal ambiguity.^{37,38} For example, while our data suggests that county-level heavy drinking prevalence is associated with liver cancer incidence/mortality rates, we are unable to confirm that individuals with liver cancer are high consumers of alcohol. On the other hand, our use of a large number of relatively small, homogeneous counties as the units of analysis and the multiple covariates available for each of those counties helped to offset the general limitations inherent in ecological analyses.

Another limitation is potential exposure misclassification at the county-level due to the spatial distribution of exposure VC. This limitation of NATA data is noted by the EPA when it is suggested that “modeling results should not be used to draw conclusions about local exposure concentrations or risk”.³⁹ Exploratory spatial analyses also revealed that there was variability in exposure VC at census tracts within the same county. County-level median concentrations may be a spatially diluted value that is not an adequate representation of exposures at specific locations, such as underestimating exposure at residences potentially near point sources or overestimating exposures at residences far away from point sources. While spatial variability in exposure VC was noted between the county-level and census tract-level, the maximum census tract-level and median county-level concentrations were not markedly different for the majority of counties. Additionally, we explored this potential dilution effect by evaluating the 95th percentile ambient VC for each county in Texas. Since the available data indicated that census tract-level and county-level 95th percentile values were similar for over 90% of the counties, we do not believe that potential spatial heterogeneity substantially impacted our conclusions. Therefore, a strength of this analysis is the examination of spatial heterogeneity through these various sensitivity analyses.

Conclusions

Overall, our findings suggest that county-level ambient VC is not associated with county-level liver cancer incidence or mortality in Texas. Strengths of this analysis include adjusting statistical analyses for potential confounding by known risk factors for liver cancer, allowing time for the development of liver cancer following VC exposure, and utilizing analytical imputation methods to address missing data. Our ecological study provides novel results regarding liver cancer risk from exposure to low-level ambient VC.

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Conflict of interest

Authors KMT, SMB, NSE, and GMM are employed by Cardno ChemRisk, a consulting firm that provides scientific advice to the government, corporations, law firms, and various scientific/professional organizations. GMM is also professor emeritus of biostatistics and founding director of the Center for Occupational Biostatistics and Epidemiology at the University of Pittsburgh, Graduate School of Public Health.

Author contributions

Participated in the study design, data analysis, and manuscript writing (KMT, SMB, NSE, GMM).

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Cytomegalovirus Hepatitis in Immunocompetent and Immunocompromised Hosts

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Abstract

Human cytomegalovirus (HCMV) infection is common and affects between 40–100% of the worldwide population. However, the majority of cases are asymptomatic and when severe disease occurs, it is usually restricted to immunocompromised patients. Liver involvement by HCMV differs significantly, accordingly to the immune status of the host. In immunocompromised patients, particularly liver transplant patients, it often causes clinically significant hepatitis. On the other hand, in immunocompetent patients, HCMV hepatitis requiring hospitalization is extremely rare. This review aims to appraise studies regarding the pathophysiology of HCMV hepatitis, including mechanisms of latency and reactivation and its contribution to disease development, clinical presentation, diagnostic modalities and treatment, with a focus on comparing different aspects between immunocompromised and immunocompetent hosts.

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Introduction

Human cytomegalovirus (HCMV) is a common pathogen, thought to affect 40% to 100% of the world population.¹ It is mainly transmitted through close contact by body fluids, such as saliva, blood, urine, breast milk, semen and cervical secretions, and also by organ transplantation. It can infect a vast number of cells within the host, including epithelial cells, endothelial cells, parenchymal cells, connective tissue cells and several types of hematopoietic cells. This facilitates both inter-host transmission and systemic transmission within the host.²

The clinical manifestations are extensive and vary particularly between immunocompetent and immunocompromised hosts. While the vast majority of immunocompetent

hosts have a completely asymptomatic course, the immunocompromised host may experience a wide range of severe complications, including esophagitis, colitis, hepatitis, encephalitis, pneumonitis, bacterial superinfection.¹ In addition, acute infection, chronic infection and reactivation of the virus generate different clinical identities.

In immunocompetent hosts, clinically significant hepatitis is rare and only case reports or small case series are available in the literature. Its presentation is usually a spectrum of malaise and fever, without the classical jaundice seen with the common hepatitis viruses.³ Other cases have reported an asymptomatic course or association with abdominal pain only. However, hepatitis is a well-known manifestation of HCMV infection in immunocompromised hosts, particularly in liver transplant patients, in which the incidence is relatively high. Indeed, fulminant hepatitis requiring living-donor liver transplant has been described in this population.⁴

Like other herpes viruses, HCMV has the ability to create a lifelong latent infection. Through a variety of complex mechanisms, HCMV modulates the host cell cycle to create an optimal environment for continuous and efficient replication.⁵ This lifecycle characteristic allows for viral reactivation and consequently HCMV-related acute disease, including acute hepatitis.

The exact mechanisms by which HCMV induces hepatitis are not well established. However, the role of the immune system appears to be important as an indirect cause of liver damage. Because of the overall rarity of the disease, especially in immunocompetent patients, delay in diagnosis is common, resulting in unnecessary and expensive diagnostic testing. Furthermore, delays can lead to incorrect management and poor outcomes.

The aim of this manuscript is to review the pathogenesis, presentation, diagnosis and management of HCMV hepatitis, with a focus on host immune status.

Epidemiology

The overall seroprevalence of HCMV has been estimated to range from 45% to 100%.¹ This large number is related to the high number of asymptomatic individuals who do not seek medical care. In addition, several risk factors contribute to changes in seropositivity rate across various groups. According to the National Health and Nutrition Examination Surveys from 1988–2004, which analyzed the HCMV seroprevalence in the USA, HCMV prevalence was associated with increasing age and was slightly higher in women, non-Hispanic black ethnicity, and Mexican Americans. Furthermore, foreign birthplace, lack of insurance, and low income and low education households were also associated with a higher infection rates.⁶

Symptomatic HCMV infection is rare in immunocompe-

Keywords: CMV hepatitis; Cytomegalovirus hepatitis; Cytomegalovirus hepatitis immunocompetent; Cytomegalovirus hepatitis immunocompromised.

Abbreviations: D+/-, donor positive/negative; EBV, Epstein-Barr virus; LSEC, liver sinusoidal endothelial cell; HCMV, human cytomegalovirus; HLA, human leukocyte antigen; HSCT, hematopoietic stem cell transplantation; MHC, major histocompatibility complex; PCR, polymerase chain reaction; R+/-, recipient positive/negative.

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tent hosts. When present, it usually manifests as a mononucleosis-like syndrome in approximately 10% of patients.⁷ The estimated incidence of mononucleosis-like syndrome secondary to HCMV infection in hospitalized immunocompetent patients in Hong Kong was reported to be 9.54 per million patient discharges in 2005–2007, and 19.52 per million patient discharges for the period of 2014–2016.⁷

Even though liver dysfunction is not uncommonly associated with HCMV mononucleosis in immunocompetent hosts, there are only case reports of clinically significant HCMV hepatitis available in the literature. To date, there have been 26 descriptions of either case reports or case series of HCMV hepatitis in a total of 44 immunocompetent hosts.

On the other hand, clinically significant HCMV hepatitis is more frequent in the immunocompromised population, particularly in liver transplant patients. For instance, Seehofer *et al.*⁸ observed a 2.1% rate of HCMV hepatitis in 1,146 consecutive liver transplantations. In terms of incidence of viremia, in a study of 182 liver transplant patients to whom pre-emptive therapy was used but no antiviral prophylaxis was employed, Singh *et al.*,⁹ observed a HCMV infection rate of 32.5% (38 of 117) of recipient positive (R+) patients, 84.6% (33 of 39) of donor positive (D+)/recipient negative (R–), and 3.8% (1 of 26) of donor negative (D–)/R– patients.

Pathogenesis of HCMV

Systemic viral dissemination

The HCMV possesses glycoproteins that can interact with a vast number of different cell surfaces within the human body and initiate its life cycle.¹⁰ This unique characteristic allows for a broad cellular tropism. The hematogenous spread of the virus allows for its systemic dissemination.¹¹ Recent observations have shown that polymorphonuclear leukocytes can more efficiently carry and disseminate the virus.^{11,12} Nonetheless, Sinzger *et al.*¹³ also observed infected macrophages in the lung and gastrointestinal tissues. Later studies supported this by suggesting that monocytes carry a comparable amount of viral load, thus contributing to systemic viral dissemination.¹⁴ Once successful termination of acute infection is achieved, a period of latency/persistence is initiated, during which multiple episodes of viral reactivation and transmission can occur.

HCMV infection of hepatic cells

Theise *et al.*¹⁵ studied liver biopsies from seven patients with HCMV hepatitis and detected that the infection started in the cells lining the sinusoids (including Kupffer and endothelial cells), proposing that hematogenous spread to the liver occurs first. Furthermore, hepatocytes were noted to be infrequently infected. On the contrary, Sano *et al.*¹⁶ found that hepatocytes were the most frequently infected cell line, and bile duct involvement was only identified in one case.¹⁶ However, they did not provide evidence of infection in Kupffer cells or other sinusoidal cells. Sinzger *et al.*¹⁷ studied HCMV infection in cultured human liver cells. They detected viral antigens from all phases of viral replication, suggesting that the tissue allowed for complete viral replication. In this study, various target cells were identified by immunocytochemical double-labelling, including bile duct cells, fibroblasts, and hepatocytes. They observed that hepatocytes were the primary cell target and supported the late stages of viral replication, indicating that this cell line participates in production of progeny virus. Olver *et al.*¹⁸

also found that hepatocytes were the predominant cell target in HCMV hepatitis in their mice studies.

Indirect vs. direct cytopathogenicity of HCMV in the liver

It has been reported that the HCMV exhibits both direct and indirect cytopathogenicity in various organs, including the liver. It is believed that liver dysfunction occurs primarily from indirect cytopathogenicity of cytotoxic T-lymphocyte (CD8+) lineage. Pape *et al.*¹⁹ identified accumulations of cytolytic T lymphocytes in the areas of liver tissue injury caused by HCMV, by means of monoclonal antibodies. This provided evidence of indirect pathogenicity by immune-related cytotoxicity and cytokine damage from the host immune system defense against the virus. Several hypotheses have emerged to explain the mechanism of tissue damage through indirect cytopathogenicity, including activation of cytotoxic T cell reactions against HCMV-infected cells,^{2,20,21} vasculitic alterations and subsequent localized necrosis,²² and in relation to allograft transplantation.^{2,20–22} In the latter, the purposed mechanism comprises a possible enhancement in the frequency of lymphocytic activation or increased MHC expression, which further exacerbated the immunological detrimental effects.^{2,20}

Sinzger *et al.*¹³ observed lysis of cultured liver cells infected with HCMV, supporting the role of the virus in direct cytopathogenicity. They concluded that HCMV can cause direct liver parenchyma damage through cytolytic mechanisms. However, despite the fact that the virus can be present in hepatocytes and bile ducts, its presence in the majority of cases has been shown to be moderate and not correlated to the degree of liver dysfunction.^{2,23}

Livingston-Rosanoff *et al.*²⁴ compared the immunological aspects involved in HCMV hepatitis in both immunocompetent and immunocompromised mice to better understand the extent of indirect cytopathogenicity caused by this virus. In their study, they injected a highly virulent strain of murine HCMV to each of the mice groups and observed that the immunocompetent group developed faster lethal hepatitis than the other. This could be related to a combination of high immune response and direct viral cytotoxic activity (given the highly lethal viral strain). They also concluded that not only CD8+ T cells but also CD4+ T cells might play an important role by producing several cytokines (*i.e.* IL-17, IFN- γ , and TNF) that contribute to the further enhancement of the host adaptive immune response. From another perspective, other mouse studies demonstrated the critical importance in the role of T lymphocytes in murine cytomegalovirus. Stahl *et al.*²⁵ demonstrated that liver damage and consequent release of liver enzymes in immunocompetent mice occurred earlier than in immunocompromised. This observation can be explained by the early immune response that mainly involves T lymphocytes and natural killer cells and contributes to early tissue damage. However, there was a decrease in liver enzymes at day 6 after infection, which reflects the host immune control over the virus. In contrast, in immunocompromised mice, the elevation of liver enzymes was observed later and lasted longer. Consequently, the liver damage in this host occurred later and was attributed to direct cytopathic effect caused by the virus.

Liver involvement by the HCMV has been reported as hepatitis alone, granulomatous hepatitis, necrotizing hepatitis, and hepatic dysfunction associated with portal vein thrombosis. As mentioned before, although direct cytopathogenicity does play a role in these identities, the inflammatory response with continuous cytokine release appears to be the predominant hepatopathic mechanism, especially in immunocompetent patients. Thus, there is an important

balance between the protective effects and extent of tissue damage caused by a natural host immune response.

Mechanism of HCMV latency and reactivation

Latency

During HCMV infection in the liver, liver sinusoidal endothelial cells (LSECs) do not function as a barrier to the virus. Rather, they allow for dissemination to the rest of the liver.²⁶ Seckert and colleagues²⁷ studied the function of these cells in mice when exposed to HCMV. They observed that LSECs were sites for murine HCMV latency and potential reactivation. However, the same was not observed in hepatocytes.¹⁷

Other functions of LSECs have been hypothesized. Namely, their role in modulating T cell recruitment and activation, and thus in promoting immune activation in the liver. Specifically, it has been shown that these cells facilitate the transendothelial migration of ICAM-1 and CXCL10-dependent CD4+ T cells. Furthermore, in that study, recruited T cells were primarily non-virus-specific effector memory T cells and activated regulatory T cells with a suppressive phenotype. Thus, this cell type contributes to viral persistence.²⁸

Hepatocytes play a major role in viral production and dissemination, but do not directly contribute to viral latency. On the contrary, LSECs have a very small capacity to allow viral reproduction and for this reason are less susceptible for direct viral cytopathogenicity and function as an optimal environment for viral latency.

Reactivation

HCMV is able to escape both innate and adaptive immunity. Several genes have the ability to down-regulate major histocompatibility complex (MHC) class I and MHC class II and may be involved in inhibition of antigen presentation.²³

Furthermore, HCMV can activate or down-regulate receptors found on natural killer cells, natural killer T cells, and T cells.² Several factors can influence reactivation of the virus, including immune cell depletion, allogenic transplantation, ischemia/reperfusion injury, sepsis, and other inflammatory states.²⁹

Although HCMV reactivation results in systemic viremia, subsequent hepatitis as a result of viral reactivation has not been clearly reported in immunocompetent patients. However, in liver recipient patients, HCMV reactivation can cause hepatitis, but at a much lower risk compared to primary infection.³⁰ From among 93 liver transplant cases, Paya *et al.*³⁰ reported that 19 of the cases developed HCMV infection. However, from the group of HCMV-seronegative-donor/HCMV-seropositive-recipients, only one developed hepatitis. Patients undergoing liver transplant are at increased risk of HCMV reactivation, particularly if receiving antilymphocyte preparations, which are highly potent reactivators of HCMV. On the other hand, immunosuppressors such as cyclosporine and corticosteroids do not cause reactivation but can contribute to increased viral replication.^{30–32}

Reactivation of the virus in liver transplant can be both the cause and the consequence of allograft rejection.³² Razonable *et al.*³³ studied the clinical predictors of late-onset HCMV disease in liver and kidney transplant recipients who received oral ganciclovir prophylaxis. They observed that allograft rejection was a significant risk factor for occurrence of HCMV disease, including hepatitis. Furthermore, its incidence was higher among liver transplant recipients. This might be explained by the release of multiple cytokines,

particularly TNF- α , which has been shown to induce HCMV reactivation.^{34,35} On the other hand, immunosuppressive therapy inhibits viral cell-mediated immunity, allowing increased viral replication rates.³⁶

Clinical presentation

The clinical presentation of HCMV infection varies among immunocompetent and immunocompromised hosts, as well as between acute and chronic stages. The spectrum is wide and can range from an asymptomatic infection to life-threatening. Nonetheless, the majority of patients, both immunocompetent and immunocompromised, undergo an asymptomatic disease course from the acute phase until the persistent and latent phases. The main at-risk immunocompromised hosts are fetuses, allograft recipients (due to cytotoxic anti-rejection agents), and human immunodeficiency virus infection. In these hosts, severe end-organ dysfunction can occur, such as hepatitis, retinitis, thrombocytopenia, and neurologic disease.^{37,38}

Immunocompetent patients

Immunocompetent hosts with HCMV infection may experience a mononucleosis-like syndrome with fevers, malaise, presence of lymphocytosis with atypical lymphocytes, occasionally a rash, and abdominal pain. Furthermore, associated hepatic dysfunction and splenomegaly are common. In contrast with Epstein-Barr virus (commonly known as EBV), this presentation usually does not involve tonsillitis and cervical lymphadenopathy, and there is no detectable heterophile antibody.³⁹

Given the overall rarity, only case reports or small case series of HCMV-induced hepatitis in immunocompetent patients have been published. We found 26 studies reporting HCMV-induced hepatitis in immunocompetent hosts, comprising a total of 44 patients. A total of 34 (77%) patients either had fever at home or upon presentation or malaise ($n=13$) and abdominal pain ($n=10$). On exam, only 10 (23%) patients had jaundice. Similarly, lymphadenopathy was only present in 10 (23%) patients, while 5 (11%) patients presented with a non-specific rash. Furthermore, 22 (50%) of the patients had either splenomegaly and/or hepatomegaly. Table 1 summarizes the clinical presentation of immunocompetent patients with HCMV hepatitis.^{3,4,40–62}

Immunocompromised (liver transplant patients)

The main groups of adult immunocompromised hosts susceptible to HCMV disease include allograft recipients and patients with human immunodeficiency virus infection with loss of CD4+ lymphocytes. More recently, the use of anti-TNF therapy has also resulted in severe HCMV disease with end-organ dysfunction.⁶³ Amongst the different types of immunocompromised hosts, there is a common viral syndrome with fever and malaise and possibly elevated liver enzymes. Studies on significant HCMV-induced hepatitis in the immunocompromised population have only been well described in liver transplant patients.

