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Editorial Board Member of *World Journal of Hepatology*, Fátima Higuera-de la Tijera, MD, MSc, PhD, Academic Research, Doctor, Professor, Department of Gastroenterology and Hepatology, Hospital General de México, Dr. Eduardo Liceaga, Mexico City 06726, Mexico. fatimahiguera@yahoo.com.mx

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REVIEW

Hepatitis C virus: A critical approach to who really needs treatment

Elias Kouroumalis, Argyro Voumvouraki

ORCID number: Elias Kouroumalis 0000-0002-6875-906X; Argyro Voumvouraki 0000-0002-2725-6028.

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Elias Kouroumalis, Department of Gastroenterology, University of Crete Medical School, Heraklion 71500, Crete, Greece

Argyro Voumvouraki, First Department of Internal Medicine, AHEPA University Hospital, Thessaloniki 54621, Greece

Corresponding author: Elias Kouroumalis, MD, PhD, Emeritus Professor, Department of Gastroenterology, University of Crete Medical School, Voutes, Heraklion 71500, Crete, Greece. kouroumi@uoc.gr

Abstract

Introduction of effective drugs in the treatment of hepatitis C virus (HCV) infection has prompted the World Health Organization to declare a global eradication target by 2030. Propositions have been made to screen the general population and treat all HCV carriers irrespective of the disease status. A year ago the new severe acute respiratory syndrome coronavirus 2 virus appeared causing a worldwide pandemic of coronavirus disease 2019 disease. Huge financial resources were redirected, and the pandemic became the first priority in every country. In this review, we examined the feasibility of the World Health Organization elimination program and the actual natural course of HCV infection. We also identified and analyzed certain comorbidity factors that may aggravate the progress of HCV and some marginalized subpopulations with characteristics favoring HCV dissemination. Alcohol consumption, HIV coinfection and the presence of components of metabolic syndrome including obesity, hyperuricemia and overt diabetes were comorbidities mostly responsible for increased liver-related morbidity and mortality of HCV. We also examined the significance of special subpopulations like people who inject drugs and males having sex with males. Finally, we proposed a different micro-elimination screening and treatment program that can be implemented in all countries irrespective of income. We suggest that screening and treatment of HCV carriers should be limited only in these particular groups.

Key Words: Hepatitis C, Comorbidities, Screening and treatment policy; Hepatitis C virus; Review

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Core Tip: Elimination of hepatitis C virus (HCV) by 2030 according to the World Health Organization policy seems highly unlikely because of the funding re-direction due to the coronavirus disease 2019 pandemic. It is important therefore to re-evaluate the treatment policies based on a more realistic and feasible approach. HCV disease has a very prolonged natural course, and even HCV-related cirrhosis has a lower mortality compared to other cirrhosis etiologies. However, liver related morbidity and mortality is increased when certain comorbidities accompany the initial HCV infection. A review of the current knowledge allows for a more or less accurate identification of these comorbidities. Therefore, an eradication program is proposed based on screening and treating only these particular groups.

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INTRODUCTION

Hepatitis C virus (HCV) is genetically variable. Seven genotypes and more than 60 subtypes have been identified so far. Genotype 1 is the most prevalent worldwide[1]. A 30% difference among genotypes and a 15% difference among subtypes of the same genotype exist at the nucleotide level. In addition, an enormous diversity within the same infected individual may be present in the form of the so called quasi-species differing by 1%-10% in the nucleotide sequence[2,3]. They are categorized on the basis of the hypervariable regions in the envelope protein and the nonstructural 5A protein. These quasi-species are generated through error-prone replication and pose the major obstacle in the development of an effective vaccine. The E2 variability is mainly located in three highly variable regions designated as HVR1 (aa384–409), HVR2 (aa460–485) and igVR (aa570–580)[4,5].

With an estimation of approximately 2 million new infections each year, the real number of individuals harboring HCV may well be underestimated[6]. Most new cases go undetected because they are mostly asymptomatic. Underestimation is not limited to low-income countries. In the United States the incidence of new HCV cases has nearly doubled in the past 10 years primarily because of people who inject drugs (PWID). It is suggested that the recorded increase is only a fraction of the true number as the majority probably escapes detection[7,8].

Intravenous drug use is not the main route of transmission in low and middle income countries. In Egypt, PWID was a risk factor only in urban areas, while the main risk factor was hospital care[9,10] and intra-familial transmission of the virus[11]. This is also true for Greece[12]. Increased incidence of the disease has also been reported in China but without an epidemiological explanation[13]. In the European Union one of the additional problems is the increased immigration from countries with a high prevalence of HCV. A crude estimation has shown that approximately one in seven adults infected by HCV in the 31 countries of the European Economic Area is a migrant, and at the same time HCV prevalence is also high in some Eastern European countries. These facts pose a challenge for the budgets of many European Union countries[14].

In 2016, the World Health Organization (WHO) initiated a campaign to reduce HCV infection rates by 90% by 2030 using the new and highly effective direct acting antivirals (DAAs). The campaign called for an increase of HCV screening and unlimited access to DAA treatment[15].

However, there are inherent limitations to this approach[16,17]. Even in the most favorable trials, DAAs do not achieve viral elimination in as many as 2%-10% of cases. Moreover, there is mounting concern that DAAs can select resistant variants that may reduce their effectiveness. Also, DAAs are expensive for most developing countries, while asymptomatic cases pose a worldwide problem as only 20% of infections are diagnosed and of those only 15% are properly treated[18]. This is further complicated by the fact that many new cases are found in marginalized populations like PWIDs and males having sex with males (MSM). These groups pose an additional problem as they are prone to reinfection[16].

As a result, only a few countries will be able to eliminate HCV by 2030. High-income countries will not achieve HCV eradication before 2050 as 80% of them are not on track to meet HCV elimination targets by 2030 and 67% are off target by at least 20 years[19,20]. The vast majority of low and middle-income countries are at a very preliminary stage[21-23]. Unfortunately, the latter are countries like Pakistan, Egypt, China and India, which have the largest numbers of chronic HCV infections.

The coronavirus disease 2019 (COVID-19) pandemic has dramatically changed health priorities around the world. It is not surprising that financial resources have been redirected in an effort to fight the new enemy. Therefore, major problems in the different programs of HCV elimination are to be expected even among high-income countries. Disruptions to hepatitis programming have already been reported in Egypt and Italy, two countries with different incomes and COVID-19 problems, and many more are certain to follow[24]. Moreover, the odds are against the control of HCV infection without an effective and widely available vaccine[25,26].

Therefore, in the present review, the problems of managing chronic hepatitis C will be analyzed, the populations that are in real need for treatment will be identified and a new, more realistic target of treatment will be proposed that may overcome the difficulties in the global effort to eliminate the disease.

CAN WE ERADICATE HCV BY 2030 WITH DAAS TREATMENT?

The number of new HCV infection rates is increasing worldwide despite the use of the highly effective DAAs. Most of the new infections go undetected as they are asymptomatic. Only 50% of infected people are aware of their infection in the United States[27]. DAAs do not protect from reinfection. Therefore, certain marginalized groups like intravenous drug users may be reinfected[17].

Yet, many investigators are convinced that eradication is possible by treating all HCV carriers, including pediatric patients[28], even in low-income countries provided that cost reduction policies will be adopted[29]. An even more optimistic view has been presented. Since 2015, after the wide use of DAAS in Spain, a profound reduction in HCV cirrhosis hospitalization has been reported. The investigators predicted that by 2025, HCV-cirrhosis will have practically disappeared as a cause of hospital admissions[30]. However, in most instances conditions are set and reservations are expressed (Table 1).

A model projection of the impact of DAA treatment on the elimination of HCV infection showed that the WHO target for 2030 could be achieved only after an annual net regression of 7%.Currently the projections made indicate that the annual regression is only 0.4% worldwide, and therefore elimination will be impossible[31]. Projection studies of different scenarios[32], such as those including countries with a high prevalence of HCV such as Pakistan[33], have indicated that HCV elimination may be feasible but only after substantial new investments from national budgets even in Europe[34]. Conditions that should be fulfilled before elimination can be on target have been described for special populations like HIV + PWID or HIV + MSM. Harm and behavior risk reduction interventions in addition to DAA treatment and a substantial increase of screening, showed improved results[35].

Treatment failure and non-compliance

One hundred percent sustained virologic response (SVR) is not feasible even with the latest DAAs. The best treatment results are reported as 90%-97% in most series. This means that 3%-10% of treated patients do not achieve an SVR and may become resistant to available regimens[36]. Response rates may even be lower than 90% in certain groups. Thus, in a recent large meta-analysis reviewing 49 reports, SVR was lower than 90% in 11/49 studies of patients without hepatocellular carcinoma (HCC) [37]. In a real-life cohort of cirrhotic patients, viral clearance was obtained by 88% (175/199) of patients with varying responses among genotypes[38].

Compliance with treatment is another obstacle in the eradication of HCV. This is particularly evident in special groups. In a recent study, compliance was associated with the time of drug dependence. Those with shorter periods of drug dependence had the highest compliance with an inadequate 47% compared with the even worse 38% of longer-time users[39]. In France, drug uptake was low in HCV patients with PWID and alcohol use disorders (AUD) despite an improvement after DAA introduction as compared to interferon (IFN)-based treatments[40].

Table 1 Reasons for failure of the World Health Organization 2030 hepatitis C virus elimination program

Treatment failure and non-compliance
Resistance of old and new subtypes
Occult HCV
Reinfection: People who inject drugs; Males having sex with males
Cost of treatment

HCV: Hepatitis C virus.

Resistance of old and new subtypes

Quasi-specie is the concept that explains the development of resistance to DAAs[41]. Low levels of resistance-associated substitutions < 15% in the NS5A region have no significant effect on treatment outcomes, but proportions greater than 15% at baseline are associated with treatment failures[42]. Furthermore, presence of NS5A resistance-associated substitutions may be responsible for the low 83% SVR reported in the ASTRAL-4 trial in certain genotypes[43]. Similarly, in a Chinese study of sofosbuvir (SOF)/velpatasvir (VEL), NS5A resistance was responsible for 89% SVR in non-cirrhotic patients with subtype 3b, while only 50% of cirrhotic patients achieved SVR after 12 wk of SOF/velpatasvir[44].

It is clear today that baseline resistance-associated substitutions to DAAs can have a significant effect on the treatment of "older" genotypes preventing SVR in many patients[45].

However, the real problem occurred when newly identified subtypes appeared in many countries. HCV classification now includes a larger number of subtypes, like 1 L, 2r, 3k, 4w, 6xa, 7a and 8a, 6 and probably many more[1]. They were extremely rare in countries where research is usually done, but they are relatively common in some low and middle-income countries as well as in immigrants in Europe and North America originating from these countries. Some of those subtypes are inherently resistant to several NS5A inhibitors[46,47].

In line with these findings, a Rwandan study with SOF/ledipasvir (LDV) showed an SVR rate of only 56% in the subtype 4r, much lower than the 93% SVR in patients with other genotype 4 subtypes[48]. A very recent report studied infections with unusual subtypes from Africa and South East Asia. The report claims that only pibrentasvir (PIB)was effective against all, and the recommendation given is to use a combination of glecaprevir (GLE) and PIB as first-line treatment for these patients. However, no data that these subtypes were indeed sensitive to GLE were presented [49].

It should be noted that in these countries only generic DAAs with an inherent resistance against these subtypes exist. It will therefore not be long before these resistant subtypes spread across Europe or North America. In an unselected cohort of African immigrants infected with an unusual genotype, including the novel subtype 1p, the SVR was 75%[50]. These studies indicate that the need for research on new drugs should not stop because these failures could jeopardize all HCV elimination efforts[51,52].

Occult HCV

Occult HCV infection (OCI) refers to the presence of HCV RNA in hepatocytes or peripheral blood mononuclear cells (PBMCs), without HCV RNA in serum. OCI has been demonstrated in hemodialysis patients, HCV/HIV coinfection and HBV-HCV coinfection but also in 3% of the general population[53]. In a recent study, the prevalence of OCI in HIV-infected individuals was 11.4%. Most patients were infected with the subtype 3a followed by the subtypes 1a and 1b[54]. OCI is also common in patients with malignant lymphoproliferative disorders. Ultrastructural examination of PBMCs demonstrated intracytoplasmic vacuoles enclosing viral-like particles[55]. The prevalence of OCI after treatment with DAAs was found to be considerably high. As a consequence, a dual testing for HCV RNA done in both PBMCs and serum at the end of treatment with DAAs as well as during validation of the SVR was recommended [56], a reasonable suggestion that will however increase the cost. Parts of the HCV 5′-noncoding region genomic RNA sequences were demonstrated at the DNA level in the extrachromosomal circular DNA fraction of PBMCs resulting in OCI[57].

Reinfection

The problem of reinfection may become a real obstacle in the near future. Reinfection mostly occurs in patients with persistent risk factors like PWID. A 10%-15% risk of reinfection after 5 years of SVR has been repeatedly reported[58]. China, Russia and the United States have the highest number of PWID with a very high HCV prevalence, and therefore the risk of reinfection is also very high. An additional issue is that reinfection rates have not been studied in low-income countries where both intra-community transmission and transmission through medical practice are still very common[59]. A recent meta-analysis of 36 studies has demonstrated a relapse rate of around 6/100 person years among drug users. There was no difference between IFN-based and DAA treatment, but patients receiving opioid agonist therapy had a lower reinfection rate[60].

Most HCV cases are reinfected with a different strain of the virus after SVR. However, some patients relapse with the same strain they had at the commencement of treatment[61,62]. Interestingly enough, reinfection rates seem to be higher after spontaneous clearance of the virus than after successful DAA treatment (at least for the first few years of the reported follow-up)[63].

Relapse or reinfection by a new strain is possibly related either to exhaustion of HCV-specific T cells or to the emergence of escape mutations. In this respect, it is interesting that DAAs do not lead to a reversion of the T cell exhaustion observed in chronic HCV infection[64].

Cost of treatment

An additional serious problem in the HCV elimination project is the cost of treatment. Most studies consider eradication programs cost-effective with almost all of them using Markov mathematical models.

A study from Switzerland claimed that a break-even for the health system will be achieved by 2031 if all fibrotic stages were treated[65]. In the United States, treatment of all eligible HCV patients with SOF/LDV would require investing 65 billion dollars over the next 5 years[66].

A more recent study in the United States reported that the pangenotypic SOF treatments (SOF/VEL, SOF/VEL/voxilaprevir) were considerably more cost saving than the equally effective GLE/PIB treatment[67]. In a Hong Kong study, elbasvir/grazoprevir was the least costly DAA treatment, dominating over most other DAAs for genotype 1 patients at a quality-adjusted life-year of 9000-11000 United States dollars[68].

In India, the use of SOF/VEL is proposed over SOF/ledipasvir or SOF/daclatasvir (DCV) to all HCV-infected people but only if there are no budget constraints. If budget is a problem, SOF/VEL is recommended for cirrhotic patients only[69]. In a recent study from Japan, GLE/PIB generated higher quality-adjusted life-years and lower lifetime costs compared to all other DAAs[70].

Even in low income-countries like Vietnam, all DAA treatments for patients with genotypes 1 and 6 were cost-effective, but the combination of SOF/VEL was the most cost-effective among them[71].

In contrast, SOF/VEL treatment was not the most cost-effective option for patients with genotype 1b compared with other oral DAA agents in China. Therefore, a price reduction of SOF/VEL would be necessary to make it cost-effective and simplify treatment, achieving thus the goal of HCV elimination[72].

In Italy, a Markov model has shown that treating all HCV patients at an early stage of the disease is cost effective for the Italian Healthcare System[73].

A different approach was adapted in another Italian study that estimated the break-even point in time. This is the period of time required for the total saved costs to be recovered by the Italian National Health System investment in DAA treatment. The break-even point in time was not achieved for those treated in 2015 due to high DAA prices and severity of disease of treated patients. On the other hand, the estimated break-even point in times were 6.6 years and 6.2 years for those treated in 2016 and 2017, respectively. The total cost savings after 20 years would be €50.13 million and € 55.50 million/1000 patients treated in 2016 and 2017, respectively[74].

In the elimination scenario, viremic cases would decrease by 78.8% in 2030 compared to 2015. The direct and indirect costs would range from €3.2-3.4 billion by 2030, but elimination of HCV would be impossible without an extensive screening program. The WHO elimination strategy can possibly be achieved and is cost-saving despite the financial uncertainty of DAA cost in Greece[75].

In Spain, incremental cost-effectiveness ratio for screening of the general population plus treatment was reported to be below the accepted willingness-to-pay thresholds in

most studies, and therefore screening plus DAA strategy is considered to be cost-effective[76]. The same results were reported from Belgium where a policy of broad screening plus DAA treatment was advocated[77].

In Germany by contrast, after a cost analysis, the recommendation was to screen all of the PWID population while applying a less extensive screening in the MSM and general population[78]. Screening all South Korean patients twice followed by SOF/LDV treatment was cost-effective as compared to the current high-risk screening, while GLE/PIB was not cost-effective[79].

However, there are many reservations as to the cost-effectiveness of the WHO elimination program[80]. The amount required for Pakistan has been estimated at 3 billion United States dollars[33]. A price analysis of some of the most commonly used DAAs across 30 countries has shown that DAAs are unaffordable in many countries. Prices at that time were variable and unaffordable, being a threat to the sustainability of many health systems[81]. Treatment prices have since fallen and are currently 25000 dollars in the United States. Even so, this cost is prohibitive without medical insurance. Only 37% of patients were treated in the United States for financial reasons [82]. Pharmaceutical companies are allowed to sell DAAs at higher prices in high-income countries and at low prices in low-income countries[83]. In Pakistan, generic drugs brought the cost of DAAs down to 60 United States dollars per treatment, which is the lowest price in the world[31].

The cost of quality-adjusted life-year gained in 60-year-old patients was approximately 9000 United States dollars at current DAA prices in Japan. HCV treatment would only be cost-effective within the next 5-20 years after a price reduction of 55%-85%[84].

In low-middle income countries of Africa, elimination programs cannot be financially viable without a substantial increase in health funding or a gross decrease in assay prices. Screening strategies would require 8%-25% of the annual health budget in these countries only to diagnose 30% of HCV-infected individuals[83].

However, an alternative low cost approach is usually neglected. A study in Dundee, Bristol and Walsall has demonstrated that needle and syringe programs are effective and low-cost interventions in the highly vulnerable PWID population, at least as far as transmission reduction is concerned[85].

An earlier report has indicated that Markov models have many flaws. Alcohol drinking is a factor that is not acknowledged in many cost analyses. Yet alcohol influences the outcome of HCV infections[86].

A serious event has changed all estimations. The severe acute respiratory syndrome coronavirus 2 pandemic has redirected a substantial amount of national health spending in almost all countries worldwide. Even if recent estimations expect approximately 72000 more liver-related deaths after 1 year of the elimination program delay, the number is small compared to more than 1 million deaths attributed to COVID-19 disease in less than a year[24].

In view of the above, one question still remains. Why are more expensive recommendations proposed when all reported trials indicate > 90% SVR? This is evident if one compares the European Association for the Study of the Liver (EASL) recommendations of 2017, 2018 and 2020. Resistance cannot be the sole answer[87-89]. Three SOF-based treatments not only had SVR > 90% but their effects were equally fast in a series of patients with advanced fibrosis or cirrhosis[90]. Even low-priced generic DAAs were both clinically effective and cost-effective[91,92]. Similar efficacy between SOF/DCV and SOF/VEL in genotypes 2 and 3 was also reported in a real world study [93]. In a very recent study on genotype 2, SOF in combination with DCV, LDV or VEL and GLE/PIB had similar high SVR rates, irrespective of cirrhosis or chronic kidney disease[94], and similar results were reported in a real-life recent study of three regimens including SOF/DCV, SOF/VEL and GLE/PIB tested in genotype 3[95]. SOF/LDV SVR rate was 97% compared to 100% of SOF/VEL in GT6 after 12 wk of treatment[96,97]. In a meta-analysis of 34 studies, SOF/DCV, SOF/VEL, SOF/VEL/voxilaprevir and GLE/PIB were found to have similar SVRs in both non-cirrhotic (95.24%) and cirrhotic (89.39%) patients[98]. Better results for SOF/LDV compared to SOF/VEL were reported in PWID[99,100].

It should be noted however that no real direct comparisons exist in most reported studies as exemplified in an emulated randomized trial where SOF/VEL were equally effective as SOF/LDV in genotype 1 patients[101].

Although cause-specific analysis demonstrated that persons with SVR were less at risk for liver-related mortality than those without SVR or never treated patients, the expected decrease in overall mortality has not yet been observed. These findings may raise hopes for the future, but the final elimination result remains to be proven[102].

No analysis of difficulties in HCV eradication would be complete without mentioning HCV vaccines. It is almost impossible to achieve complete HCV elimination without a vaccine, but the real problem is that we cannot have a vaccine, at least not for the time being. An excellent review on the reasons why HCV cannot be eradicated without an efficient vaccine has been recently published[25].

A model based on 167 countries showed that only between 0 and 48 countries could achieve an 80% HCV incidence reduction without a vaccine. The number went up to 15–113 countries with a 75% effective vaccine and 10-year duration of protection, while billions of United States dollars could be saved across 78 countries at a cost of 50 United States dollars per vaccination[103].

Spontaneous clearance of HCV has been correlated with the development of neutralizing antibodies[104] targeting the E2 envelope protein of the virus[105]. An earlier study showed that induction of neutralizing antibodies was feasible in chimpanzees and human volunteers[106]. CD4 T cell responses against envelope proteins E1 and E2 were also observed in humans[106,107]**.** For the time being vaccines based on proteins of the E2 hypervariable region are not producing satisfactory results[108].

A different vaccine based on the use of a replication defective adenovirus containing the entire non-structural proteins was also developed[109,110]. This vaccine elicited a broad and durable CD4/CD8 T cell response, IFN-g production and memory cell development in humans. The same group developed and tried in a phase 1 human trial a vaccine based on a replication defective simian adenoviral vector and a modified vaccinia Ankara vector containing the NS3, NS4, NS5A and NS5B proteins of the genotype 1b. An effector and T cell memory response were developed[111].

However, a randomized, double-blind, placebo-controlled phase I and II clinical study mostly on PWIDs who received either the vaccine or placebo did not show a protective effect against the development of viral persistence. The protection provided was no greater than that provided by the placebo[112]. Recent investigation on HCV vaccine adjuvants demonstrated that the induction of a strong T and B cell immunity is enhanced by choosing the right adjuvant[113]. The subject of vaccines producing neutralizing antibodies has also been briefly reviewed in two recent editorials[114, 115].

HCV INFECTION: HOW DANGEROUS IS IT REALLY?

It is estimated that 5%–20% of patients with chronic HCV will develop liver cirrhosis [116,117], while approximately 25% of subjects will clear the virus after the initial infection[118]. It is also stated that approximately 27% of liver cirrhosis and 25% of HCC cases are attributable to chronic HCV[119]. This estimate is based on studies of patients with advanced liver disease.

Efforts to determine the natural history of HCV are not easy because of the inherent characteristics of the disease. Its onset cannot be verified with certainty and its course may be unusually prolonged. Disease outcome reports were mainly based on retrospective studies concluding that at least 20% of chronic infections develop cirrhosis within 20 years of the disease onset. By contrast, studies that used a retrospective/prospective approach have produced different results at least in certain population groups. Among young people, particularly young women, spontaneous resolution was more common than previously thought, and cirrhosis developed in only 5%of infections or less. The major drawback of most studies is that natural history studies rarely exceed the first 20 years so that evolution beyond this time is usually the result of predictions through models. An additional problem is that many confounding factors that influence outcome are not taken into account[120].

The first paper that raised questions about the real natural course of HCV was reported almost 30 years ago. An accurate infection time was ascertained for over 94% of the 568 patients, as participants were infected by transfusion. More importantly, two control groups (526 first controls and 458 second controls) were also included. After an average of 18 years, life-table analysis showed that all-cause mortality was 51% for those with transfusion-associated non A non B hepatitis compared to 52% and 50% for the control groups. The survival curves for the three groups were almost identical. Liver-related mortality was 3.3%, 1.1% and 2.0%, respectively. (P = 0.033 between hepatitis and the combined control group). Most importantly, 71% of liver-related deaths occurred in patients with chronic alcoholism. There is no specific mention of the causes of non-liver mortality in all groups, but one can assume that controls had more cerebrovascular accidents than the hepatitis group[121].

As a result of this study, one could further assume that if a curative treatment was given leading to a reduction of liver-related deaths in the hepatitis group, then survival would be more than the general population, which is rather odd**.** However, there may be a plausible explanation after the demonstration of changes in the lipid profile of HCV patients[122,123]. HCV-infected patients show significantly lower levels of cholesterol with a reduction of low-density lipoprotein levels compared to healthy controls irrespective of the degree of hepatic fibrosis[124,125]. Viral clearance increases levels of low-density lipoprotein and total cholesterol[126]. Therefore, it is reasonable to assume that after treatment more cerebrovascular accidents will equate the overall mortality of patients with general population mortality.

Mortality and morbidity data of the same patients and controls after approximately 25 years of follow-up were presented. All-cause mortality was 67% among 222 HCV cases and 65% among 377 controls. Yet again, liver-related mortality was 4.1% and 1.3%, respectively (P = 0.05). Interestingly, among the 129 living persons with transfusion-associated hepatitis, 70% had proven HCV and 30%, non-A-G hepatitis. In addition, 17% of all those originally HCV infected had cirrhosis[127].

There have been more studies verifying the fact that HCV may be a rather benign condition unless a harmful confounding factor like alcohol is added. Thus, many children acquired HCV infection after cardiac surgery before blood donors were screened for HCV in Germany. After about 20 years, the virus had spontaneously cleared in many patients, and the clinical course in those with chronic infection was more benign than expected when compared to infected adults[128].

A very long follow-up of 45 years was reported in a group of military recruits. HCV-positive persons had very low liver-related morbidity and mortality rates suggesting that healthy HCV-positive persons may be at low risk for progressive liver disease[129].

Older patients with tuberculosis sequelae, transfused at a younger age, entered a study approximately 30 years after the infection and were followed up for an average of 5.7 years after entry. Overall, 64% of HCV-infected and 57% of non-infected people had died. The main cause of death was tuberculosis sequelae in 42% and 46% of patients, respectively. Although liver-related deaths during follow-up were higher in HCV-infected patients, the overall mortality was similar between the two groups[130].

Similar findings were reported in a group of transfusion recipients from Denmark. After 22 years of follow-up no difference was found in all-cause mortality between HCV-exposed and non-exposed patients, but liver-related mortality was higher in the HCV group[131]. Even in specific high risk groups similar results have been reported. In a cohort of community-acquired HCV among mostly PWIDs, only 8% had overt cirrhosis, while no HCC cases were identified after 25 years of follow-up. Moreover, HCV-infected individuals were 8 times more likely to have committed suicide or die from drug overdose than from liver-related disease[132].

In a cohort of 1667 PWID with HCV infection for a mean of 14 years and followed up for an additional mean of 8.8 years, a low incidence of liver-related mortality of 3/1000 years was found. The risk of end-stage liver disease was higher for persons who ingested more than 260 g of alcohol per week. Two hundred and ten of these patients without advanced liver disease were randomly selected to have a liver biopsy. Only 2 had cirrhosis (1%)[133].

In another PWID cohort, an all-cause high mortality rate of 1.85/100 person-years after 33 years of follow-up was found, but it was not related to persisting HCV infection. Intoxication and suicide were the main causes of death[134].

Using statistical models for untreated HCV-infected PWIDs, the time to cirrhosis was estimated to be 46 years, and estimations for the time required to reach the F3 fibrosis stage was 38 years using stage-specific estimates[135].

Nine hundred and twenty-four patients with a known date of transfusion-acquired HCV were followed up for 16 years after infection in the United Kingdom. They were compared to 475 not infected transfusion recipients. All-cause mortality was not different between cases and controls, but the risk of liver-related mortality was higher among cases. It should be noted that nearly 30% of the HCV-infected cases that died directly from liver disease had also reported excessive alcohol consumption[136].

Almost identical data were reported from a cohort of East German women who were also exposed to HCV *via* contaminated Rh immunoglobulin. Clinical cirrhosis was identified in 4 (0.4%) of 917 infected women, 2 of whom died, 1 from HBV coinfection and 1 as a result of heavy alcohol use. Among the 403 chronically infected women in this study who underwent liver biopsy after a mean duration of infection of 20 years, only 3% exhibited bridging fibrosis, and none had cirrhosis[137].

Interestingly, more women of the initial group were later identified and followed up for 25 years. In total, 1980 women representing 70% of the total cohort were re-examined; 46% were positive for HCV RNA. Only 0.5% had overt liver cirrhosis, 1.5% developed pre-cirrhotic stages and only one HCC was diagnosed. Ten died of HCV-related complications, but in five of those an additional comorbidity was present[138].

Since this was an ideal homogeneous population to investigate the course of HCV infection, 718 patients were further evaluated at 35 years after inoculation. Four groups were compared for liver disease and mortality: self-limited HCV, untreated chronic HCV and treated condition with or without SVR. Overall, 9.3% of patients had clinical signs of cirrhosis. End stage liver disease was higher in the non-SVR group (15.3%), whereas the overall survival was significantly higher after SVR compared to untreated patients or non-SVR. However, looking at the details of the study, it is obvious that the survival probability is significantly decreased only in overweight women and even more so in cases of obesity. The same is true for the development of extensive fibrosis or cirrhosis[139].

A similar study from Ireland included 376 women with anti-D contamination, infected with HCV and followed up for about 17 years. Liver biopsies showed inflammation in 356 of 363 women (98%). The inflammation was mild (41%) or moderate (52%). Only 7 women (2%) had probable or definite cirrhosis, and of those only two reported excessive alcohol consumption[140].

After 5 additional years of follow-up, inflammatory scores were increased in 18% and decreased in 28% of patients. Forty-nine percent of patients had no change in fibrosis, 24% showed regression and only 27% showed progression, while 4 patients (2.1%) developed cirrhosis. Given the age of these women, currently in their fifth decade, some may still be at risk for more advanced liver disease, but for most of these patients it appears unlikely[141].

A third follow-up paper appeared presenting data from 36 years after contamination. In total, 682 patients were studied, including the 374 women chronically infected with HCV. Nineteen percent of them had developed cirrhosis, while all-cause mortality was 13% (4.9% died from liver-related causes) as opposed to 6.8% of the non-infected women. Liver-related, but not all-cause mortality, was significantly higher between the two groups. At face value, the results indicate that cirrhosis and liver-related deaths had increased during the last 5 years of follow-up. However, when factors associated with cirrhosis were examined, high alcohol intake, diabetes mellitus and obesity were present in the majority of cirrhotic women. It should also be noted that over two-thirds of cirrhotic patients were still alive[142].

Hemophiliacs were a group of patients heavily infected with HCV prior to the introduction of viral inactivation of factor concentrates and blood screening. Only hemophiliacs with HCV/HIV coinfection had a lower survival compared with HCV mono-infected who had a similar mortality with non-infected hemophiliacs over 10 years. There was no survival gain from anti-HCV treatment (IFN) or from achievement of an SVR[143].

One hundred and six HCV RNA positive hemophiliacs were followed up for approximately 30 years, and 34% were HIV coinfected. All-cause mortality was 44% in HIV positive patients and 29% in HIV negative patients. Liver-related mortality was 12.5% and was not different between the two groups. In this cohort as well the probability of cirrhosis was significantly increased when either HBV coinfection or substantial alcohol consumption were present[144].

There have been studies indicating that HCV infection is associated with both a high rate of cirrhosis and/or high mortality. However, these are in disagreement with studies where the contamination time point can be ascertained. Most mortality studies report on cirrhotic patients, *i.e.* the final stage of HCV infection usually comparing mortality without and with viral elimination. Thus, an Italian study showed that patients with compensated HCV cirrhosis achieving SVR by IFN obtained a significant benefit as they level their survival curve to that of the general population. But the controls were based on the standardized mortality ratio of the age and sex matched general population. Importantly, no mention of drinking or metabolic syndrome was made[145].

Higher mortality rates compared to the general population even after SVR were reported from Denmark. The time point of contamination was not ascertained. Half of the patients were heavy drinkers[146]. Similar results were also reported in 1824 patients from Scotland, followed up for an average of 5.2 years after SVR. In total, all-cause mortality was 1.9 times more frequent for SVR patients than the general population. Characteristics associated with increased mortality were markers either of heavy alcohol consumption or intravenous drug use. Without these behavioral markers an equivalent survival to the general population was noted[147].

A reduction in serious liver-related events was also noted in a prospective study of patients with HCV-compensated cirrhosis who achieved an SVR. SVR reduced overall mortality and risk of liver-related deaths. However, metabolic features were associated with a higher risk of HCC in patients with SVR. No alcohol consumption was reported [148].

In a study from Japan, the presence of HCV viremia increased mortality, mainly due to liver-related deaths. There were no biopsies at the beginning of the study, and the cause of death was based on death certificates[149].

Paid blood or plasma donors were studied after a median of 27 years from the last blood donation to the time of survey. HCV RNA was detected in 592 individuals. A high 27.2% of them were considered to have cirrhosis by liver stiffness measurement. Almost 50% of the total were overweight. No intravenous drug use was mentioned, and insulin resistance (IR) was significantly higher in HCV-infected blood donors compared to the non-infected ones. More importantly, the time point of infection was not known[150].

In another study of paid plasma donors with HCV infection from China, liver cirrhosis or HCC developed in 10.00% of individuals, with a liver-related mortality of 8.18% after 12-19 years of follow-up. Alcohol was again a risk factor involved in the outcome of HCV infection[151].

In a recent study, age-sex standardized mortality ratios for patients with an HCV infection were calculated and compared to the general population. All-cause age-sex standardized mortality ratio were 2.3 times higher. No confounding factors like alcohol, obesity or HIV coinfection were reported in detail[152].

The significance of confounding factors is clearly exemplified in a recent study by the same group where the median age of death was lower in persons with evidence of HCV than the general population (53 years *vs* 81 years). A significant proportion of persons with HCV died of external causes, liver disease and HIV compared to the general population[153].

A recent investigation reported on the natural history of HCV in children and young adults after a median follow-up of 33 years. The modes of transmission were intravenous drug use (53%), blood product exposure (24%) and perinatal infection (11%); 55% of them were treated with IFN and the rest with DAAs. SVR was obtained in 75%. Mortality rate was higher in patients without SVR *vs* those with SVR, but SVR did not abolish mortality altogether. Almost 40% of patients consumed alcohol heavily or were HIV coinfected. No mention of the presence of metabolic syndrome was reported[154].

Mortality is not high even in patients with advanced/decompensated HCV cirrhosis with Model for End-Stage Liver Disease (MELD) > 10. In a recent long-term follow-up after initiation of treatment with DAAs, a meaningful decrease of 3 or more in MELD occurred in 29% of patients, while a final MELD score of < 10 was obtained in 25%. Only 11% died after a median follow-up of 4 years. In view of the marginal changes that were achieved, the authors concluded that the low mortality was certainly not due to the favoring effect of DAAs[155].

HCV cirrhotic patients survived longer than those with alcoholic cirrhosis, while HBV patients were over twice at risk of dying compared with HCV patients. Patients with alcoholic cirrhosis had a higher risk of decompensation compared to those with HCV, while patients with HCV plus alcohol use had a significantly higher rate of decompensation compared with HCV alone. The highest median decompensation-free time was noted in the HCV patient group[156].

A large recent meta-analysis updated prognostic estimations of important patient subpopulations. Findings indicate that HCV's natural course is influenced by factors like infection, age, duration of infection and population studied. Fibrosis progression is grossly heterogeneous across study populations. It is suggested that HCV prognosis should be examined in homogeneous populations[157].

WHAT SVR REALLY REPRESENTS

SVR is a marker of viral eradication in HCV infection. The all-oral,] DAAs have drastically increased SVR rates compared to IFN-based treatment[158,159]. In addition, SVR achieved after DAAs treatment has been shown to persist long term[63,160]. SVR is indiscriminately characterized as a surrogate marker of clinical cure.

The National Institutes of Health (United States) defined surrogate endpoint or surrogate marker as a biomarker intended to substitute for a clinical endpoint. A first step for establishing a new surrogate marker is the investigation of the degree to which

the candidate biomarker can explain or predict the effect of treatments on clinical endpoints measured in randomized clinical trials[161].

A Cochrane systematic review published in 2017 evaluated 51 trials comparing DAAs with placebo and/or no interventions. Most trials primarily reported on sustained virologic response and provided relatively limited data on clinically important outcomes and none at all on long-term effects. Meta-analysis of the effects of all DAAs showed no evidence of a difference in all-cause mortality between DAA recipients and controls with very low-quality evidence. Furthermore, all trials and outcome results were at high risk of bias.

It has been suggested that DAAs may reduce the number of patients with detectable virus in their blood, but no sufficient evidence from randomized trials exists that could allow us to understand how SVR affects long-term clinical outcomes. Therefore, SVR was considered to be an outcome that needs proper validation in randomized clinical trials. A laboratory measurement cannot be characterized as a surrogate marker without solid proof of a clinical outcome[162]. Within a month, an unusual response to the Cochrane review appeared with EASL as both the author and the corresponding author! No names were mentioned, although they should have since EASL is a scientific society and cannot certainly write papers[163]. At about the same time, the Cochrane review was fiercely criticized, but the critics admitted that the end point for most clinical trials of DAA therapies conducted has been SVR. They also pointed out that there were a few DAA trials that have examined clinical outcomes in decompensated patients with cirrhosis[164].

The Cochrane team in fact substantiated their argument on both SVR and on the effect of DAAs on mortality[165]. They correctly pointed out that SVR had never been validated and even failed such validation in one analysis of long-term IFN intervention[166]. Moreover, it was pointed out that quality of life is a completely subjective outcome, and its assessment needs blinded random controlled trials with unbiased patients[167]. This is very difficult to be implemented in the case of DAAs as advertisements have promised a cure even for asymptomatic patients. It was also pointed out that the use of the word 'cure' was inappropriate. They argued that although SVR is a good prognostic sign, it has no validation as a surrogate outcome [168].

There are two critical points that require further analysis. The first is the question of randomized controlled trials, and the second is the use of the word 'cure.' The second is easier to answer as patients who achieve SVR can relapse years later with genetically identical viruses, suggesting that the virus possibly existed in a latent form inside the body, and so patients who achieve SVR can still progress to end-stage liver disease [169,170]. A certain number of patients with compensated or decompensated cirrhosis show a deterioration of liver function[43,171,172] or display persistent hepatic inflammation or progress to cirrhosis despite SVR[173]. Not only fibrosis or inflammation but also the development of HCC in patients with advanced fibrosis or cirrhosis may continue in the presence of SVR[174,175] and in non-cirrhotic patients with a FIB-4 score ≥ 3.25[176].

In addition, achievement of SVR, characterized as 'cure,' has indeed been associated with reduced mortality among cirrhotic patients, but nonetheless it remains higher than general population mortality[146]. Recent reports indicate that HCV patients may also die from liver-related complications even after SVR[154,177]. Likewise, all-cause mortality is still high after SVR in cirrhotic patients[178]. SVR after IFN-based treatment offered no survival benefit after 10 years of follow-up even in the specific group of hemophiliacs[142].

The other question is more difficult to answer, as prospective randomized controlled studies are extremely difficult, if not impossible, to organize in the case of DAAs. The very existence of many confounding factors intervening in the progress and outcome of HCV infection makes the whole project very difficult indeed. Two recent follow-up studies may exemplify the situation. In patients studied for the incidence of HCC, age, gender, cirrhosis and aspartate aminotransferase to platelet ratio index were predictors of HCC in multivariate analysis, while SVR was not[179]. In the same group of patients, diabetes proved to be a significant risk factor for disease progression in certain subgroups of patients without cirrhosis or compensated cirrhosis. Moreover, the absence of SVR was not an important risk factor[180].

A beneficial effect of SVR after DAA treatment on diabetes prevention and the short-term outcome of metabolic alterations has been reported by some studies. A recent review however argued that this effect may not be maintained in the long term, or more importantly this effect may not have any real clinical impact in liver disease [181].

The best conclusions that can be presented so far come from a recent prospective study but not a randomized trial comparing patients who achieve SVR with those that fail. After an extensive adjustment for a large number of variables, administration of DAAs was associated with a decrease in all-cause mortality and HCC but not with decompensated cirrhosis[182].

Data from the extensive Italian RESIST-HCV cohort similarly indicated that in patients with Child-Pugh B cirrhosis, the rate of liver decompensation was not associated with SVR[183]. The need for extensive adjustments in different groups of patients may produce conflicting results. In a different study of patients with HCC from the same group, SVR was also a significant predictor of hepatic decompensation apart from being a predictor of survival and HCC recurrence[184].

SVR was a favorable prognostic marker of fibrosis decrease in a study based on liver elasticity measurements. However, progression of liver stiffness despite viral clearance was observed in 17% of patients[185].

Similarly, despite improvements in MELD score with DAA treatment that allowed the removal of almost a fifth of candidates from the transplantation list, many HCV-infected patients with SVR and decompensated cirrhosis still die or have a liver transplantation[186]. In a longer follow-up of the same delisted patients due to their clinical improvement after DAAs, approximately 4% were either relisted because of disease progression or died due to the development of HCC[187].