Liver transplant patients had a reported high incidence of HCMV infection which led to a viral syndrome with fever, malaise and some degree of bone marrow suppression, or tissue invasive disease. The latter mainly affects the gastrointestinal tract (*i.e.* gastritis, esophagitis, enteritis, and/or colitis) and with a relatively high incidence, the liver, and consequently hepatitis.^{8,30,64} An important aspect that is regarded as a risk factor for HCMV disease in liver transplanta-

Table 1. Most common presenting signs and symptoms of HCMV hepatitis in immunocompetent patients

Study	n	Age	Gender, M:F	Fever	Malaise	Abdominal pain	Jaundice	Hepato and/or splenomegaly	Lymphadenopathy	Additional diagnosis
Ates et al. ⁵¹	1	28	1	1	1	-	-	1	1	-
Bonkowsky et al. ⁴³	2	27.5	1:1	2	2	1	0	0	-	-
Chan et al. ⁴⁷	1	29	0	1	1	1	0	0	0	Pancreatitis
Clarke et al. ⁵²	3	48	0:3	3	1	-	1	1	1	-
Fernandez-Ruiz et al. ⁶²	1	32	0	-	1	1	0	0	1	Myocarditis
Groza et al. ⁵³	1	12	1	1	-	-	1	1	1	-
Prashant-Gupta et al. ⁵⁴	1	20	1	1	-	-	-	-	-	-
Jensen et al. ⁵⁵	1	35	1	-	-	1	-	-	-	-
Kanno et al. ³	11	28	11:0	8	-	-	0	11	4	-
Ma et al. ⁵⁶	1	19	1	0	1	-	0	-	-	Guillain-Barré
Ladd et al. ⁴⁸	1	17	1	1	1	1	0	0	0	Portal vein thrombosis
Migueluez et al. ⁴²	1	32	1	1	1	-	-	1	-	-
Oku et al. ⁵⁷	1	55	0	0	-	-	1	-	-	Pancreatitis
Puccia et al. ⁴⁶	1	30	0	1	-	1	-	1	-	Portal vein thrombosis
Qian et al. ⁴⁹	1	66	1	0	-	-	1	-	-	Ascites and pancytopenia
Reller et al. ⁴¹	1	30	1	1	-	-	1	1	0	-
Sacks et al. ⁴⁰	1	59	1	1	1	1	1	1	1	-
Serna-Higuera et al. ⁶¹	1	39	0	1	-	1	1	1	-	-
Shusterman et al. ⁴⁵	1	33	0	1	1	1	1	1	1	Hepatic necrosis
Ten Napel et al. ⁴⁴	6	46	2:4	4	-	-	-	-	-	-
Toghill et al. ⁵⁸	2	40	2:0	2	-	-	2	2	-	-
Tzavella et al. ⁵⁹	1	34	0	1	1	1	-	-	0	-
Hooi et al. ⁵⁰	1	38	0	1	1	-	-	-	-	Transverse myelitis
Yu et al. ⁴	1	39	1	1	-	-	-	-	-	Fulminant hepatitis
Zubiaurre et al. ⁶⁰	1	36	0	1	-	-	-	-	-	Myocarditis
TOTAL, n	44	35	25:19	34	13	10	10	22	10	
TOTAL, %				77	30	23	23	50	23	

tion is the serological status of both the donor and recipient. HCMV-seropositive-donor/HCMV-seronegative-recipient are at increased risk of HCMV hepatitis, whereas HCMV-seropositive-recipients have a moderate risk and HCMV-seronegative-donor/HCMV-seronegative-recipients have a lower risk.³⁰ Paya *et al.*³⁰ reported an incidence of 17% of acute hepatitis following 93 liver transplantations. However, Seehofer *et al.*⁸ evaluated 1,200 liver transplant patients and of these only 2.1% developed acute hepatitis. This difference could be due to not only the difference in sample size but also to dissimilarity in immunosuppressive therapy after transplantation. For this reason, a precise number is difficult to obtain. In both studies, the incidence of acute HCMV hepatitis was higher in seronegative recipients compared to seropositive recipients. Paya *et al.*³⁰ found that HCMV hepatitis was most common in liver transplantation for cholestatic disease (*i.e.* primary biliary cirrhosis and primary sclerosing cholangitis). Unfortunately, that study did not clearly describe the number of patients for each specific etiology requiring a liver transplant. In that same study, HCMV hepatitis was more frequent in patients who required retransplantation (38%) than in those who received one hepatic allograft (12%).^{8,30}

As in immunocompetent patients, patients commonly had a mononucleosis-like syndrome including fever and malaise.^{31,65,66} In both studies discussed above, fever occurred in 24% and 84% of the patients.^{8,30} Furthermore, Paya *et al.*³⁰ described myalgia in 31% of patients. Extrahepatic involvement was somewhat infrequent in the two studies, pneumonitis occurred in four patients (three in Paya *et al.*³⁰ and one Seehofer *et al.*⁸) and generalized organ involvement in one patient.

It has been noted that HLA-donor/recipient matches were significantly higher in patients that developed HCMV hepatitis.⁶⁷ Moreover, HCMV hepatitis was reported in a patient with Crohn's disease who was previously on mercaptopurine and switched to infliximab 1 year prior to presentation.⁶³ This case shows that the role of TNF- α in HCMV infection is complex. As previously mentioned, it has been associated with induction of HCMV reactivation in allograft rejection.^{34,35,68} However, a signaling cascade is also important in inducing an antiviral state.^{69,70}

Role of HCMV in chronic liver disease

To understand whether HCMV infection could play a role in unexplained cases of chronic liver disease, Toghiani *et al.*⁷¹ analyzed 70 patients with cirrhosis with the following diagnosis: alcoholic cirrhosis, cryptogenic cirrhosis, primary biliary cirrhosis, hemochromatosis, drug-induced jaundice, secondary biliary cirrhosis, and congenital hepatic fibrosis. They did not find any evidence for HCMV as the cause of liver disease. There was no significant difference in the antibody titers of these patients compared to that of the general population.

On the contrary, HCMV infection in post-liver transplant patients has been associated with chronic rejection.^{72,73} The main suggested cause of chronic rejection is the vanishing bile duct syndrome. Lautenschlager *et al.*⁷² investigated the relationship between HCMV and chronic liver rejection with vanishing bile duct syndrome in 10 patients and verified that all the patients had persistence of HCMV genome in the graft. Furthermore, HCMV reactivation was associated with late acute rejections. Moreover, Faivre *et al.*⁷⁴ verified that HCMV was associated with an increased risk of liver-related death in patients with liver cirrhosis.

Although HCMV infection has not been directly seen as the cause of liver cirrhosis, there appears to be evidence in support of a higher mortality in these patients. In addition, it is an important cause of chronic liver rejection in transplant patients.

Diagnosis

The diagnosis of HCMV hepatitis requires liver tissue biopsy for confirmation of HCMV presence in the liver. The best modality for identification of viral inclusions or viral antigens is immunohistochemistry.⁷⁵ However, detection of HCMV by means of acute serology or polymerase chain reaction (PCR) can provide faster results when the suspicion of HCMV-induced hepatitis is high, allowing for an earlier management plan when biopsy results are pending. In many circumstances, particularly in immunocompetent patients who present with acute elevation of liver enzymes, acute HCMV serology may be sufficient for diagnosis of HCMV-induced hepatitis when other causes have been ruled out. Furthermore, it can be used to monitor disease progression and treatment response in combination with liver function results.

Liver enzyme tests

Elevation of hepatic aminotransferases in HCMV infection is non-specific but levels are on average lower than those seen in hepatitis caused by hepatitis viruses.³ In immunocompromised patients, 1.3-fold elevations in mean alanine aminotransferase has been reported.⁸ Paya *et al.*³⁰ observed 2–30 times higher (mean 9.3-fold above upper limits of normal) levels of gamma-glutamyltransferase and 1–10 times higher (mean 3.6) in alkaline phosphatase levels compared to aminotransferases. Furthermore, the elevation of gamma-glutamyltransferase and alkaline phosphatase may persist longer than that of alanine aminotransferase and aspartate aminotransferase.^{8,30} The levels of bilirubin elevation in this study were relatively low, 1–4 times higher than the normal levels.

From the analysis of independently reported cases of HCMV hepatitis in immunocompetent patients, the mean aspartate aminotransferase was 422 (± 582), $n=38$, and the mean alanine aminotransferase 521 (± 579), $n=37$. The mean total bilirubin value was 5 mg/dL (± 9), $n=33$. Overall, there was a higher elevation of aminotransferases in immunocompetent patients compared to immunocompromised. This observation could be related to the early and robust immune response in immunocompetent patients, and the consequent indirect cytopathogenicity. These immunocompromised patients underwent liver transplantation and were closely monitored for HCMV hepatitis, resulting in earlier diagnosis and subsequent treatment with ganciclovir. Bilirubin elevation was minimal in both population groups.

Serology

While serology in the immunocompetent patient plays an important role in the diagnosis of HCMV hepatitis, it has a limited role in the immunocompromised because of an immune system impairment in mounting an antibody response.⁷⁶ However, serological testing can provide a good assessment of recipient risk prior to transplant.^{31,77} In immunocompetent patients, it provides a fast, non-invasive and less expensive test when used in the context of hepatitis and when other etiologies have been ruled out. However, IgM antibody assays may be falsely positive due to persistence of high IgM levels long after primary infection. It can also represent viral reactivation.⁷⁸ Furthermore, HCMV IgM might be falsely positive in the presence of a positive rheumatoid factor or with infection of other herpes virus.⁷⁹

The sensitivity and specificity of serology have been reported between 70.7–84.4% and 99.3–100%, respec-

tively.^{80,81} Another study that compared five different commercial immunoassays for the serologic diagnosis of HCMV showed significant differences in sensitivity and specificity between the different tests as well as in cross reactions with EBV-IgM and rheumatoid factor.⁸²

Antigenemia

The pp65 HCMV antigenemia assay detects HCMV antigens in peripheral blood leukocytes.^{39,83} It has a good utility for monitoring disease progression and treatment response. However, given its limitation of detecting the virus only in leukocytes, it may not be reliable in patients with leukopenia,⁷⁸ as this may contribute to false negative results. Its sensitivity and specificity have been reported as 64% and 81%, respectively.⁸⁴

Culture

The utility of viral culture is limited because of the long time required for results.⁸⁵ In 65 patients with HCMV hepatitis, Brand *et al.*⁸⁵ were able to confirm the diagnosis in 63 patients through histology and early antigenemia, but viral culture only contributed to the management of 1 patient among 2,508 liver biopsies. A study comparing several diagnostic techniques for HCMV detection in liver transplant patients using 108 hepatic tissue specimens also showed an overall low sensitivity (52%) of cell culture for detecting the virus.⁸⁶ However, the use of shell vial assay provided results within 12 h and not only had a similar specificity to traditional culture but also higher sensitivity.⁸⁷

PCR

PCR can identify HCMV from body fluid or tissue. Furthermore, it can provide both qualitative and quantitative measurements. Quantitative PCR is generally used in immunocompromised patients to determine which patients need preemptive therapy and for monitoring disease response.^{78,88} Methods using real-time PCR have better precision, are easier to perform, faster and less risk of contamination compared to conventional PCR.^{89,90} It is a reliable, sensitive and very specific method, with sensitivities ranging from 61–92% and specificities 75–99%.^{91,92}

Histopathological findings

The most consistent finding of HCMV hepatitis in liver biopsies of both immunocompetent and immunocompromised patients is a mononuclear infiltrate.^{3,40–43,86,93,94} However, the degree of inflammatory infiltrate differs. Immunocompromised patients have an overall low degree of inflammatory mononuclear infiltrate. For instance, Sano *et al.*¹⁶ studied immunocompromised patients with underlying malignancy, and from three liver biopsies with detected HCMV, there was hardly any inflammatory infiltrate around the infected cells. Similar findings were observed in two patients after renal transplantation and one patient with Hodgkin's disease.⁴⁴

Ten patients assessed by Ten Napel *et al.*⁴⁴ showed relative similarities to inflammation and viral expression in portal and periportal regions between both immunocompetent and immunocompromised. On the other hand, the immunocompetent patients had a higher level of mononuclear infiltrate in the liver parenchyma. However, the magnitude

of hepatocyte damage was lower compared to that in immunocompromised patients. Although focal necrosis is more common in the immunocompromised population, there is one isolated report of fatal hepatitis in a previously healthy patient for whom liver biopsy had showed broad bands of necrosis.⁴⁵

Lautenschlager *et al.*⁷⁹ reviewed biopsies of 26 livers from patients who had a liver transplant and subsequent HCMV liver infection. The most common observation was the presence of micro-abscesses, which have been described before.¹⁵ Another finding that has been more frequently seen in immunocompetent patients are granulomas. In addition, these are more commonly seen in HCMV hepatitis in contrast to other viral causes of hepatitis.^{3,41,43}

The degree of the host immunodeficiency likely affects the extension of the inflammatory reaction observed in the specimens. As discussed above, lack of immune response might allow an increased direct viral cytopathogenicity, which leads to more extensive necrosis. Despite an overall lower magnitude of inflammatory infiltrate observed in the liver of these patients, the presence of either HCMV inclusion bodies or the detection of HCMV antigens confirms the diagnosis. Table 2 describes, in more detail, the biopsy findings of 22 previously healthy immunocompetent patients.^{40–45,47,52,53,58,61} Table 3 summarizes the main biopsy findings of immunocompetent and immunocompromised patients.

Treatment

The recommendations for antiviral initiation in HCMV hepatitis differ according to the patient population. As a result of a high incidence of HCMV hepatitis in patients undergoing liver transplantation, the main approach of management relies on preventing disease occurrence.³² There are two approaches in prevention, prophylaxis and preemptive therapy. In prophylaxis, antivirals are started just after transplantation occurs and last for at least 3 months. In preemptive therapy, the recipients are closely monitored for the presence of HCMV replication before any symptoms occur; if HCMV replication is identified, antiviral treatment is promptly initiated.

Antiviral prophylaxis has been the preferred method for high-risk allograft recipients (D+/R-).³² Treatment is started within 10 days of transplantation, regardless of the existence of HCMV replication or not. In these patients, acyclovir, valacyclovir, intravenous ganciclovir, and valganciclovir can be used.⁷⁵ In a large study from Paya *et al.*,⁹⁵ high-risk allograft patients receiving heart, liver, kidney or pancreas received prophylaxis with either ganciclovir or valganciclovir. The efficacy and safety of these drugs were similar but the incidence of HCMV disease was slightly lower in the valganciclovir group (17.2% vs. 18.4%). In other studies, valganciclovir has demonstrated lower incidence of HCMV disease at 6- and 12-months follow-up.^{32,33}

Preemptive therapy involves close monitoring for viral replication, followed by initiation of therapy when HCMV is detected. At this time, there is no consensus regarding the threshold of viral load and the start of therapy. A recent meta-analysis involving 2,452 liver transplant recipients demonstrated an incidence of HCMV disease of 10% in patients receiving prophylaxis versus 7% in those receiving preemptive therapy. In addition, acute cellular rejection and mortality rates were similar in both groups. Importantly, these results comprised all D/R status.⁹⁶ However, Singh *et al.*⁹⁷ performed a randomized clinical trial to compare preemptive therapy and antiviral prophylaxis in 205 HCMV-seronegative liver transplant recipients (R-) with seropositive donors (D+). In that study, the incidence of HCMV disease

Table 2. Histopathologic findings of liver biopsies in immunocompetent patients

Study	Liver biopsy findings
Bonkowsky <i>et al.</i> ⁴³	Portal triads infiltrated with lymphocytes, histiocytes, plasma cells, and neutrophils. Lobules with lymphocytes, monocytes and proliferating reticuloendothelial cells. Granulomata in the lobules. One large epithelioid granuloma with areas of necrosis. Normal lobular architecture. Many of the portal triads were enlarged, containing a small to moderate number of lymphocytes and histiocytes. Proliferation of RE cells and infiltration of lymphocytes in the sinusoids. Few necrotic hepatocytes. Small, sharply circumscribed granulomata made of closely packed epithelioid cells and rare lymphocytes. No giant cells.
Chan <i>et al.</i> ⁴⁷	Mild to moderate infiltrate of small lymphocytes in the sinusoids and a beaded sinusoidal infiltrate characteristic of HCMV infection.
Clarke <i>et al.</i> ⁵²	Focal areas of necrosis and many noncaseating epithelioid granulomas and portal triaditis. Non-caseating epithelioid granulomas, focal liver cell necrosis, portal triaditis. Prominent sinusoidal lymphocytic infiltrate and early granuloma formation.
Groza <i>et al.</i> ⁵³	Viral hepatitis in an advanced phase.
Migueluez <i>et al.</i> ⁴²	Intense mononuclear portal infiltration and severe alteration of zone 3 with confluent necrosis.
Reller <i>et al.</i> ⁴¹	Non-specific resolving hepatitis with sparse cellular necrosis and mononuclear infiltrates in portal areas. Scattered granulomas with giant cell formation.
Sacks <i>et al.</i> ⁴⁰	Acute hepatitis with focal parenchymal necrosis, periportal and sinusoidal mononuclear infiltration. Focal fatty degenerative changes.
Serna-Higuera <i>et al.</i> ⁶¹	HCMV intranuclear inclusions. Portal spaces with fibrous expansion and irregularly distributed lymphocytic infiltrate. Fibrin ring granulomas.
Shusterman <i>et al.</i> ⁴⁵	Hepatic lobules markedly disrupted by broad bands of necrosis.
Ten Napel <i>et al.</i> ⁴⁴	Enlargement of portal tracts, lymphocytic infiltrate, bile duct inflammation, focal necrosis, granulomas. Enlargement of portal tracts, lymphocytic infiltrate, lymphocytic spill-over, granulomas, liver cell degeneration. Enlargement of portal tracts, lymphocytic infiltrate, liver cell degeneration. Enlargement of portal tracts, lymphocytic infiltrate, bile duct inflammation, lymphocytic spill-over, liver cell degeneration. Enlargement of portal tracts, lymphocytic infiltrate, granulomas, focal necrosis.
Toghill <i>et al.</i> ⁵⁸	Areas of liver cell necrosis and mononuclear cell infiltration, acidophil bodies, slight portal enlargement, siderosis. Portal and periportal infiltration with chronic inflammatory cells, piecemeal necrosis, fibrosis of portal areas extending to lobules.

was significantly lower with pre-emptive therapy (9%) than with anti-viral prophylaxis (19%). Opposing these results,

Bodro *et al.*⁹⁸ analyzed 74 D+/R- liver recipient patients. Thirty-five patients (47%) received prophylaxis, and thirty-

Table 3. Summary of the main histological findings of liver biopsies in immunocompetent and immunocompromised patients

	Immunocompetent	Immunocompromised
Portal tracts	Enlarged portal tracts Prominent mononuclear portal and peri-portal infiltrate (frequent) Reported inflammatory cells: lymphocytes, histiocytes, plasma cells, neutrophils Fibrosis of portal areas (rare)	Mild to low mononuclear portal infiltrate Reported inflammatory cells: mainly lymphocytes
Parenchyma	Giant cell granulomas (frequent) Lymphocytes, monocytes Sinusoidal lymphocytic infiltrate (frequent) Few necrotic hepatocytes Focal areas of necrosis (rare)	Micro-abscesses (frequent) Giant cell granulomas (very rare) Parenchymal and sinusoidal inflammatory reaction Extensive focal liver necrosis
Presence of viral inclusion bodies	Extremely rare	Moderate (in inflammatory cells of portal mononuclear infiltrate and in hepatocytes)

nine patients (53%) followed a pre-emptive strategy based on CMV antigenemia. They observed an increased rate of HCMV disease in the group that received pre-emptive therapy (33.3%) compared to the group that received prophylaxis (8.6%). Nonetheless, late-onset HCMV disease was only found in patients receiving prophylaxis (5.7%).

Another study from Weigand *et al.*,⁹⁹ analyzed 211 liver recipients for the occurrence of CMV infection. From these, 51.7% received prophylaxis with ganciclovir or valganciclovir. Overall, 32.7% had CMV infection despite antiviral prophylaxis. It is important to note that antiviral prophylaxis was started in cases of high-risk donor-recipient status, retransplantation, and according to clinical decision. In addition, the authors did not mention if CMV disease occurred.

If HCMV disease (including end-organ damage such as HCMV hepatitis) develops in immunocompromised patients, the main treatment is intravenous ganciclovir or valganciclovir. In addition, immunosuppressive regimens should be reduced as much as possible. The length of treatment varies according to individual response, which can be monitored by clinical and laboratory data.

Importantly, infections by ganciclovir-resistant HCMV has been rising, particularly in patients receiving pre-emptive therapy. In a study with 561 patients who underwent 616 hematopoietic stem cell transplantations (HSCTs), drug resistance was solely observed in haploidentical (haplo)-HSCT recipients receiving pre-emptive therapy and was as high as 14.5%.¹⁰⁰ In such patients, treatment is challenging and depends on several factors, including which mutation has led to the viral resistance.¹⁰¹ Foscarnet is currently recommended as the first-line option, followed by cidofovir. Of note, both of these drugs have a certain degree of ganciclovir cross-resistance, particularly the latter.^{83,101}

The majority of immunocompetent patients with symptomatic HCMV infection had spontaneous resolution of both symptoms and laboratory abnormalities (elevated aminotransferases).⁴⁶ For this reason, there are no specific guidelines for treatment. Furthermore, there are no major studies on antiviral therapy in immunocompetent patients with HCMV disease. Among the 45 immunocompetent patients with HCMV hepatitis that we found in the literature, management with antiviral therapy varied. Only nine cases reported the use of antiviral medications. From these, five received ganciclovir only, two received valganciclovir only, one received ganciclovir followed by foscarnet, and one received ganciclovir followed by valganciclovir. Except for one case,⁴³ all other cases that received antiviral therapy comprised patients with additional organ involvement or clinical deterioration; these included pancreatitis, myocarditis, acute pulmonary embolism, encephalopathy, transverse myelitis, pancytopenia, and fulminant hepatitis.^{4,42,47–50,61,62} Overall, from among all of the 45 five reported patients with HCMV hepatitis, only 1 died. In this patient, unfortunately, the diagnosis was made just prior to his death and many days after his admission.

Despite the fact that there is no clear indication for treatment of symptomatic HCMV infection in these populations, treatment should be considered when the liver function or overall clinical status of the patient is not improving, or if there is another organ involvement which can be an indicator of disease severity.

Conclusions and recommendations

Although slightly elevated aminotransferases in the setting of HCMV mononucleosis are common in immunocompetent patients, clinically significant HCMV hepatitis is uncommon, with only few cases reported. In the immunocompromised population, liver transplant patients have an increased risk

of HCMV hepatitis.

Hepatocytes play a major role in HCMV replication but do not contribute to viral latency. On the contrary, LSECs have a very low capacity for viral reproduction, and for this reason are less susceptible for direct viral cytopathogenicity and function as an environment for viral latency.

Indirect cytopathogenicity, due to the host immune response, plays a major role in early liver damage, particularly in immunocompetent patients. Furthermore, in this population, hepatitis typically occurs earlier than in immunocompromised patients but also subsides earlier. This is due to the robust immune activation of immunocompetent patients. However, the poor immune response in the immunocompromised patients can lead to a prolonged state of direct cytopathogenicity and, consequently, marked detrimental effects to the liver. Hepatitis as a result of HCMV reactivation has not been reported in immunocompetent patients. In addition, in liver recipient patients, HCMV reactivation can cause hepatitis but at a much lower risk compared to primary infection.

The clinical presentation is non-specific with fever, malaise and/or myalgias being the most common signs/symptoms regardless of immune status. Treatment of HCMV hepatitis with antiviral therapy in the immunocompetent population is not generally recommended but should be considered in patients with severe disease and/or extra-hepatic manifestations. On the other hand, the management approach in immunocompromised patients relies on disease prevention.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

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Celiac Disease and Elevated Liver Enzymes: A Review

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Abstract

Aminotransferases are commonly found to be elevated in patients with celiac disease in association with two different types of liver dysfunction: cryptogenic liver disorders and autoimmune disorders. The purpose of this review is to discuss the mechanisms by which aminotransferases become elevated in celiac disease, clinical manifestations, and response to gluten-free diet. Many studies have shown that celiac patients with cryptogenic liver disease have normalization in aminotransferases, intestinal histologic improvement and serologic resolution after 6–12 months of strict gluten-free diet. In patients with an underlying autoimmune liver disease, simultaneous treatment for both conditions resulted in normalized elevated aminotransferases. The literature suggests that intestinal permeability may be at least one of the mechanisms by which liver damage occurs. Patients with celiac disease should have liver enzymes routinely checked and treated with a strict gluten-free diet if found to be abnormal. Lack of improvement in patients who have strictly adhered to gluten-free diet should prompt further workup for other causes of liver disease.

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Introduction

Aminotransferases are commonly found to be elevated in patients with celiac disease, due to multiple reasons. The purpose of this review is to discuss the mechanisms by which aminotransferases become elevated in celiac disease, clinical manifestations, and response to gluten-free diet (GFD).

Celiac disease

Celiac disease (or gluten-sensitive enteropathy) is an au-

toimmune condition triggered by consumption of the gliadin fraction of gluten and other cereals.¹ The prevalence is around 0.5–1% in the general population.^{2,3} The small bowel is primarily affected, causing symptoms such as diarrhea, flatulence and weight loss from malabsorption. However, celiac disease is a systemic disorder that can be associated with diseases of organs other than the small intestine, such as the colon, thyroid, skin, pancreas, and liver. For instance, the prevalence of celiac disease among children with type 1 diabetes is high, likely related to expression of HLA risk genotypes (DQ2 or DQ8) in up to 90% of patients with type 1 diabetes.⁴ The prevalence of celiac disease in patients with autoimmune thyroid conditions, such as Grave's disease and Hashimoto's thyroiditis, has been found to be around 2–7%, while the risk of thyroid disease in celiacs has been estimated at 3-fold higher compared to controls.⁴ Similar associations have been made with Sjogren's syndrome, psoriasis, microscopic colitis and dermatitis herpetiformis.⁴ In regards to the liver, the common hepatic manifestation is isolated aminotransferase elevations.³

The pathogenesis of celiac disease is not entirely understood, but it is thought to be due to the combination of genetic, environmental and immunological factors. The strongest genetic susceptibility factors are HLA-DQ2 and HLA-DQ8 from the HLA class II genes. However, the presence of these alone is insufficient for disease development. Gluten is the primary trigger of an immune response in the gut epithelium.⁵ Intestinal enzyme tissue transglutaminase (tTG) 2 modifies gluten peptides, which bind to HLA-DQ2 or HLA-DQ8 on the surface of antigen-presenting cells. These trigger a T-cell response, with release of pro-inflammatory cytokines that lead to mucosal inflammation and damage to epithelium. These can also induce a B cell response, leading to production of anti-tTG antibodies as well. Peptides are capable of directly activating epithelial cells to produce cytokines, such as IL-15. This enhances cytolytic activity of intraepithelial lymphocytes and ultimately disrupts the lining and increases the intestinal permeability.⁶

It has also been proposed that infections are related to development of celiac disease.⁷ Beyerlein *et al.*⁸ studied infants born between 2005 and 2007 retrospectively. Based on a search for ICD codes for infections and celiac disease, they found that risk of developing celiac disease was higher in children with a gastrointestinal infection during the first year of life (hazard ratio of 1.32). There was a weaker association with respiratory infections in the first year of life (hazard ratio 1.22). As the authors did not have information on how celiac disease was diagnosed or whether it was confirmed with biopsy, these results were limited. However, Marild *et al.*⁹ showed similar results with increased risk of development of celiac disease in children with 10 or more infections before 18 months of age, compared to those with 4 or less infections. That study was prospective, making the results more convincing.

Keywords: Celiac disease; Gluten free diet; Celiac hepatitis; Autoimmune liver disease.

Abbreviations: AIC, autoimmune cholangitis; AIH, autoimmune hepatitis; AILDs, autoimmune liver diseases; ALP, alkaline phosphatase; ALT, alanine aminotransferase; ASMA, anti-smooth muscle antibody; AST, aspartate aminotransferase; GFD, gluten-free diet; GGT, gamma-glutamyl transferase; NAFLD, nonalcoholic fatty liver disease; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis; SIBO, small intestinal bacterial overgrowth; tTG, tissue transglutaminase.

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Liver damage in celiac disease

Liver dysfunction in patients with celiac disease was first described in studies from the 1970s.¹⁰ Celiac disease is associated with two different types of liver dysfunction: cryptogenic liver disorders, usually with positive response to GFD; and autoimmune disorders.

Cryptogenic liver disorders can range from mild to severe hepatitis, and usually present with isolated increase in aminotransferases.^{11,12} Histology of the liver typically shows preserved architecture with a mild mononuclear infiltrate of the portal and lobular tract, and hyperplasia of the Kupffer cells. Intraepithelial lymphocytes can be seen in interlobular bile ducts as well as small bowel.¹³ Hyperplasia of Kupffer cells is typical of nonspecific reactive hepatitis, also known as celiac hepatitis.^{11,12,14} The term 'celiac hepatitis' specifically refers to liver injury in patients with confirmed celiac disease that resolves after introduction of GFD.³

Conversely, in autoimmune liver diseases (AILDs), histology of the liver shows mononuclear and eosinophilic infiltration of the portal tract in the presence of characteristic circulating autoantibodies (anti-nuclear ANA, anti-smooth muscle antibody (ASMA), anti-liver kidney microsomal LKM1), suggestive of autoimmune disease. AILDs usually require combination of GFD and immunosuppressive therapy as treatment. It is unknown whether cryptogenic and autoimmune liver disorders have different pathogeneses or constitute a spectrum of the same disorder.¹¹

Liver dysfunction in celiac disease can manifest with nonspecific symptoms of hepatitis, such as malaise and fatigue. However, patients are usually asymptomatic and may not have any celiac disease manifestations or symptoms.^{3,15} Elevations in aminotransferases are usually mild to moderate, with an aspartate aminotransferase (AST):alanine aminotransferase (ALT) ratio usually less than 1. Alkaline phosphatase (ALP) can be normal or elevated in around 4–20% of cases. Bilirubin and gamma-glutamyl transferase (GGT) are often normal, but prothrombin time and albumin levels are nonspecific and could be altered due to malabsorption.³ Signs such as jaundice, ascites, encephalopathy or portal hypertension usually indicate advanced liver disease, which can be from another co-existing liver condition.

Wakim-Fleming *et al.*¹⁶ tested for celiac disease in 204 patients with biopsy-proven cirrhosis. Five patients showed positive for celiac disease with duodenal biopsy. These patients had cirrhosis secondary to non-alcoholic steatohepatitis, cryptogenic liver disease, primary sclerosing cholangitis (PSC), autoimmune hepatitis (AIH), and alcoholic liver disease. Four of these patients were started on GFD and followed for 2 years, the last patient with alcoholic liver disease passed away. Only the patient with AIH received additional treatment with prednisone. All experienced biochemical/serological resolution and normalization of small bowel histology after treatment. Model for end-stage liver disease scores improved in three of the patients.¹⁶ That study suggested that liver damage from celiac disease can be simultaneous to liver damage from another pathology, and biochemical abnormalities can be corrected after GFD incorporation. However, it cannot be determined whether GFD alone would have corrected the elevated aminotransferases. Other measures could have played a role, such as weight loss in the non-alcoholic steatohepatitis patient or cessation of alcohol or hepatotoxic drugs.

Cryptogenic liver disease

Celiac disease has been found in up to 9% of patients with elevated liver enzymes.^{3,17} Vajro *et al.*¹⁵ studied six pediatric patients with long-standing aminotransferase eleva-

tions. One of them had presented with fatigue, another with hepatomegaly, while the others had incidental findings of elevated aminotransferases. Workup was negative for infectious, infiltrative and toxic causes of liver injury. Other liver tests, including those for alkaline phosphatase, gamma-glutamyl transpeptidase and bilirubin, were normal. However, they tested positive for anti-gliadin serum antibodies, and histological findings from intestinal biopsies were consistent with celiac disease. Liver biopsies in five of the patients yielded nonspecific results (Table 1).^{14,15,18–23} All of them experienced biochemical resolution after implementation of GFD, and two of the patients with repeat liver biopsy had histological resolution as well. Three patients received a gluten challenge and experienced elevation of aminotransferases once again, along with increase in anti-gliadin antibodies and histological relapse of intestinal mucosa. After re-introduction of GFD, once again there was biochemical resolution, which persisted at 1–3 year follow-up.¹⁵ The association between isolated elevation of aminotransferases from liver injury and presence of gluten in diet seemed convincing, as other causes of liver damage were ruled out, and there was a positive association between gluten and biochemical/histological abnormalities.