In another study of HCV cirrhosis, SVR was associated with an improved MELD score in 37% of patients, but MELD was aggravated in 22% and stayed unchanged in the remaining 41%[38]. These results were verified in another real world study of patients with advanced/decompensated cirrhosis treated with DAAs and a median follow-up of 4 years. After SVR, only marginal improvements in MELD at a clinically meaningful decrease were found in 29% of patients who might still remain at high risk of decompensation[155]. These are examples of SVR use as a surrogate marker of a laboratory index rather than a specific clinical outcome.

Despite the previous reservations as to the use of SVR as a surrogate marker, recent guidelines of the two major liver disease associations equate SVR with successful clinical treatment[89,188]. In accordance to these guidelines, investigators concluded that successful treatment of HCV translates into a significant mortality benefit in a very large study of HCV mono-infected patients without advanced fibrosis. Patients were stratified according to FIB-4 measurements. SVR patients with FIB-4 < 1.45 had a 46% reduction in mortality compared to no SVR patients, while patients with a FIB-4 between 1.45 and 3.25 had a 63.2% reduction in mortality rates. However, data were not based on liver biopsies as a basal stratification point, and this is a serious disadvantage. Another finding is that patients with no SVR die less compared to untreated patients, which is hard to explain[189]. Conversely, SVR was associated with a reduction in fibrosis, but no association of fibrosis with mortality was reported. Finally, since only 14% of patients were re-biopsied, steatosis was not assessed[190].

A review on the reinfection rates after SVR with DAAs clearly states that SVR corresponds to a definitive cure of HCV infection as the incidence of late recurrent viremia is low[61]. This is clearly different from another recent review stating that DAAs have been efficient in curing patients with chronic hepatitis C[191]. A recent report on long-term results of DAAs avoids the words cure and surrogate marker, stating that despite excellent SVR rates there was a considerable overall mortality and liver-related mortality as well as decompensation incidence after 28 mo of follow-up [192]. Even those supporting that SVR has been proven to be associated with reduced liver events and reduced overall mortality cannot support that SVR is a surrogate marker of the clinical cure of the patient[193].

The advent of liver stiffness measurements with the resultant substantial reduction of liver biopsies has not allowed for an accurate estimation of the effects of DAAs in patients with F1-F2 fibrosis. As rightly pointed out in a very recent review, only after long-term follow-up of large real-life cohorts of patients with mild to moderate fibrosis will we be able to confirm the real impact of SVR[194].

A very recent prospective multicenter study of 226 patients with HCV-related cirrhosis and clinically significant portal hypertension has shown that liver stiffness markedly decreased after SVR but did not correlate with hepatic venous pressure gradient changes. Ninety-six weeks after SVR, clinically significant portal hypertension may persist in up to 53%–65% of patients indicating a persistent risk of decompensation[195]. These findings verify previous reports from the same group, also confirmed by other investigators, indicating that hepatic venous pressure gradient measurement is a better prognostic factor than SVR in advanced liver disease[196,197].

The conclusion is that no randomized trials exist so far, and only a few trials are actually prospective. Furthermore, it is clear that a considerable number of liver-related incidents may occur despite SVR. We feel therefore that there is no justification of referring to SVR as a surrogate marker or as an equivalent to clinical cure for HCV patients. SVR should only be used as a favorable prognostic marker or as a marker of viral cure for HCV.

As mentioned before, HCV infection may have a relatively benign outcome without the presence of harmful confounding factors, namely alcohol, metabolic syndrome, HCV/HIV coinfection and liver iron.

EFFECT OF ALCOHOL CONSUMPTION IN HCV-RELATED FIBROSIS AND CIRRHOSIS

Earlier studies have shown that the prevalence of HCV is 3-30 times higher in alcoholics than the general population. Alcohol abuse is an independent factor of HCV progression. Advanced fibrosis and higher probability of developing cirrhosis and HCC have been reported for those with heavy alcohol consumption compared with non-drinkers[198-203].

In Dionysos, an extensive study of HCV in the general population in Northern Italy reported an association between alcohol and severity of HCV-associated liver disease. Alcohol consumption higher than 30 g/d aggravated the natural course of the disease significantly[204]. Overall, at least in Western countries, approximately 60% of HCV patients have a past history of alcohol use. Chronic consumption of more than five drinks per day increases the rate of liver fibrosis[205].

Even lower alcohol intake increases HCV viremia and hepatic fibrosis[206]. An interesting report of HCV patients was based on pairs of liver biopsies with a median time of 6.3 years between them. Alcohol consumption during the period between the biopsies was low (median 4.8 g ethanol/d. Deterioration of fibrosis was associated with higher total alcohol intake and higher drinking frequency between the biopsies [207]. The fact that even low amounts of alcohol intake may lead to fibrosis progression has been recently reviewed[208].

An early study of HCV patients with elevated alanine aminotransferase has convincingly demonstrated that these patients had severe fibrosis associated with high alcohol consumption. Interestingly, all 3 patients who had cirrhosis and persistently normal alanine aminotransferase were also heavy drinkers[209]. Other studies have also confirmed that total life-time alcohol consumption was independently associated with cirrhosis[210]. A large observational study in five European countries confirmed the reports on the detrimental effects of alcohol on liver fibrosis irrespective of differences in biopsy use and preferred scoring systems[211]. There was a 2-3-fold greater risk of liver cirrhosis and decompensated liver disease in the alcohol-consuming group. Progression to cirrhosis and decompensation was much faster in alcoholics with HCV compared to non-drinkers[212]. A liver biopsy was performed on 86 heavy alcohol drinkers (80 g or more of ethanol/d for at least 10 years) with or without HCV infection. Higher intralobular necrosis and periportal inflammation was found on the liver histology of drinkers. Importantly, the development of cirrhosis was related more to the amount of alcohol intake than to the presence of HCV infection [213]. Among 1620 HCV patients, the fraction of cirrhosis attributable to heavy alcohol intake was 36.1% and exceeded 50.0% among those who had engaged in heavy alcohol use at some point. AUDs contributed to approximately 70% of liver-related complications in young and middle-aged adults with HCV infection. An additional 15% was attributed to metabolic syndrome. Importantly, alcohol rehabilitation and abstinence reduced liver complications by 60% and 78%, respectively[214].

In addition to fibrosis and cirrhosis the risk of decompensation is also increased after alcohol. Data suggested that heavy, but not moderate, alcohol intake was associated with a higher risk for hepatic decompensation in patients with cirrhosis than HCV infection was[215], a finding confirmed in a population study of HCV-infected individuals. Age-adjusted decompensated cirrhosis incidence was considerably higher in people with AUDs in British Columbia, New South Wales and Scotland; AUD was present in 28%, 32% and 50% of those with decompensated cirrhosis, respectively[216]. A very recent meta-analysis also verified that alcohol is strongly associated with HCV cirrhosis decompensation. Data from 286641 people with chronic HCV infections, of whom 22.3% with AUDs, showed that AUD diagnosis was associated with a 3.3-fold risk for progression of liver disease. Almost 4 out of 10 decompensated liver cirrhosis cases were attributable to an AUD[217]. Interestingly,

similar findings were reported in a very recent study on the effects of alcohol in patients with HBV[218].

The effect of alcohol on response to treatment with DAAs was recently reported. The baseline risk factors related to the success of DAAs were studied in 4946 HCV patients. They found that obesity, diabetes and alcohol consumption were associated with persistent liver enzyme elevation after SVR[219].

Earlier research also showed that HCC development increased as a result of the combined effect of alcohol and HCV infection[220]. Alcohol intake was almost universally considered as a critical factor in the still unresolved dispute of HCC occurrence and recurrence after treatment with DAAs[221,222].

The most important aspect of the involvement of alcohol consumption in the natural history of chronic HCV infection is its effect on mortality. The risk of death in HCV patients is increased by 40% if alcohol abuse is also present[223]. HCV patients admitted to the hospital with alcohol-related problems have doubled in-hospital mortality rates[224]. Mortality rate was worst for alcoholic cirrhosis with concomitant HCV even after adjustment for age and gender[225]. In a long-term study of cirrhosis of different etiologies from Sweden, the lowest 10-year transplantation-free survival was found in cryptogenic cirrhosis (11%) and in alcoholic cirrhosis combined with hepatitis C (12%)[226]. The attributable risk of AUDs was 68.8% of 6677 liver deaths of patients infected by HCV. Moreover, liver-related mortality increased faster for individuals with AUDs[214].

The all-cause mortality was 1.9 times higher compared to the general population in patients with HCV followed up for an average of 5.2 years after SVR. Increased mortality was associated with either heavy alcohol use or injection of drugs. Patients without these behavioral markers had equal survival to the general population[146]. Similar findings were also reported in a very recent study from the United States. Survival analysis demonstrated that there was a significant association between unhealthy drinking and lower survival compared with non-drinking[227].

Conclusions are similar when patients with AUDs were evaluated for the effects of the concomitant presence of HCV infection. In a small study of hospitalized alcoholic hepatitis patients, the presence of HCV was a significant risk factor for a poor outcome at 6 mo, even after adjustment for disease severity and treatment[228]. The same group reported that patients with alcoholic hepatitis had a higher prevalence of HCV compared with the general population and that the presence of HCV infection also predicted a higher mortality[229]. In a more recent investigation, the overall mortality rate was significantly higher among HCV-positive alcoholic patients than among HCV-negative patients, and the same was true for their respective liver-related mortality. Survival time for the HCV-infected patients was 34% less[230]. These findings are usually not taken into account in Markov models of cost-effectiveness, and therefore estimates may be exaggerated[86].

Research has focused on the mechanism of the synergistic effect of alcohol and HCV in view of the above findings. An increase of apoptotic cell death in hepatocytes of HCV-infected patients has been demonstrated. Apoptosis was further upregulated by active ethanol consumption[231]. Another critical mechanism of HCV-alcohol synergy is the effect both have on innate immunity. Dendritic cells, the critical cell type in antigen presentation, have shown to be a major target for HCV and ethanol with a resultant dysfunction of CD4 and CD8 T cells[232,233]. The common molecular mechanisms of the synergy between alcohol and HCV also include the interference with cytokine production, lipopolysaccharide-TLR4 signaling and reactive oxygen species production. Increased oxidative stress seems to be the dominant mechanism for this synergism between alcohol and HCV[203,234]. Recently, the synergistic effect of alcohol and HCV on alcohol-induced 'leaky gut' as well as their effects on miR-122 and immune dysregulation have been investigated[235].

An important mechanism still under intense investigation is autophagy. Both alcohol and HCV infection could induce cellular autophagy in liver cells, a process that is considered to be essential for productive HCV replication. It would seem, at least from experimental studies, that alcohol promotes HCV replication through activation of autophagy[236].

The problem of HCV-alcohol interconnections has been extensively reviewed[237, 238].

HCV AND METABOLIC SYNDROME

Other factors that might interfere with the outcome of HCV infection are related to

metabolic syndrome.

Previous reports have indicated that obesity and diabetes occur more frequently in HCV patients. Both conditions result in fatty liver. Steatosis is associated with either metabolic alterations like IR and visceral obesity (metabolic steatosis) or a direct cytopathic effect of the virus mostly genotype 3 (viral steatosis), which is strongly related to serum viral load[239-243].

There is evidence that metabolic syndrome is directly linked to HCV infection[244]. Visceral adiposity index is a marker of adipose dysfunction in HCV patients. It is associated with steatosis and necro-inflammatory activity and has a direct correlation with viral load and SVR. In fact, visceral adiposity index represents a measure of the metabolic syndrome[245,246].

There is also indirect evidence connecting metabolic syndrome with HCV. Hyperuricemia is associated with the metabolic syndrome. Its association with HCV infection is additional indirect evidence of their relationship. As expected, body mass index (BMI) is also a factor associated with hyperuricemia in HCV patients[247]. Hyperuricemia has been independently associated with severity of steatosis, indirectly affecting liver damage[248]. There are conflicting recent reports on the effect of DAA treatment on hyperuricemia. Uric acid levels were significantly decreased in chronic HCV patients after viral eradication. The improvement was particularly enhanced in patients with mild liver disease[249]. On the contrary, uric acid levels were moderately increased after HCV eradication in patients with hyperuricemia. Thus, it was considered an adverse effect to DAAs containing ribavirin, potentially leading to side effects such as renal impairment[250]. The reason for this discrepancy is unknown.

Carotid plaques are also an indirect indication of metabolic syndrome, although other pathophysiological mechanisms may be involved. The risk of a person with HCV infection developing carotid plaques is approximately 3.94 times the risk of an uninfected person[251]. More importantly, metabolic syndrome is an independent risk factor of HCV mortality[252,253]. An extensive review on the association between HCV and metabolic syndrome has recently been published[254].

Obesity-steatosis

According to the WHO, obesity is an excessive or abnormal accumulation of fat. It has been suggested that this condition is a 21st century pandemic, with a prevalence of 1.9 billion cases worldwide, while almost 40% of the adult population in industrialized countries is overweight[255].

Liver steatosis affects up to 80% of patients with HCV infection[256]. In a recent cross-sectional study, 66% of HCV patients were obese and almost one-third fulfilled the criteria of metabolic syndrome. Of note is the fact that 67% of them were either current or past heavy drinkers[257]. HCV has been closely associated with obesity and steatosis. Obese HCV patients had higher grades of steatosis and advanced fibrosis [258]. Earlier and more recent studies have proven beyond any doubt that obesity, steatosis and liver inflammation are interconnected[240,259-262]. At the same time, the fibrosis progression rate was higher when excessive steatosis was present in the first liver biopsy[263]. The association between steatosis and fibrosis was dependent on a simultaneous association between steatosis and liver inflammation[264].

The association of BMI with steatosis and fibrosis may have important therapeutic implications[265] because weight reduction improved both biochemistry and the Knodell fibrosis score[266]. In the era of IFN-based treatments, obesity and steatosis were associated with reduced SVR[267-271].

Cirrhosis-HCC

A recent Swedish population-based study of cirrhosis identified that irrespective of etiology the most common comorbidities at diagnosis were arterial hypertension (33%), type 2 diabetes (T2DM) (29%) and obesity (24%)[272]. The metabolic syndrome and liver stiffness measurements were independent risk factors of HCV progression to cirrhosis[273]. Indirect evidence that cirrhosis in HCV infection is related to metabolic syndrome comes from a prospective study indicating that dysregulation of various metabolic profiles preceded the ultimate development of cirrhosis[274].

There are data connecting the appearance of HCC with components of metabolic syndrome. The risk of HCC in HCV patients increases in proportion to their BMI, from underweight to obese[275]. Individuals with a high BMI (≥ 25.0 kg/m^2) accompanied by low triglyceride levels (< 160 mg/dL) had a significantly increased risk for liver cancer related mortality[276]. In the DAA era, increase in HCC incidence after treatment has been associated with higher BMI and cirrhosis[277]. A systematic review demonstrated a significant association between BMI and HCC risk. As expected steatosis was also associated with a higher risk of HCC[278].

In IFN-based treatment, response was diminished in overweight patients without other comorbidities. The group included children and teenagers[279]. This however is not the case with DAAs as reported recently but is worth noting that the separation between obese and non-obese patients was set at a rather high BMI of 30[280].

Diabetes mellitus and IR

Several studies have verified that T2DM, IR and hepatic steatosis are highly prevalent in patients with genotype 1 HCV infection[246,278,281-283]. In a large study of 710 patients with a known duration of infection, both overt diabetes and high serum glucose levels were associated with advanced fibrosis and a high fibrosis progression rate independent of alcohol consumption and other risk factors such as the duration of infection[244]. IR was also associated with fibrosis[284]. HOMA-IR was higher in advanced fibrosis than in mild. The number of lipid laden hepatocytes was also higher in cases of advanced fibrosis with increased HOMA-IR and BMI > 25.0 kg/m^2[285,286]. IR is associated with HCV infection in up to 80% of cases, and the risk of developing T2DM is twice as high compared to subjects without HCV[287].

Not only is HCV natural course aggravated by diabetes, but HCV infection is a significant risk factor for developing T2DM as well. Spontaneous or treatment-induced HCV clearance may reduce the risk of the onset of T2DM[288].

Another important aspect of the interaction between T2DM and HCV is the association with HCC development. Diabetics with HCV has a 2-3-fold increase in HCC risk[289-291]. Maintenance of glycated hemoglobin level below 7.0% reduced the development of HCC[292]. Diabetes was independently associated with both de novo HCC occurrence and HCC recurrence after DAA treatment[178,293]. A systematic review of seven cohort and two case-control studies has confirmed the significant contribution of T2DM in the development of HCC in HCV-infected patients[278].

The presence of IR or overt diabetes has implications in the treatment of HCV[242] as it adversely affects the response to treatment with IFN-based therapies[294-296]. However, HCV patients who achieved SVR after IFN-based therapy had an improvement of both HOMA-IR and HOMA-b[297]. SVR12 rates are not affected by the presence of T2DM in DAA treated HCV patients[298]. Recent evidence indicates that viral elimination by DAAs improves the increased IR and T2DM incidence by restoring alterations of glucose homeostasis induced by HCV. It should be noted however that IR may persist after SVR, particularly in patients with high BMI[299,300].

HCV patients have an altered serum lipid profile characterized by a reduction of total cholesterol, low-density lipoproteins and very-low-density lipoproteins[301]. Viral eradication with DAAs may have improved HOMA-IR, but serum lipids were increased and the lipid profile worsened in a follow-up study of 24 wk after SVR. BMI did not change in this study[302]. This was not the case in a larger study of 343 HCV patients with the same follow-up. In addition to increased serum cholesterol and low-density lipoprotein cholesterol, an increase in BMI was also observed. Serum glucose, HOMA-IR and HOMA-b were decreased. More importantly, one-third of patients with fasting hyperglycemia normalized serum glucose values, and almost half of diabetics improved glycemic control[303]. A temporary IR increase during treatment with DAAs that went back to normal after treatment was reported, in contrast to lipids that remained increased[304].

In general, HCV steatosis occurs in association with multiple metabolic abnormalities like hyperuricemia, hypocholesterolemia, IR, arterial hypertension and expansion of visceral adipose tissue referred to as "HCV-associated dysmetabolic syndrome" and shares many underlying abnormalities with nonalcoholic liver disease [305].

There are some mechanistic explanations for the aforementioned findings. HCV-associated metabolic steatosis accelerates liver fibrosis progression and development of HCC by inducing liver inflammation and oxidative stress[306]. Both pathways lead to increased fibrosis through induction of the connective tissue growth factor[307,308]. HCV core protein and nonstructural protein 5A are implicated in the disturbance of lipid and glucose pathways that lead to steatosis, lipid abnormalities and metabolic syndrome[309]. Moreover, HCV interferes directly and indirectly with insulin signaling that results in the production of proinflammatory cytokines[310].

HCV/HIV COINFECTION

HIV patients are very often coinfected with HCV. Prevalence of coinfection varies in different countries and among different subpopulations like PWID or hemophiliacs

[311-313]. HCV/HIV coinfection may interfere with some aspects of HCV natural course[314,315]. HIV antiretroviral therapy (HAART) alone did not fully correct the adverse effect of HIV infection on HCV progress[316].

Mortality

In the HAART era, HCV coinfection increased the risk of mortality compared with HIV mono-infection possibly due to a more rapid progression of HCV in the coinfection group[317].

In a long-term follow-up study of HCV-infected hemophiliacs, HIV coinfected patients were compared to non-HIV patients for mortality after an average of 24 years. The adjusted risk ratio for death was significantly greater among HIV-positive than among HIV-negative patients after adjustment for alcohol use and HAART use was applied, indicating that HIV accelerates HCV disease progression[318]. Failure to clear HCV led from rapid progression to decompensation in HCV/HIV coinfected patients [319]. These findings were confirmed as HCV infection was independently associated with all-cause and liver-related mortality in HIV patients with alcohol problems, even when adjusting for alcohol and other drug use[320]. In a very large retrospective study, a higher mortality of HCV/HIV coinfection compared to HCV mono-infection was reported. Moreover, the presence of HCV cirrhosis or complications from it were associated with four times greater mortality risk in HIV patients[321]. A very recent study of people living with HIV (PLWH) and of those injecting drugs demonstrated the highest odds of HCV-positivity, which was an independent predictor of greater mortality[322].

An individual-based model of disease progression in HIV/HCV coinfected MSM has been developed. There was a gradual increase of liver-related deaths according to fibrosis state and the time treatment was initiated. Two percent of treated patients would die if treatment was initiated at stage F0 and 22% if treatment was deferred until F4. Similar gradual mortality increments were associated with the length of time individuals replicate HCV[323].

HCC development is associated with increased HCV mortality rates. Older age, cirrhosis and low current CD4 cell count were associated with a higher incidence of HCC in HCV/HIV coinfection[324,325]. Furthermore, a recent prospective study demonstrated that HCV/HIV patients with compensated cirrhosis have similar risks for further end-stage liver disease and HCC with HCV mono-infected patients[15] provided they receive both HAART and DAA treatment[326].

Fibrosis

In the pre-DAA era, many studies based on paired liver biopsies demonstrated that hepatic fibrosis progressed more rapidly in HCV/HIV coinfection than in HCV mono-infected patients even after adjustment for alcohol consumption or duration of HCV infection[327-331]. In a retrospective cohort study focusing on a PWID group of patients, HIV coinfection worsened the outcome of chronic HCV infection, increasing liver damage and decreasing sustained SVR after IFN therapy. Age and alcohol were cofactors associated with cirrhosis and mortality[332]. Fibrosis progressed in a significant number of HCV/HIV coinfected patients even after DAA-induced SVR [333].

Not all studies agree with the above findings. After adjustment for daily alcohol use, HIV patients with HCV coinfection and BMI greater than 25 kg/m^{2}had equal liver fibrosis to those without HIV but at an average onset of 9.2 years earlier[334]. Hepatic steatosis increased faster and was associated with fibrosis progression only in HIV mono-infected patients but not in HIV/HCV coinfected ones. Diagnosis was based on liver stiffness measurements for fibrosis and controlled attenuation parameter for steatosis without histological documentation[335]. Histological abnormalities were usually significantly milder in HCV/HIV coinfection with persistently normal alanine aminotransferase levels than those found in patients with high alanine aminotransferase, but this was not always the case as patients with persistently normal alanine aminotransferase levels also developed significant fibrosis[336].

The risk of advanced fibrosis increased at high levels of alcohol consumption[337]. In this group of patients even low alcohol consumption was associated with advanced hepatic fibrosis[338]. The impact of alcohol was recently verified. HIV/HCV coinfected patients had a higher prevalence of intermediate and advanced liver disease markers than HIV mono-infected patients. Advanced markers of liver disease were strongly connected to hazardous drinking for both men and women[339].

Genetic factors are also involved. Cirrhosis was more prevalent in IL28B CC genotype HCV/HIV infected patients than in patients with CT/TT genotypes, possibly indicating that IL28B CC carriers have a more rapid progression of HCV-

related fibrosis[340].

A number of studies have reported on the achievement of SVR in either observational studies or clinical trials, and the results were conflicting. A statistically significant difference in SVR12 response was observed between HCV mono-infection and HCV/HIV coinfection after DAAs (94% and 84%, respectively)[341]. HCV/HIV coinfection response to DAAs was worse (86%) compared to HCV mono-infection (95%). This was possibly due to a higher rate of relapses among HCV/HIV coinfected subjects[342].

A high SVR12 was similar in a review of 11 real-world observational studies (90.8%) and 8 clinical trials (93.1%). There was no control group of HCV-mono-infection[343]. A recent multicenter study of SOF/DCV from Brazil showed an SVR12 rate of 92.8% in an intention-to-treat analysis. There was no comparison with HCV mono-infected patients[344]. A similar SVR12 of 94% was reported in a retrospective study from the United States. Substance abuse and diabetes, but not obesity, had a negative effect on treatment[345]. Importantly, a recent paper stressed the fact that both adherence to HAART treatment and alcohol consumption should be carefully monitored in this group of patients. Furthermore, higher alcohol consumption per day was positively associated with HAART non-adherence[346]. Interestingly, high coffee intake is probably associated with reduced liver fibrosis even in HCV/HIV coinfected patients with high alcohol abuse[347].

Clinical consequences after successful treatment with IFN-free regimens are limited. A Spanish group reported that successful SVR in HCV/HIV coinfected patients led to the same probability of liver complications with HCV mono-infection after a median follow-up of 21 mo[348]. In addition, the same group reported that only successful SVR patients with > 14 kPa on liver stiffness measurement are among the few who develop a liver-related complication[349].

Strangely enough, HCV/HIV coinfected patients had a lower risk of HCC development compared to HCV-mono-infection after successful SVR in a follow-up study from the same group. This is hard to explain particularly because alcohol consumption and diabetes were the same, and the HIV-positive group included significantly more PWID[350]. This is in slight disagreement with another study where HCV/HIV coinfected patients had a greater mortality risk and a similar risk of HCC development indicating that DAAs do not produce complete resolution of inflammatory and profibrogenic stimuli[326].

Similar SVR rates, as well as risk of liver-related deaths and events, were also reported between HCV-mono-infected and HCV/HIV coinfected individuals, but a higher risk of all-cause and non-liver related cancers were observed in HIV coinfected patients[351].

There are some mechanistic explanations to account for the discrepancies mentioned before. An opposite effect between HCV mono-infected and HCV/HIV coinfected patients was observed after an increase in HCV viral load and CD4+ T cell count. HCV viral load in HAART-treated patients was associated with greater natural killer cell dysfunction than the same HCV viral load alone in HCV mono-infection. This may influence HCV disease progression in these patients[352]. Advanced liver fibrosis in coinfected individuals is associated with reduced numbers and a defective function of natural killer cells, along with an increased expression of the exhaustion/senescence marker PD-1[353]. In addition, HIV-positive patients have a persistent $CD4^+$ T cell depletion in the gut, which increases gut permeability. A greater microbe translocation leads Kupffer cells to produce more proinflammatory and profibrotic cytokines[354].

Despite differences, it seems that coinfected patients have a higher mortality risk compared to HCV mono-infected patients even after DAA administration. This implies the existence of unique pathways that continue to promote accelerated liver disease in these patients compared with those with HCV mono-infection. An article on the pathobiology of liver disease in HCV-HIV coinfection in the DAA era was very recently published[355].

There are some recent reports addressing the final question that needs to be answered: can we eliminate HCV/HIV in the real world?

A model analysis from Spain showed that screening and treating all PLWH every year from 2020 onwards would probably lead to an increase of the number of new HCV infections among PLWH by 28% as the majority belong to the PWID and MSM groups with ongoing infection and reinfection. As for the general population, only a reduction of new HCV infections by 39% will be achieved by 2030[356].

A program of HCV elimination among PLWH has been reported from Scotland. As in Spain, the most common mode of HCV transmission was injection of drugs. DAAs increased the number of treated patients and produced higher SVR12 results compared to pegylated IFN. However, the number of the patients involved was small

[357]. Rather disappointing results came from Austria where despite the fact that HCV testing was successful in the Viennese HIV-positive patients, HCV prevalence was stable in HIV-positive PWIDs and even doubled in HIV-positive MSMs. New HCV infections occurred mostly in MSM and HCV, while reinfections were mostly observed in PWIDs. HCV treatment adherence was not adequate since 42.8% remained HCV-RNA positive at follow-up[358].

The last findings lead to the problem of PWIDs. Can we eliminate HCV in PWIDs in the real world?

Efforts to reach PWIDs are unsuccessful in many countries. Better organized interventions are urgently needed since additional factors, like HIV infection and alcohol abuse that contribute to the progress of fibrosis, are commonly involved[359, 360]. This is exemplified by a longitudinal study of 501 patients from an opioid agonist therapy program. Prevalence of HCV and HIV infection was 70% and 34%, respectively. Almost half of those infected with HCV used alcohol, cannabis and cocaine. Current drug use was a strong independent risk factor for not receiving treatment against HCV[361]. Treatment costs and reduced treatment uptake are additional prohibitive factors. Screening and treatment for high-risk populations like PWIDs and MSMs are considered cost-effective in high-income countries, but DAAs remain expensive and a barrier worldwide despite lower prices in many of the low-income countries. Although universal DAA availability led to a 50% reduction in acute HCV incidence among HIV + MSM in the Netherlands, this cannot be achieved in low-income countries. Close monitoring for HCV reinfection, harm reduction and behavioral interventions are also required among others[35]. In a large French study of 27127 individuals, DAA treatment uptake was still lower in women despite an increased uptake after DAA introduction compared with IFN[362]. Alcohol, HIV and PWID interactions were extensively reviewed[363].

Frequent testing has been suggested as a means to achieve HCV elimination in PWIDs. If the prevalence of HCV approaches 75% or more, like it does in Indonesia, Iran, Italy, Malaysia, Mexico and Pakistan[364], it is possible that even a frequency of one HCV test every 3 mo would not contribute to major reductions in incidence[365]. In low-middle income countries the situation would be much worse.

HCV-infected PWIDs may achieve high SVR12 rates with DAAs, but their response rates are lower compared to patients who have never used drugs. This is because they are more frequently lost to follow-up[366]. This was verified in an Austrian study where GLE/PIB was administered under direct observation by doctors as part of an opioid substitution therapy. Similar high SVR rates (94.6%) as in patients with excellent compliance were obtained[367].

Left untreated, PWIDs with chronic HCV infection will develop serious liver disease (including HCC) in mid to late adulthood. Models estimate the average time to cirrhosis to be between 36 years and 46 years after infection[135]. Strangely enough, a notable exception was the reported lower incidence of HCC in users of illicit drugs. In fact, while cirrhosis, male gender and diabetes are risk factors for HCC development in both SVR and non-SVR patients, the use of illicit drugs seemed to be a protective factor in both SVR and non-SVR patients. Moreover, despite the fact that HCC incidence after IFN treatment was significantly less in SVR patients, deaths were twice more frequent after SVR compared with non-SVR (4.9% *vs* 2.0%)[368].

Reinfection is another serious problem in this group of patients. Reinfection rates were higher among recent and former PWIDs than among non-PWIDs. Among recent PWIDs, reinfection rates were higher among those born after 1975 and those coinfected with HIV[369]. These findings are in agreement with a previous study where higher reinfection risk was found in HIV coinfection and PWID. Both opioid-substitution therapy and engagement with mental health counselling services were negatively associated with risk of reinfection[63]. An analysis of reinfection rates in PWIDs from seven middle-income countries was recently reported. The follow-up time from cure to reinfection ranged from 12 wk to 6.6 years. The pooled reinfection rate was 2.8 cases/100 person-years that dropped to 1 case/100 person-years when differentiation between relapse and reinfection was feasible[370].

HCV AND IRON

The presence of iron in the liver may interfere with the natural course of HCV infection. Chimpanzees on high iron diets had histological abnormalities only if coinfected with HCV[371]. Minor increases in iron stores in heterozygous carriers of C282Y or H63D mutations for hemochromatosis are associated with more fibrosis in

chronic HCV[372,373]. HCV patients have low serum hepcidin levels, a fact leading to enhanced necro-inflammation and fibrosis[374]. Increased serum aminotransferases were found only in HCV patients with stainable iron in the Kupffer cells but not in those with hepatocyte iron[375]. Genotype 3-infected patients had more frequently elevated liver iron, and this condition was related to viral-induced hepatic steatosis [376].

Thalassemia patients coinfected with HCV prove that iron is indeed harmful. Liver iron overload affects the prognosis of liver disease negatively, leading to severe hepatic inflammation and fibrosis[377].

Some studies however do not support the linkage between advanced fibrosis and increased iron in hepatocytes[378]. Iron overload was not common in HCV patients, and hepatic-iron content was not related to the liver damage process[379]. Others reported that the presence of high serum ferritin and stainable liver iron had no significant role in the progression of liver disease[380].

Whatever the differences may have been, there is a uniform agreement that liver iron stores adversely affect the response to IFN-based treatments[381-383]. This is not the case with DAAs. Thus, in IFN-treated patients, ferritin levels increased 24 wk after treatment regardless of SVR. Ferritin levels decreased at about 3 years post-treatment. Conversely, among DAA-treated SVR patients, ferritin levels were permanently decreased and remained stable thereafter[384]. Now that treatment with DAAs seems to be effective, achieving SVR 90%-98% irrespective of iron status, long-term follow-up studies will delineate the role of iron and HCV in the progress of liver disease[385-388].

Many studies have addressed the reasons for iron overload in certain patients with HCV as well as the possible detrimental effect it may have on the disease progress. A possible direct mechanism for iron overload has been proposed based on experiments in transgenic mice harboring the HCV polyprotein. The presence of HCV proteins led to reduced hepcidin transcription and increased ferroportin expression, leading to hepatic iron accumulation[389]. Another explanation is that extravascular hemolysis may play a role in the development of secondary iron overload since alterations in red blood cell indices indicate hemolysis in end-stage liver disease[390]. Some studies found that iron favors HCV replication and more specifically HCV translation due to increased expression of factors involved with HCV internal ribosome entry sites[391]. Alternatively, HCV alters expression of the iron uptake receptor transferrin receptor 1. Transferrin receptor 1 is possibly involved in HCV particle internalization at the level of glycoprotein-mediated entry[392,393]. Increased iron can feed the Fenton reaction to produce excess amounts of free radicals that cause serious cellular and tissue damage and contribute to fibrosis[394].

As a general rule, intracellular iron overload, induced by upregulation of hepcidin, promotes the progression of viral infections[395]. Seemingly, HCV infections represent a peculiar exception with a downregulation of hepcidin, which following antiviral therapy is then upregulated again[374,396]. Low levels of hepcidin have been attributed to reactive oxygen species-mediated decrease in *HAMP* gene expression. On the other hand, HCV-driven inflammation may counteract reactive oxygen species-induced hepcidin reduction, as elevated IL-6 stimulates *HAMP* gene transcription. Details have been presented in an extensive review[397].

An additional, mechanistic explanation of the role of iron in HCV viral persistence has been reported. HCV may or may not upregulate *HAMP* gene expression and hepcidin levels depending on infection acuteness and viral load. Enhanced hepcidin expression led to enhanced HCV translation and replication. Apart from intracellular iron sequestration through hepcidin, an intercellular mobilization of iron through ferritin was also required for efficient viral replication[398]. A recent review emphasized the role of low levels of hepcidin in various liver diseases, including HCV, as hepcidin is further implicated in both iron deposition in hepatocytes and participation in stellate cell activation and liver fibrosis[399].

Recently, ferroptosis has been investigated as a type of non-apoptotic cell death in different pathological conditions, including viral hepatitis. The molecular hallmark of ferroptosis is iron-driven lipid peroxidation that interacts with cellular antioxidant systems and may play a critical role in HCV's natural course[400].

However, other studies reported opposing results stating that supra-physiological, intracellular iron damages HCV. A knockdown of hepcidin increased intracellular ferritin and inhibited HCV replication[401]. A recent review argued that iron overload is weakly fibrogenic in experimental animals, rarely causing serious liver damage in patients and only if its action is potentiated by a coexisting inflammation. However, this is exactly the situation in HCV-iron coexistence[402].

WHO REALLY NEEDS TREATMENT: THE PROPOSED POLICY

There are two reasons for a global elimination of HCV. The most important is to prevent the progression to cirrhosis, then to HCC and finally to death. Once cirrhosis has been developed, these objectives are no longer feasible as both HCC and all-mortality continue despite treatment with DAAs[403,404]. An additional reason for treating HCV infection is that even subjects with a low progression of disease are still capable of transmitting HCV, a problem of major interest for PWIDs and MSMs but also for hemodialysis patients or health care employees performing invasive procedures. As previously detailed, comorbidities such as diabetes, obesity, metabolic syndrome and alcohol consumption may play a critical role in the outcome of liver disease in SVR patients without cirrhosis[405] (Table 2).

We live in the severe acute respiratory syndrome coronavirus 2 era. All cost-effectiveness studies are based on Markov models. Such mathematical models, however accurate they may be, rely on various assumptions that may or may not be true, like the real mortality of HCV or the real outcome of HCV cirrhosis. In fact, the accepted cost of quality-adjusted life-years may be seriously reconsidered in many countries in view of the COVID-19 pandemic.

Since it is very doubtful that screening and treatment programs will be feasible even in high-income countries, the idea of micro-elimination programs has been proposed. A micro-elimination program is a concept that breaks down the national elimination targets into easier-to-accomplish, smaller tasks[406]. So far, there have been programs applied to either special populations or to small, geographically-restricted communities. Currently successful efforts are being made in Egypt[407,408] where a follow-up study of 2.4 years after the first evaluation with a re-test of the initially non-infected individuals demonstrated a significant reduction in the incidence of new cases of HCV infection. Infections through surgery or dental procedures were independent predictors of incident new cases[409]. A different approach was reported from the United States. They estimated cost effectiveness comparing screening programs targeting PWIDs with the universal screening of United States adults. They concluded that HCV screening for PWIDs could potentially decrease the risk of untreated HCV infection and liver-related mortality and may thus be cost effective[410].

New tools must be developed to implement micro-elimination. This is obviously harder in high-prevalence, low-income countries than in Western countries[411]. One of the most difficult parts of such a program would be to engage as many patients as possible in alcohol abuse treatment programs provided by liver clinics. It should also be kept in mind that successful abstinence will be limited to a minority of those treated anyway[412].

Current literature, as detailed above, has clearly identified certain subpopulations that should definitely be treated as they are prone to develop cirrhosis or HCC and may therefore have increased mortality. On the other hand, prospective studies have shown that HCV infection without an additional confounding factor will have very limited consequences. Even cirrhotic patients have a much better survival rate compared to those with cirrhosis of other etiologies. Present data allow for the proposition of a different micro-elimination program based on groups that are likely to either progress towards cirrhosis or are at risk of reinfection.

We believe that screening and treatment should be reserved only for people with AUDs, obesity or other aspects of metabolic syndrome, compromised immune system including HIV coinfection, cancer and hemodialysis patients and cirrhosis patients. Additional groups should include PWID, MSM, hemophiliacs and thalassemia patients. These are the groups that should be treated with DAAs, not necessarily those DAAs recommended by the EASL or the American Association for the Study of Liver Diseases.

Approaching these subpopulations is easier compared with approaching the entirety of the general population and obviously more cost-effective in every country irrespective of income. The proposition remains valid even after the development of an effective vaccine as these are the groups that should be vaccinated to avoid waste of valuable resources.

CONCLUSION

It is highly unlikely that the WHO target will be achieved by 2030, as among other reasons, there is only one known example of a chronic human infection that is near eradication in the absence of a vaccine: yaws, caused by *Treponema pallidum pertenue*, a

Table 2 Group targets in a hepatitis C virus micro-elimination program

Alcohol consumers
Metabolic syndrome: Obesity-steatosis; Cirrhosis-HCC; Diabetes mellitus and insulin resistance
HCV/HIV coinfection
People who inject drugs
Males having sex with males
Hemophiliacs, thalassemia patients
Immunocompromised patients

HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma.

spirochete bacterium. However, this disease is geographically localized and can be effectively treated with conventional inexpensive antibiotics[413].

The ambitious WHO goal can be achieved only after the development of a highly efficient, low-cost vaccine or by treating all carriers of HCV with DAAs. Both however are unlikely to be achieved in the foreseeable future, particularly in the era of the severe acute respiratory syndrome coronavirus 2 pandemic. Moreover, HCV is a disease with two main characteristics. First, its transmission is difficult since sexual transmission is negligible. Second, the deadly consequences are not frequent and take a very long time to appear even after cirrhosis development.

On the other hand, practically all studies indicate that in the vast majority of patients cirrhosis is associated with one or more of the following: alcohol, the presence of metabolic syndrome (including obesity, diabetes, IR and hyperuricemia), intravenous drug use or immune compromise (including HIV coinfection).

A micro-elimination program should therefore be implemented focusing on screening and treatment of people who belong to the above high-risk groups. Such a program may altogether eradicate morbidity and mortality of HCV even in the COVID-19 era. This program can still be used after any future vaccine development.

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REVIEW

Current aspects of renal dysfunction after liver transplantation

Mariana P Pacheco, Luiz Augusto Carneiro-D'Albuquerque, Daniel F Mazo

ORCID number: Mariana P Pacheco 0000-0002-3932-3195; Luiz Augusto Carneiro-D'Albuquerque 0000-0001-7607-7168; Daniel F Mazo 0000-0002-2164-2630.