Volta *et al.*¹⁷ evaluated the prevalence of celiac disease in patients with elevated aminotransferases. From 600 mostly asymptomatic patients, 55 (9%) were found to be cryptogenic after other causes of liver disease were ruled out. Viral hepatitis panel, ANA, ASMA, LKM-1, anti-mitochondrial antibody, ceruloplasmin, alpha-1-antitrypsin were negative. Toxins and iron overload were also ruled out.¹⁸ The authors measured IgA anti-endomysial and IgA/IgG anti-gliadin antibodies in the 55 patients with unexplained elevated aminotransferases. Six patients were positive for antibodies, and were offered duodenal and liver biopsies. All five patients (9%) with positivity for IgG anti-gliadin, IgA anti-endomysial and/or IgA anti-gliadin antibodies were diagnosed with celiac disease after duodenal biopsy. Of note, ALP, GGT, serum albumin and prothrombin time were normal in all five patients. The sixth patient with positivity only to IgG to gliadin did not have celiac disease demonstrated on biopsy. Three patients with diagnosed celiac disease had liver biopsies, which showed nonspecific reactive hepatitis. They also performed a liver ultrasonography on all patients, with exception of one patient with hepatic steatosis. After 6 months of GFD, 4 of them (including the patient with fatty liver) experienced histological resolution and normalization of aminotransferases. The remaining patient had persistently elevated aminotransferases, antibodies and villous atrophy, which was thought to be due to poor diet adherence. After correction of diet, they also had biochemical, histological and serological resolution. These results seem convincing, as other causes of liver damage were ruled out and four patients had regularization of aminotransferases after implementation of the GFD. The fifth patient had a delayed recovery with persistently elevated aminotransferases, as well as IgA to endomysium and to gliadin at 6 months. The authors mentioned that presence of these antibodies were suggestive of dietary transgressions. However, testing for gluten immunogenic peptide in urine or stool would have been more reliable detectors of dietary indiscretions.^{24,25}

Bardella *et al.*²⁶ screened 140 patients in Italy with cryptogenic elevated aminotransferases for subclinical celiac disease, and found 13 (9%) positive for anti-gliadin antibody and anti-endomysial IgA antibody. On endoscopy, three patients were found to have mild villous atrophy with intraepithelial lymphocytes, six to have subtotal villous atrophy, three to have total villous atrophy. It is notable that the screened patients had isolated elevation of aminotransferases on three different occasions. Drug and alcohol abuse, viral hepatitis, iron overload and autoimmune liver diseases were ruled out. The prevalence of ce-

Table 1. Liver biopsy findings in celiac disease patients with elevated aminotransferases

Study	Patients	Histologic findings on liver biopsy	Biopsies	AILD*
Vajro <i>et al.</i> ¹⁵	6	Reactive hepatitis	2	(–)
		Chronic persistent hepatitis	2	
		Chronic active hepatitis	2	
Volta <i>et al.</i> ¹⁸	5	Reactive hepatitis peri-portal inflammation	2	(–)
		Mild fatty infiltration	1	
Bardella <i>et al.</i> ¹⁹	13	Minimal changes	9	(–)
		Fatty infiltration	3	
Bardella <i>et al.</i> ²⁰	67	Chronic active hepatitis	5	(–)
		Fatty infiltration	2	
Hagander <i>et al.</i> ²¹	74	Reactive hepatitis	5	N/A
		Hepatic injury	7	
Jacobsen <i>et al.</i> ²²	62	Nonspecific hepatitis	25	(–)
		Chronic active hepatitis	5	
Kaukinen <i>et al.</i> ²³	4	Acute fulminant hepatitis	1	N/A
		Congenital liver fibrosis	1	
		Cirrhotic changes	1	
Mounajjed <i>et al.</i> ¹⁴	26	Nonspecific findings	5	N/A

Abbreviations: AILD, autoimmune liver disease; N/A, not applicable.

*Serology for autoimmune liver disease; (–), negative serology; N/A, serology not mentioned or not performed.

liac disease was higher compared to the general population (9.3%), with a relative risk of 18.6; although, these findings might not be reproducible on other countries, as celiac disease prevalence is highest in the European continent.²⁶ At the 1-year follow up, 12 patients had normal laboratory tests after being on GFD, and all of them had disappearance of celiac antibodies. These results seem to support the fact that celiac hepatitis improves with GFD, but there was no mention of the patient who did not have biochemical resolution and whether an underlying disease was found. In addition to the blood-work, liver biopsies were carried out in nine out of the thirteen patients, and six had minimal changes while three had evidence of steatosis. Documentation of body mass index and possible weight loss resulting in normalization of aminotransferases would have been useful in order to determine if non-alcoholic fatty liver disease was also likely playing a role.^{17,19}

Bardella *et al.*²⁰ had previously also evaluated 158 patients with celiac disease and found that 67 of them had elevated AST and/or ALT. All patients were followed for 1–10 years while on GFD. In patients with elevated aminotransferases, body mass index increased from 18.5 to 21 ($p < 0.001$) and aminotransferases normalized in 60 (95%) after 1 year of GFD adherence. The seven remaining patients were found to have fatty infiltration on liver biopsy (two patients) and chronic active hepatitis (five patients) from HCV, HBV and AIH. Other causes of concomitant liver disease with blood-work, abdominal ultrasound and liver biopsy were only sought in those with persistent elevated aminotransferases at 1 year. Whether testing for other causes of liver disease were done at time of celiac disease in all patients is unknown. Nevertheless, histological and biochemical improvement in those patients with celiac disease was likely due to GFD, as they did not receive any other types of treatment and had higher body mass index at 1 year. Three patients were found to have elevated ALP, attributed to hyperparathyroidism according to elevated

bone ALP isoenzymes, hypocalcemia, elevated parathyroid hormone and decreased bone mineral density. The authors raised the question of whether the persistently elevated aminotransferases observed in the seven patients, despite proven improvement (but not normalization) in small intestine histology, was due to the fact that intestinal abnormality might not be the only factor associated with liver injury. However, these patients were found to have other causes for elevated aminotransferases. A lack of improvement of elevated aminotransferases in celiac disease patients adherent to GFD should be an indicator for underlying concomitant liver pathology.²⁰

Mechanism of liver dysfunction in celiac disease

The mechanism by which celiac disease patients develop abnormal liver enzymes remains unknown. Predisposition to autoimmunity and systemic effects of abnormal intestinal permeability are thought to play pathogenic roles.³ Gliadin induces an increase in gut permeability and MyD88-dependent zonulin release by binding to CXCR3 chemokine receptor.^{10,27} Zonulin is able to reversibly regulate intestinal permeability by modulation of intercellular tight junctions. It is thought that the increased intestinal permeability allows toxins, cytokines, and antigens to reach the liver through the portal circulation and cause liver injury through release of pro-inflammatory mediators. Toll-like receptors expressed in liver cells (such as Kupffer, endothelial, dendritic, hepatic stellate and hepatocytes) can recognize lipopolysaccharides (present in Gram-negative bacteria) and mount an immune response.¹¹

Novacek *et al.*¹³ studied 178 adults with celiac disease and measured serum aminotransferases prior to initiation of GFD, and periodically thereafter for 1 year. They also measured gut permeability to assess the relationship with liver damage prior to start of GFD. Permeability index was

Table 2. Classification of duodenal histologic changes

Classification	Findings
Grade I	Normal, no shortening of villi or lengthening of crypts
Grade II	Slight partial villus atrophy; slight shortening of villi
Grade III	Marked partial villus atrophy; marked shortening of villi
Grade IV	Subtotal villus atrophy; no definite villus structure

Adapted from Scott and Losowsky.²⁸

determined from the ratio of percentage of lactulose excreted in urine to the percentage of mannitol excreted. A comparison was made between patients with elevated versus normal aminotransferases, and permeability indexes were found to be higher in those with abnormal aminotransferases ($p < 0.0001$). There was no difference in body mass index, duodenal intraepithelial lymphocytes, age, or onset of symptoms between the two groups. The authors screened for other causes of liver disease in patients with elevated aminotransferases (71 patients), and 9 were found to have underlying causes of liver diseases, such as viral hepatitis, autoimmune conditions, and alcohol abuse, amongst others.^{9,13}

After 1 year on GFD, 63 patients showed normalization of aminotransferases and significant decrease in intestinal permeability index ($p < 0.0001$). These results seem to support the concept that permeability indexes have an association with liver damage, as they correlated with AST and ALT levels, and improved with GFD adherence. However, it would have been helpful to prove that intestinal permeability indexes had not changed in the group with normal aminotransferases. From patients with persistently elevated aminotransferases, some had underlying liver conditions and four were thought to be due to dietary indiscretions disclosed by the patients.¹³ It would have been helpful to determine that there was no significant change in the permeability indexes of those who continued gluten consumption. Additionally, lack of antibody testing before and after GFD implementation makes the determination of whether autoimmunity plays a role difficult.

Bardella *et al.*²⁰ studied 158 patients with known celiac disease and reported duodenal histological changes classified in terms of severity, at the time of diagnosis, and again after 1 year of GFD. The authors used the histologic classification of Scott and Losowsky.²⁸ Within the group of patients with elevated aminotransferases, the number of grade I-II histological changes increased from 7 to 60 along with a decrease in grade III-IV histological changes from 60 to 31 after 1 year of GFD (Table 2).²⁸ There was no correlation assessment performed for levels of aminotransferases and severity of histologic changes, which could have been helpful to determine if there is such a relationship between gut and liver damage. There was also no correlation assessment performed for levels of aminotransferases and celiac antibodies, which could have supported the hypothesis of autoimmunity and liver damage. Small intestinal permeability has been used as an indicator for histological recovery in the past, but that study did not test permeability. Even though there were no differences in histological findings seen between patients with and without elevated aminotransferases, the role of gut permeability in liver dysfunction is difficult to assess without permeability tests. Biopsies for celiac activity are notoriously variable, and the specific tissue obtained might not represent mucosal changes adequately along the entirety of the gut. Additionally, histology based on Scott and Losowsky classification is subjective and operator-dependent, making these results less reliable.

Ukabam *et al.*²⁹ studied 13 patients with celiac disease before starting GFD and during treatment, and compared

them with 25 non-diseased adults. The subjects underwent permeability testing with ingestion of mannitol and lactulose, measurement of percentages of the oral dose in urine, and calculation of lactulose/mannitol excretion ratio (LMER). Small bowel biopsies were obtained without knowledge of the group which the patient belonged to or results of permeability testing. The percentage of lactulose excretion was significantly higher in celiacs compared to non-celiacs, while the percentage of mannitol excretion was significantly lower. Intestinal mucosal damage decreases transcellular absorption of small molecules (such as mannitol) and increases larger paracellular pores that allow passive permeation of larger molecules, such as lactulose.³⁰ Normally, <1% of ingested lactulose permeates the intestinal mucosa and appears in urine.³¹ Lactulose excretion improved on GFD, resulting in decreases in LMERs after GFD, but remained significantly higher compared to normal controls. There was also a positive correlation with improvement in severity of histology findings after GFD; patients with lower LMERs after treatment had mostly grade I histological grading (minor abnormalities) and higher ratios of villus height to total mucosal thickness.²⁹ That study suggested an association between histology changes and permeability. Results are convincing, as the investigators used villus height to total mucosa thickness ratio, which is a more objective measure of gut lining damage.

Greco *et al.*³¹ measured intestinal permeability in two groups (27 patients with celiac disease on GFD for 2 years and 19 healthy controls matched by gender and age) before and 6 h after ingestion of gluten. Urinary excretion of lactulose and L-rhamnose (low molecular weight monosaccharide) were quantified. Lactulose/L-rhamnose ratio was unchanged in controls before and after gluten ingestion. In contrast, the same ratio increased in all celiac patients after ingestion of gluten; although, it is worth mentioning that two of the twenty-seven total patients had abnormal permeability at the beginning.³¹ Even though the sample size was rather small, the results suggest that gluten causes changes in permeability, even after a single meal. However, this study was carried out in a pediatric population and thus results are not necessarily applicable for adults.

Lahdeaho *et al.*³² challenged 21 known celiac disease patients with low (1–3 g) or moderate (3–5 g) doses of gluten daily for 12 weeks and assessed for symptoms, intestinal histology, and celiac serology. These patients were on a strict GFD and in clinical remission. The authors performed morphometric analysis, measuring villus height/crypt depth ratio. A decrease in the ratio of 0.5 or more after gluten challenge was considered gluten sensitivity. A significant decrease in villus height/crypt depth ratio was found in 67% of patients, without correlation with dose of daily gluten intake. There was an increase in CD3 intraepithelial lymphocytes, especially in the group with moderate gluten dose, which in the past has been found to be dose-dependent. There was a serological response in 43% of patients with positivity for EMA and TG2 antibodies.³² That study suggested an association between gluten and small bowel histological damage, but there is not enough evidence to support association between histological small bowel damage

(or intestinal permeability) and antibody levels.

Lindberg *et al.*³³ prospectively studied 180 children with suspected malabsorption. Ninety-six children with elevated aminotransferases were diagnosed with celiac disease, milk protein and/or multiple protein allergy or were classified as miscellaneous after evaluation of intestinal mucosal morphology. Of 10 children with food allergy and moderate-severe mucosal damage, 6 had elevated ALT and 7 had elevated AST. Those with normal or slight mucosal damage had normal AST and ALT.³³ These findings suggested that mucosal damage is associated with elevation of aminotransferases and not exclusively due to gluten exposure. Nevertheless, these results were limited, as there was no mention of whether other causes of elevated aminotransferases were ruled out. In addition, even though aminotransferases were monitored in some children and normalized after 2–8 weeks of dietary treatment, they did not specify which group of children was followed and what dietary changes were made. For this reason the results are not reliable.

Small intestinal bacterial overgrowth (SIBO)

A study was done to determine the prevalence of SIBO in celiac disease patients who were unresponsive to GFD, symptomatic or asymptomatic on GFD.³⁴ SIBO was found in 11% of patients unresponsive to GFD and 11% who were symptomatic, while none were found in the asymptomatic group. SIBO was not the only factor associated with persistent symptoms, as 67% of these patients had underlying conditions, such as microscopic colitis. No serologic difference was found between patients with and without SIBO, and serum aminotransferases were not measured. Previous studies have found that prevalence of SIBO is increased in celiacs compared to healthy controls,³⁵ and that study showed that SIBO can be associated with persistent symptoms in patients adherent to GFD. However, no relationship has been demonstrated between SIBO and elevated aminotransferases. Therefore, an association between bacteria and liver damage in celiac patients remains speculative.

Systemic autoimmunity

The pathogenesis behind extraintestinal manifestations of celiac disease is still not entirely understood, but it is thought to be due to autoantibodies that target transglutaminase 2 (TG2). TG2 is the antigen to which celiac IgA antibody binds *in vitro* in intestinal and extraintestinal tissues. Karponay-Szabo *et al.*³⁶ performed a study to detect IgA against intestinal and extraintestinal tissues by immunofluorescence. IgA deposition on extracellularly located TG2 was found in jejunal and extrajejunal specimens of all celiac patients. Overexpression of TG2 in liver causing deposition of IgA antibodies could potentially explain liver damage in celiac disease patients.¹¹ However, it would not explain why some patients have elevated aminotransferases and others do not.

Diagnosis

In the serum of patients with celiac disease, there are various types of antibodies that target gliadin or connective tissue components. These include anti-endomysial and anti-tissue transglutaminase antibodies (anti-tTG). Sjöberg *et al.*³⁷ measured anti-gliadin antibodies (IgA and IgG) in patients with chronic liver disease and compared them to healthy controls. Anti-gliadin IgA positivity was significantly higher in the group with chronic liver disease (particularly

patients with PSC) compared to healthy individuals.³⁷ Further work-up with anti-endomysial antibody was positive in two patients out of four-hundred and sixty-five who were found to have celiac disease based on small bowel biopsy. These results showed that anti-gliadin antibodies can be positive in many chronic liver conditions without celiac disease. Anti-gliadin antibodies are no longer recommended for diagnosis due to low sensitivity and specificity.

Anti-tTG IgA is the serologic test of choice for diagnosis of celiac disease. However, Vecchi *et al.*³⁸ measured anti-tTG and anti-endomysial antibodies in a group with celiac disease and another group with chronic liver disease (including cirrhosis) and found anti-tTG positivity in the chronic liver disease group. Even though anti-endomysial antibody was negative in all patients within this group, 57.9% of the cirrhotics had positive anti-tTG, likely indicating that chronic liver disease can cause false positives. Similarly, anti-tTG can be falsely positive in diabetes mellitus, Down's syndrome, and inflammatory bowel disease.³⁸

Testing for deaminated gliadin peptide IgA or IgG is likely more accurate in children <2 years-old and who are anti-tTG negative.³⁹ IgA-endomysial antibodies have nearly 100% sensitivity and specificity in untreated celiac patients but testing is expensive and time consuming. Serologies usually normalize after 6–12 months of GFD but mucosal healing is a slower process.^{39,40}

Small bowel biopsy remains the gold standard. Pathologic findings in the duodenum can vary in severity and may have a patchy distribution, affecting certain areas more than others. Collection of multiple specimens (four to six) must be submitted to increase sensitivity for diagnosis.³⁹ Several studies have reported higher diagnostic yields for biopsy of duodenal bulb^{39–41} compared to the terminal ileum.^{42,43}

Treatment

In the previously mentioned studies performed by Volta *et al.*,¹⁵ Vajro *et al.*¹⁸ and Bardella *et al.*,¹⁹ findings seem convincing that GFD reverses cryptogenic liver disease, as evidenced by normalization of elevated aminotransferases. In the study by Novacek *et al.*,¹³ eight patients did not respond to GFD and it was thought to be either due to diet non-adherence or another concomitant liver disease. That study suggested that celiac disease patients with persistently elevated aminotransferases despite GFD could have a second liver pathology non-responsive to GFD. In the study by Bardella *et al.*²⁰ from 1995, patients who responded to dietary changes were not tested for other underlying liver disease. Thus, it could not be determined if they did have another underlying condition, despite showing improvement with only GFD.

Hagander *et al.*^{17,21} first described liver injury in association with celiac disease after they found patients with known celiac disease and elevated liver enzymes. The authors retrospectively studied 74 patients with biopsy-proven celiac disease. Histology sections were available from thirteen patients, of which five showed reactive hepatitis and seven had hepatic injury. Out of 53 patients with measured aminotransferases, 29 had elevations and 19 of them had measurements before and after starting GFD; a significant reduction was found after dietary changes. However, the authors did not mention whether other causes of liver damage were ruled out in the 29 patients and not all patients with elevated enzymes were monitored after GFD; furthermore, the follow-up period was for only 8 weeks. There was also no clarification on whether all 19 patients had reduction in aminotransferase levels after GFD. There may have been cases of non-response to GFD that were undiscovered, but the follow-up period was also too short to determine non-

response. Overall, it is difficult to draw conclusions from this study even though a significant reduction in measured enzymes after start of GFD in certain patients seem to support the previous studies' findings.

Jacobsen *et al.*²² examined 132 patients with biopsy-proven celiac disease for hepatic involvement and found that 62 (47%) had elevated AST, ALT and ALP. These patients had other causes of liver damage ruled-out. Thirty-seven patients had liver biopsies performed, of which twenty-five showed nonspecific hepatitis and five showed chronic active hepatitis.^{17,22} Only 32 patients were rechecked after 2 years on GFD and were found to have significantly lower liver enzymes, while 24 had complete normalization of laboratory values. Four patients with complete normalization of laboratory tests had repeat liver biopsies that showed additional histologic normalization of nonspecific changes. It was not mentioned why only these patients had a repeat liver biopsy, especially after normalization of enzymes. Of 38 patients with small intestine biopsies before and after implementation of GFD, 24 had improvement in gut lining after GFD. The group with intestinal histologic improvement had median duration of symptoms of 11.4 years compared to 21.1 years from those who did not respond to dietary restrictions.²² This could mean that onset of celiac disease could be a determinant of whether GFD reverses gut permeability and therefore, liver damage. Findings seem convincing that GFD improved liver damage in the study, as seen by significant improvement in aminotransferases. Lack of intestinal histologic improvement in eight patients, however, seemed to contradict these findings. There is no mention, though, of severity of histological findings, whether there was correlation with antibodies or timing of repeat intestinal biopsy, or whether it was carried out concomitantly with repeat blood-work after 2 years. Thirty-eight patients had a repeat small intestine biopsy, while only thirty-two patients with elevated aminotransferases were rechecked at 2 years. This suggests the small intestinal biopsies occurred prior to follow up at 2 years, and there are no data on whether these were the same patients who had aminotransferases rechecked. A longer period of time on GFD could have potentially led to different biopsy results.

Kaukinen *et al.*²³ showed that a GFD could cause reversal of hepatic dysfunction in patients with celiac disease and severe liver disease. The investigators retrospectively studied four patients with untreated celiac disease and severe liver failure. Liver biopsy showed acute fulminant hepatitis in one patient with known celiac disease who was non-compliant with GFD. He had no family history, denied use of drugs/alcohol, and other underlying liver diseases were ruled-out. His liver function initially improved after starting GFD but, unfortunately, he later progressed due to non-adherence to diet. Another patient was diagnosed with congenital liver fibrosis, from a biopsy after presenting with ascites. He was later found to have histologic changes in duodenum consistent with celiac disease. Within 6 months of GFD, his ascites resolved. There were no signs of liver disease and small bowel mucosal biopsy was normal. This patient was considered for liver transplant due to severity of disease but, ultimately, improved with dietary changes and transplant was not needed. Of the two remaining patients, one had other causes of liver disease investigated and ultimately improved with GFD and steroids. The remaining patient had cirrhotic changes on biopsy without clear recovery despite steroid treatment for suspected AIH. Although her lack of improvement was deemed due to poor GFD (and steroid) compliance with persistent mucosal villus atrophy on repeat small bowel biopsy, her persistent liver damage could have been due to autoimmune disorder. It is difficult to determine if GFD improved liver function based on these case reports, especially when two of them were suspected to have autoimmune liver disease and were treated with concomitant

steroids. Correlation with celiac antibodies could have been useful to determine whether the persistently elevated aminotransferases was possibly related to higher levels of antibodies.

Celiac disease and autoimmune liver diseases

Liver involvement in celiac disease can coexist with other autoimmune conditions, such as primary biliary cholangitis (PBC) or PSC.¹³ Many of the aforementioned studies showed cases of patients with celiac disease who had persistently elevated aminotransferases despite GFD and who were ultimately found to have an autoimmune liver condition. Another study found a 3% prevalence of PBC in 143 patients with celiac disease, and there have been similar findings in other studies in Denmark and Sweden.⁴⁴ Schrupf *et al.*⁴⁵ found a 3% prevalence of celiac disease in patients with PSC. The prevalence of celiac disease in AIH was reportedly higher compared to the general population (around 3–6%).⁴⁵ Mounajjed *et al.*¹⁴ studied 30 patients with celiac disease who had liver biopsies and found 9 patients with AIH, 3 with PBC and 7 with PSC.

AIH and celiac disease

Celiac disease has a strong HLDA-DQ association. Approximately 95% of patients with celiac disease express HLA-DQ2, which has a strong association with HLA-DR3 expressed in autoimmune hepatitis.¹ Prevalence of celiac disease in AIH is higher compared to that in the general population and is thought to be around 4-6.4%.¹

Iqbal *et al.*¹ presented the case of a patient with known AIH on azathioprine with flares of elevated aminotransferases. The patient was not taking any supplements or over-the-counter medications and denied drug/alcohol use. ASMA, alpha-1 antitrypsin antibody and anti-mitochondrial antibody were negative, as well as hepatitis panel and antibodies against cytomegalovirus and Epstein-Barr virus. Ceruloplasmin, thyroid stimulating hormone and iron were normal. A liver biopsy showed grade 3 portal fibrosis. The patient was started on steroids, without improvement. He was further investigated for anti-tTG and endomysial immunoglobulin A antibody, the results of which seemed consistent with celiac disease. A small bowel biopsy revealed flattening of villi with intraepithelial lymphocytes, confirming diagnosis. He had normalization of aminotransferases and bilirubin with GFD.¹ The fact that aminotransferase elevations did not improve despite being on treatment for AIH but normalized after initiation of GFD seems to suggest that persistent elevated aminotransferases was due to celiac disease.

Volta *et al.*⁴⁶ studied a case of a celiac patient who had persistently elevated aminotransferases after 1 year on GFD. She also had elevated bilirubin, high albumin and low platelets. Eventually, the patient was investigated for autoimmune conditions and was found to have positive ANA, ASMA and anti-dsDNA. On GFD, celiac disease-related antibodies were negative, while small bowel biopsy showed normal findings, suggesting adherence. Liver biopsy showed chronic active hepatitis with lymphocytic and plasma cell periportal infiltration. She was started on azathioprine and methylprednisolone, in addition to GFD, and had normalization of laboratory tests at the 18-month follow up visit.⁴⁶ Although this was a single reported case, it suggests that combination therapy for both celiac disease and AIH normalized aminotransferases.

Additionally, Di Biase *et al.*⁴⁷ studied seven children with known celiac disease on GFD and AIH diagnosed by liver his-

tology and serology. They had mild fibrosis and necrosis in all cases. They were treated with steroids and azathioprine for 5 years, with biochemical and ultrasound tests every 3 months. All patients had normalized aminotransferases at 5 years. Six underwent liver biopsy, which revealed no interface hepatitis, and only two had (minimal) inflammation. Even though there was no mention found of aminotransferases levels prior to addition of AIH therapy, the results of liver biopsies suggest that treatment for both conditions led to improvement in liver histology.⁴⁷ There is no evidence to suggest liver damage from AIH-celiac disease is reversible with GFD only.

PBC and celiac disease

The prevalence of PBC has been reported to be 3- to 2-fold higher in celiac disease patients, while celiac disease prevalence in PBC patients ranged from 3–7%.⁴⁸ Kingham *et al.*⁴⁹ determined the relative prevalences of PBC and celiac disease in a population of around 250,000 over 12 years. They found 143 patients with celiac disease (biopsy-proven and responsive to GFD) and 67 with PBC (proven with liver biopsy). In patients with celiac disease, four were found to have concomitant PBC. Of all patients with PBC, 11 underwent duodenal biopsy and 1 was diagnosed with celiac disease. Approximately 3% of patients with celiac disease might develop PBC, while around 6% of patients with PBC might have celiac disease.⁴⁹ Many cases of simultaneous PBC and celiac disease have been reported in multiple studies but a common causal association has not been proven.^{50,51} Conversely, Chatzicostas *et al.*⁵² screened 62 patients with PBC and 17 with autoimmune cholangitis (AIC) for celiac disease by testing for anti-gliadin, anti-reticulin, anti-endomysial and anti-tTG antibodies. They also tested 100 random donated serum samples and 18 biopsy-proven uncontrolled celiac disease patients as controls. Anti-gliadin and anti-tTG antibodies were significantly higher in patients with PBC and AIC compared to the healthy controls, but none were positive for anti-reticulin or anti-endomysial antibodies. However, duodenal biopsies in 15 out of 24 patients with PBC or AIC and positive antibodies did not show histologic features suggestive of celiac disease. The study had a small sample size, and did not establish an increased risk for celiac disease in PBC patients.

Ginn *et al.*,⁵³ Neuberger *et al.*⁵⁴ and Logan *et al.*⁵⁵ reported cases of patients with concomitant celiac disease and PBC where GFD alone did not normalize aminotransferases. There was a lack of evidence to prove that GFD is sufficient for histological and biochemical resolution of liver damage in patients with both conditions. Additionally, whether GFD slows down progression of liver damage is difficult to prove.

PSC and celiac disease

Brazier *et al.*⁵⁶ reported a case of a patient with elevated AST, ALT and ALP and moderate dilation of the common bile duct on ultrasound. He had an ERCP, which showed diffuse narrowing and irregularity of the intrahepatic bile ducts without any obstruction. In the absence of secondary causes of PSC, diagnosis of primary PSC was suggested. On liver biopsy, he was found to have onion skin fibrosis and mononuclear infiltrate. Colonoscopy showed findings consistent with ulcerative colitis, while upper endoscopy showed findings consistent with celiac disease. Anti-reticulin antibodies and anti-endomysial antibodies were positive. He was treated only with GFD for 14 months, with improvement in duodenal histology, normalization of antibody levels and of AST, ALT and ALP. Repeat liver biopsy showed improvement

from histological stage 2 to stage 1. Although treatment with only GFD showed improvement in liver histology and normalization of laboratory tests, it is difficult to draw conclusions from a single case report.

Nonalcoholic fatty liver disease (NAFLD) and celiac disease

Reilly *et al.*⁵⁷ studied the risk of NAFLD in celiac disease patients with matched healthy controls. They ruled out patients with previous liver disease and those with lifetime diagnosis of alcohol-related conditions. They found that patients with celiac disease had an increased risk of developing NAFLD compared to healthy controls, with a hazard ratio of 2.8.⁵⁷ Similar findings were concluded by Tovoli *et al.*⁵⁸ after studying a celiac disease group of patients compliant with GFD with matched controls. It has been shown that NAFLD patients have increased intestinal permeability and greater association with SIBO.^{35,59} The increased risk for developing NAFLD in celiac disease patients might be explained by these pathogenic mechanisms in common.

Miele *et al.*³⁵ investigated intestinal permeability in patients with NAFLD (biopsy-proven), assessed correlation with liver damage, integrity of tight junctions and prevalence of SIBO, and compared to a group of celiacs and group of healthy controls. They found that patients with NAFLD had a higher prevalence of SIBO and increased permeability compared to healthy patients, but lower compared to the celiac patients. Increased intestinal permeability and SIBO prevalence correlated with severity of steatosis in patients with NAFLD. This group also had lower intensity of duodenal ZO-1 staining, suggesting less intact tight junctions, possibly causing increased permeability. These findings suggest that bacterial translocation may be related to increased gut permeability and steatosis; although, the mechanism remains unproven.

Conclusions

Patients with celiac disease should have liver enzymes routinely checked. If abnormal laboratory tests are found, it is reasonable to implement a strict GFD and monitor for response with repeat testing over the next 6–12 months. Many studies have shown improvement or normalization in aminotransferases with GFD and relapse with a gluten challenge. Lack of improvement should prompt a search for evidence of dietary transgressions. Following IgA anti-endomysial and IgA anti-gliadin levels might be useful in assessing compliance with diet, but if the patient is strictly adherent to GFD, further workup for other causes of liver disease and/or liver biopsy should be considered.¹⁸

On the other hand, patients with elevated liver enzymes without known history of celiac disease should be screened for this disease, regardless of symptoms, as the evidence shows most patients are asymptomatic.¹⁷ These patients should be screened with celiac disease serology, and if found to be positive, undergo a confirmatory small-bowel biopsy. If diagnosed with celiac disease, implementation of GFD is recommended, with follow-up testing in 6–12 months.

In patients with known celiac disease and an autoimmune liver disorder, treatment of concomitant autoimmune hepatobiliary disease is suggested in addition to GFD, as there is insufficient evidence to suggest biochemical or histological normalization with GFD only. Previous studies do show that GFD can improve aminotransferase levels in patients with PBC and PSC. These enzymes might help in slowing down progression of disease, but further longitudinal studies are needed to prove this hypothesis. Assuming systemic auto-

immunity is the main mechanism of liver damage in celiac disease, assessing response to steroids alone in patients with simultaneous AIH and correlating aminotransferase levels and antibodies could be useful.

The mechanism of liver damage in celiac disease patients is likely multifactorial. It is possible that gut lining damage in general, and not exclusively due to gluten-induced damage, could cause elevated aminotransferases. SIBO and local (rather than systemic) endotoxemia could be related to increased intestinal permeability and liver damage. However, this specific association remains speculative and further studies would be needed to determine if there is a significant association.

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Conflict of interest

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Author contributions

Wrote the manuscript (JVK), proposed the idea for the review and revised the manuscript with critical revisions (GYW).

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Efficacy and Safety of Glecaprevir/Pibrentasvir in Patients with Chronic HCV Infection

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Abstract

Hepatitis C virus (HCV) infection is a major cause of end-stage liver disease, including decompensated cirrhosis and hepatocellular carcinoma. Over 95% of patients with HCV infection have achieved sustained virologic response at 12 weeks under the treatment of several pan-genotypic regimens approved for patients with HCV infection. The glecaprevir/pibrentasvir (G/P) regimen has some features that distinguish it from others and is the only 8-week regimen approved for treatment-naïve patients and patients experienced in regimens containing (peg)interferon, ribavirin, and/or sofosbuvir, without an HCV NS3/4A protease inhibitor or NS5A inhibitor (except those with genotype 3). This review aims to summarize the efficacy and safety of G/P in HCV-infected patients from clinic trials and real-world studies, including those who have historically been considered difficult to cure.

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Introduction

Hepatitis C virus (HCV) infection threatens the health of people around the world. According to the World Health Organization, about 71 million people worldwide suffer from chronic HCV infection, and in 2016 approximately 399,000 patients died from end-stage HCV infection, mainly from cirrhosis and hepatocellular carcinoma.¹ Direct-acting antiviral agent (DAA)-induced sustained virologic response (SVR) has been associated with a reduction in the risk of cirrhosis, hepatocellular carcinoma, and mortality.^{2,3}

All of the three pan-genotypic antiviral regimens [sofos-

buvir/velpatasvir, sofosbuvir/velpatasvir/voxilaprevir, glecaprevir/pibrentasvir (G/P)] approved for the treatment of patients with chronic HCV infection in recent years have high efficacy, and more than 95% of patients administered them have achieved SVR at 12-week post-treatment (SVR12).^{3–7}

However, the fixed-dose combination of Glecaprevir (300 mg) and pibrentasvir (120 mg), an all-oral, once-daily, ribavirin-free pan-genotypic regimen approved in 2017, has certain characteristics that distinguish it from other pan-genotypic antiviral regimens, including shorter duration of therapy for most patients; it has been approved for use in patients with end-stage renal disease and adolescent patients aged 12–17 years.⁸

Both glecaprevir and pibrentasvir have potent anti-HCV activity across genotypes 1 through 6 *in vitro*, which harbor a high genetic barrier to resistance.⁸ The former is a second-generation NS3/4A protease inhibitor (PI), essential for the cleavage of the HCV unprocessed polyprotein and viral replication; the latter is a second-generation NS5A inhibitor, critical for viral RNA replication and virion assembly.⁹

Glecaprevir and pibrentasvir are mainly excreted through the biliary-fecal route, with only a minor fraction (less than 1%) excreted in urine; renal impairment and hemodialysis appeared to have no significant influence on glecaprevir or pibrentasvir exposures.^{8,10} Compared with patients with normal renal function, HCV-infected patients with end-stage renal disease, including dialysis, were observed to have an 86% increase in glecaprevir and a 54% increase in area under the curve for pibrentasvir.⁸ In chronic HCV-infected patients with compensated cirrhosis, exposure to glecaprevir was 160% higher and pibrentasvir exposures showed little difference in outcome compared to patients without cirrhosis.¹⁰ Meanwhile, there was no statistically significant difference in the exposure rates of glecaprevir and pibrentasvir between Japanese or Han Chinese and Whites.¹¹ But, we should note that patients with decompensated cirrhosis are not recommended for treatment with a PI-containing regimen (*e.g.*, glecaprevir, grazoprevir, and voxilaprevir).

Most patients with chronic HCV infection are at risk of drug-drug interactions (DDIs) with co-medication.¹² Both glecaprevir and pibrentasvir are substrates and inhibitors of P-glycoprotein and breast cancer resistance protein. Moreover, glecaprevir is a substrate and inhibitor for organic anion transporting polypeptide (OATP) 1B1 and OATP1B3. Pibrentasvir is an inhibitor of OATP1B1/3. Therefore, coadministration of G/P with drugs that inhibit hepatic P-glycoprotein, breast cancer resistance protein, or OATP1B1/3 may increase the plasma concentrations of glecaprevir and/or pibrentasvir.⁸ As per the drug label, G/P is contraindicated in combination with atazanavir or rifampin.⁸

The main purpose of this review is to comprehensively evaluate the efficacy and safety of G/P in patients with HCV infection, including those in the so-called “special population” that have historically been considered difficult to treat.

Keywords: Hepatitis C; Mavyret; Glecaprevir; Pibrentasvir; Treatment outcome.