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Mariana P Pacheco, Daniel F Mazo, Division of Clinical Gastroenterology and Hepatology, Department of Gastroenterology, University of São Paulo School of Medicine, Sao Paulo 05403-900, Sao Paulo, Brazil

Luiz Augusto Carneiro-D'Albuquerque, Division of Digestive Organs Transplant, Department of Gastroenterology, University of São Paulo School of Medicine, Sao Paulo 05403-900, Sao Paulo, Brazil

Daniel F Mazo, Division of Gastroenterology, Department of Internal Medicine, School of Medical Sciences of University of Campinas, Campinas 13083-878, Sao Paulo, Brazil

Corresponding author: Daniel F Mazo, MD, PhD, Professor, Staff Physician, Division of Clinical Gastroenterology and Hepatology, Department of Gastroenterology, University of São Paulo School of Medicine, Av. Dr. Enéas de Carvalho Aguiar nº 255, Instituto Central, # 9159, Sao Paulo 05403-900, Sao Paulo, Brazil. daniel.mazo@hc.fm.usp.br

Abstract

The development of chronic kidney disease (CKD) after liver transplantation (LT) exerts a severe effect on the survival of patients. The widespread adoption of the model for end-stage liver disease score strongly impacted CKD incidence after the procedure, as several patients are transplanted with previously deteriorated renal function. Due to its multifactorial nature, encompassing pre-transplantation conditions, perioperative events, and nephrotoxic immunosuppressor therapies, the accurate identification of patients under risk of renal disease, and the implementation of preventive approaches, are extremely important. Methods for the evaluation of renal function in this setting range from formulas that estimate the glomerular filtration rate, to non-invasive markers, although no option has yet proved efficient in early detection of kidney injury. Considering the nephrotoxicity of calcineurin inhibitors (CNI) as a factor of utmost importance after LT, early nephroprotective strategies are highly recommended. They are based mainly on delaying the application of CNI during the immediate postoperative-period, reducing their dosage, and associating them with other less nephrotoxic drugs, such as mycophenolate mofetil and everolimus. This review provides a critical assessment of the causes of renal dysfunction after LT, the methods of its evaluation, and the interventions aimed at preserving renal function early and belatedly after LT.

Key Words: Liver transplantation; Acute kidney injury; Chronic kidney disease; Calcineurin inhibitor; Mycophenolic acid; Everolimus

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Core Tip: Post-liver transplantation renal dysfunction is a frequent complication that has a major impact on the survival rate of the graft and the patient. Due to the multifactorial nature of post-transplantation chronic kidney disease, the ability to accurately identify patients under risk and the development of preventive approaches are paramount. This review presents the state-of-the-art on the topic: Its causes, renal function assessment methods, and the most studied nephroprotective strategies.

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INTRODUCTION

Liver transplantation (LT) changed the natural history of cirrhotic patients. It is considered the gold standard treatment for liver diseases on terminal stages, including hepatocellular carcinoma[1,2]. Significant advances were achieved in immunosuppression, in the treatment of acute and chronic cellular rejection, in the prevention of infections, and in preoperative preparation, organ preservation, surgical procedure, and anesthesiologic techniques[1]. Therefore, short-term mortality, which was due mainly to intraoperative causes, infection, and acute rejection, has considerably decreased[1]. On the other hand, long-term mortality has not been altered for the past few years[3]. Longer survival rates have, in turn, increased the so-considered late complications of LT, such as diabetes mellitus, cardiovascular diseases, malignance, and renal dysfunction[1,4].

Chronic kidney disease (CKD) develops in the majority of patients who survive the first 6 postoperative months[5,6]. The cumulative incidence of post-LT CKD is significantly higher than those following cardiac and lung transplants[5]. The presence of CKD post-LT, defined by the Chronic Kidney Foundation as the reduction of the glomerular filtration rate (GFR) to values lower than 60 mL/min/1.73 m² for 3 mo or longer, is a frequent complication and has a negative impact on the graft's and patient's survival rates[7]. A recent study from the United States assessed 602 liver transplanted patients between 2010 and 2016 and reported a prevalence of CKD in its distinct stages in 41.5% of recipients[8]. In addition, renal failure was responsible for 6% of deaths of patients who survived the first 6 post-transplantation months[8].

Prevalence and incidence studies of post-LT renal dysfunction show wide variations, attributable mainly to different criteria used for CKD definition, and to the various follow-up periods evaluated[9]. The first consensus of the International Liver Transplantation Society reported that the prevalence of post-LT CKD ranged between 30% and 90%, and terminal CKD that required renal replacement therapy (RRT) was described in 2% to 5% of patients per year[10]. According to the Scientific Registry of Transplantation Recipients, the incidence of stage 4 or 5 of CKD after 1, 3, and 5 years of transplantation was 8%, 14%, and 18%, respectively, reaching up to a quarter of recipients within 10 years after the transplant[5,7]. An extensive prospective study evaluated the prevalence of CKD through measurement of GFR by iothalamate clearance and the associated mortality in 1211 patients over 25 years[9]. The authors reported that after 4 mo of LT, 40% of the patients already had CKD stage ≥ 3, and the risk of death increased when the GFR decreased to values below 30 mL/min/1.73 m² or worse[9]. Only 18% of the subjects had normal renal function after 25 years, in opposition to 39% of age-group matched individuals from the general population[9].

When the whole spectrum of renal dysfunction after LT is evaluated, few studies exhibit data of its occurrence in the early stages after the procedure. A Spanish study with 230 patients revealed that 30.8%, 28.8%, and 26.4% of patients had stage 3 CKD after 12, 24, and 30 mo of transplantation, respectively[11]. It is interesting to highlight that, despite a mild reduction in GFR (60-89 mL/min/1.73 m²) is not considered CKD, this same Spanish study observed that a significant percentage of patients had this GFR range (46.2%, 41.9%, and 46.2% within 12, 24, and 30 mo after the transplant, respectively), even with normal GFR prior to LT[11]. In the American cohort, this mild

reduction in GFR occurred in 21.7% of patients who had controlled blood pressure, and in 24.9% of patients with uncontrolled blood pressure within 1 year after LT[8].

Renal dysfunction etiologies in LT are multifactorial and are related to the period of its occurrence. Therefore, the main factors leading to renal dysfunction can be grouped into pre-LT, intraoperative, and post-LT periods (Table 1). Though efforts have been made to reduce or avoid such predisposing factors of renal dysfunction, many of them are not modifiable, such as, for instance, pre-existing conditions.

CAUSES OF RENAL DYSFUNCTION IN LT

Pre-transplantation

Acute kidney injury (AKI) is a common and increasing clinical event in subjects with cirrhosis[12]. It is estimated to occur in 20% to 57% of hospitalized subjects with decompensated liver disease, with a significant impact on survival[12-14]. AKI in this setting is an underestimated problem because the main assessed parameter is the serum creatinine (Scr). Scr overestimates GFR for several reasons, such as muscle mass, frequently reduced in patients with cirrhosis[15]. In advanced liver disease, the presence of AKI is common, often secondary to infection, hypovolemia, use of vasodilators, and other nephrotoxic drugs, such as non-steroidal anti-inflammatory agents and contrasts. Nonetheless, particularly in those with advanced decompensated liver disease, one of the main causes of loss of renal function is the circulatory dysfunction induced by portal hypertension[16]. Activation of the renin-angiotensin-aldosterone system (RAAS) leads to kidney vasoconstriction, which may be reverted with the resolution of the portal hypertension, as seen in cases of hepatorenal syndrome (HRS)[16]. The understanding of the pathophysiology of HRS-AKI has evolved and currently encompasses, in addition to circulatory dysfunction, systemic inflammation, microvascular dysfunction, and direct tubular damage[17-19]. The combination of albumin and terlipressin can restore renal function in 40% to 73% of patients with HRS-AKI[20-23]. Moreover, response to terlipressin and albumin was associated with a reduction in the need for RRT after LT and reduced the risk of CKD at 1 year after LT, as recently reported by Piano *et al*[24]. However, LT remains the definitive treatment for this condition. On the other hand, renal vasoconstriction for extended periods, associated with the intrinsic kidney damage caused by the surgical procedure, may lead to organic and less reversible renal injury, which explains why some patients with HRS develop worse renal function after LT[16]. Indeed, non-recovery of renal function is associated with the duration of pre-LT dialysis in HRS patients[25]. In addition, renal recovery and patient survival post-LT are better in those with HRS than in those with acute tubular necrosis[26].

It is noteworthy that AKI is an increasingly recognized risk factor for CKD development and progression[27]. A recent Spanish study reported that in a cohort of patients with cirrhosis who survive an episode of AKI, 25% of them developed CKD, and this passage from AKI to CKD was associated with an increased risk of AKI, complications of cirrhosis, and hospital re-admissions[28]. According to Francoz[29] (2020), cirrhosis, in addition to being a risk factor for AKI, would also be a predisposing condition for the development of CKD, with an impact on those needing LT.

Some renal parenchymal chronic diseases relatively specific to cirrhosis, such as immune complex glomerulonephritis (seen in hepatitis B and C) or immunoglobulin A nephropathy (seen in cirrhosis due to alcohol), are increasingly found in candidates for LT[16]. However, non-specific causes of CKD, mainly secondary to metabolic syndrome, are also increasingly seen in this population[30]. Special attention must be paid to subjects with non-alcoholic steatohepatitis (NASH)/metabolic dysfunction-associated fatty liver disease (MAFLD), which represents a growing LT indication worldwide[31-33]. These patients have additional risk factors for renal injury, such as diabetes, hypertension, and obesity, which in turn are associated to some degree with kidney injury in the pre-LT period[34]. In addition, it has already been demonstrated that NASH is an independent predictor of stage 3 renal injury post-LT[35].

Finally, the model for end-stage liver disease (MELD) score itself, used for the allocation of organs, which includes Scr in its estimation, favors candidates with renal function impairment for LT. Therefore, its widespread use may increase the number of patients who require RRT and simultaneous liver and kidney transplants, impacting the long-term renal function of the recipients[16,36]. The risk of post-LT end-stage renal disease, which is related to post-LT mortality, was 15% higher in the MELD era, as shown by Sharma *et al* in 2011[37]. Interestingly, the proportion of MELD sodium score attributable to creatinine ≥ 50% was associated with advanced renal dysfunction

Table 1 Main causes of kidney dysfunction in liver transplantation according to the period of its occurrence

Pre-transplantation	Perioperative	After transplantation
Hypovolemia; Infections; Nephrotoxic drugs; Hepatorenal syndrome; High MELD; NASH/MAFLD; Renal parenchymal diseases associated with hepatitis B, C and alcohol	Hemodynamic instability; Reperfusion injury; Nephrotoxic drugs	Calcineurin inhibitors; Diabetic nephropathy; Hypertensive nephropathy

MELD: Model for end-stage liver disease; NASH: Non-alcoholic steatohepatitis; MAFLD: Metabolic dysfunction-associated fatty liver disease.

at 1 year post-LT in a recent United States retrospective study using the United Network for Organ Sharing (UNOS) database[38].

Altogether, these factors in the pre-LT period contribute to the increased finding of renal dysfunction after LT.

Perioperative

The development of renal injury in the perioperative period leads to extended hospitalization, increases the risk of acute rejection and infection, and impacts global mortality[39]. Renal dysfunction during this period has a reported incidence of 11% to 94%, depending upon the definition and the assessment method applied[40], with acute tubular necrosis as the most frequent etiology[6,16]. Risk factors for perioperative AKI include sepsis, nephrotoxic drugs, impairment of renal perfusion associated with hemodynamic instability during surgery, and the harmful effect of the ischemia-reperfusion injury[6,41].

High perioperative aminotransferase aspartate peak is independently correlated with the risk of renal injury after LT[6,42]. The use of blood transfusion in the intraoperative period, especially above 10 units, increased the risk of renal dysfunction when combined with diuresis lower than 100 mL/h[43]. The excessive use of blood products may be related to large blood losses and, consequently, hypotension, but could also induce a pro-inflammatory state that impairs oxygen supply to tissues and increases the concentration of free hemoglobin and iron, both nephrotoxic[44].

In addition, the lack of grafts forces surgeons to use more marginal grafts (of older patients, with steatosis, and organ donors with circulatory causes of death), which is directly related to reperfusion-ischemia injury[45]. This may result in more renal dysfunction posteriorly[46]. Predictive models for renal dysfunction have already been assessed, but none of the candidates was capable of adequately predicting the outcome within a time frame suitable for appropriate intervention[40].

Post-transplantation

Up to 1 year: The most common histopathological findings in subjects with CKD 1 year after the LT included calcineurin inhibitors (CNI) toxicity, diabetic nephropathy, and thrombotic microangiopathy[7].

Early nephrotoxicity by CNI is in most part functional, and a dose-dependent mechanism. CNI induce vasoconstriction of afferent and efferent arterioles, with reduction of renal perfusion and of the ultrafiltration coefficient and, consequently, reduction of the glomerular filtration[10,47]. Therefore, early renal alteration may be reversible with the reduction of the CNI dose[5]. The accurate vasoconstriction mechanism is still unclear, but it is known that there is a disequilibrium of vasoactive substances that lead to the increase of vasoconstrictors, for instance, endothelin, angiotensin II, and thromboxane, and the decrease in the generation of vasodilators, such as prostaglandins and nitric oxide[10,48] (Figure 1).

A comprehensive study using the UNOS database evaluated 1720 patients with pre-LT renal dysfunction and demonstrated that the most important independent predictor of recovery of renal function, defined as creatinine < 1.5 mg/dL and survival rate greater than 29 d, was the absence of liver graft dysfunction[49]. Induction with anti-thymocyte globulin, decreasing the use of CNI, was also considered a protective factor[49]. Interestingly, the authors showed that the need for RRT for up to 8 wk was not a contributing factor to CKD evolution[49]. In Taiwan, Lin *et al*[50] (2012) reported that the Scr in the 4th wk after LT was a good predictive variable for CKD over 5 years, which implies that the aggressive management of early kidney injury may avoid the development of CKD. Ye *et al*[51] (2020) described that the estimated GFR at 1 year after LT, beyond the stage at which postoperative complications may occur and with greater immunosuppression stabilization, had a good correlation with the estimated GFR in 5 years.

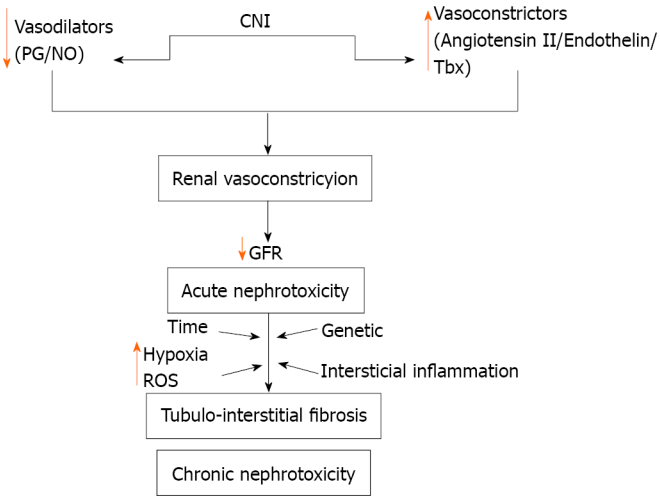

Figure 1 Calcineurin inhibitors nephrotoxicity mechanism. CNI: Calcineurin Inhibitors; PG: Prostaglandins; NO: Nitric oxide; Tbx: Thromboxane; GFR: Glomerular filtration rate; ROS: Reactive oxygen species.

After 1 year: Unquestionably, the main mechanism of CKD evolution is CNI nephrotoxicity. It is estimated that in about 50% of patients who develop renal dysfunction in the postoperative period, CNI nephrotoxicity is the root cause[6]. Gonwa *et al*[52] (2001) evaluated 843 liver-transplanted patients for up to 13 years, and the presumed etiologies of end-stage renal disease that occurred in 45 patients were CNI toxicity (73.3%), progression of subjacent renal disease (11.1%), focal segmental glomerulosclerosis (6.66%), non-recovered HRS (6.66%), and acute tubular necrosis/toxicity of amphotericin (2.22%). In the longer term, diabetes mellitus and high blood pressure worsen renal damage even further[16]. As standard immunosuppressive therapy is based upon CNI [tacrolimus (FK) and cyclosporine] monotherapy or is associated with other agents (for instance, mycophenolate)[53], handling such complications is one of the core challenges of physicians who manage liver-transplanted patients.

The chronic renal damage that CNI causes is characterized by the development of irreversible structural injury and may culminate in terminal stages of kidney disease [10]. Upon histology evaluation, obliterative arteriopathy, glomerular ischemic collapse, tubular vacuolization, and focal areas of tubular atrophy and interstitial fibrosis may occur[10]. The development of chronic nephropathy induced by CNI is also influenced by genetic variability[54]. The factors responsible for chronic injury by CNI are complex and not completely understood, and involve interstitial inflammation and renal vasoconstriction, with activation of the RAAS in a relevant manner (Figure 1). Consequently, there is an imbalance between vasodilator and vasoconstrictor factors, leading to renal damage[10]. Another possible mechanism generated by CNI nephrotoxicity is the direct injury of tubular epithelial cells, derived from the blocking of mitochondrial permeability and inhibition of prolyl isomerase, the enzyme responsible for the interconversion of the cis- and trans-isomers of peptide bonds, which can speed up or slow down protein cleavage[55].

The previously mentioned study from Ye *et al*[51] (2020) also evaluated several predictive factors of the evolution to CKD in post-LT patients under the FK regimen [51]. The authors reported that the GFR found 1 or 2 years after LT showed a good correlation with the one found after 5 years, and demonstrated that those subjects with GFR < 60 mL/min/1.73 m² are those who will probably develop an irreversible renal injury in the following years. It is important to highlight that while statistical significance was not found in the annual reduction of the GFR between FK-using and FK-free groups, the serum concentration of FK influenced the progression to CKD within 1 and 2 years, with receiver operating characteristic curves of 0.73 and 0.78, respectively[51].

The development of post-LT metabolic syndrome is frequent[56]. In addition to lingering pre-LT risk factors, there is an increase in post-LT risks due to immunosuppression with corticosteroids and CNI[57]. Moreover, it is expected that metabolic syndrome components after LT will continue to rise due to the increase of NASH/MAFLD as an indication for LT[31-33,58]. VanWagner *et al*[8] (2020) reported that hypertension was observed in up to 92% of patients and diabetes in 53%, adding risk to the development of CKD in such patients. Moreover, there is the possibility of

an evolution to advanced liver fibrosis/cirrhosis, which adds renal dysfunction components of cirrhotic patients to those associated with immunosuppression.

METHODS OF ASSESSMENT OF RENAL FUNCTION

Due to the multifactorial nature of CKD in the post-LT period, the ability to accurately identify patients under risk and the development of preventive strategies are crucial [59]. The discovery of a more sensitive biomarker would make it feasible to quickly detect renal damage factors and implement early therapeutic interventions. To assess renal function after LT, the GFR measurement is the most used laboratory tool[60]. For such, the most commonly used test in clinical practice is the dosage of Scr, which supplies information about GFR and is widely available and inexpensive[7,60]. Nevertheless, besides considerations concerning analytical aspects of the test, there are individual characteristics that may interfere with the results. Reference Scr values are influenced by non-renal factors, such as body weight, muscle mass, race, age, gender, and protein intake[61]. In this way, Scr values differ among children and adult women and men[61]. Scr is also considered a late renal dysfunction marker, requiring a reduction above 50% of glomerular ultrafiltration before an increase in Scr is observed [60]. Accuracy can be improved through measurement of 24 h creatinine clearance, but it also brings limitations: Higher costs, the need to store urine for 24 h (subject to errors in sample collection and incomplete bladder emptying), and the effect of tubular secretion of creatinine[61]. As it is a small molecule and does not bind to serum proteins, creatinine is freely filtrated by glomeruli; however, about 10% to 20% of the creatinine excreted in urine comes from its secretion by the proximal tubular cell. Tubular secretion is the main determinant of the overestimation of renal function when creatinine clearance is used[62]. This secretion by the tubular cell is variable in the same individual and increases with the reduction of the glomerular filtration[60-62].

Equations specifically developed for the estimation of creatinine clearance, such as Cockcroft-Gault[63] or the Modification of Diet in Renal Disease (MDRD)[64], have been widely used in clinical practice[60]. MDRD-4 (simplified MDRD) is the equation usually employed to compute GFR because it is considered to be as accurate as MDRD-6, the original equation[65]. Indeed, there are undeniable advantages to its use, but despite its generalized use in clinical practice, the measurement of GFR through formulae is not accurate, particularly in patients with uncommon biotypes or diet alterations, in the presence of rapid deterioration of renal function, or when the GFR values are above 60 mL/min/1.73 m^2[62].

The 2012 joint guideline of the American Society of Transplantation along with the American Association for the Study of Liver Diseases (AASLD) recommended the use of the MDRD equation in any of its four variations as superior to the use of the isolated Scr and of the 24 h creatinine clearance[66]. Nevertheless, in 2016 the American Society of Transplantation developed a document that specifically endorsed the use of MDRD-4 and CKD-epidemiology-creatinine[67] as the formula that yields the most accurate results of GFR in this population[68] (Table 2). The 2019 British guideline for post-LT management advised that close monitoring of renal dysfunction after LT is necessary, but did not cite which method to use in the evaluation[69].

Due to the antiproteinuric effect of CNI, proteinuria may be absent even in advanced stages of CKD[66]. The AASLD guideline recommends its measurement in isolated samples at least once per year[66].

Cystatin-C is placed as an alternative glomerular filtration marker because, as it can be completely eliminated in the circulation, its serum concentration could properly reflect the GFR[70]. Unlike creatinine, it is not influenced by muscle mass, diet, or the presence of infection or malignance[60,70]. However, other factors, such as age, male gender, weight, height, tobacco use, steroid use, and thyroid disease, are independently associated with elevated cystatin-C levels, suggesting low specificity in detecting renal impairment[60]. The 2016 guideline of the American Society of Transplantation states that among all blood-based estimates of GFR, the cystatin-C equations are the most accurate in post-LT patients[68].

Renal clearance of inulin is the gold standard of GFR measurement, but the necessity of performing a test in standardized conditions, with continuous intravenous injection of the marker, its elevated cost, and peculiar aspects of laboratory dosage limit its use in clinical practice, restricting it to research settings[71]. The use of renal and plasma clearance of radioactive isotopes, such as ^{51}Cr-ethylenediamine tetraacetic acid (EDTA), is growing in clinical practice, as they are safer and simpler methods -

Table 2 Main formulas for measurement of glomerular filtration rate

Formulas	
Cockcroft Gault	[(140 – age) × weight]/[(72 × Scr) × (0.85 if female)]
MDRD 4	$175 \times (Scr)^{-1.154} \times (age)^{-0.203} \times (0.742 \text{ if female}) \times (1.212 \text{ if black})$
MDRD 6	$198 \times (Scr)^{-0.858} \times (age)^{-0.1678} \times (0.822 \text{ if female}) \times (1.178 \text{ if black}) \times (Ur)^{-0.293} \times (\text{urine urea nitrogen excretion g/d})^{0.249}$
CKD-EPI creatinine equation	$141 \times \min(\text{creat}/\kappa, 1)\alpha \times \max(\text{creat}/\kappa, 1)^{-1.209} \times 0.993^{age} \times (1.018 \text{ if female}) \times (1.159 \text{ if black})$

Age in years; Weight in kg; κ is 0.7 for females and 0.9 for males; α is -0.329 for females and -0.411 for males. MDRD: Modification of diet in renal disease; CKD-EPI: Chronic kidney disease epidemiology collaboration; Scr: Serum creatinine; Ur: Urea concentration; Min: Minimum of creat/κ or 1; Max: Maximum of creat/κ or 1.

and were sufficiently accurate - to measure GFR[71]. Despite the ongoing discussion about the underestimation of renal clearance of ^{51}Cr-EDTA in comparison to renal clearance of inulin, the determination of GFR by ^{51}Cr-EDTA and by inulin had comparable results in kidney-transplanted patients[72]. The authors have shown elevated correlation coefficients between both methods (0.9516)[72]. Only a few studies used ^{51}Cr-EDTA clearance measurement to evaluate GFR in children and adults post-LT[73-76].

Neutrophil gelatinase-associated lipocalin (NGAL), a protein expressed in the renal tubular cells, has been gaining attention as an early marker of AKI, including the immediate post-LT period, though there is still considerable variation among studies [77]. In a Japanese study, Tsuchimoto *et al*[78] (2014) reported that NGAL was the best urinary marker in comparison to 5 other assessed candidates [liver-type fatty acid binding protein, monocyte chemoattractant protein-1, interleukin (IL)-18, cystatin-C, and osteopontin], with its values in the 1st and 7th postoperative days being helpful to predict AKI by FK in liver-transplanted patients. In 2019, Lima *et al*[79] evaluated the urine and plasma NGAL elevation pattern in the LT perioperative period of 100 patients and showed that these measurements were able to predict AKI diagnosis earlier in this setting. Urinary NGAL levels evaluated just after the LT procedure could accurately predict AKI development in 27 subjects in the United Kingdom, as Robertson *et al*[80] reported in 2019. NGAL could also be useful in the context of chronic renal injury, not only to predict its progressions but also to monitor the response to treatment aimed at protecting renal function[60,81,82]. However, changes in urinary NGAL are not specific to CKD, and more studies are required to further explore its potential in the context of LT[83]. The utility of NGAL and other urinary and serum biomarkers for the prediction of AKI in patients undergoing LT has yet to be defined because AKI pathogenesis in this context is complex[84]. Moreover, optimal cut-off values and source of confounding factors must be addressed prior to routine clinical use in LT[60,77].

The use of imaging tests for kidney evaluation, under several clinical situations, is a well-established method. In adults, the ultrasound exam finding of a more echogenic renal cortex as compared to liver echogenicity clearly suggests renal disease[85-87]. It is a very sensitive marker of renal parenchymal disease and correlates well with some glomerular and tubular-interstitial injuries[87]. Recent studies displayed the role of magnetic resonance imaging (MRI) in evaluating hypoxia and fibrosis of the renal parenchyma through 2 techniques (blood oxygen level-dependent MRI and diffusion-weighted MRI). Both provided information on the progression of kidney disease[88]. The standardization of acquisition and processing protocols is required, as current methodological differences exist across studies and pose difficulties in comparing the results[88]. In addition, kidney evaluation data in liver-transplanted patients are awaited, as the non-invasive assessment of renal changes by magnetic resonance diffusion imaging has so far been evaluated only after lung transplantation[89].

Acoustic radiation force impulse (ARFI) is a recently developed noninvasive technique. It is safe and convenient to assess the elasticity of tissues[90]. The technique is capable of identifying the parenchymal elasticity by measuring the speed of the shear wave, and it is integrated into conventional ultrasound devices[91]. It has been used mainly in the determination of hepatic fibrosis and cirrhosis in chronic viral hepatitis and displays a good correlation with the degree of liver fibrosis[92,93]. In recent years, the ARFI technique has also been applied to other organs, such as the muscles[94], prostate[95], and breast[96]. Fibrosis is the core process of the progression

of CKD and this method has also been evaluated in this scenario. Despite the inability to predict pathological alterations, ARFI results were significantly correlated with GFR and the stage of CKD in several studies[97]. However, kidney hemodynamical alterations may affect the renal parenchymatic elasticity during CKD progression[97]. In a pilot study, Bob *et al*[98] (2015) reported that ARFI measurements diminished with the decrease in GFR, suggesting a cutoff at 2.26 m/s or less as a predictor of stage 4 or 5 CKD. Structurally, the final *via* of post-LT CKD culminates in kidney fibrosis and, similar to what happens in other organs such as the liver, the collagen deposition may culminate in an increase in tissue stiffness. Therefore, elastography techniques could play a role in this setting.

STRATEGIES FOR PREVENTING POST-TRANSPLANTATION CHRONIC RENAL DISEASE

Patients developing CKD, besides having limitations regarding the use of immunosuppressing drugs, exhibit an increased risk of hospitalization, infectious complications, and graft dysfunction. Moreover, they have a 2 to 4 times greater risk of death[5,99]. Thus, preventive strategies to preserve kidney function after LT are paramount. The management of comorbidities and other general factors leading to CKD must be remembered. Therefore, it is possible to extrapolate non-transplanted CKD orientations to these patients[100]. It is advisable to, at least once per year, measure or calculate GFR *via* formulae, besides performing albuminuria or proteinuria tests and, if necessary, referring the patient to a nephrologist (Table 3).

Regarding arterial hypertension, the pressure target should be below 140/90 mmHg in the absence of proteinuria, and below 130/80 mmHg when it is present[66,68]. These objectives must be reached with a combination of lifestyle changes and pharmacological options. The choice of the anti-hypertensive must be based upon safety and drug interaction[69]. Dihydropyridine calcium channel blockers, such as amlodipine, are considered first-choice agents, as they reduce systemic vascular resistance and improve renal blood flow, thus blocking CNI's vasoconstrictor action[68,69]. Drugs that block the RAAS, such as angiotensin converting enzyme inhibitors and angiotensin receptor blockers, must be avoided in the immediate postoperative period because, in this period, the activity of plasmatic renin is decreased and their use may worsen the hyperkalemia observed with FK use[69]. After this period, these drugs are of choice for patients with diabetes, significant proteinuria, and CKD[66,68,69]. Beta blockers are safe, but diuretics must be employed with caution, as they may further affect renal function[69]. Cardiovascular complications are frequent non-graft-related causes of mortality after LT, and it has already been shown that the mean GFR is inversely proportional to the time of the first cardiovascular event[6,101]. In these cases, immunosuppression based on everolimus, with the withdrawal or reduction of FK, improved both renal function and the risk of major cardiac events in comparison to standard therapy, as shown by Saliba *et al*[101].

Post-LT diabetes treatment must target an Hb1AC below 7.0%[66]. Though there is no consensus about which is the best antidiabetic, it is advisable to decrease or interrupt corticosteroids as soon as possible[66,69]. When corticosteroids are administered at higher doses, the use of insulin is safer and more efficient[66].

Dietary interventions may help to slow down CKD's progress. Salt intake should be restricted to less than 2 g of sodium per day to better control blood pressure and proteinuria[69,102]. Other interventions with less evidence would be to avoid high protein intake (less than 1.3 g/kg/d) in subjects at risk of CKD and to further reduce it to 0.8 g/kg/d in those with GFR < 30 mL/min/1.73 m²[102]. Post-LT weight gain is also associated with the development of metabolic syndrome, cardiovascular events, and renal dysfunction[103,104]. Therefore, weight gain should be avoided. Charlton *et al*[105] (2017) demonstrated that the introduction of everolimus as an attempt to reduce the FK dosage decreased the weight gain of patients within 1-2 years after LT.

Liver-transplanted patients are particularly vulnerable to hemodynamic insults and present an increased risk of developing AKI after exposure to nephrotoxins, such as non-steroidal anti-inflammatory drugs, amphotericin B, aminoglycosides, and contrast agents. Whenever possible, therapy with CNI before and after exposure to potential nephrotoxins should be reduced or suspended, and a temporary switch to other non-nephrotoxic immunosuppressors should be considered, due to rejection risks, in addition to other nephroprotective measures established for other patients[100].

Table 3 Referral to specialized kidney care services

Indication
AKI or abrupt sustained fall in GFR
GFR < 30 mL/min/1.73 m^2
Consistent significant albuminuria (albumin/creatinine ratio ≥ 300 mg/g or albumin excretion rate ≥ 300 mg/24 h, equivalent to protein/creatinine ratio ≥ 500 mg/g or protein excretion rate ≥ 500 mg/24 h)
Progression of CKD (a drop in GFR from baseline by 25% or a sustained decline in GFR of more than 5 mL/min/1.73 m^2/yr)

AKI: Acute kidney injury; GFR: Glomerular filtration rate; CKD: Chronic kidney disease.

CNI-induced nephrotoxicity contributes to the worsening of renal function in both the short and long term; the greatest challenge is the choice of a strategy that minimizes renal dysfunction without simultaneously affecting the survival rate of the liver graft. In the immediate postoperative stage (< 1 mo), a strategy to spare the renal function has been the administration of short-term induction therapy (mono- or polyclonal antibodies), with delayed introduction of CNI[6,69]. Several clinical trials have shown that in individuals with preoperative renal dysfunction, this approach resulted in a better renal outcome, as it avoided the vasoconstrictor risks of CNI in synergy with other perioperative risk factors associated with AKI[6,69,106]. Basiliximab and daclizumab, which have a selective target upon activated T-cells blocking CD25, the IL-2 receptor, are the most used. The use of belatacept has also been studied, in addition to other standard strategies of induction, but the study had to be terminated due to a higher mortality rate of the belatacept group[107].

Early usage of mycophenolate mofetil (MMF) has also been assessed in subjects without preoperative kidney dysfunction, with an improvement of GFR without disadvantages in terms of graft rejection[108]. Thus, reduced FK doses in combination with MMF are capable of protecting renal function more efficiently than isolated FK use[109].

The use of the mammalian target of rapamycin inhibitors (mTor-I) everolimus has also been demonstrated to be a protective renal strategy[110]. Everolimus was capable of promoting an early decrease of FK dosage with similar efficiency and safety, and with preservation of renal function[111,112]. A continued effect was observed after 1, 2, and 3 years of LT[112]. In these studies, in which everolimus was introduced 4 wk after LT, thrombosis of the hepatic artery and impaired wound healing were not observed[111,112]. In the PROTECT randomized trial, it was shown that the monotherapy with everolimus displayed better results regarding GFR within 12 mo after LT, with similar mortality, graft rejection, and therapeutic failure rates[111]. In addition, maintenance of the GFR benefit in the extension of the study of 24 and 36 mo was observed[113,114]. However, infections, leukopenia, dyslipidemia, and treatment discontinuation were higher in the everolimus group[111].

The use of sirolimus, another mTor-I, was also evaluated as a nephroprotective option after LT. A large randomized prospective trial assessed the conversion of CNI to sirolimus in 607 liver-transplanted patients, and a higher rate of acute rejection and discontinuations was observed in the sirolimus group, with no gains regarding the GFR[115]. Also, the early use of sirolimus in *de novo* LT in a phase II trial did not exhibit nephroprotection and showed higher graft loss rates, mortality, and sepsis as compared to the use of tacrolimus at standard doses alone[116]. The FDA added a black box warning for the use of sirolimus and belatacept in LT recipients[68].

Therefore, strategies for the primary preservation of long-term renal function are based upon precocious post-operative CNI reduction in combination with non-nephrotoxic immunosuppressing drugs, such as MMF or everolimus, and the use of induction therapy in selected patients[6,66,68,69].

Despite the evidence that early MMF and mTor-I usage minimizes renal dysfunction, this strategy does not seem to be as effective when performed after 1 year from the LT[68]. The significant reduction of the CNI dose (below 50% of the original dosage) with the addition of MMF resulted in an improvement of GFR without negatively affecting the graft's survival rate, and it did not increase the incidence of adverse events, even when it was implemented after 1 year of LT, but with a weaker effect[117-120]. Complete withdrawal of CNI increased the risk of rejection and graft loss, without adding gains to the GFR[120,121]. Unfortunately, these studies demonstrated that once the renal function is markedly affected (GFR < 60

mL/min/1.73 m²), changing to a kidney preservation approach is less efficient to improve GFR, possibly due to irreversible kidney structural damage[6]. Regarding everolimus, the studies revealed no increase in the rate of liver rejection, but they also reported little to no improvement in the GFR (about 4 mL/min)[122-125], with a discontinuity rate above 10% and the development of proteinuria in some recipients [125]. Therefore, there is little evidence that the substitution of CNI for mTor-I after 1 year has some benefit for the improvement of renal function[68]. Indeed, early rather than late conversion of CNI to everolimus after LT was shown to be a safe approach to preserve long-term renal function, as recently reported by Saliba *et al*[126] (2020) in the EVEROLIVER Registry.

To minimize the use of CNI, new drugs are currently being tested, such as CFZ533, an IgG1 anti-CD40 antibody, which blocks the signaling pathways implicated in rejection; however, the majority of such studies were in renal transplantation[127,128]. Finally, for those patients who develop end-stage renal disease with a need for dialysis, there is a benefit from renal transplantation from either living or deceased donors, with a mortality reduction of 44% to 60% in comparison to those patients who stay in RRT[66,129,130].

CONCLUSION

Post-LT renal dysfunction is a frequent and severe problem, impacting patients' morbimortality. Its etiology is multifactorial, with pre-, intra-, and post-LT factors. Its incidence is increasing, mainly after the changes in organ allocation by MELD score. Early diagnosis is paramount, but the most conventional methods of estimating GFR have limitations, and there is currently no accurate, non-invasive marker ready to use in clinical practice. Taking into consideration that CNI's toxicity is an important post-LT cause of renal dysfunction, strategies to minimize its use, such as induction therapy followed by a reduction in CNI levels, and the introduction of less nephrotoxic drugs, such as MMF and everolimus, are still the best options to preserve renal function. Also, aggressive treatment of other comorbidities that can negatively impact GFR is important. Nonetheless, once the renal function is significantly compromised, the adoption of a nephroprotective immunosuppression approach is less efficient. New immunosuppressing drugs that do not lead to GFR impairment and do not increase liver rejection rates are eagerly awaited.

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REVIEW

Hepatitis C: Problems to extinction and residual hepatic and extrahepatic lesions after sustained virological response

Sara Cuesta-Sancho, Mercedes Márquez-Coello, Francisco Illanes-Álvarez, Denisse Márquez-Ruiz, Ana Arizcorreta, Fátima Galán-Sánchez, Natalia Montiel, Manuel Rodriguez-Iglesias, José-Antonio Girón-González

ORCID number: Sara Cuesta-Sancho 0000-0002-9890-1063; Mercedes Márquez-Coello 0000-0003-3948-6258; Francisco Illanes-Álvarez 0000-0002-0837-8265; Denisse Márquez-Ruiz 0000-0001-5183-8872; Ana Arizcorreta 0000-0001-8655-3164; Fátima Galán-Sánchez 0000-0003-0101-6701; Natalia Montiel 0000-0003-2503-3960; Manuel Rodriguez-Iglesias 0000-0002-7097-9651; José-Antonio Girón-González 0000-0002-7482-5828.

Author contributions: Cuesta-Sancho S and Márquez-Coello M are equal contributions; Cuesta-Sancho S, Márquez-Coello M, Girón-González JA wrote the draft; All authors conceived and designed, analyzed the data; all authors contributed to critical revision of the manuscript, and saw and approved the final version.

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Sara Cuesta-Sancho, Mercedes Márquez-Coello, Francisco Illanes-Álvarez, Denisse Márquez-Ruiz, Ana Arizcorreta, José-Antonio Girón-González, Medicina Interna, Hospital Universitario Puerta del Mar, Facultad de Medicina, Universidad de Cádiz, Instituto para la Investigación e Innovación en Ciencias Biomédicas de Cádiz (INiBICA), Cádiz 11009, Spain

Fátima Galán-Sánchez, Natalia Montiel, Manuel Rodriguez-Iglesias, Microbiología, Hospital Universitario Puerta del Mar, Facultad de Medicina, Universidad de Cádiz, Instituto para la Investigación e Innovación en Ciencias Biomédicas de Cádiz (INiBICA), Cádiz 11009, Spain

Corresponding author: José-Antonio Girón-González, MD, PhD, Doctor, Medicina Interna, Hospital Universitario Puerta del Mar, Facultad de Medicina, Universidad de Cádiz, Instituto para la Investigación e Innovación en Ciencias Biomédicas de Cádiz (INiBICA), Avda Ana de Viya, s/n, Cádiz 11009, Spain. joseantonio.giron@uca.es

Abstract

Loss of follow-up or reinfections hinder the expectations of hepatitis C eradication despite the existence of highly effective treatments. Moreover, the elimination of the infection does not imply the reversion of those chronic alterations derived from the previous infection by hepatitis C virus (HCV). This review analyzes the risk factors associated with loss to follow-up in diagnosis or treatment, and the possibility of reinfection. Likewise, it assesses the residual alterations induced by chronic HCV infection considering the liver alterations (inflammation, fibrosis, risk of decompensation, hepatocellular carcinoma, liver transplantation) and, on the other hand, the comorbidities and extrahepatic manifestations (cryoglobulinemia, non-Hodgkin lymphoma, peripheral insulin resistance, and lipid, bone and cognitive alterations). Peculiarities present in subjects coinfected with human immunodeficiency virus are analyzed in each section.

Key Words: Hepatitis C virus; Sustained virological response; Direct antiviral agents; Human immunodeficiency virus; Cirrhosis decompensation; Hepatocarcinoma; Extrahepatic complications

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Core Tip: The excellent hepatitis C virus (HCV) response to direct acting agents should not obviate certain obstacles to eradicate this pathology, especially the loss to follow-up and the possibility of reinfections. Chronic hepatitis C determines persistent alterations despite the elimination of HCV, such as liver dysfunction and continued risk of decompensation and hepatocarcinoma, especially in subjects treated in advanced stages of the disease. Weight gain after sustained virological response (SVR) may favor liver steatosis, increasing the risk of progression of hepatic disease. The probability of complications after SVR in human immunodeficiency virus coinfected patients is similar to that of those HCV-monoinfected.

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INTRODUCTION

In 2015, 71 million people were estimated to be infected by hepatitis C virus (HCV) worldwide[1]. Based on the release of curative treatment for chronic hepatitis C infection[2], the World Health Assembly set the target of a 90% reduction in new infections and a 65% reduction in viral hepatitis related mortality by 2030 as compared to 2015[3].

These expectations should not let us forget some problems that underlie the current situation and that could be classified into the following (Figure 1): (1) Patients whose HCV infection has not been eradicated or those who have been reinfected; and (2) Organic injuries associated with chronic hepatitis C, whether hepatic or extrahepatic, whose normalization is not reached by the elimination of the virus.

FAILURE TO ERADICATE HCV INFECTION

Both American[4] and European[5] Associations for the Study of Liver Diseases (AASLD and EASL, respectively) recommend combinations of direct acting agents (DAAs) against HCV, such as an NS5A inhibitor with either an NS3/4 protease inhibitor (grazoprevir/elbasvir or glecaprevir/pibrentasvir), or a nucleotide analogue plus an NS5A inhibitor (sofosbuvir/velpatasvir), for eight to twelve weeks. The preferred regimens to simplify HCV therapy are pangenotypics combinations (sofosbuvir/velpatasvir or glecaprevir/pibrentasvir)[6].

Treatment should be offered to all HCV RNA-positive patients. The efficacy of these combinations has been higher than 90%[6].

The proportion of patients who do not achieve a sustained virological response (SVR) is lower than 10%, considering those who have virologic relapse and those who are lost during the follow-up[7,8]. The combined therapy with sofosbuvir, velpatasvir and voxilaprevir is recommended for retreating patients with previously failing DAAs regimens[9,10].

There are few contraindications to therapy with DAAs. The use of certain cytochrome P450/P-glycoprotein inducing agents are contraindicated with all regimens, because of the risk of reducing DAAs concentrations. In patients with Child-Pugh B or C decompensated cirrhosis, NS3/4a protease inhibitors are contraindicated due to the increased concentrations of protease inhibitor in these patients and its associated toxicity risk. In patients with a glomerular filtration rate lower than 30 mL/min/1.73 m^2, increased serum levels of sofosbuvir are detected[8]. Interactions between DAAs and other drugs need to be addressed in patients, mainly in those with human immunodeficiency virus (HIV)/HCV-coinfection and those with central nervous system-acting drugs[11].

A major barrier to HCV elimination is the loss to follow-up, defined as nonattendance to any appointment in the care cascade at any time[12]. A review about

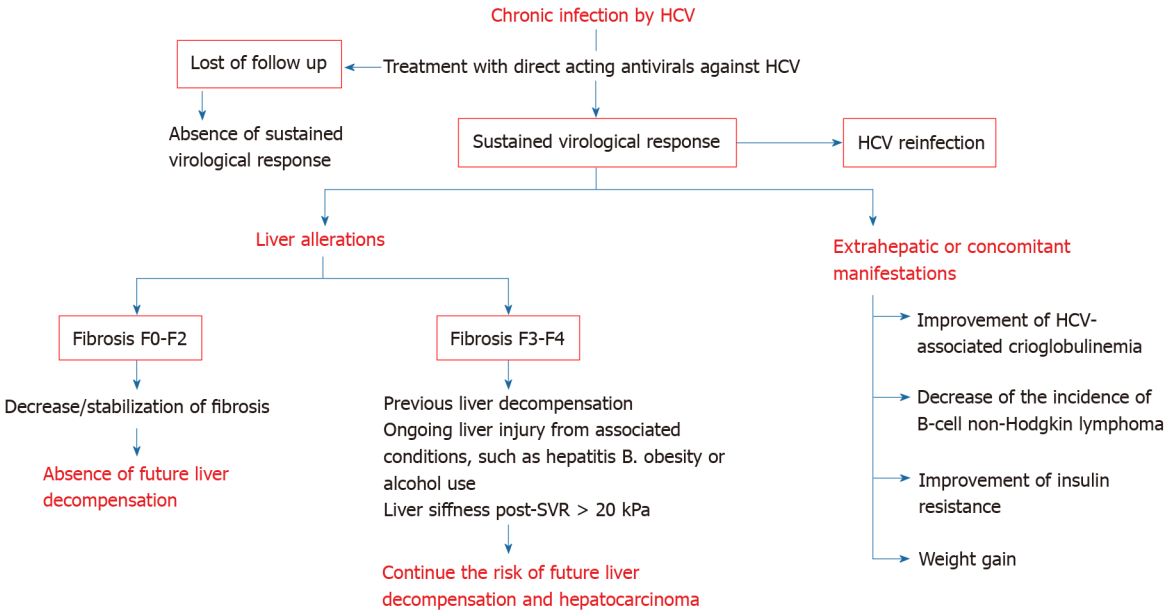

Figure 1 Modification of hepatic and extrahepatic manifestations of chronic hepatitis C after therapy with direct acting agents. Figure represents the main alterations modified by this treatment, as well as those factors that influence the risk: absence of follow-up, previous fibrosis stage, previous decompensation, ongoing liver injury or liver stiffness after sustained viral response. HCV: Hepatitis C virus; SVR: Sustained virologic response; Kpa: Kilopascals.

the loss to follow-up in HCV care has been recently published[13]. Factors associated with the loss to follow-up are younger age (< 45 years old)[14], treatment in hospital [15], a history of homelessness[15,16], mental illness[16,17] and injecting drug use, either past[18] or ongoing[17]. In contrast, factors associated with retention in care are older age (≥ 60 years old) and HIV coinfection[19].

Several strategies have been proposed to overcome the loss to follow-up in HCV care[20]: (1) Enhancing HCV identification and linkage to care for vulnerable populations (injecting drug users) through intensified outreach screening. A large-scale intensified screening initiative across Europa (Hep-Check and Hep-Link) has been started up[21,22]; (2) HCV micro-elimination strategies, focused on collectives with a high prevalence and/or increased risk of loss of follow-up and/or in patients with worst short-term prognosis[23,24]; (3) Reflex testing, a strategy of hepatitis C diagnosis in a single step, based on the detection of HCV RNA or HCV core antigen when the anti-HCV antibody test proves to be positive[25] and referral to an HCV specialist for further evaluation[26]; (4) Use of pan-genotypic HCV drug regimens; and (5) Inclusion of HCV-infected patients who use drugs on opioid agonist therapy programs can reach elevated HCV elimination rates with current DAAs[27].

Reinfection following SVR has been documented in several studies in drug users [28], prisoners[29], and men who have sex with men (MSM)[30]. After SVR, the incidence of reinfection is 2 to 6/100 person-years in subjects who inject drugs and 10 to 15/100 person-years in HIV-infected MSM[30-33]. Elevated rates of reinfection may compromise the benefits of treatment.

EFFECTS OF SVR

The analysis of the changes in the hepatitis C evolution after SVR will be considered in several sections: (1) Overall survival; (2) Changes in liver disease (liver fibrosis, liver function, decompensation of chronic liver disease, hepatocarcinoma); and (3) Modifications of extrahepatic alterations. In each section and whenever evidence is available, the changes in HIV/HCV-coinfected patients will be discussed.

Global survival

Chronic HCV infection is associated with a substantially impaired overall survival, both by liver-related and extrahepatic causes[34].

Individuals with compensated cirrhosis who reach SVR with interferon-based treatments have an improved long-term outcome[35-37]. Real-world cohorts have confirmed this significant reduction in the liver-related death risk after DAAs: In the

HEPATHER study, the annual incidence of liver-related mortality in subjects with SVR was 0.36% *vs* 0.96% in non-SVRs; and in individuals with cirrhosis, the respective incidence was 0.64% *vs* 1.57%[38]. Even though in other studies[39-41], the risk reduction was more pronounced, the risk still existed. Therefore, a proportion of subjects (small but worrisome) dies because of liver-related disease after viral clearance.

Symptoms and mortality from severe extrahepatic manifestations, such as cryoglobulinemic vasculitis, renal-related effects[42,43] and some lymphoproliferative disorders[44,45] decrease with HCV eradication as well. Moreover, subjects with SVR have a better physical and emotional health and an improved life quality[46].

In HIV/HCV coinfected patients with compensated cirrhosis, benefits from SVR due to interferon-based regimens in the incidence of liver-related decompensation, some extrahepatic manifestations and the overall mortality, have been demonstrated [47,48]. In addition, decreases of HIV reservoirs[49] and HIV progression[48] have been observed after HCV eradication.

Hepatic modifications after SVR

Inflammation, fibrosis, and liver function previous to treatment against HCV affect the prognosis of chronic liver disease[50].

Liver inflammation and fibrosis: Several factors contribute to liver inflammation, acting on macrophage receptors. They include viral particles; pathogen-associated molecular patterns, such as lipopolysaccharides, which can translocate from the intestine into the circulation because of increased intestinal permeability; and damage-associated molecular patterns released by hepatocytes[51]. These factors induce and amplify hepatic inflammation by activating macrophages[52]. Macrophage activation promotes hepatic stellate cell activation and extracellular matrix accumulation[53]. After antiviral therapy of chronic hepatitis C, macrophage activation is diminished, as indicated by biomarkers[54] or aminotransferase levels[55], in parallel with the amelioration of hepatic inflammation observed in liver biopsies[56]. Only patients who achieve a SVR solve inflammation in liver biopsies[57].

Biopsy-proven fibrosis regression is developed when SVR is achieved[58]. In patients treated with interferon-based regimens, a 39%-73% of subjects who reached SVR had decreased liver fibrosis and necrosis, as assessed by liver biopsy[55]. Likewise, several studies have reported a significant diminution in liver stiffness after treatment, either by interferon- or DAAs-based schemes[59-64].

HCV-induced fibrosis progresses more rapidly in HIV-coinfected patients than in monoinfected individuals[65]. The impact of SVR on liver fibrosis (biopsy-proven) or liver stiffness within HIV/HCV patients treated with interferon- or DAAs-based regimens has been also proved[66].

Liver function: In routine clinical practice, Child-Pugh and Model for End-stage Liver Disease (MELD) scores are often used for the evaluation of liver function. Although improvement of liver function is not uniformly demonstrated in studies with HCV-induced liver cirrhosis, a Child-Pugh decrease ≥ 1 and/or MELD decrease ≥ 2 between baseline and SVR has been demonstrated in a 56%-57% of DAAs-treated patients. Factors independently related with liver function improvement are male gender, bilirubin < 1.2 mg/dL and international normalized rate < 1.3 at baseline[67].

Short-term outcomes after DAAs in individuals with decompensated cirrhosis showed a decrease of the MELD score in the majority, while it did not change in 17%, and worsened in 25% of them[68-73]. Subjects with low MELD scores can even be removed from liver transplantation lists[74-76], although the clinical improvement may not necessarily persist, and they may be still in risk of relisting on the transplant list or even death[76-78].

A more detailed analysis of liver function changes can be obtained by using other methods[79]. Thus, it has been demonstrated that amelioration of inflammation improves the ureagenesis[80].

Decompensation of liver cirrhosis: Decompensated cirrhosis is characterized by the development of new ascites, spontaneous bacterial peritonitis, hepatic encephalopathy, bleeding gastro-esophageal varices, hepato-renal syndrome or hepato-pulmonary syndrome[81].

With DAAs, subjects with compensated cirrhosis achieve SVR rates over 95%[82, 83]. Among subject with ongoing or previous decompensation, SVR rates of approximately 80% are reached with DAAs treatment[69,73,84].

Hepatic venous pressure gradient (HVPG) improves shortly after DAAs therapy in patients with HCV-related cirrhosis[85-87]. However, an HVPG more elevated at baseline is linked with smaller reductions in portal pressure and practically all of those with an HVPG ≥ 16 mmHg remain with clinically significant portal hypertension after SVR[85,88,89]. These findings contribute to explain the persistence of risk of decompensations due to portal hypertension in patients with decompensated liver cirrhosis after SVR.

The severity of liver disease prior to the therapy is a predictor of liver decompensation after SVR. The Scottish real-world study[39] informed that the risk of decompensation decreased by 86% compared to non-SVR in patients with compensated cirrhosis. In those patients with previously decompensated cirrhosis, viral clearance was linked to a lower incidence rate of decompensations[73]. In the Krassenburg's series, the cumulative 2-year event-free survival was 89.0% for those with Child-Pugh class A cirrhosis compared to 45.2% for those with Child-Pugh class B/C cirrhosis. Furthermore, while SVR was independently associated with an improved event-free survival in patients with Child-Pugh class A cirrhosis, it did not in patients with Child-Pugh class B/C cirrhosis[90], although controversial results have been published[41].

Attending to these contradictory findings in patients with decompensated cirrhosis, in a retrospective study of patients with Child-Pugh class B or C, El-Sherif *et al*[77] analyzed those factors related with the reduction of Child-Pugh score to class A (implicating the absence of new decompensations) after DAAs. During a follow-up of 255 d, 31.6% of subjects with baseline Child-Pugh class B cirrhosis and 12.3% of subjects with Child-Pugh class C cirrhosis met the primary study end point. The presence of complications such as ascites or encephalopathy, serum concentration of albumin < 3.5 g/dL or alanine aminotransferase < 60 U/L, and body mass index (BMI) > 25 kg/m^2 were related to a higher risk of not achieving a decrease in Child-Pugh to class A, regardless of SVR[77].

Bleeding from esophageal varices is uncommon after SVR[91]. However, subjects with compensated cirrhosis who achieve SVR should follow on receiving endoscopic surveillance for esophageal varices, according to the AASLD guidance[92]: (1) In those without known varices, surveillance endoscopy is indicated every 2 years if there are associated conditions, such as obesity or alcohol use; and every 3 years if liver injury is suppressed, such as after alcohol abstinence; and (2) In those with known varices, surveillance endoscopy is indicated every 12 mo if there is proof of present liver injury from associated conditions and every 24 mo if liver injury is quiescent[92]. However, in our opinion, these recommendations could be modulated by the knowledge of liver stiffness, as will be analyzed later on.

In individuals with compensated cirrhosis, HIV coinfection was not related with an increased probability of liver complications after viral eradication than those HCV-monoinfected[93,94]. In the series of Corma-Gómez *et al*[95], the likelihood of staying free of hepatic complications or transplant at 12 and 24 mo was 99% and 96% in HCV-monoinfected patients and 99 and 98% in HIV/HCV coinfected patients with predominantly (> 95% of individuals) Child-Pugh class A cirrhosis (P = 0.648). In the multivariate analysis of the overall population, liver decompensation before SVR, drug use as the risk factor for HCV infection –reduced healthcare adherence and ongoing use of toxics may underlie this finding– and liver stiffness at SVR were independently linked to the presence of a hepatic complication or requiring a liver transplant[96].

The importance of the liver stiffness at SVR has been remarked. Post-treatment liver stiffness > 20 kPa is significantly associated with developing cirrhosis decompensation, either ascites, variceal bleeding or hepatic encephalopathy (Hazard ratio 8.04)[97]. This cutoff point has been supported by other authors[98].

Furthermore, liver stiffness–based strategies recognize subjects with reduced risk of developing esophageal variceal bleeding episodes, in whom esophagogastroduodenoscopy screening can be unnecessary[95,99-102]. Corma-Gómez *et al*[95] demonstrated that subjects with a liver stiffness < 30 kPa and a platelet count > 110000/mm^3 after SVR are not at risk of variceal bleeding[102].

Hepatocellular carcinoma: Hepatocellular carcinoma (HCC) incidence has been growing over the last two decades and is expected to rise until 2030 in several countries[103]. HCV-infected patients have a lifetime risk of approximately 5%. HCC is expected to occur 30 years after infection. In patients with hepatitis C, HCC is almost invariably present in the setting of cirrhosis[104].

Antiviral treatment of chronic HCV infections significantly reduces the risk of HCC [104-109]. Meta-analyses have shown that DAAs therapy is associated with a decrease of *de novo* HCC incidence close to 80%, similar to that achieved with interferon-based

therapies[108,109]. However, it is the most frequent liver-related event after SVR[98,106]. The incidence rate of HCC after SVR is 1.1-1.9/100 patient-years[94,98].

A Spanish series including 1035 HCV-infected patients, of which 667 (64%) were coinfected with HIV, has demonstrated that HIV-coinfection appears to be associated with an inferior risk of HCC occurrence among patients with HCV and advanced fibrosis who reach SVR due to DAA[110], although these data are controversial[94]. HIV/HCV coinfected patients have an earlier onset and aggressive HCC, with associated higher mortality risk[93].

Post-SVR surveillance by liver imaging and alpha-fetoprotein (AFP) tests every six months after SVR is recommended in cirrhotic population by international guidelines [111,112]. This recommendation has also been extended to patients with advanced fibrosis (F3) by EASL guidelines[113]. Unless they are affected by liver comorbidities, patients with lower stages of fibrosis may be discharged from specialized care. Age, male sex, lower baseline albumin or higher bilirrubin levels, a FIB-4 score > 3.25, hepatitis B coinfection or a liver stiffness post-SVR ≥ 20 kPa have been associated with a higher risk of developing HCC[94,98,114]. A scheme of the factors that could influence the need of surveillance of HCC is shown in Table 1.

A low sensitivity of usual screening methods of HCC occurrence has been observed. Ultrasonography has been reported to have 60% sensitivity and 97% specificity as a screening method of HCC in cirrhotic patients; it has been proved to be cost-effective [115]. The performance of ultrasound surveillance of HCC is even worse in HIV-coinfected patients with cirrhosis[116]. AFP by itself is not adequate for screening purposes: A low sensitivity (40%-75%), as well as a high false positive rate in active hepatitis, precludes its use as screening method[117].

Liver transplantation: Liver transplantation is an appropriate treatment option for individuals with acute liver failure, end-stage liver disease, and primary hepatic malignancy. Patients with cirrhosis are typically candidates for liver transplantation once MELD score is ≥ 15[118].

A decrease of MELD score is expected in a proportion of patients with cirrhosis after SVR[90]. However, although a clinically significant decrease in MELD score is achieved by a 25% of DAAs-treated patients across a short follow-up, after a longer period (median follow-up of 4 years), the average MELD variations are not significant [78]. Yet, these data suggest that certain patients with decompensated cirrhosis may benefit from therapy with DAAs.

Both International Liver Transplantation Society Consensus Statement[113] and EASL guidelines[114] only advise against antivirals when MELD score > 20, based on the ELITA study[74]. Treatment of subjects with more elevated scores could make MELD score improves at such point that they could no longer be eligible for liver transplantation, but they would still be at risk of fatal complications and/or low life quality. Below this threshold, the recommendations suggest offering antiviral therapy with the hope of a stable improvement in liver function.

Nowadays, the 5-year overall survival after liver transplantation in individuals with chronic HCV infection is expected to be approximately 75%, since HCV recurrence does not limit anymore the liver transplantation outcome because of possibility of SVR after DAAs therapies[119,120].

Extrahepatic modifications

Two-thirds of patients with chronic hepatitis C present extrahepatic manifestations [75]. These include autoimmune and lymphoproliferative disorders, ranging from cryoglobulinemia vasculitis to malignant B-cell lymphoma[42-45], cutaneous, metabolic, cardiovascular, neurological, and bone conditions[40,78,105,106].

Cryoglobulinemia and B cell non-Hodgkin lymphoma: There is evidence that SVR after treatment with peginterferon α and ribavirin is related to improvements in cryoglobulinemia associated to HCV infection and possible regression of B-cell non-Hodgkin lymphoma. In Cacoub's meta-analysis, SVR was confirmed to be linked to notably more elevated proportion of complete remissions in subjects with cryoglobulinemia vasculitis [odds ratio (OR) 20.76] and objective response in those with malignant B-cell lymphoproliferative diseases (OR 6.49)[121].

Some data with DAAs therapy in the scenario of vasculitis end-organ disease related to cryoglobulinemia, including renal disease, have demonstrated responses in 20% to 90% of subjects[122,123]. Notwithstanding, subjects with severe end-organ disease are likely to still need plasmapheresis and/or rituximab[123].

Regression of marginal zone lymphomas in HCV-infected individuals after interferon-based therapies has been noticed[124]. In addition, HCV infection treatment

Table 1 Factors that influence the surveillance of hepatocarcinoma

Factors existing previous to sustained virological response	Factors existing after sustained virological response
Comorbidities (steatosis, diabetes mellitus, excessive alcohol consumption)	Persistently elevated ALT, AST, GGT, alpha-fetoprotein, liver stiffness, FIB-4, APRI, or VITRO
Male gender	Hypoalbuminemia
Age > 64 years	Increasing body weight (?)
F4	
Portal hypertension	
Elevated FIB-4, APRI, alpha-fetoprotein	
History of decompensation	
History of IFN therapy (?)	
HCV genotypes 1 and 3 (?)	

Reference[115], modified. FIB-4: Fibrosis 4 index (includes serum levels of AST and ALT, platelet count and age); APRI: Aspartate aminotransferase to platelet ratio; IFN: Interferon; HCV: Hepatitis C virus; ALT: Alanine amino-tranferase; AST: Aspartate aminotransferase; GGT: Gamma-glutamil transferase; VITRO: Von Willebrand Factor Antigen to Platelets ratio.

diminishes the incidence of lymphomas in HCV-monoinfected individuals[45]. HCV treatment with interferon does not change the incidence of lymphomas in patients coinfected with HIV[125].

There are few data about the effects of an SVR achieved with DAAs therapy on extrahepatic diseases apart from potential regression of cryoglobulinemia and B-cell non-Hodgkin lymphoma[126,127].

Extrahepatic dermatologic manifestations: Approximately 50% of individuals with porphyria cutanea tarda present HCV infection[128]. Amelioration of this metabolic condition during interferon-based therapy has been described repeatedly[129]. Currently, there are not enough data to ascertain the effect of DAA therapy on porphyria cutanea tarda.

Between 10% and 40% of patients with lichen planus present HCV antibodies[130,131]. Contradictory data have been reported about resolution of lichen planus with interferon-based regimens[130,131], but promising perspectives with DAAs are present[131].

Comorbidities: Liver steatosis: Metabolic dysfunction-associated fatty liver disease (MAFLD)[132] is the main chronic liver disorder[133]. Subjects with chronic HCV infection and MAFLD display accelerated liver fibrosis progression, and a higher risk of developing HCC[134,135]. HCV clearance can lead to amelioration (and even to regression) of liver steatosis, at least when directly related to HCV genotype 3 infection[135,136]. However, a meaningful percentage of patients with SVR may still have continued to have steatosis not related to HCV, but to other factors associated with it, especially overweight/obesity[135].

An additional problem in patients with hepatitis C is the weight gain after SVR. In a prospective study on more than 11000 patients, 52.6% gained weight and 19.8% gained excess weight (defined as at least 9 kg gain after 24 mo). SVR was an independent weight gain predictor[137,138]. The mechanisms behind body weight modifications could involve neuropsychiatric alterations or diminished circulating levels of inflammatory cytokines[137]. Increased BMI after viral clearance has clinical impact on fibrosis evolution of HCV-infected patients. The long-term evaluation of the German HCV-contaminated anti-D cohort demonstrated that a 6% of patients with SVR after DAAs developed advanced liver fibrosis after 35 years from infection; BMI and viral clearance independently predicted the evolution to cirrhosis[138].

Comorbidities: Insulin resistance and diabetes mellitus, lipid alterations: HCV perturbs glucose metabolism inducing insulin resistance, which may progress to type 2 diabetes[135]. Furthermore, type 2 diabetes is one of the main risk factors of progression to chronic hepatitis C[134,135].

SVR has been shown to improve insulin resistance, as measured by the Homeostatic Model Assessment of Insulin Resistance score; it provides a significant protective effect on the incidence of diabetes[122,139]. However, patients with diabetes mellitus

type 2 diagnosed previously to DAAs remain diabetic despite to SVR, although the doses of antidiabetic drugs could be smaller[139]. Moreover, antiviral therapy may reduce renal and cardiovascular (ischemic stroke, acute coronary syndrome) complications in HCV-infected patients with established diabetes, as has been demonstrated in a prospective cohort[140].

Type 2 diabetes mellitus could continue affecting progression of hepatitis C after SVR[134]. Pre-treatment diabetes has been linked to a higher risk of cirrhosis, liver decompensation and HCC in a population of 33000 patients without baseline cirrhosis, treated with DAA and followed up for 3 years. The effect of diabetes mellitus was independent of the attainment of SVR[141].

DAAs increase triglyceride and cholesterol release through very low-density lipoproteins, thus normalizing hepatic lipid homeostasis[142]. An increase in total cholesterol and low- and high-density lipoproteins is observed during treatment and after treatment completion[143].

Comorbidities: Osteoporosis: Viral hepatitis has been linked to decreased bone mineral density (BMD). Diverse factors have been hypothesized to contribute to it: elevated serum levels of inflammatory cytokines, decreased hepatic hydroxylation of vitamin D, altered hepatic production of insulin-like growth factor 1 and osteoprotegerin, and hypogonadism[144]. Osteoporosis and bone fractures are usual among individuals with liver cirrhosis, especially in those with other risk factors[145].

The knowledge about the effects of SVR on BMD in HCV-infected subjects is limited. Studies have included usually samples of less than 50 patients treated with peginterferon plus ribavirin regimens. Although limitations of these studies are evident, they have demonstrated that BMD values at the lumbar spine and the femoral neck improve after the treatment[146,147], but controversial data have been also reported[148].

In HIV coinfected patients, a higher risk of osteoporosis and bone fractures has been communicated. Meaningful modifications in BMD and bone remodeling biomarkers plasma levels have not been observed after HCV eradication[149].

Comorbidities: Cognitive alterations: Improvements of neurocognitive dysfunction are observed after interferon-based SVR[150,151], including self-reported mood outcomes[152]. This finding is corroborated by DAAs-induced improvements in brain magnetic resonance spectroscopy[153]. However, controversial data have been published[154]. These discrepancies could be due to the distinct methods used to assess cognitive dysfunction and the timing of the tests.

CONCLUSION

The excellent HCV response to DAAs treatment should not obviate certain obstacles to eradicate this pathology, especially the loss of follow-up and the possibility of reinfections.

Apart from the above, chronic hepatitis C determines several alterations whose normalization is not expected despite the elimination of HCV, especially in subjects treated in advanced stages of the disease. These include persistent liver dysfunction and continued risk of decompensation and HCC, although certainly less frequently than in individuals without SVR. Furthermore, weight gain after SVR may favor MAFLD in these patients increasing the risk of progression of liver disease. In HIV coinfected patients, SVR is not associated with a higher probability of liver complications than that of those HCV-monoinfected.

To summarize, DAAs administration reduces the death risk by cirrhosis and HCC and also reduces common comorbidities among people with HCV. Nevertheless, we are still far from eradicating the disease, but the World Health Organization goal by 2030 (a 90% reduction in new infections and a 65% reduction in viral hepatitis related mortality as compared to 2015)[3] is hopefully feasible.

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REVIEW

Metabolic and nutritional triggers associated with increased risk of liver complications in SARS-CoV-2

Rosangela Passos de Jesus, Jozélio Freire de Carvalho, Lucivalda Pereira Magalhães de Oliveira, Carla de Magalhães Cunha, Thaisy Cristina Honorato Santos Alves, Sandra Tavares Brito Vieira, Virginia Maria Figueiredo, Allain Amador Bueno

ORCID number: Rosangela Passos de Jesus 0000-0002-2276-0452; Jozélio Freire de Carvalho 0000-0002-7957-0844; Lucivalda Pereira Magalhães de Oliveira 0000-0003-4822-5930; Carla de Magalhães Cunha 0000-0002-9644-7194; Thaisy Cristina Honorato Santos Alves 0000-0001-6286-497X; Sandra Tavares Brito Vieira 0000-0002-3773-1531; Virginia Maria Figueiredo 0000-0002-6641-1203; Allain Amador Bueno 0000-0002-9456-8558.

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Rosangela Passos de Jesus, Lucivalda Pereira Magalhães de Oliveira, Carla de Magalhães Cunha, Thaisy Cristina Honorato Santos Alves, Sandra Tavares Brito Vieira, Postgraduate Program in Food, Nutrition and Health at the School of Nutrition of the Federal University of Bahia, Salvador 40.110-150, Bahia, Brazil

Jozélio Freire de Carvalho, Institute of Health Sciences, Federal University of Bahia, Salvador 40231-300, Bahia, Brazil

Thaisy Cristina Honorato Santos Alves, Institute of Life Sciences, State University of Bahia, Salvador 41.150-000, Bahia, Brazil

Virginia Maria Figueiredo, Department of Gastroenterology, IPEMED, Ipemed Faculty of Medical Sciences, Salvador 40170-110, Bahia, Brazil

Allain Amador Bueno, College of Health, Life and Environmental Sciences, University of Worcester, Worcester WR2 6AJ, United Kingdom

Corresponding author: Jozélio Freire de Carvalho, MD, PhD, Adjunct Professor, Institute of Health Sciences, Federal University of Bahia, Av. Reitor Miguel Calmon, s/n - Canela, Salvador 40231-300, Bahia, Brazil. jotafc@gmail.com

Abstract

Obesity, diabetes, cardiovascular and respiratory diseases, cancer and smoking are risk factors for negative outcomes in severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which can quickly induce severe respiratory failure in 5% of cases. Coronavirus disease-associated liver injury may occur during progression of SARS-CoV-2 in patients with or without pre-existing liver disease, and damage to the liver parenchyma can be caused by infection of hepatocytes. Cirrhosis patients may be particularly vulnerable to SARS-CoV-2 if suffering with cirrhosis-associated immune dysfunction. Furthermore, pharmacotherapies including macrolide or quinolone antibiotics and steroids can also induce liver damage. In this review we addressed nutritional status and nutritional interventions in severe SARS-CoV-2 liver patients. As guidelines for SARS-CoV-2 in intensive care (IC) specifically are not yet available, strategies for management of sepsis and SARS are suggested in SARS-CoV-2. Early enteral nutrition (EN) should be started soon after IC admission, preferably employing iso-osmolar

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polymeric formula with initial protein content at 0.8 g/kg per day progressively increasing up to 1.3 g/kg per day and enriched with fish oil at 0.1 g/kg per day to 0.2 g/kg per day. Monitoring is necessary to identify signs of intolerance, hemodynamic instability and metabolic disorders, and transition to parenteral nutrition should not be delayed when energy and protein targets cannot be met *via* EN. Nutrients including vitamins A, C, D, E, B6, B12, folic acid, zinc, selenium and ω-3 fatty acids have in isolation or in combination shown beneficial effects upon immune function and inflammation modulation. Cautious and monitored supplementation up to upper limits may be beneficial in management strategies for SARS-CoV-2 liver patients.

Key Words: COVID-19; SARS-CoV-2; Enteral nutrition; Parenteral nutrition; Hepatic failure

Core Tip: Coronavirus disease-associated liver injury may occur in the progression of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in patients with or without pre-existing liver disease. Patients with cirrhosis-associated immune dysfunction are particularly vulnerable. Strategies for management of sepsis and SARS are suggested in SARS-CoV-2 for intensive care patients, including early enteral nutrition soon after intensive care unit admission. Transition to parenteral nutrition should not be delayed when energy and protein targets cannot be met *via* EN. In outpatient settings, micronutrient and ω-3 fatty acids have shown beneficial effects upon immune function and inflammation modulation and may be beneficial in management for SARS-CoV-2 liver patients.

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INTRODUCTION

In December 2019 a new viral infection was identified and observed to quickly induce severe respiratory failure. Its aetiological agent was described as a new beta-coronavirus, responsible for inducing severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), distinct from SARS-CoV and Middle East respiratory syndrome coronavirus (MERS-CoV). The disease spread extremely quickly around the globe, and the World Health Organization declared in March 2020 a pandemic[1]. It is believed that the SARS-CoV-2 may have mutated in wild sources including bats and snakes, and passed onto humans through direct contact, including their consumption, or indirect contact, for example exposure to faeces[1]. Anthroponotic transmission occurs mainly through saliva droplets, aerosols and through direct or close physical contact. Measures aimed at blocking droplets and aerosols such as efficient face covering, social distancing and hand and surface cleaning are imperative in reducing transmission[2].

Recent studies have described the angiotensin-converting enzyme receptor (ECA2) as a gateway for viral penetration into the host cells[3]. The ECA2 receptor is abundant in lung alveolar cells, which explains the significant pulmonary component of this infection. The gastrointestinal tract, heart and blood vessels are also target organs[4].

Around 81% of patients with coronavirus disease 2019 (COVID-19) infection will develop mild or mild to moderate disease. However, a study examining over 70000 cases in China showed that evolution to more severe forms is prone to occur in the elderly and in individuals suffering with comorbidities, such as systemic arterial hypertension, type 2 diabetes mellitus, heart disease, obesity, smoking, chronic pulmonary disease and cancer. Children are not statistically present as a risk group[5], and the male gender may be a potential risk factor[6]. Severe cases will account for

approximately 14% of infected patients, who often need mechanical ventilation and present high mortality rates, reaching 80% within the severe case category. The overall risk of mortality in the general population varies from less than 1% to 3%[7].

CLINICAL AND HEPATOLOGICAL ASPECTS OF SARS-COV-2

A recent study recruiting 745 patients suffering with chronic liver disease and diagnosed with SARS-CoV-2 showed that cirrhosis was strongly associated with worsened outcomes, and of the 150 patients who died during the study, 82% had a diagnosis of cirrhosis. Although in that study the most common cause of death was infection-associated lung injury, and in only 19% of cases the primary cause of death was liver failure, such findings suggest that cirrhosis and its consequent immune dysfunction are potential facilitators of lung injury[8].

Hyperglycaemia, a metabolic disarrangement observed not only in type 2 diabetes but also in other conditions characterized by an important pro-inflammatory background, such as cardiovascular disease, hepatic steatosis, cancer and chronic respiratory disease, including respiratory disease induced by smoking, is known to impair the immune response and consequently lead to a worsened clinical evolution in SARS-CoV-2 patients[9]. Hyperglycaemia is a common feature of severe SARS-CoV-2 infection, usually induced by glucocorticoid hypersecretion associated with metabolic stress, and manifested in approximately 51% of cases[10]. The hyperglycaemia observed in severe cases has also been associated with transient impairment of pancreatic islet cell function[11]. Hyperglycaemia should not be neglected in SARS-CoV-2 therapies as it can induce additional immune suppression.

The presence of metabolic disturbances, inflammation and exacerbated oxidative stress are features associated with a rapid clinical deterioration in SARS. Although all populational groups can be affected by the viral disease, the elderly and patients with underlying clinical conditions, especially obesity and type 2 diabetes mellitus, are more vulnerable and are at greater risk of developing the more severe forms of SARS-CoV-2[12].

Individuals with obesity present a range of metabolic disturbances that facilitate worsened clinical outcomes, including alterations in different stages of their innate and adaptive immune response, a state of mild chronic inflammation, chronically higher levels of pro-inflammatory adipokines and lower levels of anti-inflammatory adipokines, and a strong association with type 2 diabetes. It is known that such unfavourable biochemical environment contributes to immune dysregulation and has been described as an important determinant in the severity of viral influenza infection [13,14]. Chronic inflammation associated with obesity jeopardizes macrophage activation by antigen presentation and pro-inflammatory cytokine synthesis[13]. In addition, B and T cell response are attenuated in obesity, contributing to increased susceptibility and delayed resolution of viral infection[13,15]. A recent study that examined the medical records of 37121 patients diagnosed with SARS-CoV-2 showed that age, the male gender and body mass index were unadjusted risk factors for disease severity[16].

A meta-analysis covering 46248 patients and examining the prevalence of comorbidities and underlying diseases in SARS-CoV-2 patients showed that the most prevalent comorbidities were arterial hypertension (17%), diabetes (8%), cardiovascular diseases (5%) and diseases of the respiratory tract (2%). Statistical analysis of each subgroup demonstrated that the presence of comorbidities in SARS-CoV-2 patients may increase the risk for greater severity and unfavourable clinical outcomes[17]. A diagnosis of diabetes was associated with a more severe clinical evolution (19.3%) when compared to non-diabetic individuals (11%) in severe case patients. The worsened clinical evolution cases showed more comorbidities including chronic obstructive pulmonary disease (4.8%), coronary heart disease (10.4%) and hypertension (38.7%). It has also been shown that hyperglycaemia at SARS-CoV-2 admission was associated with more severe evolution and higher mortality[18].

Some clinical and observational studies have described a “cytokine storm” in SARS-CoV-2 infection, which is characterised by significantly exacerbated systemic inflammation and immune dysfunction[19,20]. A study found that the pro-inflammatory cytokines related to macrophage function, mainly interleukin (IL) 6, IL-10 and tumour necrosis factor-α, increase significantly in most severe cases, in relation to cases of lesser intensity. Meanwhile, IL-6 levels remain high even in patients who have had moderate SARS-CoV-2 infection[19].

A meta-analysis evaluated clinical data obtained from 10 studies with 1995 cases in total and found that the immunological response of the patients included in the study was consistent with viral respiratory tract infection: 64.5% of patients showed lymphocytopenia, 29.4% leukocytopenia, 44.3% increased C reactive protein (CRP) and 28.3% showed increased lactate dehydrogenase (LDH)[20]. Furthermore, patients with more severe clinical presentation showed on hospital admission significantly increased inflammatory markers including CRP, ferritin, alanine aminotransaminase (ALT), aspartate aminotransferase (AST), LDH, gamma-glutamyl transferase (gamma-GT), as well as hypoalbuminemia, lymphocytopenia, neutropenia and eosinopenia[19-21].

Patients with chronic liver disease, particularly cirrhosis, show multiple mechanisms of immune dysfunction that together can increase the vulnerability to infection and an inadequate inflammatory response, defined as cirrhosis-associated immune dysfunction[22,23]. Elevation of transaminases and other biomarkers of liver dysfunction are common in SARS-CoV-2 patients, occurring in approximately 15% to 65% of cases. Abnormalities in liver biochemistry are commonly observed in SARS-CoV-2 patients regardless of the presence or not of pre-existing liver disease. However, the mechanisms underlying the impact of COVID-19 infection on liver function are not fully understood and may be multifactorial[24,25].

COVID-associated liver injury is defined as any liver injury that occurs in SARS-CoV-2 progression and treatment in patients with or without pre-existing liver disease. Generally, 2% to 11% of SARS-CoV-2 patients show underlying liver disease, and 14% to 53% show elevated AST and ALT[26]. Damage to the liver parenchyma in SARS-CoV-2 patients can be caused directly by infection of liver cells, once the ACE2 protein is expressed in hepatocytes and cholangiocytes[26,27]. Viral binding to ACE2 allows the virus to penetrate hepatocytes, inducing cytokine activation, apoptosis and necrosis, with resulting liver damage[28]. However, in addition to COVID as primary cause of liver disease, it is clinically important to note that pharmacotherapies including macrolide or quinolone antibiotics and steroids can also induce liver damage [29].

A study recruiting 1099 SARS-CoV-2 patients showed that 23.7% of the cohort had some pre-existing liver disease, including hepatitis B infection, non-alcoholic fatty liver disease and alcohol-related liver disease[30]. Hepatitis B was the most prevalent disease, identified in 2.1% of that sample population, with the majority of those patients presenting a more severe SARS-CoV-2 clinical evolution. Other studies have found hepatitis B to be more frequent in male individuals and in patients taking a worsened clinical outcome[26,29,31].

However, a recent retrospective study assessing the clinical presentation and specific biomarkers of 158 hospitalized SARS-CoV-2 patients showed that 42.4% of the cohort had elevated AST, ALT, alkaline phosphatase (AP), gamma-GT and total bilirubin at admission, and that 31.6% of the patients developed liver biomarker abnormalities in the course of their hospitalization. The liver changes were correlated with the oxygenation index, and at the time of discharge 40.5% of the patients were still showing abnormal liver biomarkers. In addition, factors such as younger age, hypertension and lymphocytopenia were independent risk factors for the persistence of abnormal liver markers during hospitalization[32]. A study carried out with 288 patients hospitalized for COVID-19 and previously established chronic liver disease caused mainly by viruses, metabolic fatty liver disease and alcohol intake, showed that from the 43 patients diagnosed with cirrhosis, 57% of them had the disease decompensated at the time of admission for SARS-Cov-2, and that mortality rate was extremely high in those patients, particularly in those with Child-Pugh cirrhosis score of ≥ 9[33].

Patients diagnosed with COVID-19 confirmed by computed tomography during the subclinical phase, that is, before the onset of symptoms, had a significantly lower incidence of AST abnormality than patients diagnosed after the onset of symptoms [34]. In addition, COVID-19 can worsen underlying chronic liver disease, inducing disease decompensation and acutely exacerbated chronic liver failure, a condition associated with high mortality rate. Recently, Cai *et al*[35] proposed a classification for the various typical liver test abnormalities found in SARS-CoV-2 patients. The study defined three patterns of injury based on ALT, AST, gamma-GT, AP and total bilirubin: first category: hepatocellular lesion usually progressing with predominant elevation of AST and ALT; second category: Cholestatic-type lesion with predominantly elevated gamma-GT and AP; and third category: mixed injury associated with an increase in all hepatocellular markers. It was observed that the presence of abnormalities in liver tests at hospital admission significantly increased the risk of severe pneumonia in the studied population, especially among those with hepatocellular or mixed lesions[35].

The SARS-CoV-2 virus was detected by *in situ* hybridization in 68% of liver sample biopsies of 48 patients who died of severe lung disease attributed to the infection[36]. Histological examination also identified abnormalities in intrahepatic vascular structures, mainly portal and sinusoidal microthrombosis (100% of cases), macrovesicular steatosis (50%), mild portal inflammation (66%) and portal fibrosis. The finding of steatosis was predominant in patients with obesity and overweight. The study of Sonzogni *et al*[36] reinforces the hypothesis that disturbances in the coagulation cascade or impaired blood circulation or endothelial damage may trigger mechanisms in the pathogenesis of COVID-19 damage in the liver.