Abbreviations: AE, adverse event; ARA, acid-reducing agent; CKD, chronic kidney disease; DAA, direct-acting antiviral agent; DDI, drug-drug interaction; G/P, glecaprevir/pibrentasvir; HCV, hepatitis C virus; ITT, intention to treat; OATP, organic anion transporting polypeptide; OST, opioid substitution therapy; PI, protease inhibitor; PPI, proton pump inhibitor; PRS, prior treatment experience with regimens containing (peg)interferon, ribavirin, and/or sofosbuvir, but no prior treatment experience with an HCV NS3/4A PI or NS5A inhibitor; SVR, sustained virologic response; SVR12, SVR at 12-week post-treatment.

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Efficacy

The clinic trials and real-world studies that have evaluated the efficacy of G/P against HCV are summarized in Tables 1 and 2,^{13–47} respectively.

Treatment-naïve or patients experienced in regimens containing (peg)interferon, ribavirin, and/or sofosbuvir, without an HCV NS3/4A PI or NS5A inhibitor, without cirrhosis

In a pooled analysis of nine clinical trials, treatment-naïve or patients experienced in regimens containing (peg)interferon, ribavirin, and/or sofosbuvir, without an HCV NS3/4A PI or NS5A inhibitor (PRS) with HCV genotypes 1–6 infections but without cirrhosis achieved an overall SVR12 rate of 98% (943/965) in the intention-to-treat (ITT) population when treated for 8 weeks, which showed no significant difference from patients treated with 12 weeks (1,060/1,076, 99%).⁴⁸ But, all 478 patients infected with genotype 3 were treatment-naïve in that study. CRETIN-1 and CRETAIN-2 were not included in the pooled analysis described previously, and the authors also demonstrated that G/P treatment for 8 weeks was highly effective in patients with HCV genotypes 1–2 infections without cirrhosis, of which 99.2% (128/129) and 98% (88/90) patients achieved SVR12, respectively.^{13,14} The ENDURANCE-5,6 trial, an open-label, multicenter, phase 3b trial, demonstrated that 98.6% (74/75) of non-cirrhotic patients infected with HCV genotype 5 or 6 treated with G/P for 8 weeks achieved SVR12.¹⁵

Data from a combined analysis of 18 real-world studies showed that the SVR rate for treatment-naïve patients without cirrhosis who received G/P treatment for 8 weeks was 98.2% ($n=697$) in the ITT population and 99.3% ($n=3,657$) in the modified ITT population.⁴⁹ For patients with treatment experience, the SVR rate was 90.7% (49/54), among which 4 patients were lost to follow-up.¹⁶

Treatment-naïve or PRS-experienced patients with compensated cirrhosis

The efficacy of 8-week G/P reported in the EXPEDITION-8 clinical trial is comparable to the 12-week regimen for treatment-naïve patients with genotypes 1–6 infections and compensated cirrhosis.¹⁷ The SVR rate was 97.7% (335/343) in the ITT population and 99.7% (334/335) in the per protocol population after treatment with G/P once daily for 8 weeks.¹⁷ After 12 weeks of treatment with G/P, the SVR12 rates of HCV genotype 3- and non-genotype 3-infected compensated cirrhosis patients without treatment experience were 97.1% (67/69)⁵⁰ and 100% (110/110),¹⁸ respectively.

For PRS-experienced patients with compensated cirrhosis and non-genotype 3 infection, the EXPEDITION-1 trial reported that 97.2% (35/36) of patients treated for 12 weeks achieved SVR12, with one genotype 1a-infected patient relapsing at post-treatment week 8.¹⁸ The CRETAIN-1, subgroup 2 trial evaluated the efficacy of a 12-week course of G/P in 38 genotype 1-infected patients with compensated cirrhosis. All of the 38 patients (100%) achieved SVR12, of which 12 were interferon-experienced.¹³

Data from real-world studies showed that the SVR12 rate for patients with compensated cirrhosis who received G/P treatment was 97.8% ($n=676$) in the ITT population and 98.2% ($N=822$) in the modified ITT population.⁴⁹ For treatment-naïve patients with compensated cirrhosis treated for 12 weeks, the SVR12 rate was 99.0% ($n=362$).⁴⁹ To our knowledge, currently there is no real-world data to evalu-

ate the efficacy of G/P treatment for 8 weeks in treatment-naïve patients with compensated cirrhosis.

“Special population”

Patients with HCV genotype 3 infection

Patients with HCV genotype 3 infection are among the most difficult to treat in the DAA-era. According to the studies described above, treatment-naïve and genotype 3-infected patients treated with G/P for 8 weeks had high SVR12 rate, regardless of cirrhosis, but the efficacy in PRS-experienced patients is still uncertain.

In the SURVEYOR-II trial, parts 1 and 2, the efficacy of G/P for 12 weeks was studied in PR-experienced patients with genotype 3 infection and without cirrhosis. In total, 91.7% (22/24) achieved SVR12, with 1 patient having a breakthrough at treatment week 6 and 1 other relapsing at post-treatment week 8.¹⁹ The SURVEYOR-II trial, part 3, a partially-randomized, open-label, phase 3 study, assessed the efficacy of G/P in patients with genotype 3 infection with prior treatment experience, and found that 91% (20/22) and 96% (21/22) of PRS-experienced patients without cirrhosis achieved SVR12 for 12 weeks and 16 weeks, respectively. Among the 47 treatment-experienced patients with compensated cirrhosis, SVR12 were achieved by 96% (45/47) of patients treated with G/P for 16 weeks.²⁰ An integrated cirrhosis of five phase 2 or 3 trials that evaluated G/P in patients with chronic HCV genotype 3 infection also demonstrated G/P was efficacious for those patients, regardless of cirrhosis or prior treatment experience.⁵⁰

Data from real-world studies also demonstrated that over 95% of HCV genotype 3-infected patients treated with the G/P regimen achieved SVR.^{21,49} But, we should point out that almost all of the above-mentioned research studies were conducted in areas where subtype 3a is dominant, and subtype 3b accounts for less than 1% of all genotype 3 cases. Therefore, more attention should be paid to the efficacy of G/P in HCV subtype 3b-infected patients. Nozaki *et al.*²² recently reported that only 33.3% (2/6) of patients with HCV subtype 3b infection achieved SVR12. Another study conducted by Tamori *et al.*²³ found that only 50% (2/4) of patients with HCV genotype 3b infection achieved SVR12. Moreover, one out of three patients with genotype 3a/b experienced virologic failure in the study of Toyoda *et al.*²⁴

Patients with severe renal impairment

The investigators of the EXPEDITION-4 study examined the efficacy of G/P administered for 12 weeks in adults with chronic HCV genotypes 1–6 infections and stage 4 or 5 chronic kidney disease (CKD).²⁵ Of the 104 patients enrolled, 44 (42%) had prior treatment experience for HCV, but treatment-experienced patients who had genotype 3 infection were excluded and 20 (19%) had compensated cirrhosis at baseline. Up to 98% (102/104) of the patients achieved SVR12, with no patients experiencing virologic failure.²⁵ In the phase 3 EXPEDITION-5 trial, the rate of SVR was 97% (98/101) in the ITT population and 100% (98/98) in the modified ITT population after treatment with G/P. Of the 101 enrolled adults with CKD stages 3b, 4 or 5, 84 patients without cirrhosis were assigned to receive G/P for 8 weeks and 4 PRS-experienced patients with genotype 3 infection were assigned to 16 weeks of treatment; all others were in the 12-week treatment group.²⁶ Real-world studies also demonstrated G/P was highly effective for patients with CKD stages 4–5 (including patients undergoing hemodialy-

Table 1. Efficacy of G/P in clinical trials

Study	HCV genotype	Population	Number	Duration	SVR12, n/total (%) ^a
EXPEDITION-1 ¹⁸	1, 2, 4, 5, 6	TN with CC PRS-exp with CC	110 36	12 w	110/110 (100) 35/36 (97.2)
EXPEDITION-2 ²⁹	1–6	TN or PRS-exp ^b , HIV coinfection, ±CC	153	8/12 w	150/153 (98.0)
EXPEDITION-4 ²⁵	1–6	TN or PRS-exp, ±CC, CKD stages 4–5	104	12 w	102/104 (98.1)
EXPEDITION-5 ²⁶	1–6	TN or PRS-exp, ±CC, CKD stages 3b, 4, 5	101	8/12/16 w	98/101 (97.0)
EXPEDITION-8 ¹⁷	1–6	TN with CC	343	8 w	335/343 (97.7)
Fontana ⁴¹	1–6	TN with NC	230	12 w	222/230 (96.5)
ENDURANCE-5, 6 ¹⁵	5, 6	TN or PRS-exp, NC CC	84	8 w 12 w	82/84 (97.6) 74/75 (98.7) 8/9 (88.9)
ENDURANCE-1 ³⁹	1	TN or PRS-exp, NC	703	8 w 12 w	348/351 (99.1) 351/352 (99.7)
ENDURANCE-2 ³⁸	2	TN or PRS-exp, NC	202	12 w	201/202 (99.5)
ENDURANCE-3 ³⁹	3	TN with NC	390	8 w 12 w	149/157 (94.9) 222/233 (95.3)
ENDURANCE-4 ³⁸	4–6	TN or PRS-exp, NC	121	12 w	120/121 (99.2)
SURVEYOR-I, II, part 1, 2 ¹⁹	1–6	TN or PRS-exp, NC	230	8 w 12 w ^e	114/117 (97.4) 108/113 (95.6)
SURVEYOR-II, part 3 ²⁰	3	TN or PRS-exp, ±CC	131	12 w 16 w	59/62 (95.2) 66/69 (95.7)
SURVEYOR-II, part 4 ³⁸	2, 4–6	TN or PRS-exp, NC	203	8 w	196/203 (96.6)
CERTAIN-1, substudy 1, 2 ¹³	1	TN or PRS-exp, without CC with CC	167	8 w 12 w	166/167 (99.4) 128/129 (99.2) 38/38 (100)
CERTAIN-2 ¹⁴	2	TN or PRS-exp, NC	90	8 w	88/90 (97.8)
MAGELLAN-1, part 1	1	TE with NS3/4A and/or NS5A inhibitor, NC	22	12w	19/22 (86.4)
MAGELLAN-1, part 2 ³²	1, 4 ^c	TE with NS3/4A and/or NS5A inhibitor, ±CC	91	12 w 16 w	39/44 (88.6) 43/47 (91.5)
MAGELLAN-2 ³⁰	1–6	TN or PRS-exp, NC, with liver or kidney transplant	100	12 w	98/100 (98.0)
Lok <i>et al.</i> ⁴²	1	TE with sofosbuvir plus an NS5A inhibitor, ±CC	177	12 w ^d 16 w	88/99 (88.9) 74/78 (94.9)
DORA part 1 ³⁴	1–4	TN or PRS-exp, ±CC, adolescent	47	8/16 w	47/47 (100)

^aSVR12 rates in the ITT population.^bPatients with treatment experience and genotype 3 infection were excluded.^cIn MAGELLAN-1 part 2, only four patients had the genotype 4 infection.^dPatients with CC received G/P+ribavirin for 12 weeks.^eGenotype 3-infected patients with TN received G/P for 8 weeks, with PR-exp for 12 weeks.

Abbreviations: ±CC, with or without compensated cirrhosis; HIV, human immunodeficiency virus; NC, non-cirrhotic; PRS-exp, prior treatment experience with regimens containing (peg)interferon, ribavirin, and/or sofosbuvir, but no prior treatment experience with an HCV NS3/4A PI or NS5A inhibitor; TE, treatment-experienced; TN, treatment-naïve; w, week.

Table 2. Efficacy of G/P in real-world studies

Study	Country/ region	HCV genotype	Number	Population	Duration	SVR, n/total (%)
D'Ambrosio et al. ³⁵	Italy	1-4	723	TN or PR-exp, ±CC	8/12/16 w	680/723 (94.1)
Atsukawa et al. ²⁷	Japan	1-3	141	With CKD stages 4-5	8 w 12 w	90/91 (98.9) 50/50 (100)
Hsu et al. ³⁶	Taiwan	1-3, 6	110	With hepatic fibrosis F3-4	8 w 12/16 w	45/45 (100) 65/65 (100)
Ikeda et al. ³⁷	Japan	1, 2 ^a	571	DAA-naïve, NC	8 w	530/571 (92.8)
Osawa et al. ⁴⁰	Japan	1-3	30	DAA-exp, ±CC	12 w	28/30 (93.3)
Persico et al. ⁴³	Italy	1-4	1,177	TN (85%), genotype 3 (10%), CC (9%)	8/12/16 w (1,061/109/6)	1,163/1,177 (98.8)
Tamori et al. ²³	Japan	1-4	423	NC, DAA-naïve CC/DAA-exp/genotype 3 or 4	8 w 12 w	220/246 (89.4) 164/177 (92.7) ^d
Toyoda et al. ²⁴	Japan	1-3 ^a	509	DAA-exp/CC/genotype 3	12 w	504/509 (99.0)
Ueda et al. ³¹	Japan	1, 2	25	With liver transplant	8/12 w	24/25 (96.0)
Liu et al. ²⁸	Taiwan	1, 2, 3, 6	108	With CKD stage 4 or 5	8/12 w	107/108 (99.1)
Nozaki et al. ²²	Japan	1-3 ^a	1,439	TN or TE, NC or CC	8/12 w	1,397/1,439(97.1)
Liu et al. ⁴⁴	Taiwan	1-3, 6	658	TN or TE, ±CC	8/12/16 w	646/658 (98.2)
Soria et al. ²¹	Italy	3	152	TN or TE, ±CC	8/12/16 w	147/152 (96.7)
Berg et al. (DHC-R) ¹⁶	German	1-6	552 ^b	TN or TE, ±CC	8/12/16 w	534/552 (96.7)
Sugiyama et al. ⁴⁵	Japan	1-3	182	TN or TE, ±CC	8/12/16 w	178/182 (97.8)
Uemura et al. ³³	Japan	1-3	42	DAA-exp, ±CC	12 w	39/42 (92.9) ^c
Kusakabe et al. ⁴⁶	Japan	2	28	PRS-exp	12 w	28/28 (100)
Sezak et al. ⁴⁷	Japan	1-3 ^a	271	TN or PR-exp DAA-exp	8/12 w 12 w	180/183 (98.4) 85/88 (96.6)

^aPatients with baseline P32del were excluded.^bPatients with on-label treatment.^cThree patients with genotype 1b HCV with NS5A P32del experienced virologic failure.^dThe SVR rate of genotype 3 patients is 4/7 (57%), and there are 2 genotype 3b patients among patients who do not reach SVR12.

Abbreviations: ±CC, with or without compensated cirrhosis; NC, non-cirrhotic; PRS-exp, prior treatment experience with regimens containing (peg)interferon, ribavirin, and/or sofosbuvir, but no prior treatment experience with an HCV NS3/4A PI or NS5A inhibitor; TE, treatment-experienced; TN, treatment-naïve.

sis), and more than 98% of patients achieved SVR.^{22,27,28,51}

Patients with human immunodeficiency virus-1/HCV coinfection

EXPEDITION-2 was a phase 3, multicenter study to evaluate the efficacy of G/P in 156 human immunodeficiency virus/HCV-coinfected adults with compensated liver diseases.²⁹ All patients with genotype 3 infection were treatment-naïve for HCV. Patients were either antiretroviral therapy-naïve or on a qualifying antiretroviral therapy regimen for at least 8 weeks. The antiretroviral therapy drugs used by more than 20 patients included tenofovir disoproxil fumarate, abacavir, emtricitabine, lamivudine, raltegravir, dolutegravir, and rilpivirine. As high as 99.3% (136/137) of the patients without cirrhosis treated for 8 weeks achieved SVR12, and 87.6% (14/16) of the patients with compensated cirrhosis treated for 12 weeks achieved SVR12. No one experienced virologic failure, except one HCV treatment-naïve patient with genotype 3 infection and compensated cirrhosis.²⁹

Patients with liver or kidney transplantation

MAGELLAN-2, a phase 3, open-label trial, evaluated the efficacy of G/P for 12 weeks in patients who had chronic HCV genotypes 1-6 infections and had received a liver ($n=80$) or kidney ($n=20$) transplant at least 3 months prior.³⁰ Patients with cirrhosis were excluded and all genotype 3-infected patients were treatment-naïve. Overall, SVR was achieved in 98% (98/100) of the patients, with one genotype 3a-infected patient who had received a liver transplant experiencing virologic relapse.³⁰ Ueda *et al.*³¹ reported that 96% (24/25) of patients with recurrent HCV infection after liver transplantation treated with 8 or 12 weeks of G/P achieved SVR12, including patients with severe renal impairment, liver cirrhosis, prior DAA experience, or jaundice after liver transplantation. But, no virologic failure occurred.