NUTRITION AND CHRONIC LIVER DISEASE IN THE CONTEXT OF SARS-COV-2

Chronic consumption of westernised diets (WD), which typically contain high levels of saturated fats and simple carbohydrates, contributes to the incidence of obesity and type II diabetes, which are conditions positively associated with the more severe forms of SARS-CoV-2 infection and higher mortality rate[37]. WD chronic consumption activates the innate immune system and compromises adaptive immunity, leading to chronic inflammation and impaired host immune defence against the virus[12]. In addition to impaired innate immunity, WD chronic consumption is known to inhibit T and B function in the adaptive immune system, potentially *via* increased oxidative stress[12,38].

WD are also associated with intestinal dysbiosis and unbalanced pro/anti-inflammatory-associated T function in the intestine, with consequent immune incompetence, intestinal and extra-intestinal inflammation. The immune imbalance resulted from gut dysbiosis can worsen infectious conditions and dysregulate metabolic pathways, increasing the risk for liver complications[37,39-41].

A study evaluated a dataset from 188 countries to identify effects of diet, malnutrition and obesity upon the global SARS-CoV-2 cases and their underlying circumstances, as well as mortality and recovery rates[40]. The results suggest that populations that consume predominantly WD showed higher SARS-CoV-2-associated mortality. Such findings may be explained by the disturbances induced by WD upon intestinal microbiota, affecting the phenotype and function of intestinal T CD4+ cells, which can result in greater susceptibility to infections[41].

In summary, individuals suffering with systemic inflammation induced by overweight or obesity and associated chronic diseases such as diabetes, heart, kidney, liver and lung diseases, are more likely to develop the most severe forms of SARS-CoV-2. Therefore, a broader access to nutritional knowledge to the wider population, with the subsequent adoption of healthier eating behaviours, are Public Health priorities. Populations in general need to be made aware that healthier eating behaviours are important protective factors against long-term complications and negative outcomes in SARS-CoV-2[12]. In general, nutritional recommendations ought to focus on reduction of saturated fats and simple sugars, combined with the adequate consumption of dietary fibre, whole grains, polyunsaturated fats, and antioxidant and bioactive nutrients that enhance immune function[12,42]. Figure 1 illustrates the main factors associated with the pathophysiology of SARS-CoV-2 disease that contribute to the most severe forms of the infection.

NUTRITIONAL THERAPIES FOR PATIENTS WITH LIVER COMPLICATIONS

Nutritional therapy (NT) recommendations for critically ill patients with a diagnosis of acute liver failure (ALC) or acute-on-chronic liver failure (ACLF) follow the same principles as NT aimed at critically ill patients. Early enteral nutrition (EN) is recommended, starting with trophic rates (10 mL/h to 20 mL/h) containing approximately 15 kcal/kg BW per day to 20 kcal/kg BW per day, due to increased risk of diet intolerance secondary to mesenteric ischaemia, vomiting, or adynamic ileus, which may occur during the first week of hospitalization[43]. Energy content can be gradually increased up until reaching 30 kcal/kg BW per day to 35 kcal/kg BW per day[44]. In severely ill ALC or ACLF patients who are malnourished, EN alone or associated with parenteral nutrition (PN) should be started immediately[44]. The enteral route should be preferred whenever possible, but PN should be initiated if there is a need to reach nutritional requirements, especially when EN is not safe or

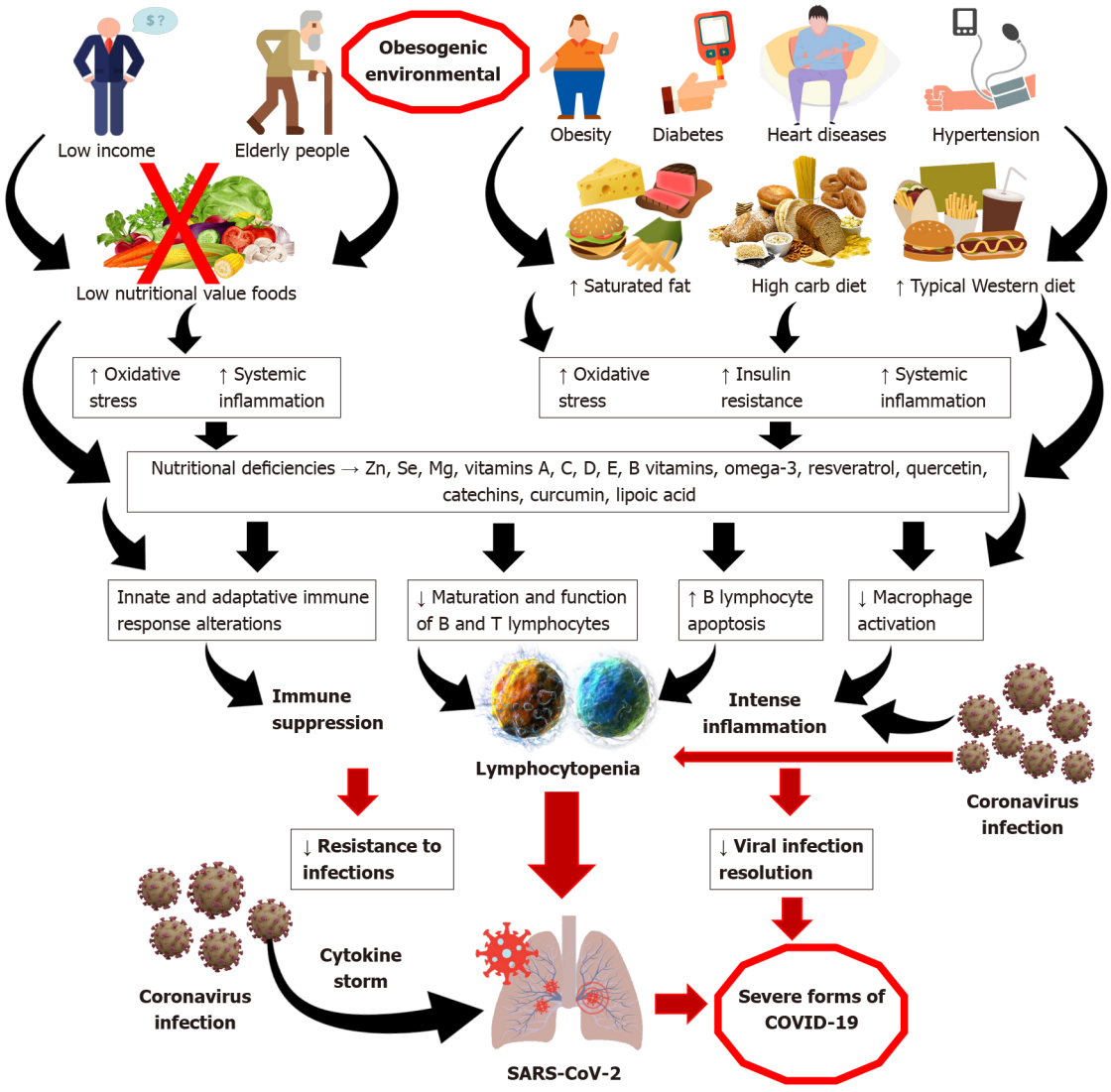

Figure 1 Health, diet and lifestyle practices associated with clinical outcomes in SARS-CoV-2 infection. Individuals suffering with systemic inflammatory background associated with overweight, obesity, diabetes, heart disease and hypertension, and chronic liver disease, as well as elderly individuals, are more susceptible to develop the most severe forms of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. Chronic consumption of typical westernised diets (WD) diets, which are rich in saturated fat, carbohydrates with high glycaemic index, and low in fresh fruits and vegetables, is relatively common amongst individuals who present worsened clinical outcomes. A typical WD dietary pattern features low nutritional value, facilitating deficiencies of vitamins, minerals, polyunsaturated fatty acids and bioactive compounds such as resveratrol, quercetin, catechins, curcumin and lipoic acid, amongst others. Nutritional deficit can facilitate the exacerbation of oxidative stress, inflammation and insulin resistance, with consequent disturbances in the innate and adaptive immune response, resulting in suppression of the immune response and greater susceptibility to infections. Coronavirus infection is usually associated with a "cytokine storm", intense inflammation, leukopenia and lymphocytopenia. Individuals with preestablished pro-inflammatory background and impaired immune system due to poor diet are at greater risk of evolving more rapidly to the more severe forms of SARS-CoV-2 infection[12,37,81]. SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; COVID-19: Coronavirus disease 2019.

tolerated[43,44,45].

Continuous EN is recommended over bolus infusion to reduce the incidence of diarrhoea, improve glycaemic control, and reduce healthcare worker interaction, thereby limiting their exposure to SARS-CoV-2[43]. A daily prescription of 1.2 g protein/kg BW is recommended for patients with liver disease without malnutrition, and 1.5 g of protein for malnourished or sarcopenic patients. However, in patients with severe hyperacute disease with hepatic encephalopathy and high arterial ammonia, or at risk of developing cerebral oedema, protein nutritional support can be delayed for 24 h to 48 h until the hyperammonaemia is controlled[44]. Additionally, the use of standard formulae may be suggested as no robust scientific evidence appears to be available as yet on the proven benefits of formulae supplemented with branched chain amino acids in critically ill patients with liver disease[44,45].

NUTRITIONAL THERAPIES FOR SARS-COV-2 PATIENTS WITH LIVER COMPLICATIONS

As NT guidelines for SARS-CoV-2 in intensive care specifically are not yet available, nutrient recommendations are centred on the principles of nutrition in intensive care, which must be adapted to the patient considering their clinical conditions and associated complications. Therefore, as the patient with severe SARS-CoV-2 generally presents manifestations similar to patients admitted to intensive care unit (ICU) with pulmonary impairment, the employment of strategies for the management of conditions such as sepsis and severe acute respiratory distress syndrome is suggested [46].

Decision making regarding the initiation and progression of NT, as well as the selection of the feeding route and the type of diet during hospitalization of moderate and severe SARS-CoV-2 patients, need to consider the patient's clinical presentation [47]. EN is less expensive and inherently presents overall lower risks for the patient than PN, in addition to mimicking a more physiological form of feeding. However, both types of nutritional administration can have adverse effects and long-term complications[48]. Early EN shows several benefits including increased splanchnic blood flow, maintained enterocyte barrier and stimulated immunity. Although current evidence suggests that the use of early EN in critically ill patients results in the preservation of splanchnic blood flow, high calorie EN can induce complications in patients with hypovolemic shock. However, it has been shown that EN infusion at low dosage to allow for intestinal trophism is associated with better clinical outcomes in critically ill patients[49].

Thus, it is recommended that EN should be started as soon as possible after admission to ICU, preferably employing an iso-osmolar standard polymeric formula designed for gradual administration, starting with low flow and evolving according to gastrointestinal tolerance. Monitoring is necessary to identify signs of intolerance, hemodynamic instability and metabolic disorders. EN with intragastric location can be safely provided even for patients positioned in pronation and oxygenation by extracorporeal membrane[46].

Sepsis is associated with an initial state of systemic and hypermetabolic inflammation, with subsequent worsening of immunosuppression characterized by apoptosis and lymphocyte depletion. This later phase is also characterised by reduced capacity of monocytes and macrophages to release pro-inflammatory cytokines, further facilitating infection[50]. SARS-CoV-2 patients who develop sepsis, with or without liver complications, can benefit from early EN in combination with supplementary PN as ideal strategy to reach at least 80% of calorie needs by the third day of hospitalization. It is also recommended that protein be administered initially at low dosage (0.8 g/kg per day), progressively increased until reaching 1.3 g/kg per day after control and resolution of sepsis[48].

The transition to PN should not be delayed when it is not possible to reach energy and protein targets through the gastrointestinal tract or where contraindications for EN exist. Such patients may present intolerance to EN, characterized by classical clinical manifestations such as nausea, vomiting and abdominal distention, which evidences the need for concomitant PN administration. Another factor that may limit EN in critically ill SARS-CoV-2 patients is the placement of the post-pyloric tube, which would involve an additional aerosol generation procedure. In addition, the use of non-invasive positive pressure ventilation can prevent the use of feeding tube due to the difficulty of establishing an efficient seal with a comfortable fit[46,51].

The indication of PN for SARS-CoV-2 patients is common in intensive care, which must follow the principles and recommendations for NT in critically ill patients[46, 51]. However, frequently in SARS-CoV-2 patients there is a need to maintain PN for extended periods, which unfortunately increases the risk of metabolic disturbances and liver disease associated with intravenous (IV) infusion of nutrients. Thus, the development of hyperglycaemia, hyperlipidaemia and fatty liver disease must be considered when PN as exclusive route is used for extended period[48].

The reduction in luminal content and the absence of trophic stimuli from the intestine to the liver induced by PN may contribute to PN-associated liver disease (PNALD), or liver disease associated with intestinal failure[52]. PNALD, in addition to hyperglycaemia, steatosis and dyslipidaemia, can also induce liver fibrosis or cirrhosis if PN exclusive use is prolonged, especially without concomitant EN[53]. Although there is variability, PN-associated cholestasis can induce elevations in transaminases, AP, gamma-GT and conjugated bilirubin, which is similar to other cholestatic diseases and therefore should be investigated for their differential diagnosis. Considering the adverse effects associated with prolonged PN, EN may be relevant in preventing liver

disease as to preserve the integrity of the intestinal mucosa and maintenance of the liver gut axis[52].

Several mechanisms can explain hepatic changes induced by PN, including deterioration of the intestinal mucosa, facilitated bacterial translocation through the intestinal epithelium, inflammation, and hepatic endotoxicity. It is believed that the lack of activation of enterocyte receptors by luminal agonists, attributed to the absence of enteral feeding, may decrease signalling to the liver *via* portal circulation, interrupting the gut liver axis cross-communication[52]. The reduced blood flow to the small intestine and portal vein that occurs in PN is associated with lowered liver mononuclear cell counts, potentially contributing to hepatocellular dysfunction[54].

Hepatobiliary receptors, including the farnesoid X receptor (FXR) and the Takeda G protein-coupled receptor 5, appear to have an important role on PNALD pathogenesis. Reduced activity of the human orthologous fibroblast growth factor-19 (FGF19), which exerts hepatoprotective action, as well as decreased activity of the intestinal trophic factor glucagon-like peptide-2, may be associated with PN. FXR signalling is a pathway that regulates the secretion of FGF19, a protein that modulates cholesterol 7 alpha-hydroxylase 1, which in turn limits the synthesis of bile acids. PN has been shown to inhibit this signalling pathway, inducing changes in bile acid metabolism, hepatocyte apoptosis, hepatocellular injury and liver fibrosis[53].

Hyperglycaemia is commonly observed in EN and PN and has been associated with increased risk of clinical complications and mortality during hospitalization[55]. To date, there are no specific guidelines that recommend glycaemic targets and effective strategies for the management of EN or PN-associated hyperglycaemia in SARS-CoV-2 patients. However, it is known that the elevation of inflammatory markers such as IL-6, CRP, ferritin and D-dimer are more persistent in hyperglycaemic patients during hospitalization for SARS-CoV-2. In addition, patients with hyperglycaemia or diagnosed with diabetes are at higher risk for the progression of SARS-CoV-2 disease severity when compared with normoglycaemic and those without a diagnosis of diabetes[56,57]. Glycaemic control in SARS-CoV-2 patients must include optimization of the carbohydrate content and continuous IV insulin to offer the best possible glycaemic control[56].

The source of lipids in PN is often derived from soybean oil, a source of ω-6 polyunsaturated fatty acids (PUFAs). Arachidonic acid is bioconverted to prostaglandins, thromboxanes and leukotrienes of series 2 and 4, which are pro-inflammatory and may contribute to the increased incidence of cholestasis, steatosis, sepsis, changes in neutrophil function and positive regulation of matrix metalloproteinases (MMPs)[58]. Preservation of the matrix is essential for reducing the progression of liver disease, and it is known that increased MMP activity can cause damage to the liver parenchyma [59]. It is therefore recommended that the PN prescription in SARS-CoV-2 should prioritize lipid emulsions enriched with fish oil, with doses of approximately 0.1 g/kg per day to 0.2 g/kg per day[60].

SARS-CoV-2 patients with history of liver disease require attention to the provision of NT as they may present impaired digestion and absorption of nutrients, altered protein metabolism, insulin resistance and previous nutritional deficiency. Such manifestations can negatively influence tolerance to NT, posing an additional obstacle for the daily nutritional requirements[61,62]. Table 1 presents the summary of the main general guidelines for NT for patients with liver disease or liver disorders developed in the clinical course of SARS-CoV-2 severe infection.

NUTRITION AND IMMUNE RESPONSE IN SARS-COV-2

The link nutrition — immune response is very well established: malnutrition is a risk factor for respiratory infection[63]. Previous scientific evidence shows that vitamins A, C, D, E, pyridoxine (B6), cyanocobalamin (B12), and folic acid, trace elements including zinc, iron, selenium, magnesium and copper, as well as the functionally essential ω-3 PUFAs eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, all have a paramount effect on immune function[64-67]. Evidence shows that the regular intake of functional foods containing anti-inflammatory and immunomodulatory nutrients and bioactive agents are associated with optimum immune function[12,42,68].

Nutritional recommendations for SARS-CoV-2 therapies specifically are not yet available. However, as the SARS-CoV-2 virus shares some functional similarities with other coronaviruses identified before the current pandemic, it may be expected that the scientific evidence gathered previously may be relevant for COVID-19. A summary

Table 1 Nutritional recommendations for patients suffering with chronic hepatic disease[44,45,95]

Nutrients	ESPEN 2019	ESPEN 2020		Critical care medicine 2020
		Non-obese	Obese	
Calories	30 kcal/kg per day to 35 kcal/kg per day	30 kcal/kg per day to 35 kcal/kg per day	25 kcal/kg per day	30 kcal/kg per day to 35 kcal/kg per day; Glycaemic target at 110 mg/dL to 180 mg/dL
Protein	1.2 g/kg per day to 1.5 g/kg per day	1.2 g/kg per day to 1.5 g/kg per day	2.0 g/kg per day to 2.5 g/kg per day	1.5 g/kg per day to 2.0 g/kg per day
EN + BCAA	0.20 g/kg to 0.25 g/kg	Not routinely recommended		0.2 g/kg to 0.25 g/kg

BCAA: Branched chain amino acids; EN: Enteral nutrition.

of the main scientific evidence available is presented in Table 2.

Vitamins

A recent narrative review encompassing 204 studies summarised the beneficial effects of vitamins A and E against COVID-19, mainly through their antioxidant and immunomodulatory effects, as well as activation of innate immunity and local paracrine signalling[69]. Furthermore, the beneficial effects of thiamine, vitamin C and vitamin D in respiratory diseases similar to SARS-CoV-2 and sepsis have also been identified[69]. Despite the current lack of clinical trials investigating the effects of vitamin supplementation on SARS-CoV-2, when considering pathophysiological pathways related to viral replication and the immune system, the scientific evidence available to date encourages the recommendation for said nutrients in supplemental form in the event of nutrient deficiency.

Vitamin A and its plant-derived precursor beta-carotene are often referred to as "anti-infective agents". Studies have shown a higher risk of measles and worsened outcomes in vitamin A deficiency. Vitamin A supplementation is believed to have reduced morbidity and mortality from measles, diarrhoea, pneumonia, HIV and malaria[70]. Vitamin A is known to possess antiviral properties in measles, reducing viral replication possibly due to its role in innate immunity[71]. Regarding coronavirus specifically, one study identified reduced effectiveness of coronavirus vaccine in cattle when in conditions of vitamin A deficiency[72].

Regarding the B group of vitamins, studies have shown that the combination of vitamin B2 (riboflavin) and ultraviolet radiation was able to reduce the viral load of MERS-CoV in human plasma products[73]. Vitamin B3 (nicotinamide) has shown bactericidal properties against *Staphylococcus aureus*[74]. Nicotinamide has been associated with reduced inflammation in mechanical ventilation-associated pneumonia, mainly attributed to reduction in neutrophilic migration, although hypoxemia was a negative outcome identified[75].

A recent study using *in silico* technology simulated the binding of the main protease (Mpro) of the coronavirus with a range of molecules that could potentially possess antiviral and or therapeutic effects against SARS-CoV-2[76]. The results showed that the chemical structure of cyanocobalamin (B12) and nicotinamide presented some interaction with the Mpro active site. Cyanocobalamin and nicotinamide were ranked in the 4th and 6th position, respectively, in the list of molecules tested, placed after the antivirals ribavirin and telbivudine. The authors suggest that the combined use of ribavirin, telbivudine, cyanocobalamin and nicotinamide could be tested as a potential treatment for SARS-CoV-2. A study that investigated the effects of cyanocobalamin in hepatitis C patients showed a sustained virological response (SVR) when this vitamin was added to standard antiviral therapy, suggesting that cyanocobalamin supplementation may be an independent factor associated with SVR in difficult-to-treat genotype (genotype 1) hepatitis C and in those with a higher baseline viral load[77].

Ascorbic acid has been studied widely, and previous research has reported beneficial effects in the prevention and treatment of coronavirus infections[78]. Ascorbic acid has also been associated with antihistamine effects, which alleviate some cold symptoms[79]. A clinical study carried out with 19357 individuals without pre-existing lung diseases investigated the association between plasma vitamin C levels and the risk of respiratory diseases after a three-year follow-up. The results showed that higher vitamin C plasma levels, which is an indicator of greater consumption of fruit and vegetables, were related to lower risk for chronic respiratory diseases, including pneumonia. The authors suggest that a daily intake of 3 to 5 servings of fruit

Table 2 Vitamins, nutraceuticals and bioactive compounds in supporting therapies for coronaviruses, including severe acute respiratory syndrome coronavirus, Middle East respiratory syndrome coronavirus, bovine and avian coronavirus[107]

Nutrients and bioactive compounds (RDA, when available)	Effects in humans[1]	Dose	Antiviral action	Ref.
Vitamin A, [2]RDA: 700-900 μg/d, UL: 3000 μg/d or 10.000 IU	Yes (Measles, Ebola)	Until 3000 IU μg/d (children from 6 mo to 11 mo) and 60000 μg/d children 1-5 yr; Adults: 60000 μg in 2 consecutive days	Measles, Ebola, Bovine coronavirus	Mayo-Wilson *et al*[96], 2011; Aluisio *et al*[97], 2019; Jee *et al*[72], 2013
Vitamin B_2, [2]RDA: 1.1-1.3 mg/d, UL: ND (not determined)	No	2-3 times RDA[3]	MERS-CoV + UV radiation (antiseptic)	Keil *et al*[73], 2016
Vitamin B_{12}, [2]RDA: 2,4 μg/d, UL: ND	Yes	5000 μg IM (intramuscular) monthly	SARS-CoV-2 (molecular modelling), HCV	Kandeel *et al*[76], 2020; Rocco *et al*[77], 2013
Vitamin C, [2]RDA: 75-90 mg/d, UL: 2000 mg/d	Yes (ICU, pneumonia)	1-3 g/d; Inpatient: 50 mg/kg IV (intravenous) 6/6 h for 4 d; Elderly: 200 mg/d-2 g/d	Pneumonia, MERS-CoV	Hemilä[78], 2003; Field *et al*[79], 2002; Myint *et al*[80], 2019; International Society for Immunonutrition[98]
Vitamin D, [2]RDA: 5-15 μg/d, UL: 50 μg/d or 2000 UI	Yes (pneumonia, acute upper respiratory infection)	30 μg/d; Or Bolus: 2500-5000 μg/mo; Elderly: 10-100μg/d	Pneumonia, UAI, bovine coronavirus	Martineau *et al*[65], 2017; Jayawardena *et al*[67], 2020; Grant *et al*[81], 2020; International Society for Immunonutrition[98]
Vitamin E, [2]RDA: 15 mg/d, UL: 1000 mg/d or 1490 UI	No	300 mg 2xd for 3 mo or 365 mg/d for 6 mo; Elderly: (134- 800 mg/d)	Bovine coronavirus, Coxsackie	Andreone *et al*[99], 2001; Look *et al*[100], 1999; Nonnecke *et al*[83], 2014; International Society for Immunonutrition[98]
Zinc, [2]RDA: 8-11 mg/d, UL: 40 mg/d	Yes (measles, SARS-CoV)	75-100 mg/d; Elderly: 30-220 mg/d	Measles, SARS-CoV	Awotiwon *et al*[92], 2017; te Velthuis *et al*[91], 2010; International Society for Immunonutrition[98]
Selenium, [2]RDA: 55 μg/d, UL: 400 μg/d	Yes (influenza)	200 μg/d	Influenza, Avian coronavirus	Hoffmann and Berry[86], 2008; Ma *et al*[85], 2019
Omega-3	Yes (influenza)	1-3 g/d[3]	Influenza, HCV	Cai *et al*[66], 2018
Quercetin	No	1 g/d	SARS-Cov (*in vitro*), IVAS	Chen *et al*[101], 2006; Heinz *et al*[102], 2010
Green tea/catechins (EGCG)	No	4 cups/d or 225 mg de EGCG[4]	Bovine coronavirus (*in vitro*)	Clark *et al*[103], 1998
Resveratrol	No	100-150 mg 2 × d[3]	MERS-CoV (*in vitro*)	Lin *et al*[104], 2017
Curcumin	No	0.5-1 g/d[3]	SARS-CoV(*in vitro*)	Wen *et al*[105], 2007
Lipoic acid	No	600 mg/d[3]	Human coronavirus 229E (*in vitro*)	Wu *et al*[106], 2008

[1]We did not find any work related to this substance and anti-viral or anti-infectious action in humans.
[2]For adult patients, according to age group and gender.
[3]Usual dose employed in clinical practice.
[4]Epigallocatechin.
RDA: Recommended dietary allowance; UAI: Upper airway infection; UL: Tolerable upper intake levels; ICU: Intensive care unit.

and vegetables may provide adequate intake of vitamin C and promote significant health benefits for the general population[80].

Vitamin D, with its important hormonal actions, acts on several components of the immune system, including the synthesis of cathelicidin, an endogenous antimicrobial peptide. A meta-analysis covering over 11000 individuals showed that vitamin D supplementation, even when its serum levels are adequate, can reduce by 25% (adjusted OR: 0.75; 95%CI: 0.60-0.95) the chance of contracting infections of the upper and lower airways, with a more expressive result (70% of cases) for those with baseline levels of 25-hydroxyvitamin D (25(OH)D) below 25 nmol/L (adjusted OR: 0.30; 95%CI: 0.17-0.53)[65]. A systematic review of 42 clinical studies testing the effects of vitamins, minerals, nutraceuticals and probiotics on immunological markers of patients with

viral and respiratory infections showed that vitamins A and D were associated with better outcomes, an effect that was more predominant in populations deficient in those nutrients[67].

A recent narrative review discussed the relationship between vitamin D deficiency and the risk for influenza and COVID-19, and further emphasized the increased risk in aged individuals and in those suffering with chronic comorbidities[81]. Their recommendations to reduce the risk of influenza and COVID-19 infections involve 10,000 IU vitamin D3 supplementation daily for a few weeks to rapidly increase 25(OH)D serum levels. This period would be followed by a maintenance daily dose of 5000 IU to maintain 25(OH)D above 40 ng/mL to 60 ng/mL (equivalent to 100 nmol/L to 150 nmol/L). However, randomized clinical trials and large population studies must be conducted to evaluate the effectiveness of such recommendations.

Vitamin E, including α-tocopherol and tocotrienol, is a potent fat-soluble antioxidant found in significant concentration in immune cells. Some evidence suggests that the currently recommended vitamin E intake may be low, and that its supplementation above current dietary recommendations favoured immune function and reduced the risk of infection, especially in the elderly. Vitamin E contributes to T cell membrane integrity, signal transduction and cell division, and also indirectly by attenuating inflammatory mediators released by other immune cells. The modulation of immune function by vitamin E has clinical relevance, as it affects the host's susceptibility to infectious diseases and respiratory diseases such as asthma and pneumonia [68].

Vitamin E supplementation was found to have different responses upon the incidence of pneumonia when factoring smoking and levels of physical activity[82]. Vitamin E reduced the risk of pneumonia by 69% in participants who had lower exposure to smoking and performed more physical exercise. In the opposite direction however, vitamin E supplementation increased the risk of pneumonia by 68% in the individuals who had greater exposure to smoking and did not exercise. Interestingly, a study in calves has shown greater risk of coronavirus infection in the vitamin E-deficient animals[83]. Overall, such findings suggest that the effects of vitamin E supplementation upon pneumonia risk are likely to be positive but may not be uniform and therefore caution is recommended when supplementing, balancing benefit against risk.

Trace elements

Selenium deficiency has been associated with reduced immune response and greater virulence of some benign viruses[84]. An experimental study in birds showed that selenium supplementation associated with ginseng increased the immune response against avian coronavirus[85]. Selenium deficiency was associated with mutations in genomic RNA that can potentially influence the virulence of certain RNA viruses, such as influenza A virus[86]. Clinical benefits associated with the potential immunomodulatory effects of selenium supplementation have also been demonstrated in other viral infections, including HIV-1[87]. A randomized controlled study found that selenium supplementation reduced the viral load in HIV patients[88]. Lastly, a systematic review assessing the effects of mineral supplementation upon immunological markers in patients diagnosed with respiratory infections of viral origin showed that selenium and zinc had favourable immunomodulatory effects, improving the clinical evolution in viral respiratory infections[67].

It is known that the risk of developing pneumonia associated with mechanical ventilation is high, especially when the use of respirators is prolonged, as in the most severe cases of SARS-CoV-2. A randomized clinical study carried out with 99 critically ill patients investigated the effects of selenium infusion compared to isotonic saline for 10 d. Selenium infusion increased not only its serum levels but also the concentration of glutathione peroxidase-3, but it did not reduce the incidence of pneumonia or mortality of the critically ill patients evaluated[89]. In view of the evidence presented, there is a possibility that selenium levels may influence the clinical evolution of SARS-CoV-2, but such suggestion can only be confirmed or rejected through well-designed clinical research.

Zinc is the second most abundant trace element in the body; it is present in the cytoplasm as a cation and in the blood it is associated with metalloprotein[90]. It is an important trace element for cell maturity in both the innate and acquired immune systems, in addition to having antiviral properties. It has been shown that the replication of SARS-coronavirus, hepatitis C virus and influenza virus (H1N1) can be inhibited by zinc oxide or salt[90,91]. In addition, zinc supplementation in children diagnosed with measles reduced morbidity from pneumonia associated with this infection[92]. The antiviral properties of zinc are not yet well defined, but they are

possibly related to the inhibition of viral binding to the mucosa, inflammation suppression, synthesis of antiviral interferon and inhibition of the enzyme necessary for viral replication[90,91].

Several clinical trials are currently being developed investigating the effectiveness of chloroquine as anti-coronavirus agent. It has been hypothesized that the mechanism of action of chloroquine may involve the induction of zinc uptake into the cytosol, a mechanism that could be associated with inhibition of the viral RNA polymerase inside the infected cell [90]. Future clinical trials shall explore any potentially synergistic role of zinc and chloroquine in SARS-CoV-2 patients.

Essential fatty acids

Essential fatty acid deficiency can result in late or insufficient resolution of inflammation, which can be a determining factor for the evolution of SARS-CoV-2 to the most severe forms, characterized by intense inflammation[63,93]. A meta-analysis assessing clinical studies that employed nutritional formulae containing EPA and DHA for patients with ARDS identified a significant improvement in blood oxygenation and a reduction in the need for mechanical ventilation, organ failure, ICU length of stay and mortality at 28 d of hospitalization[94]. Such results suggest an important effect of EPA and DHA in improving inflammation and lung injury, probably due to anti-inflammatory mediators including resolvins, protectins and maresines and others, which are derived from EPA and DHA[63,94].

CONCLUSION

Hyperglycaemia, a common feature of severe SARS-CoV-2 infection induced by glucocorticoid hypersecretion associated with metabolic stress, should not be neglected in SARS-CoV-2 therapies due to the additional risk of immune suppression. The unfavourable biochemical environment observed in obesity and diabetes contributes to immune dysregulation and has been described as an important determinant in SARS-CoV-2 outcomes.

COVID-associated liver injury often occurs in the evolution of SARS-CoV-2 into more severe stages and can affect patients with or without pre-existing liver disease. Furthermore, pharmacotherapies including macrolide or quinolone antibiotics and steroids can also induce liver damage. The presence and exacerbation of liver disease are directly associated with more negative outcomes in SARS-CoV-2.

NT guidelines for liver patients affected by SARS-CoV-2 in intensive care, specifically, are not yet available. For that reason, strategies for the management of conditions such as sepsis and severe acute respiratory distress syndrome are suggested. EN poses several advantages, namely lower cost, overall lower risk, preservation of splanchnic blood flow and intestinal trophism, and maintenance of enterocyte barrier. NE with intragastric location can be provided for patients positioned in pronation and oxygenation by extracorporeal membrane. An iso-osmolar standard polymeric formula designed for gradual administration with initial protein content at 0.8 g/kg per day progressively increasing up to 1.3 g/kg per day and enriched with fish oil at 0.1 g/kg per day to 0.2 g/kg per day is often recommended. Transition to PN should not be delayed when it is not possible to reach energy and protein targets through the gastrointestinal tract or where contraindications for EN exist. As prolonged PN may contribute to PNALD, EN should always be considered whenever possible. As EN or PN-associated hyperglycaemia is another factor to consider, glycaemic control in SARS-CoV-2 patients must include optimization of the carbohydrate content and continuous IV insulin to offer the best possible glycaemic control.

For patients who have not developed the more severe forms of SARS-CoV-2, the following recommendations can be made: (1) Optimal nutrient intake can help reduce the impact of SARS-CoV-2 and possibly limit the evolution to more severe forms; (2) Early health interventions in obesity, type 2 diabetes, heart, lung and liver disease are effective preventative strategies against SARS-CoV-2; (3) Supplementation with the micronutrients and omega-3 fatty acids described in the present study is a safe, effective and low-cost strategy to help stimulate optimal immune function; (4) Supplementation beyond the Recommended Dietary Allowance can be considered, but only within the upper safe limits (maximum tolerable intake — tolerable upper intake level — UL) for specific nutrients such as vitamins C and D; and (5) Public health authorities are encouraged to include nutritional strategies in their recommendations so that public health policies can assist in the efforts against respiratory diseases of

viral origin.

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REVIEW

Recent updates on progressive familial intrahepatic cholestasis types 1, 2 and 3: Outcome and therapeutic strategies

Seema Alam, Bikrant Bihari Lal

ORCID number: Seema Alam 0000-0003-0888-8625; Bikrant Bihari Lal 0000-0001-6275-7736.

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Seema Alam, Bikrant Bihari Lal, Department of Pediatric Hepatology, Institute of Liver and Biliary Sciences, New Delhi 110070, India

Corresponding author: Seema Alam, MD, Professor, Department of Pediatric Hepatology, Institute of Liver and Biliary Sciences, D-1 Vasant Kunj, New Delhi 110070, India. seema_alam@hotmail.com

Abstract

Recent evidence points towards the role of genotype to understand the phenotype, predict the natural course and long term outcome of patients with progressive familial intrahepatic cholestasis (PFIC). Expanded role of the heterozygous transporter defects presenting late needs to be suspected and identified. Treatment of pruritus, nutritional rehabilitation, prevention of fibrosis progression and liver transplantation (LT) in those with end stage liver disease form the crux of the treatment. LT in PFIC has its own unique issues like high rates of intractable diarrhoea, growth failure; steatohepatitis and graft failure in PFIC1 and antibody-mediated bile salt export pump deficiency in PFIC2. Drugs inhibiting apical sodium-dependent bile transporter and adenovirus-associated vector mediated gene therapy hold promise for future.

Key Words: Genotype; Biliary diversion; Gene therapy; Liver transplantation

Core Tip: The spectrum of clinical manifestations in progressive familial intrahepatic cholestasis varies from mild to severe leading to end stage liver disease necessitating liver transplantation. Medical therapy forms the mainstay of treatment of pruritus with surgical biliary diversion reserved for refractory cases. Apical sodium bile salt co-transporter inhibitors are among the most promising newer drugs. This article describes the recent advances in understanding the clinical course and emerging therapies.

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INTRODUCTION

Progressive familial intrahepatic cholestasis (PFIC) is estimated to affect 1 in 50000-100000 births[1]. With improved awareness about the various presentations of these bile transport disorders, we now know that the clinical presentation can vary from early-onset severe liver disease to episodic late-onset occurrence triggered by an external stimulus. Figure 1 describes the various transporters which are involved in different types of PFIC. This article will focus on the PFIC types 1, 2 and 3. Table 1 depicts the phenotype and genotype of these three types of PFIC.

FAMILIAL INTRAHEPATIC CHOLESTASIS 1 DEFICIENCY

Prevalence of familial intrahepatic cholestasis 1 (FIC1) deficiency is 10.4%-37.5% amongst the cholestatic disorders[2]. Earlier known as Byler's disease, seen predominantly in children of Amish descent, this disease is now known to occur in all racial and ethnic groups.

Pathogenesis

FIC1 is encoded by the ATPase phospholipid transporting-8B1 gene (ATP8B1), located on chromosome 18 and responsible for maintaining the asymmetric distribution of phospholipids across the lipid bilayer by the flippase movement of phospholipids from the outer lipid leaflet of the canalicular membrane to the inner lipid leaflet. Earlier it was believed that FIC1 was responsible for the flippase movement of phosphatidyl-serine, but it is now proven that the preferred substrate is phosphatidyl-choline[3]. Phosphatidyl-choline helps in protecting the canalicular membrane from hydrophobic bile acids (BA). Hence, in the absence of FIC1, proteins in the canalicular membrane such as the bile salt export pump (BSEP) can have impaired function. ATP-binding cassette subfamily B member 4 (ABCB4) and ATP8B1 maintain lipid asymmetry which is important for the integrity of the canalicular membrane. When ABCB4 flops the phosphatidyl-choline, there is destabilization of the canalicular membrane which is counteracted by the flippase activity of FIC1. Hence by maintaining the integrity of the canalicular membrane, FIC1 maintains the function of multidrug-resistant type 3 (MDR3) which is also known as ABCB4[4]. The mechanism by which loss in ATP8B1 leads to cholestasis is poorly understood, but it has been proposed that an abnormal lipid packing (due to the loss in ATP8B1 flippase activity) makes the outer leaflet of the canalicular membrane susceptible to bile salt-mediated extraction of cholesterol and phospholipids. This results in reduced activity of ABCB11, a major bile salt exporter that has also been linked to PFIC2[5-7]. Further, the lower expression of FIC1 down-regulates farnesoid X receptor (FXR) expression, encoded by the NR1H4 gene. FXR has a dual action. FXR in hepatocytes regulates the secretion of bile through BSEP and FXR in enterocytes regulates bile salt reabsorption through apical sodium-dependent bile acid transporter (ASBT)[8].

FIC1 is expressed in a variety of tissues, including the canalicular membrane of hepatocytes, apical membrane of cholangiocytes, brush border of enterocytes and cochlear hair cells[9]. Hearing loss is attributed to defects in the composition of membranes of inner ear cilia. Knockdown ATP8B1 in Caco-2 cell model of intestinal epithelium leads to an unorganized apical actin cytoskeleton and post-transcriptional defect in apical protein expression. This prevents the movement of the ATP8B1 into the apical membrane further causing inhibition of ASBT through the intestinal FXR, eventually resulting in bile malabsorption and diarrhea[8]. FIC1 is also expressed in the pneumocytes where ATP8B1 inhibits cardiolipin. Hence deficiency of ATP8B1 increases the cardiolipin which can disrupt surfactant function within pulmonary alveoli and increase the risk for pneumonia[10]. ATP8B1 deficiency is also associated with down-regulation of cystic fibrosis transmembrane conductance regulator (CFTR) and increased sweat chloride test in 15% of patients.

Clinical and laboratory presentation

Based on the patients' clinical, laboratory profile, and liver histology, the presentation of ATP8B1 deficiency ranges from a severe (earlier known as PFIC1) to a milder phenotype [earlier known as benign recurrent intrahepatic cholestasis (BRIC)][11].

Severe ATP8B1 deficiency presents in children with refractory cholestasis (typically beginning in early infancy) manifesting as jaundice, severe failure to thrive (disproportionate to the degree of cholestasis), delayed puberty and fat-soluble vitamin deficiency (*e.g.*, coagulopathy of vitamin K deficiency). The jaundice can often be

Table 1 Genotype, phenotype and histopathological differentiation of the various types of progressive familial intrahepatic cholestasis

	PFIC1	PFIC2	PFIC 3
Locus/gene/protein	18q21-22/ATP8B1/FIC1	2q24/ABCB11/BSEP	7q21/ABCB4/MDR3
Known mutations (*n*)[1]	50	200	300
Clinical profile			
Onset	Early onset	Early onset	Second decade
Age of presentation pruritus	60% by 3 mo	72% by 3 mo	2-3 yr
Jaundice	Severe	Severe	Mild to none
Cirrhosis	Severe; By end of first decade	Severe; Majority within first 2 yr of life	Mild to moderate; By end of first decade
Growth failure	Present 90%	Present 59%	
Others	Diarrhea 61%; Pneumonia 13%; Pancreatitis 12%; Deafness 31%	Gall stones in 32%	Delayed puberty
Progression	Moderate rate of progression	Rapidly progressive	Highly variable rate of progression
Associations with other cholestatic presentations	BRIC; ICP	BRIC, DIC; ICP, HCC	DIC, LPAC; ICP
Laboratory profile			
TBA	High	Very high	High
GGT	Low to normal	Low to normal	High
AST/ALT	Mild elevation	Moderate elevation	Mild elevation
AFP	Normal	High	Normal
Histopathology	As disease progresses, periportal & pericentrilobular fibrosis develops; Leads to bridging fibrosis and micronodular cirrhosis	Canalicular cholestasis, lobular/portal fibrosis and inflammation with giant cells; Severe hepatocellular necrosis	Portal inflammation, portal fibrosis, cholestasis, ductular proliferation
Immunohistochemistry	Canalicular BSEP is normal or faint and MDR3 is normal bland intralobular cholestasis	BSEP expression decreased to absent in the canalicular membrane	MDR3 decreased to absent in the canalicular membrane

[1]Medline genetics. BRIC: Benign recurrent intrahepatic cholestasis; BSEP: Bile salt exporter pump; ICP: Intrahepatic cholestasis of pregnancy; HCC: Hepatocellular carcinoma; DIC: Drug induced cholestasis; FIC1: Familial Intrahepatic Cholesatsis-1; LPAC: Low-phospholipid-associated cholelithiasis; MDR3: Multidrug resistant protein-3; PFIC: Progressive familial intrahepatic cholestasis; ABCB: ATP-binding cassette subfamily B.

intermittent. The liver disease is progressive causing secondary biliary cirrhosis and end-stage liver disease. Pruritus is often disproportionate to hyperbilirubinemia but correlates with the serum BA levels[1]. Symptoms begin in the first month of life in 15% of patients and by 3 mo of age in 61% of patients with PFIC1[12]. Undernutrition is generally responsible for poor growth. However, proportionate growth failure and delayed puberty together suggests a likely systemic manifestation of ATP8B1 deficiency. BA is high and serum cholesterol is characteristically low. ATP8B1 deficiency is associated with extrahepatic manifestations such as hearing loss, pancreatitis (8%) or pancreatic exocrine insufficiency, kidney stones, resistance to parathyroid hormone and diarrhea (61%) not attributable to fat malabsorption[9,13]. The severe form of ATP8B1 deficiency may be associated with congenital hypothyroidism (both clinical + subclinical) which can be treated[14]. Coarsely granular canalicular bile may be found on electron microscopy which may improve after the administration of ursodeoxycholic acid (UDCA). A recent multi-centric study included 130 patients with pathological ATP8B1 mutations and low-gamma-glutamyl transpeptidase (GGT) cholestasis phenotype[15]. Among these patients, 15 initially presented as BRIC that later evolved into a severe FIC1 phenotype. Age at first presentation to the tertiary center was 0.6 (0.3-2.2) years, illustrating an early onset of disease. Hepatocellular carcinoma (HCC) was not seen in any of the patients, either before transplantation or in the explants.

Mild ATP8B1 deficiency: Episodic cholestasis beginning later in childhood, adolescence or young adulthood usually rules out severe disease. The presentation may

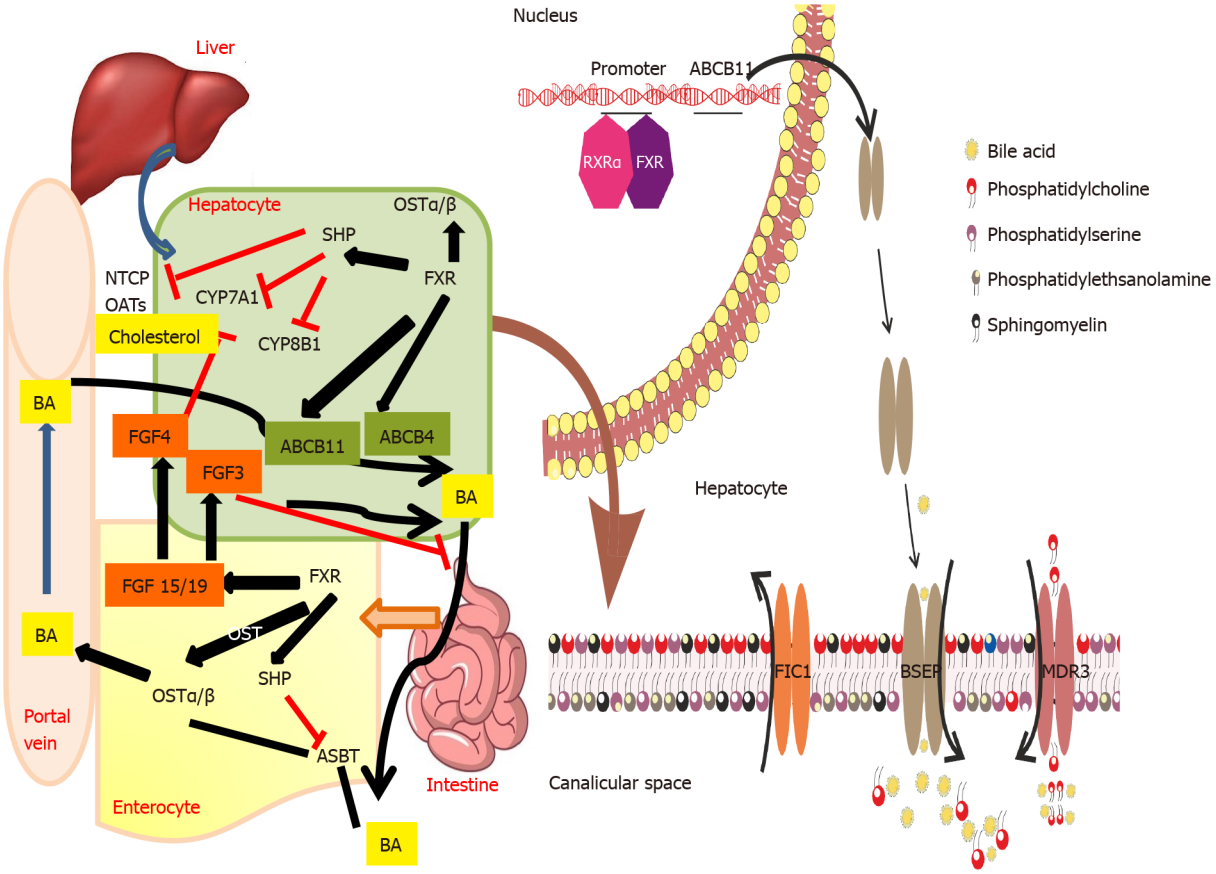

Figure 1 Bile acid secretion and transport from the hepatocyte across the canalicular membrane and enterohepatic circulation: Role of various transporters involved in transport of bile salts and phospholipids across the canalicular membrane. Enterohepatic circulation of bile salts and their regulation *via* farnesoid X receptor - fibroblast growth factor 15/19 is also depicted. ABCB: ATP-binding cassette subfamily B; FXR: Farnesoid X receptor; FGF: Fibroblast growth factor; FIC1: Familial intrahepatic cholestasis 1; BSEP: Bile salt exporter pump; MDR3: Multidrug-resistant type 3; BA: Bile acids; NTCP: Sodium taurocholate cotransporting polypeptide; OATs: Organic anion transporters.

include pruritus without jaundice or presence of mild jaundice. Episodes may last from weeks to months and symptom-free intervals may vary from months to years. The intermittent disease pattern is usually associated with different or unknown triggers which may include fever or any other inter-current illness, drugs (such as contraceptives), malignancy, hormonal changes or pregnancy. BA is high and serum cholesterol is low during symptomatic periods. Histopathological findings during the symptomatic periods may be milder, yet similar to that seen in severe form.

Genotypic variation

In a recent study with 18 potential disease-causing mutations in ATP8B1, 14 patients (4 homozygous, 6 compound heterozygous, 4 heterozygous) were below 18 years of age and had a median age of 0.75 years[16]. Overall, 28 different genetic variants were identified including two common single nucleotide polymorphisms (SNPs) (p.R952Q and c.3531+8G>T)[16]. These variants have been described earlier in European patients with intrahepatic cholestasis of pregnancy (ICP)[17] and pancreatitis[13]. Pathogenic variants with underlying severe ATP8B1 deficiency are likely to be fully penetrant but some of the milder variants may not become symptomatic even in adulthood. The p.Ile661Thr pathogenic variant, which is frequently detected in people with a mild disease of European descent, appears occasionally to be non-penetrant. However, it is also found in compound heterozygous form in persons with severe disease. A multicentric study on FIC1 patients further classified the patients based on the presence of predicted protein-truncating mutations (PPTMs) (*i.e.*, splice site, frameshift due to deletion or insertion, nonsense, duplication)[15]. FIC1-A patients harbored no PPTMs, FIC1-B patients had one PPTM, and FIC1-C patients manifested two PPTMs. Patients with FIC1-C genotype presented to the referral center at 0.4 (0.2-0.7) years, which is significantly earlier as compared to FIC1-A [0.8 years; interquartile range (IQR): 0.4-3] and FIC1-B (0.9 years; IQR: 0.4-2.7) respectively (P = 0.004). At presentation, levels of BA, aminotransferases, and GGT were comparable among the three genotype groups.

FIC1-B patients had the highest bilirubin levels. Half the cases of FIC1-A were receiving UDCA at presentation but did not show a significant improvement biochemistry in comparison to the other patient groups.

Natural history and outcome

The majority of the severe variants of ATP8B1 deficiency will have disease onset within infancy. Some may experience episodes of severe cholestasis followed by disease-free intervals with eventual persistence of cholestasis. Malnutrition and fat-soluble vitamin deficiencies can cause significant morbidity, if left untreated. Features of chronic liver disease and portal hypertension may develop leading to cirrhosis by the end of the first decade of life but variations have been noted within families[15]. Without surgical intervention, end-stage hepatic failure and death usually occur in the second decade of life. In some of those with mild disease, clinical monitoring may reveal a shift in the clinical spectrum from mild intermittent disease to more persistent cholestasis and fibrosis on the follow-up biopsies[2]. The Natural course and Prognosis of PFIC and Effect of biliary Diversion (NAPPED) consortium data revealed that 8 of 130 patients (6%) died before liver transplantation (LT), of which 3 had undergone surgical biliary diversion (SBD)[15]. Survival analysis showed that at 18 years of age, 44% of patients were alive with their native liver. Three-fourths of those alive with their native liver had undergone SBD by 18 years and a significantly lower percentage of patients with a FIC1-C genotype underwent SBD (FIC1-A 65%, FIC1-B 57%, FIC1-C 45%; P = 0.03). Native liver survival (NLS) was comparable between the three genotype sub-groups at 10 years of age (FIC1-A 67%, FIC1-B 41%, FIC1-C 59%; P = 0.15) with or without SBD. However, the proportions of total patients alive with the native liver at the age of 10 years were lower in patients without SBD than in patients who had undergone an SBD during follow-up.

BSEP DEFICIENCY

BSEP is a liver-specific transporter involved in actively transporting monovalent bile salts out of hepatocytes into biliary canaliculi against a concentration gradient. BSEP is encoded by the ABCB11 gene located on chromosome 2q31. Homozygous or compound heterozygous mutations in this gene results in PFIC2[18].

Pathogenesis

Chenodeoxycholic acid (CDCA) and the Retinoid X Receptors (FXR) α-heterodimer together activate the ABCB11[18]. Increasing bile acid concentration in the hepatocytes stimulates greater synthesis of BSEP to maintain equilibrium. There are over 200 mutations of ABCB11 causing PFIC2[18]. Common mutants that cause retention of the protein in the endoplasmic reticulum are G238V, D482G, G982R, R1153C, R1286Q, and ΔGly. The mutant protein that is retained and translocated out of the endoplasmic reticulum is then broken down by proteasomes[19]. Intrahepatic accumulation of bile salts and inflammation promote carcinogenesis through genomic modifications[19]. Metabolomic studies have shown that ABCB11 knock-out mice have impaired mitochondrial fatty acid β-oxidation with the resulting increase in reactive oxygen species that might exacerbate the liver injury[19].

Clinical and laboratory presentation

BSEP deficiency is the most common subtype of PFIC (with an estimated incidence of 1 in 50000 to 1 in 100000)[8]. It accounts for 37.5%-90.9% of cholestatic patients in the 9 studies that were analyzed in a systematic review[2]. Symptoms appear in the first month of life in 44% and by 3 mo of age in 72%[12]. Some patients with BSEP deficiency also present with early signs of vitamin D deficiency (rickets 3%-22%), vitamin K deficiency (bleeding 8%) or cholelithiasis (28%)[8,12]. High serum alanine aminotransferase and alpha-fetoprotein levels, early liver failure, cholelithiasis, HCC, very low biliary BA concentration, and negative BSEP canalicular staining suggesting PFIC2 (Figures 2A and 2B)[12]. In infancy, PFIC2 has mild to severe portal and lobular fibrosis with bridging fibrosis. Beyond infancy, advanced portal and lobular fibrosis are observed[2]. Early progression to HCC is described[19]. For more details see Table 1.

Genotypic variation

Of the 252 patients with cholestatic liver disease screened for ABCB11, 88 (34.9%) were identified with at least one disease-causing BSEP mutation with the median age of

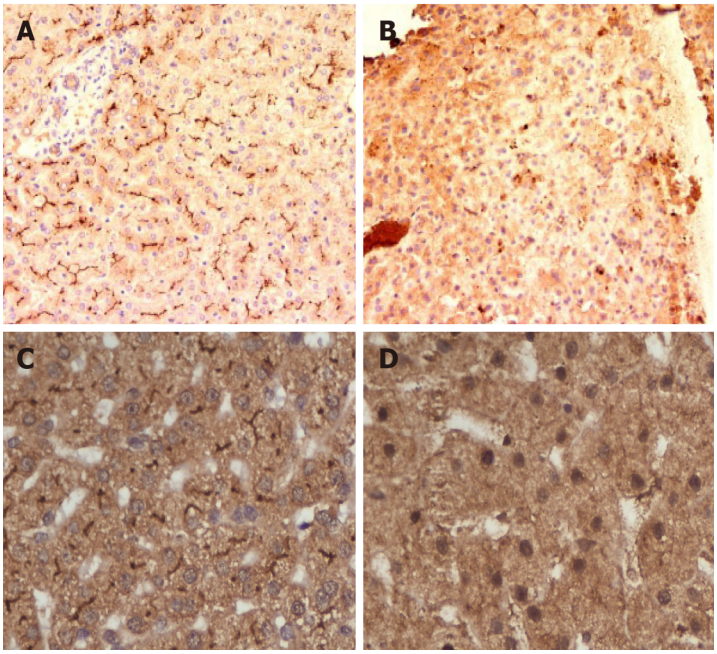

Figure 2 Immunohistochemistry of progressive familial intrahepatic cholestasis type 2 and 3. A: Normal liver showing canalicular bile salt exporter pump (BSEP) immunostaining; B: Faint BSEP immunostaining in a case of progressive familial intrahepatic cholestasis (PFIC2); C: Normal Liver showing canalicular multidrug resistant protein-3 (MDR3) immunostaining; D: Faint or absent MDR3 immunostaining in a case of PFIC3.

disease onset being 0.75 years[16]. 64.3% had no disease-causing mutation but at least one BSEP SNP. Amongst the common SNPs, p.A1028A was found in 197 of 252 families and p.V444A in 204 of 252 families of this cohort. The most common PFIC-2 mutations were p.E297G, p.D482G, and p.N591S. Apart from PFIC2, ABCB11 mutations cause BRIC2, ICP, contraceptive induced cholestasis and drug-induced cholestasis[16]. Four potential ABCB11 mutations and a donor splice site mutation (intron 19) were identified in 147 women with ICP[17]. Please also see the section on BRIC2 and ICP.

A global consortium on BSEP deficiency categorized the mutations as BSEP1 [p.D482G (c.1445A>G) or p.E297G (c.890A>G) mutation], BSEP2 (at least 1 missense mutation, not p.D482G or p.E297G) or BSEP3 (mutations leading to a predicted non-functional protein)[20]. Patients with BSEP1 genotype (p.D482G and p.E297G) have residual BSEP functionality and thus have a milder phenotype as compared to patients exhibiting BSEP2/BSEP3 genotype. There is wide geographical variations in genotype with the commonest mutations found in a study from Shanghai being c.145C>T (p.Gln49Ter) and c.2594C>T (p.Ala865Val)[21]. Severe phenotypes are often associated with mutations leading to premature protein truncation or failure of protein production. Insertion, deletion, nonsense and splicing mutations exhibit little or no detectable BSEP at the hepatocyte canalicular membrane.

Natural history and outcome

PFIC2 is likely to have more severe lobular injury, portal fibrosis and inflammation than PFIC1. Hepatocellular necrosis and giant cell transformation are also much more pronounced in PFIC2 than in PFIC1 and may persist with time. Majority of homozygous or compound heterozygous mutations present as severe disease in infancy with progressive liver disease which requires LT. Despite treatment, 76%-100% of PFIC2 continue to have pruritus with progression to severe liver disease and this may occur as early as 7 mo of age. Advanced portal and lobular fibrosis are seen in children beyond infancy[12]. Among patients undergoing LT, liver failure and/or HCC were detectable in about 60%. Liver samples obtained from 10 children with HCC aged 13-52 mo showed that 9 had evidence of BSEP deficiency and/or mutations in ABCB11. Recently, HCC and/or cholangiocarcinoma was described in 15% of those with PFIC2. Hence, close monitoring of these children particularly those carrying 2 null mutations is essential[22]. Children with transient neonatal cholestasis harbour non-null variants, express immune-histochemically detectable BSEP and have better outcomes[21].

The global NAPPED consortium reported that 23.1% PFIC2 had undergone SBD and 46.1% had undergone LT[20]. In total, 16 (6%) [BSEP1: n = 3/72 (4%), BSEP2: n = 8/136 (6%), BSEP3: n = 5/56 (9%)] died prior to LT at a mean age of 1.6 years. NLS was 32% at 18 years of age. Five patients underwent LT beyond 18 years of age (19.6-27.5 years). Patients with mutations categorized as BSEP1 had better long-term outcomes than those with BSEP2 or BSEP3, with a median NLS of 20.4 years, 7 years and 3.5 years respectively. The incidence of HCC increased with the genotype severity from 4% in BSEP1 to 7% in BSEP2 and 34% in BSEP3. HCC occurred in 3% of patients who had SBD as against 7% who had not received SBD[20].

MDR3 DEFICIENCY

Pathogenesis

This autosomal recessive condition is caused by mutations of the MDR3 glycoprotein, which is coded by the ABCB4 gene located on chromosome 7q21. The gene consists of 27 coding exons spanning-74 kb[23]. ABCB4/MDR3 P-glycoprotein consists of six intracellular domains and six extracellular loops separated by twelve transmembrane domains. The protein contains two intracytoplasmic ATP-binding domains, also known as nucleotide-binding domain. The nucleotide-binding domains provide energy for the trans-membrane transfer of the substrate against the concentration gradient. The trans-membrane domains in turn provide specificity for the substrate. A study on fetal liver demonstrated that ABCB4 mRNA levels are 16-fold lower compared to the normal adult liver with only faint and focal canalicular MDR3 immunostaining suggesting that ABCB4 normally develops late in gestation or possibly in the postnatal period. The protein is present in the canalicular membrane of hepatocytes and transports phosphatidyl-choline from the hepatocytes to the bile canaliculi. Phosphatidyl-choline is a key component of micelles which keeps the cholesterol in soluble form thus protecting the cholangiocytes from damage. In presence of abnormal MDR3, phosphatidyl-choline is not transported across to the bile canaliculi leading to abnormal micelle formation. Inadequate micellar formation results in presence of insoluble bile salts and cholesterol in the biliary canaliculi which damage the cholangiocytes. Cholesterol is more likely to crystallize into stones, damaging liver structures by obstructing small bile ducts.

Clinical and laboratory presentation

These patients present with a wide spectrum of manifestations ranging from transient neonatal cholestasis, episodic cholestasis, gall stones, cirrhosis to end-stage liver disease[24,25]. The patients with PFIC3 have elevated GGT and alkaline phosphatase. Bile salts and cholesterol values can be normal while biliary phospholipids are significantly reduced. Age of the onset of PFIC3 can vary widely from the neonatal period to adulthood. PFIC3 often presents later as compared to PFIC1 and PFIC2, as late as adulthood in some cases[25]. The early-onset disease can present with jaundice, pruritus, variceal bleeding (portal hypertension), stunted growth, reduced bone density and learning disabilities[25]. The complete absence of canalicular staining of MDR3 protein is associated with mutations leading to a truncated protein form (Figures 2C and 2D). Those with missense mutations have faint or normal MDR3 canalicular expression. The abnormal MDR3 canalicular expression combined with low levels of biliary phospholipids is highly suggestive of MDR3 deficiency. Histopathological findings associated with PFIC3 include primarily portal-based inflammatory infiltrate with marked periportal ductular reaction. They usually progress to biliary-type cirrhosis, lobular disarray, and hepato-canalicular cholestasis.

Genotypic variation

Degiorgio *et al*[26] analyzed ABCB4 gene mutations in 68 PFIC3 patients and found 29 mutations in the coding region and 10 in the transmembrane domains which involved phosphatidyl-choline translocation. Delaunay *et al*[27] devised a classification system for the various forms of these mutations: Class I (nonsense mutations that result in a defective synthesis), class II (missense variations that prevent protein maturation), class III (missense mutants resulting in defective protein activity), class IV (unstable variations) and class V mutants (unknown pathogenicity). These classifications are useful in determining potential therapies for patients. Approximately 300 disease-causing ABCB4 variants have been reported, typically in those with homozygous status who have a rapidly progressive cholestatic liver disease with potentially life-threatening complications. Those with heterozygous status have less severe manifest-

ations.

Natural history and outcome

Patients with residual phosphatidyl-choline secretion and MDR3 expression (especially those with missense mutations), respond to treatment with UDCA in 70% of cases. LT is reserved for patients with PFIC3 associated with end-stage liver disease. The mean age at LT is 9.6 (2-33) years[28]. HCC and cholangiocarcinoma have both been described with PFIC3.

BRIC

BRIC is an autosomal recessive inherited disorder characterized by the intermittent occurrence of severe and recurrent cholestasis[29]. BRIC typically appears later than PFIC and is not progressive. Two genetic forms of BRIC are known; BRIC1 is characterized by the mutations of the ATP8B1 gene while BRIC2 presents a mutation in the ABCB11 gene. BRIC shows a non-progressive course since the protein function is only partially impaired[29]. Most of them have missense mutations located in less conserved regions of the gene. The global NAPPED consortium reported 11 BRIC2 who later presented with persistent cholestasis and progressive liver disease with pathological genetic variants[20].

Association with other cholestatic presentations

ICP is the occurrence of intense pruritus in a pregnant woman without pre-existent liver disorder; beginning usually in the third trimester and resolving completely after delivery. Elevated levels of BA, aminotransferase and alkaline phosphatase are typically present. Although the disease is benign in the mothers, poor fetal outcomes have been reported. Heterozygous mutations in ABCB4, ABCB11, ATP8B1, ABCC2 and tight junction protein 2 have been associated with ICP[30]. While GGT is normal in ABCB11-related forms of estrogen-associated cholestasis, increased GGT levels could serve as a discriminating feature of ABCB4 deficiency.

The associations of heterozygous missense and nonsense ABCB4 mutations in cryptogenic liver fibrosis and biliary cirrhosis have been reported[28,31]. The histomorphological phenotype associated with ABCB4 mutations includes portal fibrosis with periportal ductular proliferation. Early diagnosis and therapy with UDCA may slow down fibrosis progression in few cases. Some cases of ABCB4 mutations may also present with chronic cholangiopathy[32].

MDR3 deficiency also leads to gallbladder disease or low phospholipid-associated cholelithiasis (GBD-1/LPAC)[32]. There is more evidence that either biallelic or monoallelic ABCB4 defects may cause or predispose patients to a wide spectrum of human liver diseases such as LPAC, small duct sclerosing cholangitis and adult biliary fibrosis or cirrhosis[28]. Liver cirrhosis accompanied by vanishing bile duct syndrome (ductopenia) in ABCB4 deficient patients have been reported[33].

ABCB11 and ABCB4 deficiency may predispose to the cholestatic liver injury induced by oral contraceptives, psychotropic drugs, selected chemotherapy drugs, statins, and antibiotics[34]. The common variant p.Val444Ala in ABCB11 is associated with an increased risk of drug-induced liver injury[35]. Most cases improve on withdrawing the drug and adding UDCA. Recent studies have demonstrated, that ABCB4 mutations (c.504C>T and c.485T>Ain exon 6 and c.2793 frameshift mutation in exon 23) are possibly involved in the development of parenteral nutrition-associated liver disease in premature infants[36].

THERAPEUTIC STRATEGIES FOR PFIC

The holistic management of patients with PFIC includes their nutritional rehabilitation, fat-soluble vitamin supplementation, control of pruritus, prevention of fibrosis progression and LT for those with end-stage liver disease.

Nutritional rehabilitation

Chronic malnutrition due to cholestasis and steatorrhoea affects almost all children with PFIC, especially those with PFIC1[37,38]. Apart from cholestasis-related malabsorption, complex interactions of the liver with the hypothalamus-pituitary-adrenal axis in chronic cholestasis have been advocated as one of the mechanisms for growth failure in these children[39]. Severe cholestasis leads to deficiency of fat-soluble vitamins (FSV) with decreased bone mineral density, often refractory to FSV supplementation[38]. Meticulous nutritional assessment should be performed and

documented at the first visit. The goal of nutritional rehabilitation should be to give calories around 125% of the recommended daily dietary allowance for the ideal weight targeting around 180-200 cal/kg/d along with high protein about 2-3 g/kg/d[38]. FSV should be supplemented orally: Vitamin A 5000-10000 IU/d; Vitamin D (cholecalciferol) 2000-5000 IU/d; Vitamin E 50-400 IU/d and Vitamin K 5 mg/wk to 5 mg/d [40]. Weekly or daily supplementation of FSV, especially vitamin D, is more effective than large bolus doses for the treatment of vitamin D deficiency in children with chronic liver disease[41]. Refractory deficiency or those with significant bony changes should be treated with calcitriol at a dose of 0.05-0.2 μg/kg[40]. The oral water-soluble liquid formulation of FSV containing vitamins A, D, E and K have shown to be more efficacious than conventional preparation in infants and children with cholestatic liver disease. Medium-chain triglycerides should be supplemented as they are absorbed directly into the portal circulation and are not affected by cholestasis[40].

Treatment of pruritus

Pruritus is the most disabling symptom of PFIC disturbing daily activities, schooling and sleep. A step-up approach of medical therapy is advocated in children with pruritus. SBD is a very useful tool in medical non-responders (Figure 3). Table 2 lists the drugs used for the management of pruritus with their mechanism of action, dose and adverse effects. Apart from medical therapy, trimmed nails, full-sleeve clothing-stockings during sleep and well moisturized skin are advocated. Various tools like visual analog scale, Whitington scale or 5D-pruritus score have been used for objective measurement of pruritus and to assess response to medications or surgical therapy [42].

Role of medical therapy

UDCA leads to complete resolution of pruritus in about a third of children with PFIC2 [38] and response is mainly dependent on the mutation and resultant residual protein expression[24]. Long-term therapy with UDCA has been shown to reverse fibrosis in MDR3 heterozygotes with residual protein expression[43]. The role of rifampicin in management of pruritus has been established by meta-analyses (pooled odds ratio of 15.2 for complete/partial relief) as compared to placebo/alternative therapy[44]. Bile acid sequestrants are less favourable in children due to non-palatability[38]. Spacing is needed from food and other drugs leading to loss of compliance and optimal effect [45]. Opioid antagonists can ameliorate pruritus, although with increased incidence of adverse events[44]. Sertraline, a selective serotonin reuptake inhibitor is also beneficial for the relief of pruritus. Sertraline has high first-pass metabolism in the liver and should be used with caution. The other drugs with equivocal efficacy for control of pruritus in PFIC include ondansetron, phenobarbitone and antihistaminics[40,41]. Newer modalities to treat pruritus of cholestasis that have not been extensively analyzed in studies include albumin dialysis using molecular adsorbent recirculating system, plasmapheresis and endoscopic nasobiliarydrainage[46]. Plasmapheresis reduces autotaxin activity thereby reducing lysophosphatidic acid and reducing pruritus in ICP.

Surgical interruption of enterohepatic circulation

Commonly used SBD include partial external biliary diversion (PEBD), ileal exclusion (IE) and partial internal biliary diversion (PIBD). Figure 4 shows a schematic representation of the various diversion surgeries, techniques and their common complications. PEBD, although being the most widely used diversion surgery is associated with a host of stoma-related complications[47-49]. Recently, external fistulae have been intubated with gastrostomy devices for intermittent drainage[49]. IE[50,51] and PIBD [52,53] are therapeutic options in those with failed PEBD or for better cosmetic results, especially in adolescents. Recent studies on SBD with their outcome and complications have been tabulated in Table 3[51-55]. Biliary diversion leads to a relatively higher ratio of cholic acid to CDCA in bile, reduced taurine-to-glycine conjugate ratio, increase in secondary bile acid pool, ultimately causing bile to become more hydrophilic[55]. Total biliary diversion involves diverting all the bile flow from the common bile duct to the exterior through a jejunal loop. It is usually reserved for pruritus refractory to PEBD or to prevent and treat allograft steatosis in post LT PFIC1 [56]. Irrespective of the technique used, biliary diversion surgeries lead to a decrease in the concentration of BA, improvement in pruritus score, growth spurt, histological improvement and even reversal of fibrosis in some cases[38,47]. There are no head-to-head trials to compare the efficacy and safety of these techniques. Post SBD, the child should be monitored for improvement in pruritus, changes in growth parameters and for decrease in BA and bilirubin in the serum. Recently, the NAPPED consortium and

Table 2 Drugs used for control of pruritus in progressive familial intrahepatic cholestasis: Mechanism of action, dose, adverse effects

Drug	Mechanism of action	Dose	Adverse effects
Ursodeoxycholic acid	Protection of cholangiocytes from the hydrophobic bile acids; Choleretic action through both bile acid dependent (cholehepatic shunt) and independent pathway; Protection of hepatocytes from bile acid induced apoptosis; Direct membrane stabilizing effect in cholangiocytes; Up-regulate synthesis, apical insertion & activation of BSEP & Mrp2 *via* Ca^{2+} and PKC-dependent mechanisms or *via* activation of p38 MAPK and Erk-1/2–dependent mechanisms in animal models	10-30 mg/kg/d	Adverse effects rare: Severe vomiting or diarrhoea
Rifampicin	Activates pregnane X receptor leading to decrease in autotoxin level thus leading to decrease in lysophosphatidic acid synthesis and down-regulation of TRP vanilloid 1; Upregulates multidrug-resistance protein 2; Activates enzymes UDP-glucuronosyltransferase-1A and cytochrome P450-3A4 and stimulates 6α-hydroxylation of bile acids, promoting urinary excretion of dihydroxy and monohydroxy bile acids	5-10 mg/kg/d	Adverse effects rare: Hepatotoxicity, vomiting
Bile acid sequestrants: Cholestyramine, colestipol, colesevelam	Non-absorbable anion exchange resins that bind bile acids, cholesterols and other compounds in the intestinal lumen and prevent their enterohepatic circulation	240-500 mg/kg/d; Usually administered mixed with juice	Palatability, steatorrhoea, constipation, intestinal obstruction from inspissations, hyperchloremic metabolic acidosis; Growth failure; Decreased absorption of other drugs (*e.g.*, UDCA) if not spaced; Need to be spaced from food
Opioid antagonists: Naltrexone	Reduces central opioidergic tone, believed to be raised in patients with cholestatic pruritus; Decreasing plasma levels of endogenous opioids like enkephalins	Gradually increment starting at 12.5 mg/d increasing every 3-7 d till pruritus reduces	Opioid withdrawal-like symptoms including abdominal pain, tachycardia and hypertension
Selective serotonin reuptake inhibitors: Sertraline	Exact mechanism of action not elucidated; Mediates its effect through serotonergic signals in the central nervous system that provide inhibitory signals to the itch pathways; Neuropharmacologic inhibition of stress	Adults: 75-100 mg/d; Children: 2.2 mg/kg/d	Adverse effects: Allergic reaction, behavioural issues, diarrhoea, insomnia, dizziness, high first pass metabolism-risk of hepatotoxicity

BSEP: Bile salt export pump; PKC: Protein kinase C; MAPK: Mitogen-activated protein kinase; TRP: Transient receptor potential; UDP: Uridine diphosphate; UDCA: Ursodeoxycholic acid.

another meta-analysis have shown improved NLS in those with common missense mutations (D482G or E297G) and in those with more than 75% reduction in BA post SBD[20,57]. Around 18%-39% of children undergoing SBD require LT in 5-10 years of follow-up, mostly those with advanced fibrosis at the time of SBD and those with failure of SBD[51-55]. Those with advanced fibrosis do not show a reduction in serum BA with biliary diversion surgeries, hence a liver biopsy should precede the surgery [57]. The choice of biliary diversion technique depends on the patient's preference and experience and comfort of the surgeon/centre. Health-related quality of life is comparable between those undergoing PEBD for PFIC and healthy controls.

LT

LT is indicated in children with end-stage liver disease, advanced fibrosis and those with refractory pruritus despite biliary diversion surgeries, yet it is fraught with its unique complications and conundrums in children with PFIC[58]. PFIC is one of the most common indications accounting for 13.4% of total pediatric LT[59]. LT in patients with PFIC2 and PFIC3, where the mutation affects only the liver has resulted in excellent graft and patient survival[37]. On the other hand, due to a wide extrahepatic tissue distribution of FIC1 expression, LT for PFIC1 has been associated with a high risk of complications due to the involvement of these extrahepatic sites after LT[60]. There are three unique issues related to LT in PFIC: (1) High rate of graft failure, allograft steatosis and diarrhea in PFIC1; (2) Antibody-induced BSEP deficiency leading to graft loss in PFIC2; and (3) Utilization of heterozygous family members as donors in living donor LT program.

Allograft steatosis, refractory diarrhea and graft failure in PFIC1: Post LT in PFIC1, the graft liver starts to secrete and excrete normal amounts of bile salts which can't be handled by the intestines as the intestinal FIC1 is still defective leading to intractable diarrhea[60]. This plausible mechanism for intractable diarrhea is further strengthened by the clinical response to bile adsorption resins and total biliary diversion. FIC1 in the

Table 3 Recent studies describing outcome and complications with biliary diversion surgeries in progressive familial intrahepatic cholestasis

Study	Type of biliary diversion	No of patients	Median follow up	Outcome	Adverse events
Yang *et al* [54] (2009)	PEBD	14 (11-PFIC)	3.1 yr (2-5.7)	Resolution of pruritus: 50%; Decrease in pruritus: 21%; Decrease in serum bile acids; Improvement in growth; Improved quality of life; No response in 2 patients with advanced fibrosis; 21.4% were listed for LT at mean follow-up 3.2 yr (all had advanced fibrosis pre-PEBD)	3 developed stoma prolapse; Post-op bleed and wound dehiscence in 1 each
Schukfeh *et al*[55] (2012)	PEBD	24	9.8 yr (1.6-14.3)	Resolution of pruritus with normalization of bile acids in 54%; 37.5% received LT at mean 1.9 yr; All of them had failed PEBD & 78% of them had cirrhosis pre-PEBD	Stomal prolapse in 2; Cholangitis, dyselectrolytemia, GI bleed and intestinal obstruction in 1 each
Wang *et al* [50] (2017)	PEBD; IE; PIBD	39; 11; 7; (38 PFIC & 20 alagillesyndrome)	24 mopostsurgery	Decrease in severe pruritus-54% in PFIC1 and 30% in PFIC2; Trend towards decreased pruritus after IE and PIBD; PEBD but not IE led to decrease in bilirubin and ALT in PFIC1; 23.7% of PFIC underwent LT post diversion	PEBD: Dehydration/dyselectrolytemia in 4; Stoma prolapse in 3; Intestinal ischemia & bowel obstruction in 1 each; IE; Dyselectrolytemia-2; PIBD: Dyselectrolytemia in 2, intestinal ischemia & intussusception-1 each
Cielecka-Kuszyk *et al*[47] (2019)	PEBD	4 (all PFIC2)	> 10 yr	Resolved cholestasis in 3; Reversal of fibrosis in 2	
Bull *et al* [48](2018)	PEBD; IE	57; 6		Sustained improvement in pruritus: PFIC1-57%; PFIC2 (D482G/E297G mutations)-76%; PFIC2 other mutations-33%; Improvement in bilirubin and bile acids; Improvement in growth; 27% of PFIC1 & 31% of PFIC2 were listed or received LT (less often in D482G/E297G)	Dehydration/dyselectrolytemia due to high stoma output seen in 6 patients (1 died); Cholangitis in 3; Bile stagnation in 2; Stoma bleed in 1
Van Wessel *et al*[20] (2020)	PEBD; IE; PIBD	47; 13; 1; (all PFIC2)	8.4 yr (1.6-12)	Relief in pruritus – sustained: 54%; Transient: 17%; None: 29%; Relief in pruritus more common in BSEP1 mutations (66%) *vs* BSEP2 (36%) & BSEP3 (0%); Decrease in serum bile acids, bilirubin, AST & ALT; A 75% reduction in bile acids or decrease to a level < 102 μmol/L post diversion predicts long term NLS of > 15 yr; Biliary diversion associated with higher NLS: HR 0.51; 95%CI: 0.29-0.91, $P = 0.02$	
Bjørnland *et al*[49] (2021)	PEBD	33; (25 PFIC)	10 yr (0.6-25.2)	Decrease in bile acids 1 wk post-op predictive of successful drainage; 39% received LT or were listed LT at a median follow up of 10 yr	42% early post op complications; Long term stoma related complications in 55%-20% secondary surgeries
Van Vaisberg *et al*[51] (2020)	IE	11	5 yr	Significant relief in pruritus: 8 (72.7%); 2/11 (18.2%) progressed to ESLD within a year and were listed for LT	Intussusception in 1; No diarrhoea
Foroutan *et al*[53] (2020)	PIBD	44	54 mo (10-105)	Significant decrease in jaundice and pruritus	Ascending cholangitis in 19.2%; No difference in cholangitis between standard procedure and PIBD with anti-reflux valve
Chen *et al* [52](2018)	PIBD	34; (PFIC1-10, PFIC2-14, PFIC3-5)	-	Decreased bilirubin and bile acids; Improved growth; 2 (5.9%) underwent LT at 20 & 39 mo post PIBD	Dyselectrolytemia/dehydration in 2; Relapse of symptoms in 4
Agarwal *et al*[38] (2016)	PIBD; PEBD	3; 1	2 yr (1-2)	PIBD: Decrease in pruritus score, improved growth & decreased serum bile acids; PEBD: Failed; One with failed PEBD needed LT in 7 yr; Rest all survived with native liver at mean follow up 8 yr	No complications with PIBD
Bull *et al* [48](2018)	PEBD; IE	57; 6		Sustained improvement in pruritus: PFIC1-57%; PFIC2 (D482G/E297G mutations)-76%; PFIC2 other mutations-33%; Improvement in bilirubin and bile acids; Improvement in growth; 27% of	Dehydration/dyselectrolytemia due to high stoma output seen in 6 patients (1 died); Cholangitis in 3; Bile stagnation in 2; Stoma bleed in 1

PFIC1 & 31% of PFIC2 were listed or
received LT (less often in D482G/E297G)

PEBD: Partial external biliary diversion; PIBD: Partial internal biliary diversion; PFIC: Progressive Familial Intrahepatic Cholestasis; IE: Ileal Exclusion; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; TBD: Total biliary diversion; LT: Liver transplantation; NLS: Native Liver Survival; BSEP: Bile salt exporter pump; ESLD: End stage liver disease.