Patients with psychiatric disorders

An integrated analysis of 10 phase 2 and phase 3 clinical trials assessed the efficacy of G/P for 8, 12 or 16 weeks in chronic HCV genotypes 1-6-infected patients with psychiatric disorders.⁵² The overall patients' treatment adherence was very high (>95%), regardless of whether there was a psychiatric disorder. Of the 2,522 patients receiving G/P, 97.3% (768/789) of those with a psychiatric disorder achieved SVR12 compared to 97.5% (1,689/1,733) of those without a psychiatric disorder. Among all patients receiving a neuropsychiatric co-medication with potential DDIs with G/P, the SVR rate was still high, including quetiapine (100%, 47/47), oxycodone (93.8%, 76/81) and hydrocodone (97.4%, 75/77).⁵² Meanwhile, patients with psychiatric disorder had overall high SVR rate (98.9%) in the modified ITT population in real-world studies.⁴⁹

Patients with NS3/4 PI and/or NS5A inhibitor treatment experience

In part 1 of the MAGELLAN-1 trial, 22 genotype 1-infected non-cirrhotic patients with past failure to NS3/4 PI and/or NS5A inhibitor were treated with G/P for 12 weeks.⁵³ SVR was achieved by 86% (19/22) in the ITT population and 95% (19/20) in the modified ITT population. Virologic failure occurred in one patient with past treatment experience

with both NS3/4A PI and NS5A inhibitor.⁵³ MAGELLAN-1, part 2, a randomized, open-label, multicenter phase 3 study, enrolled 91 HCV genotypes 1 or 4-infected patients with compensated liver disease (with or without cirrhosis) who had past treatment experience with NS3/4A PI and/or NS5A inhibitor.³² Patients enrolled were randomized to receive 12 or 16 weeks of G/P. Patients with NS3/4A PI experience alone (NS5A inhibitor-naïve) had 100% SVR12, regardless of treatment duration. Patients with NS5A inhibitor experience alone (NS3/4A PI-naïve) had 88% (14/16) and 94% (17/18) SVR rate among those treated with G/P for 12 weeks and 16 weeks, respectively. Patients with both NS3/4A PI and NS5A inhibitor experience had 79% (11/14) and 81% (13/16) SVR rate among those treated for 12 weeks and 16 weeks, respectively. All (4/4) patients with genotype 4 infection achieved SVR12 and 10.3% (9/87) of patients with chronic HCV genotype 1 infection experienced virologic failure. In CRETAIN-1 and -2 trials, The SVR rate in the HCV subtype1b-infected patients with PI and NS5A inhibitor experience was 93.3% (28/30). Virologic failure occurred in two patients with NS5A P32del.⁵⁴

Research conducted by Uemura *et al.*,³³ in which 34 of 42 patients had both NS3 and NS5A inhibitors treatment experience, reported that the SVR12 rate of chronic hepatitis c patients with prior DAA treatment experience treated with G/P for 12 weeks was 92.9% (39/42). All genotype 1b-infected patients carrying the NS5A P32del (3/35) experienced virologic failure.³³

Adolescent patients aged 12–17

The DORA part 1, nonrandomized, open-label, multicenter clinical trial assessed the efficacy of G/P in adolescent patients with chronic HCV genotypes 1-4 infections.³⁴ Of the 47 enrolled patients, 36 patients were HCV treatment-naïve and the others were interferon-based regimen-experienced. Except for three patients with HCV genotype 3 infections and treatment experience, who received G/P for 16 weeks, all patients received 8 weeks of therapy. The SVR12 rate of overall patients was 100% (47/47).³⁴

Patients who used drugs recently or were receiving opioid substitution therapy

Among 15.6 million patients who inject drugs, an estimated 52.3% are HCV-antibody positive.⁵⁵ A pooled analysis of clinic trials revealed that SVR12 was achieved by 92.9% (91/98), 97.0% (592/610), and 99.5% (1,106/1,111) of recent, former, and non-drug users, respectively. But, the overall rates of virologic failure were $\leq 1.5\%$, regardless of drug use status.⁵⁶ An integrated analysis of eight clinical trials compared the efficacy of G/P in HCV-infected patients receiving opioid substitution therapy (OST) and those not receiving OST. SVR rates were 96.2% (151/157) and 97.9% (2,055/2,099) in the OST and non-OST patients, respectively.⁵⁷ Methadone was the most commonly prescribed OST (76%).⁵⁷ An integrated analysis of 18 real-world studies demonstrated that 98.9% of chronic HCV patients using OST achieved SVR12 in the modified ITT analysis.⁴⁹

Patients with concurrent use of acid-reducing agents

Proton pump inhibitors (PPIs) were among the most frequently used co-medication in patients treated for chronic HCV infection.¹² An integrated analysis of nine phase 2 and phase 3 clinic trails showed that in patients treated with G/P, SVR rates were 97.0% (389/401) and 97.5% (1,918/1,968)

for patients using concomitant acid-reducing agents (ARAs) and those not concurrently using ARAs, respectively.⁵⁸ SVR was achieved in 96.3% (105/109) of patients taking a high-dose PPI and 97.4% (150/154) taking a low-dose PPI. The overall virologic failure rate in patients taking an ARA was less than 1% (4/401) compared with 1.5% (29/1,968) in patients not taking any ARAs.⁵⁸ The SVR rate of patients with chronic HCV infection receiving PPI was 97.9% ($n=180$) in real-world studies.⁴⁹

Tolerability and safety

HCV N3/4A PIs have been shown to have concentration-dependent hepatotoxicity and are contraindicated in patients with decompensated cirrhosis. Therefore, patients with factors known to affect the exposure to glecaprevir need to be carried out, including those on patients with renal impairment, DDIs, advanced age, female sex, and cirrhosis status.⁵⁹

A pooled analysis of nine clinical trials evaluated the tolerability and safety of G/P in chronic HCV-infected patients and studied by subgroup.⁶⁰ In general, the prevalence of adverse events (AEs) were 68% (1,603/2,369), and the most commonly reported AEs with an incidence rate exceeding 5% included headache, fatigue, nausea, and pruritus.⁶⁰ Serious AEs occurred in 73 (3%) patients but only one (<1%) was reported as DAA-related. Although 55% (11/20) of patients with compensated cirrhosis and CKD stages 4 or 5 developed serious AEs, none were reported as G/P-related.⁶⁰ No matter whether concurrent with end-stage renal disease or not, DAA-related serious AEs and AEs leading to G/P discontinuation were rare (<1%).⁶⁰ Hepatic decompensation and death occurred in 1 and 7 patients, respectively, but none were considered related to G/P. Patients with laboratory abnormalities grade ≥ 3 were rare among both patients with compensated cirrhosis and without cirrhosis.⁶⁰ Alanine aminotransferase elevations greater than 5 times the upper limit of normal occurred in two (<0.1%) patients without cirrhosis, and elevations of total bilirubin at least 3-times the upper limit of normal occurred in nine (0.4%) patients, including three (0.1%) with cirrhosis and six (0.3%) without cirrhosis. Overall, this combined analysis demonstrated that the recommended dose of G/P for patients with compensated liver disease and/or with any degree of renal impairment is safe and well-tolerated. The fixed-dose of G/P was also demonstrated safe and tolerable in patients with psychiatric disorders,⁵² those with recent drug use,⁵⁶ those receiving OST therapy⁵⁷ or acid-reducing agents,⁵⁸ and patients aged 65 years or older⁶¹ by data from clinical trials and additional subgroup analysis. The safety profile of G/P in adolescents was consistent with that in adults, as demonstrated in the DORA part 1 trial published recently.³⁴

Data from real-world studies have demonstrated that the safety is similar among clinic trials.^{16,27,35–37,59} The prevalence rate of patients with AEs was 17.7% (1,271/7,199), and the most commonly reported AEs were the same as in the clinical trials. Only 1% (55/5,522) of patients reported serious AEs, and 0.6% (33/5,595) of patients discontinued study treatment because of AEs. Only 4 of 2,333 (0.2%) patients experienced hepatic decompensation events.⁴⁹

Although patients with G/P had a high prevalence of AEs, ENDURANCE-2 reported that the frequency and severity of AEs and laboratory abnormalities in the HCV genotype 2-infected patients treated with G/P were similar to those treated with placebo.³⁸

Conclusions

Although the data from clinical trials and real-world studies described above demonstrated that G/P could help a vast

majority of chronic hepatitis c patients to safely eliminate HCV, even in patients who are not fully adherent to G/P regimen,⁶² there are still some problems that deserve attention.

Several studies have demonstrated that the A30K substitution significantly decreases the SVR12 rate in genotype 3 patients treated with the G/P regimen.^{39,63} Patients with subtype 3b naturally possess A30K, and over 95% of patients with subtype 3b harbor the paired A30K+L31M substitutions,^{64,65} which showed a >20-fold increase in 50% effect concentration for pibrentasvir.⁶⁴ Research conducted by Nozaki *et al.*²² and Tamori *et al.*²³ showed SVR rates were 33.3% (2/6) and 50% (2/4), respectively. Therefore, the efficacy of G/P in patients with subtype 3b is worthy of attention, especially in Asia, where the prevalence of subtype 3b is much higher than that in other continents.^{66,67} Nevertheless, most clinical studies have been conducted in Europe and North America, where the proportion of subtype 3b is less than 1% for genotype 3.⁶⁷ Evaluation of the efficacy of G/P in patients with HCV subtype 3b infection is still lacking and needs further study.

All patients with NS5A-P32del have experienced virologic failure after receiving G/P treatment, according to the limited data.^{23,33,40,54} P32del confers an >1,000-fold change resistance to pibrentasvir and >10,000-fold change resistance to velpatasvir in the HCV genotype 1b Con replicon⁵⁴ appearing in 5% to 10% of genotype 1b patients who experienced virologic failure with daclatasvir-containing therapies and sofosbuvir/ledipasvir treatment, but which has not been found among treatment-naïve patients.^{68,69} Therefore, patients with prior NS5A inhibitor treatment experience, especially those with genotype 1b infection, should pay attention to P32del and switch to other treatment options when P32del occurs.

Currently, although G/P has been approved for children aged 12 years and older or weighing at least 45 kg without dose changing, the efficacy and safety of G/P for adolescents with genotypes 5 and 6 infections or previous sofosbuvir experience were inferred from adult data and have not been directly evaluated.³⁴ More evidence from clinical trials and real-world studies is needed to prove effectiveness and safety in adolescents.

In conclusion, G/P is highly efficacious and well-tolerated in chronic HCV-infected patients with compensated liver disease, including patients with compensated cirrhosis, HCV/human immunodeficiency virus coinfection, end-stage renal disease, liver or kidney transplants, recent drug use or in adolescents, according to data from clinic trials and real-world studies. In the DAA era, the characteristics of HCV patients have changed greatly, including for treatment-experienced patients and patients with cirrhosis that has decreased over time.^{70,71} The fixed-dose G/P regimen for 8-week duration has been approved for treatment-naïve HCV-infected patients with compensated liver disease (with or without cirrhosis) by the European Commission⁷² and the USA Food and Drug Administration,⁸ which means a shorter duration of therapy can benefit the vast majority of chronic hepatitis c patients. However, patients with HCV subtype 3b infection or NS5A-P32del need special attention.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Wrote the manuscript (XL), critically revised the manuscript and provided study supervision (PH).

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Chronic Active Hepatitis B with COVID-19 in Pregnancy: A Case Report

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Abstract

Currently, infection with coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), during pregnancy is a problem worthy of attention, especially in patients with underlying diseases. In this case report, we present a case of chronic active hepatitis B with COVID-19 in pregnancy. A 31-year-old woman at 29 weeks of gestation who had a history of chronic hepatitis B virus infection discontinued antiviral treatment, was admitted to the hospital with chronic active hepatitis B, and tested positive for SARS-CoV-2 infection. In this case, we applied liver protective and antiviral agents, and low-dose dexamethasone therapy to successfully treat the critically ill pregnant woman suffering from chronic active hepatitis B combined with COVID-19.

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Introduction

At present, the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) caused the epidemic of coronavirus disease 2019 (COVID-19).¹ The World Health Organization has declared the COVID-19 outbreak as a pandemic on March 11, 2020.² With the expansion of the infected population, the clinical characteristics, management and prognosis of pregnant women with COVID-19 have received more and more attention, especially in the presence of underlying diseases. Elevated bilirubin and transaminase are common in patients with COVID-19,^{3,4} suggesting that liver damage is often accompanied with SARS-2 infection. As

for SARS-CoV-2 and HBV co-infected patients, it has also been reported that the proportion of abnormal liver function tests is similar to SARS-CoV-2 alone infection.^{5–7} In this case, there has been no sufficient evidence on treatment for such patients, such as whether the use of systematic glucocorticoids will slow down virus clearance, etc., especially for pregnant women. This case reports the successful treatment of antiviral, liver protection and low-dose dexamethasone to effectively treat acute chronic hepatitis B with COVID-19 in pregnancy.

Case report

A 31-year-old woman at 29 weeks of gestation presented to the hospital on March 29, 2020, with a 30-day history of malaise, diarrhea, itching, and jaundice. She also reported having experienced exertional dyspnea with cough. A nucleic acid test for SARS-CoV-2 was thus performed prior to admission, and yielded positive result.

The patient had a history of chronic hepatitis B virus (HBV) infection, for 9 years. She reported a transaminase level of nearly 1,000 U/L in September 2019. She had taken “tenofovir” once a day, which she had discontinued (by herself) after pregnancy (September 2020).

The vital signs on admission were as follows: SpO₂ 96% (no oxygen inhalation therapy); heart rate of 102 bpm; blood pressure of 119/87 mmHg; respiratory rate of 20 breaths per minute; and, temperature of 36.3°C. No swelling of the lower limbs was observed. Nasopharyngeal swabs tested positive for SARS-CoV-2 nucleic acid. Chest and abdominal computed tomography showed no obvious lesions, except for a plump pancreas.

The laboratory examination was expanded upon after admission. Blood examination showed white blood cell count of 13.06 × 10⁹/L, normal platelet count (258 × 10⁹/L), and HBV-DNA of 2.56 × 10⁵/mL. Coagulation test showed an international normalized ratio of 1.33, an activated partial thromboplastin time of 34.3 s, a fibrinogen level of 3.17 g/L and D-dimer level of 0.94 μg/mL (Table 1). Obstetric ultrasound showed a single live fetus, equivalent to 29+2 weeks, a slightly faster fetal heart rate, placenta grade I, and low placenta.

The patient was diagnosed with the following: 1. COVID-19, mild type; 2. 29 weeks of gestation, G3P1, head position; and, 3. Chronic active hepatitis B with liver function abnormality (Child grade B).

After admission, the patient was administered adenosyl-

Keywords: COVID-19; SARS-CoV-2; Pregnancy; Active hepatitis B.

Abbreviations: COVID-19, coronavirus disease 2019; HBV, hepatitis B virus; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

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Table 1. Laboratory findings

	2020/3/20	2020/3/29	2020/3/30	2020/4/2
WBC (*10 ⁹ /L)	ND	ND	13.06	10.27
Plt (*10 ⁹ /L)	ND	ND	258	274
AST (IU/L)	795	1,321	506	55
ALT (IU/L)	503	858	534	147
D-Dimer (μg/mL)	ND	ND	0.94	0.72
T-Bil (μmol/L)	183.1	117.8	105.3	45.0
D-Bil (μmol/L)	ND	ND	101.3	41.8
PT (s)	ND	ND	16.6	14.0
APTT (s)	ND	ND	34.3	29.9
INR	ND	ND	1.33	1.07
HBV-DNA	ND	ND	2.56*10 ⁵ /mL	1.67*10 ³ /mL
HCV-Ab	ND	ND	Negative	ND
HBs-Ag	ND	ND	Positive	ND
HBs-Ab	ND	ND	Negative	ND
HBe-Ag	ND	ND	Positive	ND
HBe-Ab	ND	ND	Negative	ND
HBc-Ab	ND	ND	Positive	ND

Abbreviations: Ab, antibody; Ag, antigen; ALT, alanine aminotransferase; APTT, activated partial thromboplastin time; AST, aspartate aminotransferase; D-Bil, direct bilirubin; HBc-Ab, hepatitis B virus core antibody; HBs, hepatitis B virus surface protein; HBV, hepatitis B virus; HCV, hepatitis C virus; INR, international normalized ratio; Plt, platelet; PT, prothrombin time; T-Bil, total bilirubin; WBC, white blood cell.

methionine succinate, reduced glutathione to protect the liver, tenofovir (as an antiviral), and a small dose of dexamethasone (10 mg q.d. for 3 days) to promote fetal lung maturation and reduce systemic inflammation. She was also monitored closely for liver function, coagulation function, and HBV-DNA change in trend (Fig. 1). Considering the results of the fetal ultrasound and fetal heart rate monitoring, we estimated that the fetus was normal and determined that there was no indication for emergency termination of pregnancy.

After treatment, the patient's liver function, and other indicators gradually improved. The patient's respiratory symptoms were also reduced, dyspnea relieved, and SpO₂ maintained at a stable high level. After 3 weeks, the SARS-CoV-2 nucleic acid test was re-performed twice, and both results were negative. She was discharged from the hospital on May 1, 2020.

We prescribed antiviral treatment with tenofovir and recommended regular follow-up examinations at the outpatient clinic. Throughout, her HBV-DNA level was maintained at about 1.27*10² IU/L. She gave birth to a baby boy vaginally

at 39 weeks of pregnancy, with 200 mL of vaginal bleeding. The mother was in good condition and the neonatal Apgar score was 9 and weighed 3,490g.

Ethics approval and consent to participate

Informed consent for the collection of medical history and blood samples was obtained in compliance with the Declaration of Helsinki and approved by the local ethical committee (Peking University Third Hospital, Medical Science Research Ethical Committee Approval: IRB00006761-M2020060), and the patient provided informed consent for publication of the case.