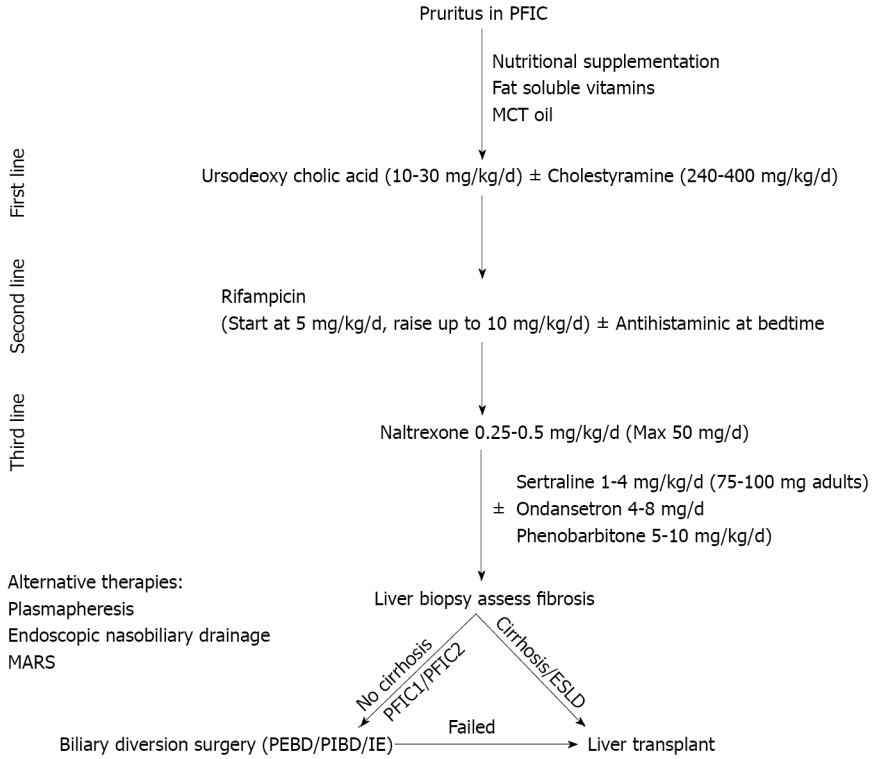

Figure 3 Algorithm for management of pruritus in children with progressive familial intrahepatic cholestasis. PFIC: Progressive familial intrahepatic cholestasis; MCT: Medium chain triglyceride; ESLD: End stage liver disease; PEBD: Partial external biliary diversion; PIBD: Partial internal biliary diversion; IE: Ileal exclusion.

intestine which has a strong correlation to SLC10A2, the ileal sodium/bile acid co-transporter is defective in PFIC1 leading to the impaired enterohepatic circulation of bile salts[61]. When the intestine of these patients which are defective in FIC1/SLC10A2 starts receiving a normal amount of BA secreted by the graft liver, it leads to bile acid diarrhea, steatosis and graft fibrosis necessitating re-transplant[60]. Hori *et al*[37] reported chronic diarrhea in 91%, grade 3 steatosis in 72.7% and significant graft fibrosis in 82%; 27% died at a median of 12 years post LT and 3 required re-transplant. Davit-Spraul *et al*[25] reported extrahepatic complications post LT in 100% of PFIC1 and one-third required re-transplant for massive steatosis. There is a recurrence of steatosis and graft failure even in the second graft[12,60]. The genetic analysis predicts the patients likely to develop these complications and thus pre-LT genetic analysis should be considered mandatory for patients with PFIC1[60]. Total internal biliary diversion during the time of LT has been described as a modality by the Kasahara group to prevent complications of diarrhea and steatosis post LT[62].

Antibody induced BSEP deficiency: Post-transplant recurrence of cholestasis in PFIC2 has been described due to *de novo* polyclonal antibodies directed against extracellular loop 1 domain of BSEP protein in the sera and on the canalicular membrane[63]. Patients with severe mutations with resultant complete absence of BSEP are typically prone to this complication as the BSEP of the transplanted liver is recognized as a foreign new antigen by the recipient's immune system, usually seen after an episode of acute rejection. Initial treatment of antibody-induced BSEP-deficiency disease is increased immunosuppression followed by either rituximab, bortezomib, intravenous immunoglobulin or plasmapheresis[64]. Those refractory to the above treatment may be considered for allogeneic hematopoietic stem cell transplantation[65]. Re-transplant

Table 4 Potential novel therapeutic drugs for treatment of progressive familial intrahepatic cholestasis

Drug	Mechanism of action	Clinical trials and current status	Notes
Maralixibat/LUM001	Apical sodium-dependent bile acid transporter inhibitor	NCT04185363: Open label phase III trial; Recruiting patients; NCT03905330: MARCH-PFIC trial; Randomized controlled trial, recruiting patients; NCT04729751: RISE trial in infants; Open label phase II safety study; NCT04168385: Long term safety study; NCT02057718; Open label phase II trial; Completed	Orphan drug designation by FDA; Breakthrough therapy for PFIC2
Odevixibat/A4250	Selective inhibitor of ileal bile acid transporter	NCT03566238: PEDFIC 1 study; Phase III, open label, randomized controlled trial; Ongoing; NCT04483531: Expanded access study including patients not enrolled in PEDFIC 1 study	Orphan drug designation by FDA; Fast track designation for PFIC
4-PB/GPA	Prolongs degradation rate & increases cell surface expression of BSEP & functions as a chemical chaperone to correct the misfolded proteins	Leads to long term reduction in serum BA, improvement in liver biochemistry as well as relief of pruritus; Increased canalicular localization of E297G and D482G BSEP mutants; GPA more palatable, has lower sodium, doesn't interact with rifampicin; Doses: 4-PB: 500 mg/kg/d; GPA: 8 g/m^2/d	4-PB FDA approved for urea cycle defect
Ivacaftor	Rescues the function of missense mutations in the nucleotide binding domains of BSEP & MDR3	*In vitro* correction of binding domain missense mutation (T463I) of BSEP; Improved phospholipid secretion activity in mutant ABCB4	*In vitro* studies; Animal studies
Oxcarbazepine	Nerve stabilizing effect; Enzyme inducer – possible role in potentiating action of 4-PB	Single case report on its combined use with 4-PB and maralixibat	
Gentamicin	Induce readthrough in nonsense mutation	*In vitro* increased readthrough in 6 common nonsense mutation of BSEP leading to increased canalicular expression of bile salt transporter	
FXR agonist (Obeticholic acid)	Farsenoid X receptor agonist	No trials in PFIC; Safe and efficacious in treatment of PSC and non-alcoholic steatohepatitis	FDA approved for PSC
Nor-UDCA	Side-chain-shortened derivative of UDCA; Increases cholehepatic shunt	No trials in PFIC; NCT03872921: Ongoing phase III randomized controlled trial in PSC	
Steroids	Possible upregulation of BSEP transporter? Up-regulation of sodium taurocholate copeptide transporterproviding increased gradient for BSEP	Only case reports and animal studies	
NGM282	FGF19 analogue	NGM282 inhibited bile acid synthesis and decreased fibrosis markers, without change in alkaline phosphatise level	
Bezafibrate	Peroxisome proliferator activated receptor agonist	Bezafibrate reduced pruritus and cholestasis in 2 out of 3 children with PFIC1 and improved lipid profile in all	

PFIC: Progressive familial intrahepatic cholestasis; BSEP: Bile salt export pump; FGF: Fibroblast growth factor; NGM282: Codename of drug which is an engineered analogue of fibroblast growth factor - 19; UDCA: Ursodeoxycholic acid; PSC: Primary sclerosing cholangitis; FDA: Food and drug administration; PB: Phenyl-butyrate; MDR3: Multidrug-resistant type 3; ABCB: ATP-binding cassette subfamily B; GPA: Glycerol phenylbutyrate; BA: Bile acids.

may be considered in those refractory to all therapy but the threat of recurrence in the re-transplanted liver is high[63].

Donor issues in living-related liver transplant: There have been apprehensions about using family members as donors, who may be heterozygous. Bassas *et al*[66] described living-related donor LT in 13 children with PFIC with good outcomes and no recurrence of symptoms in the recipients. Other studies have also reported favourable outcomes in PFIC2 and PFIC3 with living-related LT[67]. Genetically proven heterozygous family members have been used as donors without recurrence of symptoms in the recipients. Currently, there is not enough evidence to perform a genetic analysis of the related donor before LT. It remains to be seen if the heterozygous donor liver in PFIC3 patients may predispose the recipient to drug-induced cholestasis, contraceptive induced cholestasis, ICP or GBD-1/LPAC.

Gene therapy: The additional complications associated with LT warrant the consideration of innovative therapeutic modalities like gene therapy. Recent approval for Adenovirus Associated viral (AAV) vector-based gene therapy for other monogenic disorders, raises hope for such future therapies for PFIC[68]. The liver tropism of AAV

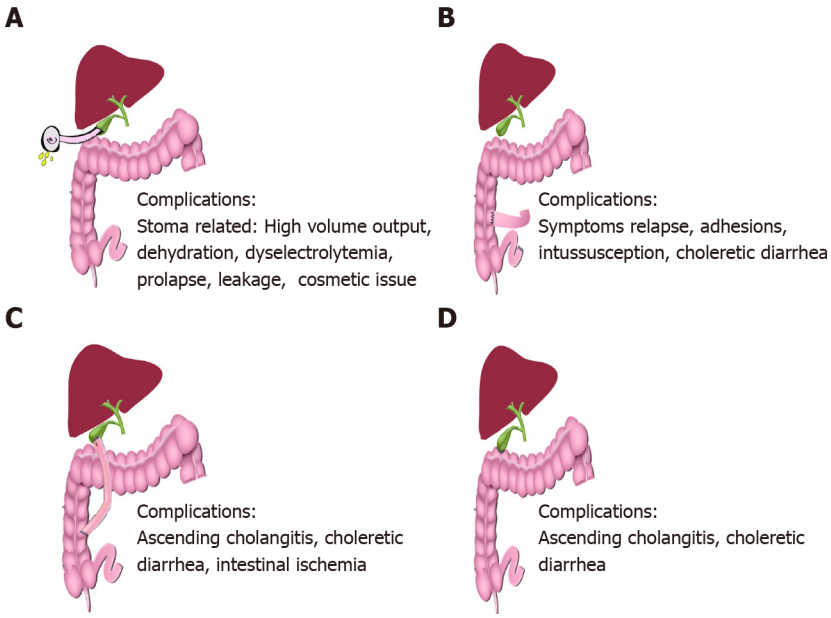

Figure 4 Schematic representation of biliary diversion surgeries. A: Partial external biliary diversion: A conduit of around 15-20 cm is made from gall bladder to an exteriorized cutaneous stoma using a jejunal conduit. This conduit diverts a variable (-50%) proportion of bile away from the intestine into the connected stoma bag which needs to be emptied regularly; B: Ileal exclusion (IE): IE involves formation of an end to side ileocolonic anstomosis in such a way that the terminal 15% of ileum is excluded, hence drastically reducing the enterohepatic circulation. The proximal end of the resected ileum is sutured to the colon around 5 cm distal to the ileocecal valve; C: Partial internal biliary diversion (PIBD): The most common technique of PIBD is a cholecysto-jejuno-colonic anastomosis. An isolated jejunal conduit of around 12-15 cm length is created and anastomosed proximally to the gallbladder and distally to the mid part of the ascending colon; D: Another commonly used technique of PIBD is cholecysto-colostomy where gall bladder is directly connected to the transverse colon to partially bypass the terminal ileum.

vectors is a major advantage in the treatment of PFIC as this would imply the need for a lower vector dose for therapeutic efficacy. Higher AAV vector doses have been shown to cause adverse effects ranging from transient liver inflammation to even progressive liver failure and deaths in few reports[69]. Gene therapy can be non-integrating where each cell division post therapy will lead to some loss of episomal AAV vector genome or integrating *in vivo* where these integrated genes are copied during cell division and passed on to the daughter cells without any loss of vector genome upon cell division[68]. The benefit of integrating gene therapy is that a limited number of corrected hepatocytes will repopulate the liver and correct the defect[68, 70]. Theoretically, *in vivo* non-integrating AAV mediated gene therapy can be successful in PFIC3 as establishing ABCB4 expression in a proportion of hepatocytes. This can partially correct phosphatidylcholine transport and prevent the progression of the disease. Gene therapy for PFIC has only been done successfully in double knockout murine models (Abcb4-/-) to date[71]. Treatment with modified mRNA variants encoding human ABCB4 encapsulated in lipid nanoparticles lead to *de novo* expression of functional ABCB4 and restored phospholipid transport in cultured cells as well as Abcb4-/-mice. Hepatocyte damage due to accumulated BA in PFIC1 and 2; and decreased membrane stability in PFIC1 would warrant the correction of the genetic defect in all the hepatocytes, thus needing an integrating gene therapy strategy [68]. Tumor genes is a possible major risk factor with integrating gene therapy as seen in trials on patients with severe combined immunodeficiency[72]. The evolution of CRISPR/Cas9 based gene-editing tools that exhibit fewer off-target effects raises hope for integrating *in vivo* controlled gene therapy in future[73].

Newer targets for therapy in PFIC: Several drugs and therapeutic molecules are currently being investigated for their therapeutic efficacy and safety in PFIC1 and 2. Table 4 and Figure 5 describes the various experimental drugs being evaluated for their therapeutic role in PFIC. Among them, the class of drugs that have generated the maximum interest are those which inhibit the ileal ASBTs.

Apical sodium bile acid transporter inhibitors: ASBTi is currently under various stages of clinical trials include maralixibat and odevixibat (A4250). Currently, there are 4 registered clinical trials for maralixibat including one in infants and two clinical trials for odevixibat. The ASBTi interfere with bile salts reabsorption in the terminal ileum and hence greatly reduce the enterohepatic circulation of bile salts which are then

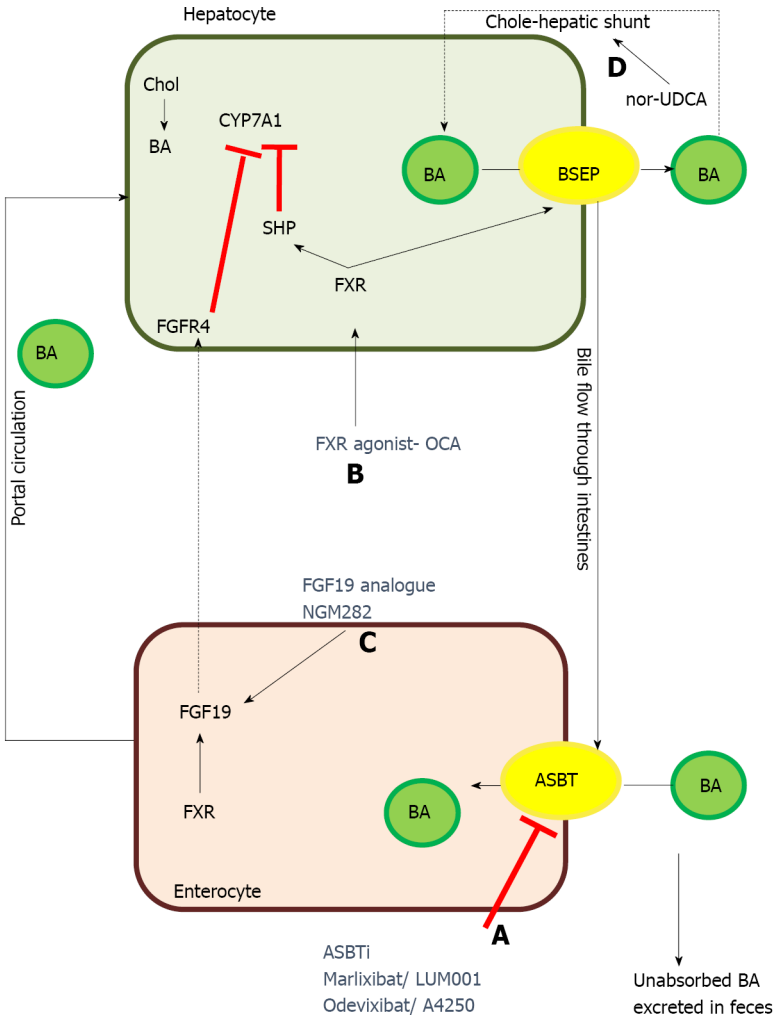

Figure 5 Therapeutic targets of some of the newer drugs for treatment of progressive familial intrahepatic cholestasis. A: Apical sodium-dependent bile salt transporter inhibitors prevent the absorption of bile acids in the terminal ileum resulting in significant decrease in enterohepatic circulation of bile acids, ultimately resulting in decreased serum bile acids; B:Farnesoid X receptor agonist acts by increasing bile salt exporter pump expression as well as by inhibiting CYP7A1, the rate limiting step in synthesis of bile acids in hepatocytes; C: Fibroblast growth factor 19 analogue act *via* fibroblast growth factor receptor-4 and inhibit CYP7A1; D:Nor-ursodeoxycholic acid increases the bile-acid dependent bile flow by increasing the cholehepatic shunt. ASBT: Apical sodium-dependent bile salt transporter; BA: Bile acid; BSEP: Bile salt exporter pump; Chol: Cholesterol; CYP7A1: Cholesterol 7-α hydroxylase; FGFR4: Fibroblast growth factor receptor-4; FXR: Farnesoid X receptor; FGF19: Fibroblast growth factor 19; nor-UDCA: Nor-ursodeoxycholic acid; OCA: Obeticholic acid; SHP: Small heterodimer partner-1.

excreted in the stool. ASBTi thus shrinks the bile acid pool and helps reduce BA and in turn improves cholestasis[74]. Odevixibat is as effective as PEBD in reducing BA as well as pruritus in a child with PFIC2 thus raising hope of a very effective medical management of cholestasis[75]. However, on stopping therapy after the completion of the trial, the patient had a relapse of symptoms and underwent PEBD. The preliminary results from the trials of maralixibat and odevixibat have shown that these ASBTi reduce BA and pruritus while improving growth[74,75].

Ivacaftor: Ivacaftor (VX-770), a CFTR/ABCC7 potentiator has been demonstrated to cause functional rescue of missense mutations of ABCB11 and ABCB4 located in a highly conserved ABC transporter motif[76]. *In vitro* molecular modelling showed that ivacaftor led to the functional rescue of mutant ABCB4 resulting in increased phospholipid secretion[76].

4 phenyl butyrate: 4-phenyl butyrate (4-PB), an FDA approved drug for urea cycle defect acts by 2 mechanisms to correct the defect in BSEP missense mutations: (1) It modulates the short-chain ubiquitination to prolong the degradation rate of cell surface resident BSEP and hence increases the cell surface expression of BSEP; and (2) Functions as a chemical chaperone to correct the misfolding of some endoplasmic reticulum-retained BSEP missense mutants[77]. *In vitro* studies in HEK293 and MDCKII cell lines showed increased cell surface expression of wild-type E297G and

D482G BSEP. This resulted in an increased taurocholic acid transport on treatment with 4-PB, long term reduction in BA, improved presence of BSEP at canalicular membrane and relief of pruritus. 4-PB therapy was effective at a dose of 500 mg/kg/d with no adverse events but with relapse of symptoms after stopping therapy[78]. However, concerns have been raised about the palatability of this drug, compliance and hepatotoxicity potential when combined with rifampicin. Glycerol phenylbutyrate was shown to be equally efficacious with good tolerance and adherence. Malatack *et al* [79] described a case report of a PFIC2 with a missense mutation in BSEP who improved with combination therapy of 4-PB, maralixibat and oxcarbazepine. They hypothesized that enzyme inducer action of oxcarbazepine augments conversion of 4-PB to phenylacetate leading to synergistic effect apart from its usual nerve stabilizing effect[79]. Although 4-PB is safe in most studies, a recent report of bipolar disorder in an adolescent on 4-PB raises concerns[80].

Readthrough therapy-gentamicin: Gentamicin has been shown to induce readthrough leading to the expression of a full-length protein in stable MDCK clones. Gentamicin in combination with 4-PB significantly increased the readthrough level of common nonsense mutation studied (p.R415X, p.R470X, p.R1057X, p.R1090X) in HEK293 cells. The maximum response was seen for the p.R1090X mutation with a 40% increase in taurocholate transport and correct localization at the cell membrane[81]. There has been an emphasis on mutation-specific therapies with: (1) Gentamicin and ataluren likely to suppress nonsense mutations by promoting the readthrough of premature stop codons; and (2) U1-small nuclear-RNA likely to rescue splicing for several ATP8B1 mutations located at donor, acceptor and splice sites[81].

Other drugs: Nor-UDCA is a side-chain shortened derivative of UDCA with relative resistance to amidation thus increasing the cholehepatic shunt and in turn increases the bile salt-dependent bile flow[82]. Peroxisome proliferator-activated receptor agonist bezafibrate has been reported to correct dyslipidemia and reduce pruritus and cholestasis in children with PFIC1 and other diseases[83]. Rapamycin restores bile acid excretion, attenuates hepatocyte damage and extends the lifespan of Abcb11b mutant zebrafish which is the ortholog of human ABCB11. Other drug categories which could theoretically be beneficial but have not yet been tried in clinical trials include FXR agonist and fibroblast growth factor-19 analogue[84]. Obeticholic acid is a very potent FXR agonist, promotes transcription of BSEP, improves histopathology and reverse fibrosis and could therefore be useful in patients with PFIC2[84].

CONCLUSION

To conclude, there has been a recent emphasis on understanding the genetic spectrum of PFIC, their phenotype, natural course and long-term outcome. Genotype correlates well with phenotype in PFIC2 but not in PFIC1. The expanded role of the heterozygous transporter defects presenting late needs to be suspected and identified even in adulthood. Medical therapy and SBD form the cornerstone of the management of pruritus. LT in these children is associated with unique issues like a high rate of intractable diarrhea, growth failure, steatohepatitis and graft failure in PFIC1 and antibody-mediated BSEP deficiency. There is a promising role of ASBT inhibitors in the management of cholestasis.

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REVIEW

Is there a role of lipid-lowering therapies in the management of fatty liver disease?

Ismini Tzanaki, Aris P Agouridis, Michael S Kostapanos

ORCID number: Ismini Tzanaki 0000-0001-6010-2509; Aris P Agouridis 0000-0002-9749-5075; Michael S Kostapanos 0000-0002-7513-5319.

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Ismini Tzanaki, School of Medicine, European University Cyprus, Nicosia, Cyprus, Nicosia 2404, Cyprus

Aris P Agouridis, School of Medicine, European University Cyprus, Nicosia 2404, Cyprus

Michael S Kostapanos, General Medicine, Addenbrooke's Hospital, Cambridge University Hospitals, Cambridge CB20QQ, United Kingdom

Corresponding author: Michael S Kostapanos, MD, MRCP, PhD, Consultant Physician-Scientist, General Medicine, Addenbrooke's Hospital, Cambridge University Hospitals, Hill's Road, Cambridge CB20QQ, United Kingdom. mk828@medschl.cam.ac.uk

Abstract

Atherogenic dyslipidemia is characterized by increased triglyceride-rich lipoproteins and low high-density lipoprotein cholesterol concentrations. It is highly prevalent in non-alcoholic fatty liver disease (NAFLD) and contributes to the increased cardiovascular risk associated with this condition. Alongside insulin resistance it plays an important pathogenetic role in NAFLD/non-alcoholic steatohepatitis (NASH) development and progression. It has been shown that cholesterol-lowering reduces cardiovascular risk more in NAFLD *vs* non-NAFLD high-risk individuals. This evidence highlights the importance of effective lipid modulation in NAFLD. In this narrative review the effects of the most commonly used lipid-lowering therapies on liver outcomes alongside their therapeutic implications in NAFLD/NASH are critically discussed. Preclinical and clinical evidence suggests that statins reduce hepatic steatosis, inflammation and fibrosis in patients with NAFLD/NASH. Most data are derived from observational and small prospective clinical studies using changes in liver enzyme activities, steatosis/fibrosis scores, and imaging evidence of steatosis as surrogates. Also, relevant histologic benefits were noted in small biopsy studies. Atorvastatin and rosuvastatin showed greater benefits, whereas data for other statins are scarce and sometimes conflicting. Similar studies to those of statins showed efficacy of ezetimibe against hepatic steatosis. However, no significant anti-inflammatory and anti-fibrotic actions of ezetimibe have been shown. Preclinical studies showed that fibrates through peroxisome proliferator-activated receptor (PPAR)α activation may have a role in NAFLD prevention and management. Nevertheless, no relevant benefits have been noted in human studies. Species-related differences in PPARα expression and its activation responsiveness may help explain this discrepancy. Omega-3 fatty acids reduced hepatic steatosis in numerous hetero-

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geneous studies, but their benefits on hepatic inflammation and fibrosis have not been established. Promising preliminary data for the highly purified eicosapentaenoic acid require further confirmation. Observational studies suggest that proprotein convertase subtilisin/kexin9 inhibitors may also have a role in the management of NAFLD, though this needs to be established by future prospective studies.

Key Words: Non-alcoholic fatty liver; Non-alcoholic steatohepatitis; Statin; Ezetimibe; Fibrates; ω-3 fatty acids; Bile acid resins

Core Tip: Statins may be beneficial against non-alcoholic fatty liver disease (NAFLD)/non-alcoholic steatohepatitis (NASH) in association with their cholesterol-lowering efficacy as well as their anti-inflammatory, antioxidant and anti-fibrotic actions. Elimination of hepatic steatosis, inflammation and fibrosis was noted with statin use in the clinical setting of NAFLD/NASH. Experimental evidence suggests that ezetimibe has similar benefits to statins against NAFLD/NASH. However, ezetimibe was beneficial only against hepatic steatosis, but not against inflammation or fibrosis in NAFLD patients. Despite their promising mechanistic potential against NAFLD/NASH through PPARα activation benefits of fibrates on liver outcomes have not been established in clinical studies. Ample heterogeneous evidence suggests benefits of ω-3 fatty acids against hepatic steatosis, but not inflammation or fibrosis.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) has become a pandemic with an estimated prevalence of 24%, 30% and 32% in Europe, America and Middle East respectively[1,2]. It has epidemiologic and pathophysiologic links with obesity, type 2 diabetes mellitus, dyslipidemia, unhealthy lifestyle patterns and the metabolic syndrome[3]. Its increasing prevalence is strongly associated with the corresponding rise in those conditions globally.

NAFLD histologic spectrum varies from steatosis alone to non-alcoholic steatohepatitis (NASH) that also encompasses various degrees of necroinflammation and fibrosis. Cirrhosis with or without portal hypertension leading to death or liver transplantation are the liver-specific endpoints of NAFLD[4-6]. Concerningly, NASH-associated liver transplantation cases increased by 170% from 2004 to 2013 in the United States[5].

NAFLD is typically asymptomatic or presents with non-specific symptoms particularly in more advanced forms. It is biochemically characterized by variable elevations in liver enzymes, mainly of elevated alanine aminotransferase (ALT), aspartate aminotransferase (AST) and γ-glutamyltransferase (γ-GT) activities. Of note, liver enzymes may not be sensitive for NAFLD diagnosis, hence many NAFLD cases may be undiagnosed *via* routine biochemical screening. It was estimated that up to 60% of patients with advanced NAFLD have normal liver enzymes[7,8]. Coagulopathy and abnormalities of albumin and bilirubin levels are typically encountered in more advance stages of the disease.

Liver imaging aims to reveal hepatic steatosis and determine whether NAFLD is accompanied by a degree of fibrosis. Liver ultrasound and magnetic resonance imaging (MRI) or computed tomography (CT) are the most frequently imaging modalities in this respect[9]. Vibration controlled transient elastography is used to grade the different levels of liver fibrosis[9]. Liver biopsy remains the 'gold standard' for the diagnosis and staging of NASH as well as for identifying cirrhosis[10].

Cirrhosis and hepatocellular carcinoma account for 4%-8% and 1%-5% of NAFLD-associated mortality respectively[1]. However, cardiovascular (CV) disease is the major mortality cause accounting for approximately 40% of all deaths[11]. NAFLD has been recognized as a risk factor for CV disease by various analyses[12]. However, it is difficult to dissociate this correlation from the high prevalence of concomitant abnormalities representing traditional CV risk factors in NAFLD. These include, but are not limited to obesity, type 2 diabetes, metabolic syndrome and atherogenic dyslipidemia. In fact, it was suggested that it is not the hepatic steatosis itself but the constellation of those metabolic abnormalities contributing to the increased CV risk in NAFLD[13].

To prevent from the progress and complications of NAFLD its early identification and management is crucial. Mainstay treatment involves lifestyle modifications, such as weight management, alcohol restriction, regular exercise and dietary intervention [14-17]. To date, there are no evidence-based drug therapies recommended for the management of NAFLD/NASH leaving a significant unmet clinical need[18]. Nevertheless, pharmacotherapy to address the increased CV risk through anti-obesity, anti-diabetic and lipid-lowering drugs is commonly used in clinical practice[19].

Dyslipidemia and NAFLD

Insulin resistance and the associated dyslipidemia play an important pathogenetic role in NAFLD. Insulin is a key player in lipid metabolism by promoting triglyceride (TG) storage into adipose tissue and by inhibiting hepatic production of the TG-rich very low-density lipoproteins (VLDL). Also, the catabolism of VLDL and their remnants *via* lipoprotein lipase is partly dependent on insulin action. In contrast, insulin resistance is associated with impaired fat storage in adipose tissue resulting in increased influx of non-esterified fatty acids (NEFAs) to the liver and subsequent hepatic fat accumulation. Besides, high cholesterol diet and increased cholesterol influx to the hepatocytes seems to promote *de novo* lipogenesis (through mechanisms that are explained further below) and hepatic steatosis. Free unesterified cholesterol further promotes pro-inflammatory and pro-fibrotic pathways which facilitate progression of NAFLD to NASH and/or cirrhosis[13].

Liver NEFAs further serve as substrate for increased VLDL hepatic production. This abnormality alongside an impaired catabolism of TG-rich lipoproteins results in increased concentrations of the atherogenic apolipoprotein(apo) B-rich lipoproteins in NAFLD. Subsequent lipolysis of these lipoproteins results in increased production of the atherogenic small dense LDL particles[13]. Furthermore, cholesteryl ester transfer protein (CETP) facilitates cholesteryl ester shift from high density lipoproteins (HDL) to TG-rich lipoproteins in exchange for TG. In insulin resistant states such as NAFLD, both TG-rich lipoprotein concentration and CETP activity are enhanced. These abnormalities result in TG-enriched HDL particles which are easily eliminated *via* the hepatic lipase. Also, in NAFLD there is reduced hepatic and intestinal production of the anti-atherogenic apoA1 (the main apolipoprotein of HDL) through decreased adiponectin levels. Through these mechanisms HDL cholesterol (HDL-C) levels are typically reduced in NAFLD. This profile is associated with impaired endothelial function and reverse cholesterol transport by HDL. Other antiatherogenic, including anti-inflammatory, antioxidant and anti-thrombotic effects of HDL are diminished too [20,21].

In summary, the serum lipid profile in NAFLD is similar to the atherogenic dyslipidemia encountered in other insulin resistant states, including the metabolic syndrome and type 2 diabetes. It is characterized by increased TG, non-HDL cholesterol and apoB levels together with low HDL-C and apoA1 Levels. Furthermore, a predominance of the small dense LDL particles further adds to atherogenicity. The same abnormalities seem to contribute to the maintenance and progression of NAFLD. All these considered, aggressive management of dyslipidemia is important to prevent CV disease, while it might be helpful in reducing liver-specific complications in NAFLD.

In this narrative review, a possible therapeutic role of the most commonly used lipid-lowering therapeutics against NAFLD/NASH is discussed.

Statins (3-hydroxy-3-methyglutaryl coenzyme A reductase inhibitors)

Statins are potent cholesterol-lowering agents and evidenced-based drugs to reduce CV outcomes. Their cholesterol-lowering efficacy is expected to be beneficial for NAFLD, which is characterized by aggregation of free cholesterol into hepatocytes[22,23]. Besides, statins have been suggested to exert variable lipid-independent pleiotropic benefits, including antioxidant, anti-thrombotic, anti-fibrotic and anti-inflammatory actions as well as endothelial function improvement. Except for their protective role against atherothrombosis these effects may play a role in the prevention

and management of NAFLD/NASH[22,24-26].

Mechanistic implications

Free unesterified cholesterol appears to be toxic to the hepatocytes by promoting liver inflammation and subsequent fibrosis. Namely, cholesterol crystals accumulation into hepatocytes results in inflammatory response *via* activated Kupffer cells surrounding the steatotic liver cells in crown-like structures. Besides, cholesterol crystals activate NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome within the Kupffer cells. Atorvastatin both alone or in combination with ezetimibe attenuated these effects in high-fat and high-cholesterol diet fed mice after 16 wk. These findings indicated a potential role of cholesterol lowering in NASH prevention[27].

Statins also inhibit the synthesis of isoprenoids, which are mevalonate pathway products. Prenylation/activation of the small guanosine triphosphate (GTP)ases through isoprenoids regulates the intracellular signaling of numerous receptors mediating liver inflammation and fibrosis. Statin-related inhibition of this isoprenoid-dependent process led to significant anti-inflammatory and anti-fibrotic effects in an experimental model of NAFLD[28].

Furthermore, statins may reduce the expression of pro-inflammatory and pro-fibrinogenic mediators. Namely, rosuvastatin significantly decreased the expression of tumor necrosis factor (TNF)α, interleukin(IL)-6, IL-1β, interferon (IFN)-γ and transforming growth factor (TGF)-β1 in a rodent model of hepatocellular carcinoma (HCC) fed with high-fat and high-cholesterol diet. These effects alongside a reduced expression of the vascular epidermal growth factor receptor (VEGFR), the epidermal growth factor receptor (EGFR) and the platelet-derived growth factor (PDGF) suggested a protective role of rosuvastatin against HCC associated with NAFLD/NASH[29].

Furthermore, antioxidant actions of statins may be beneficial against NAFLD/NASH. Peroxisomes play a key role in the maintenance of intracellular redox balance. Statins were suggested to increase the gene expression of peroxisome proliferator-activated receptor α (PPARα), a regulator of peroxisomal and mitochondrial fatty acid oxidation. Through this mechanism statin treatment limited hepatic steatosis and improved peroxisomal and mitochondrial function in an experimental rodent model [24]. Paraoxonase (PON)1 is another liver-derived enzyme, which is linked to HDL. It has a limiting role in oxidative stress and inflammation by hydrolysing peroxides and lactones associated with lipoproteins. Genetic studies have shown that reduced PON1 activity plays a significant pathogenetic role in NASH[30]. Atorvastatin 40 mg/d was associated with increased PON1 activity in 25 NAFLD patients after 8 mo. This effect was accompanied by significantly reduced serum malondialdehyde levels as a marker of lipid peroxidation, suggesting a promising role of this statin in NASH prevention [25].

Also, *in vitro* and *in vivo* experimental evidence suggested that the antioxidant and anti-inflammatory actions of statins can prevent hepatic stellate cells (HSCs) activation and subsequent fibrosis in NASH. This benefit may be mediated *via* reduced expression of pro-inflammatory genes as well as of reactive oxygen species (ROS), nicotinamide adenine dinucleotide phosphate (NADPH) oxidase gp91 phox subunit, α-smooth muscle actin (α-SMA) and nuclear factor-κ (NF-κB) p65 nuclear translocation [31]. An inhibitory effect of statins on HSCs activation may also be mediated *via* increased endothelial NO synthase (eNOS) together with reduced inducible NO synthase (iNOS) expression[32].

Furthermore, statins may exert anti-fibrotic effects and reduce portal pressure *via* improving the nitric-oxide (NO)-dependent liver sinusoidal endothelial cells (LSECs) function. LSECs dysfunction in NASH is considered to precede portal hypertension and its subsequent fibrosis *via* activation of the HSCs[33].

Clinical evidence

Due to their evidence-based anti-atherothrombotic benefits statins have a key role in addressing the increased CV risk in NAFLD. Interestingly, it was suggested that the reduction of CV events by statin use is greater in high-risk patients with *vs* without NAFLD[34]. Also, long-term statin use was associated with reduced risk of cancer in NAFLD. This protection was greater with increasing the time of statin exposure, becoming significant after 1 year of treatment[35].

Unfortunately, real-life statin use in NAFLD is often limited by the abnormal pre-treatment liver enzymes and the possibility of their further treatment-induced elevations. This is relevant despite several clinical studies showing that statins may improve liver enzyme activities and limit hepatic steatosis in NAFLD/NASH. However, these benefits have not been reflected by relevant recommendations in the

treatment guidelines, possibly awaiting further confirmation by larger scale studies. Current evidence suggests that statin use is overall safe with appropriate liver function test monitoring except for Child-Pugh B and C cirrhosis, particularly when total bilirubin is > 3 mg/dl. In this context, the vast majority of patients with increased aminotransferase activities due to an established chronic liver disease, including NAFLD/NASH, should not be exempt from statin use[36-38].

To date, statin benefits on several markers and surrogates of NAFLD/NASH have been demonstrated in several large population studies as well as in smaller prospective clinical ones. A large-population study tested whether statin therapy can lower NAFLD incidence in healthy individuals or reduce progression to hepatic fibrosis among patients with established NAFLD. Overall, 11,539,409 individuals were recruited and were followed up for 6 years. Fatty liver index (FLI) and BARD score were used for the diagnosis of NAFLD and the assessment of hepatic fibrosis, respectively. Of all study participants 5,339,901 were NAFLD-free (FLI<30); of those, 164,856 subjects were diagnosed with NAFLD at the end of the follow-up. Statin treatment was associated with reduced incidence of NAFLD (assessed by FLI > 60; adjusted odds ratio (OR:) (0.66; 95% confidence interval [CI] 0.65-0.67). Statin treatment was also associated with reduced progression to hepatic fibrosis (BARD score > 2) among NAFLD patients (adjusted OR: 0.43; 95%CI: 0.42-0.44)[39].

In a multicenter cohort study liver biopsy was performed in 1,201 individuals considered to have NASH. In the biopsy-proven NASH cases statin use was associated with a significant dose-dependent protection against hepatic steatosis, NASH and hepatic fibrosis F2-F4 stage. In statin-treated patients with genetic mutations (PNPLA3 I148M risk alleles, TM6SF2 E167K variant), impaired fasting glucose, type 2 diabetes, increased age and elevated body mass index (BMI), statin use was associated with reduced risk of steatosis (OR: 0.09, 95%CI: 0.01-0.32; P = 0.004), steatohepatitis (OR: 0.25, 95%CI: 0.13-0.47; P < 0.001), and fibrosis F2-F4 stage (OR: 0.42, 95%CI: 0.20-0.80; P = 0.017)[40].

A randomized clinical trial included 5,400 military personnel who were screened through clinical and laboratory checkup. Of those individuals, 604 were diagnosed with NAFLD/NASH and were randomized by 1:1:1:1 to diet/exercise, rosuvastatin, atorvastatin or pitavastatin treatment. After 1 year, changes of 2 non-invasive scores [NAFLD Activity Score (NAS); Fibrosis-4 score (FIB-4)] were assessed. No significant changes in any of these scores were observed in the diet/exercise group. However, statin treatment was associated with significantly reduced NAS and FIB-4 scores at the end of follow up. This benefit was relevant for all statins used in this study[41].

A possible protective role of statin treatment against NAFLD progression to HCC was also demonstrated in observational studies. A case-control study included 102 NAFLD patients with *vs* without HCC (cases, n = 34 *vs* controls, n = 68 respectively). In multivariate analysis statin treatment was associated with lower risk of HCC (OR: 0.20, 95%CI: 0.07-0.60, P = 0.004)[42]. Another retrospective cohort study investigated the likelihood of progression to HCC in 18,080 non-cirrhotic NAFLD patients identified in Taiwan's National Health Insurance Research Database between 1998 and 2012. The median follow-up period was 6.32 years. The 10-year cumulative incidence of HCC was estimated to be 2.73% (95%CI: 1.69%-3.76%). In multivariate analysis statin use was associated with reduced risk of HCC progression (hazard ratio, HR 0.29, 95%CI: 0.12-0.68)[43]. However, all these studies should be interpreted with caution due to their retrospective character. More prospective longitudinal data may be required to establish a protective role of statin treatment against HCC development in NAFLD.

Atorvastatin

The Greek Atorvastatin and Coronary Heart Disease Evaluation (GREACE) study included 1,600 patients with established coronary artery disease, low density lipoprotein cholesterol (LDL-C) > 2.6 mmol/l (100 mg/dl) and TG < 4.5 mmol/l (400 mg/dl). These patients were randomized to atorvastatin 10-80 mg/d or usual care (including statins) to achieve LDL-C < 2.6 mmol/l (100 mg/dl). A *post hoc* analysis of this study included 437 patients with suspected NAFLD due to moderately abnormal liver function tests: 227 received atorvastatin at a mean dose of 24 mg/d and 210 received usual care. The primary outcome of this analysis was risk reduction by statin *vs* non-statin treatment for first recurrent CV event in patients with moderately abnormal liver tests (AST and ALT levels < 3 x the upper limit of normal). A relative risk reduction by 68% was noted in the atorvastatin-treated group compared with usual care. Interestingly, this benefit was significantly greater by 39% in the subgroup of patients with suspected NAFLD compared with patients exhibiting normal liver function tests at baseline. This finding suggested that CV benefits of atorvastatin is

more prominent among high-risk patients who have NAFLD compared with the corresponding patients who do not have NAFLD. Also, the use of atorvastatin was safe in NAFLD patients leading to rare treatment discontinuations due to liver enzyme abnormalities (7/880 patients)[34].

Small pilot prospective studies also suggested that atorvastatin monotherapy improves liver-specific outcomes in NAFLD/NASH. Namely, atorvastatin across dose range 10-80 mg/d was given in patients with dyslipidemia and NAFLD for 3-12 mo [44,45]. Treatment improved the lipid profile and was associated with significant reductions of the baseline elevated liver function tests. A large proportion of patients (*i.e.*, 36.3%) showed liver enzyme normalization after 6 mo of treatment with additional patients exhibiting similar benefits with continuation of treatment for up to 1 year. Radiographic regression of liver steatosis assessed by CT was noted too[44].