Discussion

This case report described a young woman who was infected with SARS-CoV-2 during pregnancy and who had a history of chronic HBV infection. Pregnant women are prone to liver damage, due to changes in liver structure and function, such as high basal metabolic rate, inactivation of large amounts of estrogen, and vigorous metabolites in the liver. Previous studies have reported that pregnant women with chronic HBV infection who have high alanine aminotransferase or who have been treated for less than 1 year before pregnancy have a higher risk of severe hepatitis after stopping antiviral drugs.⁸ A systematic review of 108 pregnant women from different countries showed that compared with SARS, Middle Eastern respiratory syndrome, influenza A, hepatitis E, etc., cases of severe illness and death among COVID-19 patients during pregnancy are extremely rare. A review determined that pregnancy causes changes in immunity which are conducive to an anti-inflammatory response and reduces the mortality rate of COVID-19.^{9,10}

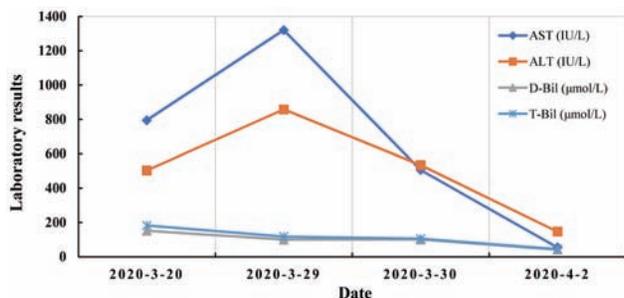


Fig. 1. Changes in laboratory tests for liver function during the infection and management course.

Clinical manifestations of COVID-19 patients during pregnancy are similar to those in non-pregnant patients, mainly including fever, dry cough, and dyspnea. In terms of treatment, since there are no specific antiviral drugs, supportive treatment is still the main method, such as sustained monitoring, maintaining hemodynamics and internal environment stability, and avoiding electrolyte disorders and dehydration.^{11,12}

Based on existing reports, SARS-CoV-2 has caused few diarrhea cases (3.8%). We consider that the patient's diarrhea was mainly caused by hepatic insufficiency.¹³ Differential diagnosis should include acute fatty liver of pregnancy and intrahepatic cholestasis of pregnancy. The main manifestations of the case included jaundice, fatigue, anorexia, and other gastrointestinal symptoms, but there was no hypoglycemia, low fibrin, nor evidence of acute kidney injury. Besides, no obvious hepatic structural lesions were observed by ultrasound, and diagnosis of acute fatty liver of pregnancy could be excluded. The transaminase level was significantly increased, and there was a history of chronic HBV infection, therefore, intrahepatic cholestasis of pregnancy was not fully supported.

There is still controversy about whether glucocorticoids should be used for COVID-19. SARS-CoV-2 has caused an excessively strong inflammation effect in COVID-19 patients, especially in critically ill patients, leading to severe multiorgan damage. Some experts believe that such anti-inflammatory effects can be applied to treat such patients. However, glucocorticoids can increase the risk of complications and delay the clearance of the virus. Even short-term use of glucocorticoids may cause venous thrombosis, fractures, sepsis, delayed virus clearance, and other steroid-related complications.¹⁴ It should also be noted that the efficacy and safety of glucocorticoids depend on the dosage. The RECOVERY trial conducted in the UK showed that the application of low-dose dexamethasone (6 mg q.d.) can reduce 28-day mortality.¹⁵ Treatment in this case revealed a small dose of dexamethasone is suitable for a short period to prevent the exacerbation of inflammation in COVID-19.

A meta-analysis showed telbivudine, lamivudine, and tenofovir to be safe during pregnancy and to not increase the incidence of adverse outcome events.¹⁶ In this case, the tenofovir antiviral treatment was promptly resumed based on adequate liver protection treatment, and a better curative effect was obtained. Coagulation dysfunction is more common among COVID-19 patients, especially for liver dysfunction in this case. Therefore, monitoring coagulation function during the treatment to prevent serious complications, such as disseminated intravascular coagulation, and emergency termination of pregnancy is considered when the condition worsens.¹⁷

In summary, we used a comprehensive treatment paradigm of antiviral, liver protection and low-dose dexamethasone to effectively treat this case of acute chronic hepatitis B with COVID-19 in pregnancy. The patient avoided termination of pregnancy and was well-healed. Pregnant women who meet the indications for viral treatment should receive standardized treatment in time to avoid severe hepatitis. This case shows that low-dose dexamethasone can be used safely and effectively in pregnant women with dual infection of HBV and SARS-CoV-2, to reduce systemic symptoms, which showed no obvious side effects that aggravated either viral infection.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Conception, design, and drafting of the manuscript (QYL), manuscript preparation with substantial contributions to intellectual content, critical editing and revisions of the manuscript (ZYA, CL, MZ, LC, NS, YYZ, JNZ, QGG).

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Occult Hepatitis B Reactivation after Liver Transplant: The Role of a Novel Mutation in the Surface Antigen

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Abstract

Occult hepatitis B infection is characterized by loss of hepatitis B surface antigen (HBsAg) and persistence of low levels of hepatitis B virus (HBV) replication that may or may not be detectable in plasma/serum. We present a case of HBV reactivation in a male patient who underwent orthotopic liver transplant for hepatocellular carcinoma secondary to active hepatitis C (HCV) infection. Pre-transplant, he was HBsAg-negative and hepatitis B core antibody-positive, with an undetectable HBV viral load that was incidentally found to be positive at a very low HBV viral load on the day of transplant. Post-transplant, his HBsAg remained undetectable, with an undetectable HBV viral load, until eradication of his HCV infection with direct acting antiviral agents. After eradication of HCV, there was reactivation of HBV, with a high viral load and emergence of serum HBsAg. A deep sequencing genetic analysis of his HBV both pre- and post-transplant revealed the presence of a mutation in the “a” determinant of the HBV surface antigen. The role of HBV genotype ‘a’ determinant mutation in HBV reactivation post-transplant is unknown and needs further examination. Our experience suggests a possible role for antiviral prophylaxis in these patients or monitoring of HBV viral loads post-transplant.

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Introduction

Hepatitis B virus (HBV) is a significant public health concern. Clinical manifestations depend on patients’ age and

immune status. Less than 5% of adult patients with acute horizontally-transmitted HBV develop chronic hepatitis, which is characterized by a persistent hepatitis B surface antigen (HBsAg) that indicates active infection. However, 90–95% of those who are infected at birth via vertical transmission will become chronic carriers.¹ Despite the ability of patients to clear HBsAg (*i.e.* revert to HBsAg seronegativity), HBV genomes can persist in liver tissue.² This state is known as occult hepatitis B infection (OHBI) and is characterized by loss of HBsAg and persistence of a very low level of HBV DNA, that may or may not be detectable in plasma/serum.³

OHBI may be the consequence of mutations that occur during viral replication. HBV replicates via an error-prone reverse transcriptase.⁴ Accumulation of mutations during this process results in a large pool of genetically distinct variants termed “quasispecies”. The ‘a’ determinant is a region of the HBsAg surface protein where most antigenic epitopes are located, and is the antigenic target of commercial hepatitis B vaccines.⁵ Mutations in this region can result in immune and vaccine escape variants of HBV. These variants can remain dormant not only in liver tissue but also in extrahepatic tissue, such as bone marrow and lymphatic tissues.⁶ These variants may not be detectable by routine immunoassays available in clinical diagnostic laboratories, necessitating advanced analysis, such as DNA sequencing.⁷

In addition to viral mutations, immune-biology and co-infections appear to facilitate OHBI.³ Up to 15% of patients with hepatitis C virus (HCV) may be co-infected with HBV.⁸ HCV often predominates, suppressing HBV replication and transcription.⁸ Several cases have demonstrated that clearance of HCV with direct acting antivirals allows HBV to resume replication and become HBsAg reactive.^{9,10} Other factors that appear to increase the risk of HBV reactivation include immune-suppressive medications.¹¹ Various interventional therapies for hepatocellular carcinoma (HCC), including surgical resection and locoregional modalities, may also increase risk of reactivation.¹²

Liver transplantation necessitates the use of post-operative immune-suppressive medications (*i.e.* “anti-rejection drugs”). Even in the absence of prophylaxis, the risk of seroconversion to HBsAg positivity after transplantation, in pre-transplant HBsAg-negative, hepatitis core antibody (anti-HBc)-positive recipients is thought to be rare, as long as the allograft is from an HBV naive donor. A French study reported an estimated risk of 1.5%,¹³ whereas in retrospective studies, both British and American centers have reported no HBV reactivations in their patients.^{14,15} There are no guideline recommendations that discuss HBV prophylaxis before or after liver transplantation in this specific group of patients. With these points in mind, we present a case of HBV reactivation after liver transplantation for

Keywords: Hepatitis; Hepatitis B; Reactivation; Seroconversion; Liver transplant; Transplant.

Abbreviations: CASL, Canadian Association for the Study of the Liver; DNA, deoxyribonucleic acid; HBsAg, hepatitis B surface antigen; HBV, hepatitis B Virus; HCC, hepatocellular carcinoma; HCV, hepatitis C Virus; OHBI, occult hepatitis B infection; OLT, orthotopic liver transplant; RFA, radiofrequency ablation; TACE, transarterial chemoembolization.

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HCC secondary to HCV cirrhosis in a pre-transplant HBsAg-negative, anti-HBc antibody-positive patient after post-transplant HCV therapy. Genotypic analysis subsequently identified a mutation in the "a" determinant within the surface antigen.

Case report

A Caucasian 60-year old male presented for orthotopic liver transplant (OLT) workup after diagnosis of HCC secondary to HCV infection. His comorbidities included Crohn's disease, for which he received monthly infusions of infliximab. He had been diagnosed with HCV infection on routine screening and developed cirrhosis 15 years after diagnosis. He carried HCV genotype 3a, with RNA level of 459,195 IU/mL. At that time, HBsAg and hepatitis B surface antibody were non-reactive. His anti-HBc was positive but HBV DNA level was undetectable (<20 IU/mL) (COBAS® Ampliprep/COBAS® Taqman® HBV v.2.0; Roche Diagnostics). He was, therefore, thought to have past HBV infection that had cleared.

Six years after developing cirrhosis, he was diagnosed with HCC and serial loco-regional therapies including transarterial chemoembolization (commonly known as TACE) and radiofrequency ablation (commonly known as RFA) were performed. Despite this, residual HCC persisted, and he received an OLT without complications. His immunosuppressants included tacrolimus, mycophenolate mofetil, and prednisone. Infliximab was held-off for the month after transplant and resumed after that. Crohn's disease remained in remission.

The patient did not receive antiviral treatment for HCV prior to the transplant. A few days post-transplant, his HCV RNA level was found to be 3,617 IU/mL. His plasma HBV DNA on the hospital admission day immediately pre-transplant was detectable for the first time at 645 IU/mL (Cobas® HBV; Roche Diagnostics) and was considered to be a contaminant, with a negative HBsAg finding. The donor liver was anti-HBc-negative, and a repeat HBV DNA determination post-transplant was undetectable (lower limit of detection, 25 IU/mL); no antiviral treatment was initiated. Two months after transplant, his HCV viral load increased to 2,621,255 IU/mL. Treatment was initiated with sofosbuvir/velpatasir (Epclusa®; Gilead Sciences) after completion of prednisone. A 12-week antiviral treatment with sofosbuvir/velpatasir was completed with achievement of a sustained viral response.

Despite previous undetectable levels of HBV DNA, the HBV DNA levels increased to 14,100,000 IU/mL 6-weeks after initiation of direct acting antiviral therapy for HCV. Additionally, there was seroconversion of HBsAg from negative to positive. Amplicon-based next-generation sequencing was utilized to investigate the presence of HBsAg mutations. Briefly, plasma samples were extracted on the MagNA Pure 24 (Roche) and eluted in 50 µL elution buffer. PCR was performed using 0.2 µM ILF (CGTGGTGGACTTCTCTCAATTTTC) and ILR (AGAAAGGCCTTGTAAAGTTGGCGA) using the Kapa HiFi enzyme on the LightCycler® 480 (Roche). A 50 ng aliquot of Agencourt bead purified PCR product was processed by 1D Native barcoding with EXP-NBD114 and SQK-LSK109 (Oxford Nanopore Technologies) and sequenced on flow cell FLO-MIN106D. FAST5 files were basecalled with Guppy V3.6.1. FASTQ files were analyzed and consensus sequence generated using Geneious V10.2.6, with 15,000 read coverage. Codons associated with immune escape were analyzed from codon 42 to the stop codon of the S gene. Genetic sequencing of HBV DNA demonstrated that a mutation in the 'a' determinant of the surface antigen, F/Y134H, was present in both pre-transplant and post-transplant plasma.

The patient currently remains HBV viral load undetectable, HBsAg detectable, with stable allograft function, while on tenofovir antiviral therapy.

Discussion

We have presented a case of HBV reactivation after liver transplantation and subsequent HCV antiviral therapy. Undetectable HBV DNA, negativity for HBsAg, and positivity for anti-HBc pre-transplant in addition to reactivation post-transplant suggests that this patient had OHBI. DNA sequencing performed on samples acquired before and after the transplant demonstrated the presence of mutations in the 'a' determinant of HBsAg, further supporting this hypothesis. This patient did not reactivate pre-transplant, despite treatment for HCC and regular infliximab therapy. He also did not suffer sustained reactivation, aside from a transient virologic breakthrough pre-transplant until eradication of his HCV infection when his HBsAg appeared for the first time with a sustained high HBV viral load. The appearance of HBsAg for the first time probably reflects the virologic heterogeneity of HBV within a given patient: the wild-type virus producing surface antigen was most likely silent prior to reactivation, but reactivation with significant viremia allowed it to re-emerge enough for surface antigen to become detectable.

In the absence of prophylaxis, the risk of seroconversion to HBsAg positivity in naive liver donors or isolated anti-HBc positive recipients is reported to be very rare.^{13–15} Studies of positive anti-HBc, HBsAg-negative patients receiving kidney transplants, which require higher level of immunosuppression, also appear to have less than a 1% risk of post-operative reactivation.¹⁶ These studies collectively appear to conclude that there is a low risk of post-transplant reactivation. The liver itself is the largest reservoir of HBV DNA, and its removal during transplantation may be a simple explanation for the low risk of post-liver transplant reactivation. However, it should be noted that these studies did not examine for 'a' determinant mutation. Although unknown at the current time, the presence of such mutations may confer a higher risk of reactivation.

Despite removal of the HBV reservoir in the liver, extrahepatic sources of HBV DNA, such as bone marrow or lymphatic tissue, may have been the source for HBV reactivation. In this case, the precipitating factors for HBV reactivation were multifactorial. Co-infection with, and therapy for, HCV likely contributed to post-transplant HBV reactivation in our patient. HCV usually predominates over HBV in co-infected patients and eradication of HCV can result in HBV reactivation.⁸ In a systematic review, Mucke *et al.*¹⁷ demonstrated that 24% of patients with positivity for HBsAg experienced HBV reactivation during direct antiviral therapy for HCV, compared to only 1.4% of patients who were HBsAg-negative. Local therapy for HCC, history of Crohn's disease maintained on infliximab, corticosteroids, and solid organ transplantation are considered to represent moderate to higher risk. Lazarevic *et al.*¹⁸ found that beside immunosuppressive factors (*i.e.* transplantation, corticosteroid use, direct antiviral therapy for HCC, and age-related immunosuppression), mutation in the 'a' determinant of HBsAg was essential for HBV reactivation in those receiving anti-CD20 monoclonal antibodies (*e.g.* rituximab). Therefore, the 'a' determinant mutation plays an important role in HBV reactivation and needs further investigation.

Multiple guidelines distinguish between chronic HBV infection and resolved HBV infection when making recommendations regarding prophylactic therapy.¹⁹ Guidelines by the Canadian Association for the Study of the Liver (commonly

referred to as the CASL) advocate for clinical monitoring without antiviral treatment in patients with normal alanine aminotransferase and HBV DNA levels <2,000 IU/mL.¹ Although patients with anti-HBc positivity are considered to be at higher risk of HBV reactivation, such categorizations are based on studies of non-transplant patients with their native liver. There are no guidelines that comment on post-transplant prophylaxis when the donor graft is HBV naive. There is also a paucity of studies that examine the clinical impact of 'a' determinant mutation and how it may affect reactivation.²⁰

Pre- and post-transplant screening with HBV DNA from serum/plasma can be utilized in the assessment of OHBI. Although OHBI can be diagnosed through HBV DNA detection in liver biopsies, validated assays are not routinely available to be incorporated into standard pre-transplant OHBI assessments.²⁰ Our case highlights the need to consider possible post-transplant prophylaxis in OHBI, but certainly post-transplant monitoring with nucleic acid testing, particularly in the context of multiple risk factors for reactivation. The identification of a surface antigen mutant may have contributed to reactivation in this case. Further studies are required to determine the relative risk of reactivation for 'a' determinant mutations post-OLT.

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None to declare.

Conflict of interest

Trana Hussaini is recipient of unrestricted research grants from Paladin Inc. Eric Yoshida is an investigator of clinical trials sponsored by Gilead Sciences, AbbVie, Merck, Intercept, Madrigal, Pfizer, Novartis, Allegan and Genfit, and is recipient of unrestricted research grants from Paladin Inc. and honoraria for CME/Ad Board lectures from Gilead Canada, Abbvie Canada, Merck Canada, Intercept Canada and Celgene Canada. The other authors have no conflict of interests related to this publication.

Author contributions

Literature review and drafting of the manuscript (HKB, DC), editing of the manuscript (DC, TH, EMY, CFL, and GR), provision of resources (TH, EMY, CFL, GR), laboratory investigations (CFL, GR), review for important intellectual content (DC, EMY, CFL, GR).

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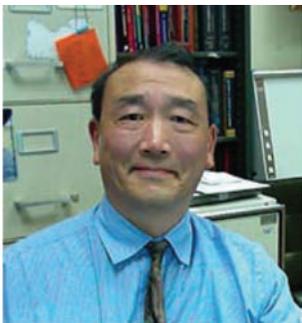
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