Atorvastatin was also beneficial when used in combination with other agents. 1005 individuals were randomized to atorvastatin 20 mg/d + vitamin C (1 g/d) + vitamin E (1000 IU/d) *vs* placebo for 4 years. The combination treatment was associated with a 71% reduced odds of hepatic steatosis development assessed by CT (liver-to-spleen ratio) compared with placebo[46]. Another prospective study included 186 non-diabetic patients with metabolic syndrome and ultrasonographic findings of NAFLD. These patients were randomized to atorvastatin 20 mg/d, (n = 63), fenofibrate 200 mg/d (n = 62) or their combination (n = 61). After 54 wk, liver enzymes and ultrasound findings of NAFLD normalized in 67% of the participants in the atorvastatin, 42% in the fenofibrate and 70% in the combination group[47].

In another phase 2 randomized placebo-controlled trial atorvastatin treatment blunted the increases in LDL-C levels and LDL particle concentration (LDL-pc) associated with obeticholic acid (OCA) in NASH patients. OCA is an agent with promising effects on liver histology and fibrosis, which adversely impacts on lipoprotein metabolism. The latter may be safely alleviated when it is used in combination with atorvastatin[48].

Rosuvastatin

Like atorvastatin, rosuvastatin effectively improved liver-specific endpoints of NAFLD in small pilot studies. Rosuvastatin 10 mg/d treatment was given in 6 non-diabetic non-hypertensive dyslipidemic patients with metabolic syndrome and biopsy-proven NASH. A second biopsy as well as liver ultrasound after 12 mo showed complete resolution of NASH characteristics (steatosis, necroinflammation and fibrosis) in 5/6 patients. Additionally, rosuvastatin was associated with significantly reduced ALT and AST activities by 76 and 61% respectively[49]. Similar were the findings of another small prospective study including 23 NAFLD patients with dyslipidemia who received rosuvastatin 10mg/d. After 8 mo liver enzymes normalized in all patients[50].

In another study rosuvastatin 10 mg/d was given in 20 patients with dyslipidemia, biopsy-proven NASH and metabolic syndrome for 12 mo. Significant improvement of the course of NASH and metabolic syndrome was confirmed through laboratory tests, repeated biopsies and ultrasound assessment at the end of follow-up. Furthermore, significant reductions in serum uric acid and fasting plasma glucose levels implied additional cardiometabolic benefits of rosuvastatin in these patients[51]. Another pilot study included 19 dyslipidemic patients with biopsy-proven NASH treated with rosuvastatin 2.5 mg/d. After 24 mo biopsies in 9 patients showed improved NAS and fibrotic stage in 33.3% patients[52].

Despite this promising evidence long-term (96 wk) rosuvastatin 10 mg/d failed to reduce hepatic steatosis assessed by the liver fat score *vs* placebo in 147 human immunodeficiency virus (HIV)-positive patients on antiretroviral treatment. Instead, hepatic steatosis progression was noted both in the rosuvastatin and placebo groups at the end of follow-up. Reassuringly, multivariate regression analysis suggested that this finding was associated with increased inflammatory biomarkers, but not with rosuvastatin treatment[53].

Simvastatin

Data on simvastatin are limited. A retrospective study evaluated the safety and efficacy of simvastatin alone or in combination with ezetimibe in 45 patients with NAFLD, metabolic syndrome, and increased CV risk[54]. Twenty-six patients received simvastatin monotherapy 20 mg/d and 19 simvastatin/ezetimibe 10/10 mg/d. After 6 mo AST and ALT activities were significantly reduced compared with pre-treatment values. Interestingly, simvastatin monotherapy was associated with significantly greater AST/ALT reductions compared with the combination therapy. This finding suggested a potential dose-dependent benefit of simvastatin in this respect[54].

In contrast, a pilot randomized study evaluating the effects of simvastatin 40 mg/d *vs* placebo in 16 patients with NASH and dyslipidemia did not show similar benefits [55]. Despite significant improvement of the serum lipid profile, liver biopsy performed after 1 year in 10 patients did not show any differences in hepatic steatosis or fibrosis stage between the simvastatin and the placebo group. No significant changes in serum aminotransferases in any of the groups were noted either[55].

Pravastatin

Limited clinical data suggest that pravastatin may be a safe and tolerable option in improving the serum lipid profile in NAFLD patients whilst improving NASH-related liver histology. A multicenter randomized clinical trial included 326 hypercholesterolemic patients with known chronic liver disease (64% with NAFLD) treated with high-dose pravastatin (80 mg/d) *vs* placebo. After 36 wk, pravastatin significantly improved the serum lipid profile. No statistically significant difference between the pravastatin and the placebo group was noted in ALT elevation events (defined as doubling from pre-treatment values): 8% *vs* 13%, respectively. These results suggest that pravastatin is a safe option to beneficially modify the lipid profile among NAFLD patients[56]. In another small study, pravastatin 20 mg/d was given in 5 participants with biopsy-proven NASH and abnormal liver enzyme activities. After 6 mo, hepatic enzymes normalized in all participants. Also, repeat biopsy in 4/5 participants showed that hepatic steatosis and inflammation were persistent only in 1 and 3 participants, respectively[57].

Pitavastatin

Pitavastatin effectively reduces LDL-C, while increasing HDL-C levels, especially in patients with pre-diabetes or diabetes[58]. Its favorable metabolic profile associated with improved insulin resistance and carbohydrate metabolism may be promising for the management and prevention of NAFLD/NASH[59]. It was suggested that these benefits are mediated through increased adiponectin levels and reduced oxidative stress[58].

However, limited clinical evidence is conflicting and cannot firmly establish a beneficial role of this statin against NAFLD. Namely, a 12 wk pitavastatin 2-4 mg/d treatment significantly reduced liver enzyme activities in 97 patients with mildly-to-moderately elevated liver enzyme tests at baseline[44]. This benefit was accompanied by reduced severity of the CT-assessed hepatic steatosis among patients with this abnormality at baseline. Events of moderate to severe ALT elevations at > 100 and > 120 IU/L at 12 wk were rare in pitavastatin-treated patients: 5/97 and 1/97, respectively[60].

Similar were the results of a 12 mo pitavastatin 2 mg/d treatment in 20 NASH patients with dyslipidemia. However, NAS and fibrosis stage in biopsy were not significantly altered[61]. In accordance with this finding a 6-month pitavastatin 4 mg/d treatment did not significantly reduce Hydrogen-1 MRI-assessed hepatic fat compared with placebo in 50 adults with Body Mass Index (BMI) ≥ 27 kg/m^2 and waist circumference ≥ 102 cm who had not used statins for ≥1 year. In the same study pitavastatin did not favorably improve indices of carbohydrate metabolism, including endogenous glucose production, whole body insulin sensitivity or insulin-induced glucose uptake[62]. Therefore, more data are needed to clarify the effects of this statin on hepatic steatosis, inflammation and fibrosis as well as on the associated metabolic abnormalities.

Lovastatin

In one multicenter study 87 patients with NAFLD/NASH and dyslipidemia received lovastatin 10 mg/d for 4 mo. Significant reductions in transaminase and cholesterol levels were noted even within the first 2 mo of treatment. Also, a decreased the AST-to-platelet ratio index (APRI) as a marker of liver fibrosis was noted[63].

EZETIMIBE

Mechanistic implications

Ezetimibe exerts its mild-to-moderate LDL-C-lowering action *via* inhibiting intestinal cholesterol absorption through the Niemann-Pick C1-like 1 (NPC1L1) protein[64]. Its additive LDL-C lowering effects to statins were associated with further significant reductions in the risk of CV events in high-risk patients[65,66].

Experimental studies suggested that dietary cholesterol uptake is associated with hepatic steatosis and, hence high cholesterol-fed animals have been extensively used as experimental models of NAFLD/NASH. Increased cholesterol absorption and hepatocyte cholesterol content results in a cholesterol-dependent activation of the liver X receptor α (LXRα). The latter enhances the expression of several transcriptional factors that promote hepatic lipogenesis, including the sterol regulatory element binding protein (SREBP)-1c and the carbohydrate response element-binding protein (ChREBP). Reducing cholesterol absorption *via* ezetimibe can help reverse this deleterious process[67].

NPC1L1 is also expressed in the human liver and facilitates cholesterol reuptake from bile to hepatocytes[67]. Further experiments suggested that NPC1L1 may be associated with impaired VLDL-TG secretion and subsequent TG hepatic accumulation[68]. NPC1L1 inhibition by ezetimibe ameliorated hepatic steatosis in genetically engineered L1-Tg mice characterized by enhanced hepatic NPC1L1 expression[69,70].

Ezetimibe-related liver de-lipidation can be facilitated through other mechanisms too. Lipoprotein secretion and lipid removal from the liver is mediated by the microsomal TG transfer protein (MTP) whose degradation is inhibited by ezetimibe [71]. Ezetimibe may also enhance the expression of cholesterol efflux transporters like Abcg5/g8[72]. Furthermore, ezetimibe was associated with improved hepatic insulin sensitivity through an upregulation of the small heterodimer partner (SHP). This effect was accompanied by an upregulation of SREBP2 and downregulation of SREBP-1c expression[73]. Also, ezetimibe was associated with a decreased hepatic expression of *Cd36* gene. Cd36 is a multifunctional scavenger receptor facilitating fatty acid uptake and oxidation, and it has been associated with dysfunctional fatty metabolism and fatty liver[74].

Besides, according to experimental studies ezetimibe can confer significant protection against inflammation and oxidative stress associated with hepatic steatosis. A proposed mechanism is through activation of the nuclear factor erythroid 2-related factor 2 (Nrf2), a transcriptional factor whose target genes are antioxidant proteins and detoxification enzymes[67].

Further *in vitro* experimental data suggested that ezetimibe exerts anti-inflammatory benefits against NASH in human liver cells. In this context, ezetimibe may promote autophagy, which plays a key role in hepatic lipid catabolism (lipophagy) and prevents from hepatic steatosis[75]. This effect may be mediated by AMP-activated protein kinase (AMPK) activation and transcription factor EB (TFEB) nuclear translocation associated with the MAPK/ERK pathway. Ezetimibe may also reduce NLRP3 inflammasome activation in macrophages by modulating autophagy and a hepatocyte-driven exosome pathway[76]. Besides, it was shown to reduce hepatic fat content and prevent from the associated NFκB pathway-mediated liver inflammation by high-fat diet[77].

Last, ezetimibe was suggested to exert protective effects against angiogenesis associated with HCC development in NASH[78]. In this context, ezetimibe was also associated with downregulated expression of SKP2 which serves as an oncogene in HCC in a high-fat diet rodent model[74].

Clinical evidence

The effects of ezetimibe on liver-specific outcomes of NAFLD/NASH were mainly evaluated in small interventional studies with similar design to the statin ones. Namely, ezetimibe 10 mg/d was associated with significantly decreased ALT, AST and γ-GT activities in 70 individuals with dyslipidemia and NAFLD. Also, the ultrasonographic progress of steatosis was limited in 38.6% of these patients[79]. Another small study included 8 non-obese individuals with NAFLD who were treated with ezetimibe. Four patients had been receiving ursodeoxycholic acid which was subsequently switched to ezetimibe, while the remaining 4 patients had been previously treatment naive. After 12 mo, ALT activity was significantly reduced by 49.3% and normalized in 4 of 8 patients. Nevertheless, ezetimibe treatment was not associated with any significant ultrasonographic changes of hepatic steatosis[80].

Long-term effects of ezetimibe monotherapy on hepatic steatosis were evaluated in 45 biopsy-proven NAFLD patients. After 2 years of treatment histologically-assessed hepatic steatosis and NAFLD activity scores were significantly reduced. However, no improvement in the fibrosis state was observed[81].

A randomized double-blind placebo-controlled study assessed whether ezetimibe reduces hepatic steatosis in NASH. It included 50 biopsy-proven patients randomized to ezetimibe 10 mg/d or placebo. After 24 wk ezetimibe was associated with reduced liver steatosis assessed by magnetic resonance imaging-derived proton density-fat fraction (MRI-PDFF) compared with baseline. However, no significant difference

between the ezetimibe and the placebo group was noted[82]. Another study assessed the effects of a 6-month ezetimibe treatment in 10 patients with dyslipidemia and NASH. Significantly improved activities of AST, ALT, γ-GT as well as reduced LDL-C, CRP and type IV collagen 7 levels were observed. Histologically-assessed steatosis also improved in every patients, whilst significant benefits on fibrosis status was noted in 6/10 patients[83].

A further randomized controlled trial included 32 NAFLD patients of whom 17 received ezetimibe, while the rest were controls. After 6 mo ezetimibe significantly reduced total cholesterol levels by 9.5% compared with control group and ameliorated hepatic fibrosis. Namely, histologically-assessed staging and ballooning scores were significantly improved in the ezetimibe group compared with baseline. In contrast, the steatosis score, lobular inflammation and NAS did not significantly change in either group[84].

The efficacy and safety of ezetimibe 10 mg/d + simvastatin 20 mg/d combination was evaluated in 19 patients with type 2 diabetes and NAFLD[85]. After 6 mo, treatment was associated with significantly reduced ALT activity by 48.9%[85]. The IMPROVE-IT (Improved Reduction of Outcomes: Vytorin Efficacy International Trial) included 18,144 patients who had been hospitalized for an acute coronary syndrome within the preceding 10 ds and had LDL-C levels of 1.3-2.6 mmol/l (50-100 mg/dl) if they were receiving lipid-lowering therapy or 1.3-3.2 mmol/l (50-125 mg/dl) if they were not receiving lipid-lowering therapy. These patients were randomized to simvastatin 40 mg/d + placebo *vs* simvastatin 40/d + ezetimibe 10 mg/d. The primary endpoint was the composite of CV death, myocardial infarction, rehospitalization for unstable angina, coronary revascularization or stroke. After 7 years the simvastatin + ezetimibe combination significantly reduced the primary endpoint compared with simvastatin monotherapy (34.7 *vs* 32.7%, p = 0.016)[65]. In a sub-analysis, NAFLD fibrosis score (NFS) was prospectively applied to 14,819 IMPROVE-IT participants. NFS is a serum-based index, originally developed for the diagnosis of advanced hepatic fibrosis in patients with NAFLD and it was found to be associated with increased CV morbidity and mortality. Using validated NFS cutoffs the effect of treatment on the primary endpoint was assessed. In the IMPROVE-IT patients with high NFS score (> 0.67) had a 30% increased risk of major CV events compared with the low-risk group. Simvastatin+ezetimibe combination was associated with a 3.7% absolute reduction in the risk of recurrent CV events, compared with placebo + simvastatin (HR 0.85 [0.74–0.98]) translating to a number-needed-to-treat of 27. This additional CV benefit was not noted in the low-risk group based on NFS. This finding suggests that ezetimibe confers additional CV protection amongst NAFLD patients with higher fibrosis score[86].

A meta-analysis of 2 randomized controlled trials (RCTs) + 4 single-arm trials included 273 participants with NAFLD and NASH. Ezetimibe treatment was not associated with improved hepatic inflammation and fibrosis, although an improvement in transaminase levels was observed[87]. Similar were the results of a more recent meta-analysis of 5 lipid-lowering treatment studies (2 ezetimibe, 1 simvastatin, 1 atorvastatin and 1 any-statin) including 199 patients with NAFLD[88]. Ezetimibe was associated with decreased NAS, but not with reduced hepatic steatosis [88].

FIBRATES

Mechanistic implications

TG reduction is the main lipid-lowering action of fibrates accompanied by moderate increases in HDL-C and mild reductions in LDL-C levels. Despite these favorable effects their role in CV disease prevention remains controversial[89]. Nevertheless, several animal studies suggested a promising role of fibrates for the management of NAFLD. Specifically, fibrates ameliorated hepatic steatosis, necroinflammation and fibrosis induced by high-fat diet in several experimental studies[90-96].

PPARα activation is their main mechanism of action associated with multiple benefits on the lipid and glucose metabolism[97]. Through this effect fibrates upregulate lipoprotein lipase, a key enzyme for the TG-rich lipoprotein catabolism. This enzyme also plays a key pathogenetic role in hepatic steatosis when downregulated[98]. Also, PPARα activation through fibrates is expected to reduce hepatic steatosis mainly *via* enhanced expression of target genes. These include fatty acid transport and binding proteins, carnitine palmitoyltransferase II, as well as medium- and long-chain acyl-CoA dehydrogenase and acyl-CoA oxidase mediating

mitochondrial and peroxisomal FA β-oxidation[97,99,100]. In the same context, fibrate use has been associated with reduced hepatic insulin resistance mostly by enhanced fatty PPARα-related acid β-oxidation. Also, PPARα activation is associated with increased expression of FGF21, a hepatokine enhancing extra-hepatic tissue insulin sensitivity through glucose transporter 1 activation[99].

Additionally, fibrates downregulate the expression of pro-inflammatory cytokines genes, such as TNFα, monocyte chemoattractant protein (MCP)-1, intercellular adhesion molecule (ICAM)-1 and vascular adhesion molecule (VCAM)-1. This suggests a promising role against NASH[97,99]. Fibrates were also suggested to exert antioxidant actions, mainly through reducing fatty acid peroxidation and ROS production. The latter effects appear to be PPARα-dependent. Furthermore, fibrate treatment was associated with amelioration of high-fat diet-induced disturbances in hepatic microvasculature. This effect seems to improve the liver microvascular environment and oxygenation and may be protective against NAFLD in a PPARα-dependent manner[97].

Many of the abovementioned effects may be also mediated by a fibrate-associated increased production of adiponectin, an adipokine that exerts multiple benefits on lipid and glucose metabolism, mainly *via* reducing hepatic insulin resistance. This adipokine also exerts variable anti-inflammatory, antioxidant and anti-fibrotic actions in the liver being potentially protective against NASH. Besides, fibrate treatment seems to preserve the liver-specific adiponectin receptor (AdipoR2), which is protective against liver steatosis and inflammation[97].

Clinical evidence

The very promising experimental evidence suggesting a therapeutic role of fibrates in NAFLD has not been confirmed by clinical studies. This inconsistency might be explained by differences between animals and humans in PPARα tissue expression, being higher in the former[101]. Another important factor is the difference in PPARα responsiveness to treatment between rodents and humans. Namely, fibrate-related human PPARα activation is less prominent in humans than in rodents at equivalent doses[99,102]. Also, in experimental rodent studies the fibrate doses used were significantly higher than the human equivalent ones in clinical studies[102]. These factors may have obscured any benefits of fibrates in human studies[99,102].

Like statins and ezetimibe, clinical data for fibrates are mainly derived from small clinical studies, in which these drugs were used as monotherapy or in combination with other agents.

In a small study, fenofibrate 200 mg/d was given in 16 individuals with biopsy-proven NAFLD for 48 wk. Fenofibrate significantly reduced triglycerides and increased apolipoprotein A1 levels, while it improved the glycemic status. Significant reductions in liver enzyme activities, including ALT, AST, ALP and γ-GT were noted at the end of follow up. Minor improvements of liver histologic abnormalities were also noted in the fenofibrate-treated patients. Namely, a significantly reduced grade of hepatocellular ballooning degeneration was observed, while the grade of steatosis, lobular inflammation and fibrosis or NAS remained unchanged[103].

Another study included 90 patients with NAFLD who were randomized to fenofibrate 300 mg/d monotherapy or fenofibrate 300 mg/d + pentoxifylline 1200 mg 3 times daily for 24 wk. Pentoxifylline+fenofibrate combination were significantly more beneficial on liver enzyme activities than fenofibrate monotherapy. Namely, ALT/AST/γGT activity was reduced by 50.0/45.6/43.2% in the monotherapy *vs* 60.6/62.0/54.2% in the combination group. Of note, liver fibrosis assessed by indirect biochemical markers, a direct marker linked to matrix deposition (hyaluronic acid), a cytokine/growth factor linked to liver fibrosis (*i.e.*, TGF-β1), the inflammatory pathway, insulin resistance and liver stiffness (assessed by Fibroscan) were improved more in the combination treatment compared with the fenofibrate monotherapy group. This finding indicated that therapy with pentoxifylline + fenofibrate may have a therapeutic role against NAFLD and progression of NASH to cirrhosis[104].

Another randomized placebo-controlled trial assessed the effects of fenofibrate *vs* nicotinic acid on intrahepatic TG levels and CV risk in 27 obese patients with NAFLD. Study participants were randomized to fenofibrate 200 mg/d for 8 wk or nicotinic acid extended release 2000 mg/d for 16 wk, or placebo for 8 wk. Both fenofibrate and nicotinic acid significantly lowered circulating very low-density lipoprotein-TG (VLDL-TG) concentrations to a similar extent: by 48.9% and 40.2% respectively. Nevertheless, the hepatic triglyceride content remained unchanged in both treatment groups[105].

Fenofibrate effects on hepatic fat was also compared with free omega-3 carboxylic acid (OM-3CA) treatment in a trial including 78 obese patients with NAFLD. Those patients were randomized to OM-3CA 4 g/d (n = 25), fenofibrate 200 mg/d (n = 27) or placebo (n = 26). After 12 wk both fenofibrate and OM-3CA significantly reduced serum TG levels by 26% ($p = 0.02$) and 38% ($p < 0.001$) respectively compared with placebo. However, none of the treatments significantly altered hepatic fat. Interestingly, hepatic fat and volume increased in the fenofibrate compared with the OM-3CA group[106].

Another study included 90 overweight patients with NAFLD who were randomized by 1:1:1 to lifestyle intervention alone or in combination with fenofibrate 200 mg/d or pioglitazone 30/d. Improved ALT and AST activities as well as significant reductions in blood pressure and BMI were noted in all study groups. However, no significant differences between groups were noted in the median changes of ALT/AST activities or BMI[107].

Also, in an abovementioned study the effects of atorvastatin and fenofibrate monotherapies or in combination were assessed in patients with metabolic syndrome and NAFLD. Normalization in the liver biochemistry and ultrasound findings of NAFLD was noted 67, 42 and 70% of participants in the atorvastatin, fenofibrate and combination group respectively[47].

OTHER LIPID-LOWERING DRUGS

ω-3 fatty acids

α-Linolenic acid (ALA), Stearidonic acid (SDA), Eicosapentaenoic acid (EPA), Docosapentanoic acid (DPA) and Docosahexaenoic acid (DHA) are ω-3 Polyunsaturated Fatty Acids (PUFAs). It was suggested that ω-3 PUFAs exert multiple benefits on the CV and nervous system, maternal and child health, cancer progression and diabetes[108]. However, their efficacy to reduce CV mortality and morbidity as well as total mortality was not supported by meta-analyses of RCTs[109]. There is some variation between different ω-3 PUFAs in this regard, with the highly purified EPA, icosapent ethyl being more beneficial[110].

An association between ω-3 PUFAs and NAFLD has also been suggested. Specifically, NAFLD patients were shown to have reduced hepatic levels of ω-3 PUFAs. This observation may be relevant for the disease development and progression. Namely, adequate levels of PUFAs were suggested to reduce liver lipogenesis and hepatic inflammation in animal studies[111]. Interestingly, a cross-sectional analysis evaluated a potential prognostic role of ω-3 PUFAs in childhood NAFLD. The study included 223 children (aged 6-18 years) who were recruited from the "*Treatment of Nonalcoholic Fatty Liver Disease in Children*" trial. Their intake on fish and ω-3 PUFA was estimated based on the *Block Brief 2000 Food Frequency Questionnaire*. Inadequate consumption of fish and ω-3 PUFA was observed in NAFLD children, and it was linked to increased inflammation of the liver. This finding suggests a significant prognostic role of limited ω-3 fatty acids intake in the development and progression of the disease[112].

The hypothesis of whether ω-3 PUFA supplementation can be beneficial on liver endpoints in NAFLD was assessed in various clinical studies. In a recent meta-analysis including 1,366 NAFLD participants of 22 RCTs, ω-3 PUFA supplements were associated with reduced hepatic fat content compared with placebo (pooled risk ratio 1.52; 95%CI: 1.09-2.13). This benefit was attributed at least in part to favorable changes in BMI, but also to effective lipid modulation by reductions in TG and total cholesterol levels in ω-3 fatty acid-treated patients. However, the heterogeneity of the RCTs (diverse ethnicity and age groups) was considered a significant limitation of this analysis. Other limitations included the small sample size as well as paucity of data from follow up, and significant differences in the treatment plans (related to therapeutic dosages, duration and regimens) of the included trials[113].

A clinical trial included 48 patients with diabetes and NAFLD who were randomized to a combination of probiotics + ω-3 PUFAs *vs* placebo for 8 wk. Fatty Liver Index and Liver stiffness were determined through Shear Wave Elastography (SWE). Combination therapy ameliorated hepatic fat, improved the serum lipid profile and decreased systemic inflammation[114].

Another study investigated potential benefits of ω-3 PUFAs on NAFLD progression. Thirty NAFLD patients were separated into 2 groups depending on the severity of their disease (moderate *vs* severe). This distinction was based on biochemical and ultrasonographic characteristics, NAS and FLI. Participants in the severe NAFLD

group received ω-3 PUFA (2 g/d) for 6 mo. At the end of the study, patients displayed increased circulating levels of EPA/DHA and reduced hepatic steatosis[115]. Similar were the results of another study that assessed whether long-term (1-year) ω-3 PUFAs use is beneficial in 56 NAFLD patients: 42 received ω-3 PUFAs (1 g/d) and 14 were controls. ω-3 PUFAs were associated with significantly reduced ALT/AST activities as well as with decreased TGs levels. Ultrasonographically assessed hepatic steatosis also improved in the treatment group[116].

Promising clinical evidence suggested a potential therapeutic role of highly purified EPA against NAFLD/NASH. A pilot study included 23 biopsy-proven NASH patients who were treated with highly purified EPA 2,700 mg/d. After 12 mo, treatment was associated with significantly improved biomarkers or NAFLD and its associated oxidative stress. Those included improved ALT activity as well as reduced fatty acid, TNF receptors 1 and 2, serum ferritin and thioredoxil levels. Also, follow up biopsy after 12 mo showed reduced hepatic steatosis and hepatocyte ballooning as well as improved lobular inflammation and liver fibrosis in 6/7 patients[117]. These benefits were evident even in the absence of any significant changes in body weight, insulin resistance and adiponectin levels[117]. Several mechanisms were proposed to mediate potential benefits of highly purified EPA on NAFLD in a mouse model. These included reduced fatty acid hepatic uptake, increased intrahepatic TG hydrolysis as well as inhibition of the SREBP-1 maturation. All those actions were proven independent of PPARα activation[118].

Bile acid sequestrant resins

Bile acid sequestrants (BAS) are lipid-lowering drugs recommended for individuals with increased cholesterol levels, but normal TGs. Both as monotherapy or in combination with other lipid-lowering drugs they may decrease LDL-C by 20% and CV risk[119].

A study including 50 NASH patients assessed whether colesevelam can reduce hepatic fat. These patients were randomized to colesevelam 3.75 g/d or placebo. After 24 wk, colesevelam was associated with slightly worsened hepatic fat and inflammation than placebo. The hepatic outcomes were evaluated by MRI-PDFF and conventional MR spectroscopy (MRS). The clinical impact and the responsible mechanism explaining these findings have not been established and more clinical trials are needed to reach safer conclusions[120].

An ongoing Phase 2 randomized trial (NCT04235205) is testing whether combination therapy of elobixibat (EXB), an ileal bile acid transporter inhibitor and cholestyramine is efficient in the management of NAFLD. This study will include 100 NAFLD patients who will be randomized to 4 treatment groups: EXB 10 mg + cholestyramine 4g, EXB 10 mg monotherapy, cholestyramine 4g monotherapy or placebo. Changes in the course of NAFLD will be evaluated by means of biochemical parameters and liver imaging after 16 wk of treatment[121].

PCSK9 (Proprotein convertase subtilisin/kexin type 9) inhibitors

To date, there are 2 available fully human monoclonal antibodies (mAbs) inhibiting proprotein convertase subtilisin/kexin type 9 (PCSK9). These drugs are highly efficacious in reducing LDL-C levels by up 60% even when given on top of existing lipid lowering therapy[122]. RCTs showed that this additional LDL-C lowering was associated with additional CV outcome benefits in high-risk populations[123,124].

PCSK9 seems to play a role in the pathogenesis of NAFLD. Increased intrahepatic and circulating PCSK9 levels were associated with enhanced hepatic fatty acid and TG content along with lipid deposition in the muscle and adipose tissue[125]. Whether PCSK9 inhibition with the currently available mAbs, but also with the upcoming small interference RNA PCSK9 inhibitor (inclisiran) can beneficially alter the development and progression of NAFLD remains unknown. There are only 2 studies suggesting a promising role[126,127].

An observational study included 26 patients with Familial Hypercholesterolemia (FH) and NAFLD maintaining increased LDL-C levels despite statins + ezetimibe treatment. These patients were divided into 2 groups based on the TG/HDL median value as a surrogate of atherogenic dyslipidemia. All patients received PCSK9 inhibitors for 6 mo. PCSK9 inhibitors significantly improved hepatic steatosis biomarkers, including triglyceride-glucose index (TyG) and HIS (by -7.5% and -8.4% respectively), particularly in individuals with low TG/HDL. This finding suggests a possible role of PCSK9 inhibitors in the management of NAFLD especially in patients who lack features of atherogenic dyslipidemia[126]. The role of PCSK9 inhibitors in NAFLD was also assessed in a retrospective chart review-based study including 29 NAFLD patients. PCSK9 inhibitors were associated with full resolution of NAFLD

radiologic features in 8 of 11 individuals with liver steatosis. Moreover, transaminase levels improved in all participants[127].

CONCLUSION

Atherogenic dyslipidemia contributes significantly to an increased CV risk in NAFLD. It also plays a key role in NAFLD development and progression. Therefore, its aggressive management through effective lipid lowering drugs is crucial. This was highlighted by the results of a *post hoc* analysis of the GREACE study suggesting that statin-related CV benefits are more prominent in high-risk NAFLD *vs* non-NAFLD patients. However, the most commonly used lipid-lowering drug classes have been associated with adverse elevations of the liver enzymes (*e.g.*, statins, fibrates). Hence, clinicians cautiously use them in NAFLD. For this reason, it is important to clarify the safety and efficacy of lipid-lowering drugs in this clinical context.

The amount of evidence in this respect varies amongst different drug classes with more data being available for statins, ezetimibe and fibrates. There is considerable evidence from pre-clinical mechanistic studies suggesting that the lipid-lowering and pleiotropic effects of statins are beneficial for NAFLD/NASH. Besides, these effects may be protective against cirrhosis/HCC development in NAFLD. Also, promising were the results from observational studies about statin effects on liver outcomes. However, due to their retrospective character these results should be viewed with caution.

Smaller prospective studies showed beneficial effects of statins on NAFLD histology, clinical (steatosis and fibrosis) surrogate scores and biochemical biomarkers, especially with atorvastatin and rosuvastatin. Data are more limited and somehow conflicting with other statins. Besides, statin use amongst NAFLD/NASH patients appears to be overall safe. In fact, liver enzyme elevations with statins are limited to a small proportion of NAFLD patients and are not accompanied by any evidence of hepatic dysfunction.

Ezetimibe is associated with more infrequent liver enzyme elevations than statins, hence clinicians comfortably prescribe it in the NAFLD/NASH clinical setting. There is considerable pre-clinical evidence suggesting that ezetimibe is beneficial on hepatic steatosis mainly through its cholesterol lowering effects and its associated reduction of *de novo* hepatic lipogenesis. Besides, ezetimibe may facilitate liver delipidation by inhibiting MTP degradation and by enhancing the expression of cholesterol efflux transporters. Experimental data also suggest significant anti-inflammatory and antioxidant effects of ezetimibe on the liver tissue through various molecular pathways. Ezetimibe may be protective against HCC development in NAFLD too.

Clinical evidence suggesting benefits of ezetimibe on hepatic outcomes in NAFLD/NASH is limited to small prospective studies, few of which were randomized placebo-controlled. Most studies showed that ezetimibe monotherapy for few mo was associated with improvements of the liver enzyme activities as well as of histologically-assessed hepatic steatosis and relevant surrogate imaging endpoints. However, evidence suggesting benefits on hepatic inflammation and fibrosis is less robust. Given the milder lipid-lowering effects of ezetimibe and its less potent pleiotropy than this of statins it is uncertain whether more longitudinal data would better establish benefits on hepatic inflammation and fibrosis too.

Fibrates exert significant lipid modifying effects against atherogenic dyslipidemia which is very prevalent in NAFLD. Their effects are exhibited mainly through activation of PPARα, which play a key role in various metabolic pathways, especially in favorably modulating key lipid and glucose metabolic pathways involved in NAFLD pathogenesis. In this context, pre-clinical studies suggested that fibrates reduce hepatic steatosis *via* increasing hepatic insulin sensitivity and by accelerating the catabolism of the TG-rich lipoproteins and fatty acid oxidation. There is also promising preclinical evidence suggesting significant liver anti-inflammatory and antioxidant effects of fibrates.

Despite this promising mechanistic potential fibrate effects on liver steatosis, necroinflammation and fibrosis were poor in clinical studies. Liver enzyme improvement was the only established improved NAFLD surrogate associated with fibrate use, though this evidence should be cautiously interpreted in view of the variability of this biomarker. Fenofibrate combination with pentoxifylline is promising but its benefits require further confirmation by larger scale longitudinal studies with histological endpoints. It should be acknowledged though that the lack of fibrate efficacy in clinical studies as compared with the experimental ones may be explained

by inter-species variation in PPARα expression and responsiveness to drug-induced activation. Also, relatively reduced human equivalent fibrate doses were used in clinical studies compared with the experimental rodent ones.

Omega-3 fatty acids can beneficially modulate lipid abnormalities encountered in NAFLD. Also, low hepatic ω-3 fatty acid levels have been associated with the development of hepatic steatosis. There have been multiple studies to suggest that ω-3 fatty acid supplementation is associated with reduced hepatic steatosis, though these are characterised by significant heterogeneity in design, study populations, type of intervention and study end points. No robust evidence exists to suggest that ω-3 fatty acid reduce hepatic inflammation or fibrosis in NAFLD.

Bile acid sequestrants have not shown any benefits against NAFLD. Instead, deleterious effects of colesevelam on hepatic steatosis and inflammation were shown in one study. Cholestyramine is currently being evaluated alone or in combination with an ileal bile acid transporter inhibitor under development in NAFLD participants of a Phase 2 RCT. Both circulating and hepatic PCSK9 seems to play a pathogenetic role in hepatic steatosis and may represent an attractive therapeutic target in NAFLD. The currently available PCKS9 inhibitor mAbs have shown promising effects only in small observational studies, particularly among patients without atherogenic dyslipidemia. However, this benefit should be established by large scale longitudinal prospective clinical trials.

Limitations of the studies discussed in this review include their relatively small size and short follow up period. For example, benefits of certain drug classes could arise with their long-term continuation. Likewise, marginal benefits could be missed due to small sample sizes of the studies. NAFLD/NASH development has long natural history and any potential reversal/regression may require long-term treatment too. Designing large-scale liver outcome studies with liver biopsy, which remains the gold-standard method for assessment of hepatic steatosis, necroinflammation and fibrosis is also challenging. Furthermore, the heterogeneity of other liver endpoints assessed in most studies hampers the pooling of data that could help reach safer conclusions. Most studies have assessed the effects of lipid lowering agents on liver enzyme activities, a biomarker which has poor correlation with disease activity and broad variability. Importantly, the predictive value of liver enzyme activity lowering on histological endpoints of NAFLD/NASH *via* certain interventions has not been validated. Identifying well-validated and easy-to-use disease surrogates remains an ongoing challenge for the assessment of various therapeutic interventions, including lipid-lowering drugs, in NAFLD/NASH.

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MINIREVIEWS

Targets of immunotherapy for hepatocellular carcinoma: An update

Vikrant Rai, Sandeep Mukherjee

ORCID number: Vikrant Rai 0000-0001-6286-2341; Sandeep Mukherjee 0000-0002-0538-3253.

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Vikrant Rai, Department of Translational Research, Western University of Health Sciences, Pomona, CA 91766, United States

Sandeep Mukherjee, Department of Medicine, Creighton University School of Medicine, Omaha, NE 68124, United States

Corresponding author: Sandeep Mukherjee, FRCP (C), MD, Full Professor, Department of Medicine, Creighton University School of Medicine, Suite 401 Education Building, 7710 Mercy Road, Omaha, NE 68124, United States. sandeep.mukherjee@alegent.org

Abstract

Hepatocellular carcinoma, the most common primary liver cancer, in an immunogenic tumor with a poor prognosis because these tumors are diagnosed at late stages. Although, surgical resection, ablation, liver transplant, and locoregional therapies are available for early stages; however, there are yet no effective treatment for advanced and recurrent tumors. Immune checkpoint inhibitor therapy and adoptive cell transfer therapy has gained the popularity with some positive results because these therapies overcome anergy and systemic immune suppression. However, still there is a lack of an effective treatment and thus there is an unmet need of a novel treatment. At present, the focus of the research is on oncolytic viral therapy and combination therapy where therapies including radiotherapy, immune checkpoint therapy, adoptive cell transfer therapy, and vaccines are combined to get an additive or synergistic effect enhancing the immune response of the liver with a cytotoxic effect on tumor cells. This review discusses the recent key development, the basis of drug resistance, immune evasion, immune tolerance, the available therapies based on stage of the tumor, and the ongoing clinical trials on immune checkpoint inhibitor therapy, adoptive cell transfer therapy, oncolytic viral vaccine therapy, and combination therapy.

Key Words: Hepatocellular carcinoma; Immunotherapy; Immune checkpoint inhibitors; Adoptive cell therapy; Oncolytic vaccines; Combination therapy

Core Tip: A significant proportion of patients with hepatocellular carcinoma (HCC) present with advanced disease and therapeutic strategies for such patients are limited. The tumor microenvironment mediating immune response suppression, immune

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tolerance, and evasion further complicate the treatment in advanced HCC. The involvement of immune response in the pathogenesis of HCC makes immunotherapy an attractive approach for the treatment of advanced HCC. Further, the recent research with beneficial results with immune checkpoint inhibition, adoptive cell transfer therapy, tumor vaccines, and combinational therapies to boost the immune response of the tumor are in development and have been discussed here.

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INTRODUCTION

Hepatocellular carcinoma (HCC), an inflammation-driven cancer, is an immunogenic tumor arising from chronically inflamed liver (liver cirrhosis) caused by risk factors including alcoholic fatty liver disease, non-alcoholic fatty liver disease, and viral and non-viral pathogenesis[1]. Liver cancer is the third most common cause of cancer deaths and sixth most common cancer diagnosis worldwide. HCC has an incidence of 9.3 cases per 100000 person-years and 8.5 deaths per 100000 person-years worldwide and incidence of 9.5 per 100000 person-years in the United States[2,3]. HCC incidence has a regional variability because of relative prevalence of key risk factors. HCC has grim prognosis and increasing incidence and with a similar existing trend, HCC has a projected rate of an increase approximately 2.8% per year through 2030 particularly in countries with a high socio-demographic index[3-5]. Current therapy for HCC based on the stage of HCC comprises of surgical, locoregional, and systemic therapies. Surgical resection or liver transplantation, depending on liver function, the presence of portal hypertension, and tumor burden, is standard of therapy with a 5-year survival rate in 70% of treated patient for early stage; radiofrequency, thermal and non-thermal ablation, and trans-arterial chemoembolization (TACE) with 3-5-year survival rates in patients with nonresectable tumor and not fit for liver transplantation; and systemic therapy with tyrosine-kinase inhibitors sorafenib and regorafenib and inhibitor of vascular endothelial growth factor (VEGF) receptors lenvatinib with a very limited survival benefit due to chemoresistance and toxicities[3,6-11]. Sorafenib, targeting VEGF is standard first line systemic therapy approved for advanced HCC patients; lenvatinib is alternative first line therapy; and regorafenib, and cabozantinib are second line systemic therapy[3,5]. The lower survival rate in unresectable HCC is due to resistance to systemic treatment modalities and chemotherapy. The recent studies suggest immunotherapy as a promising modality for the treatment of advanced HCC because immunotherapy could elicit nontoxic, systemic, long-lived anti-tumor activity. Additionally, a correlation between immune response and HCC and the paucity of available therapeutic strategies supports the notion to investigate immunotherapeutic targets and designing of better therapeutics for HCC. In this review, we have discussed the basis of resistance to therapy and various modalities for the treatment of advanced cancer along with the recent updates including ongoing clinical trials.

TUMOR MICROENVIRONMENT, IMMUNOSUPPRESSION, AND IMMUNE EVASION IN HCC

The intrinsic immune response of the liver mediated by liver sinusoidal endothelial cells (LSECs), liver resident macrophages or Kupffer cells (KCs), hepatic stellate cells (HSCs), and hepatic dendritic cells (HDCs) plays a central role in host defense, functional heterogeneity of the liver, and in the maintenance of self-tolerance (Figure 1). Natural immune response mediated by these cells to protect liver parenchyma is generated by the exposure to antigens and makes liver an immune suppressive microenvironment. LSECs, the specialized endothelial cells, are the most effective scavenger cells which also act as antigen-presenting cells (APCs) while regulating the immune response. LSECs regulate immune cell recruitment *via* specific

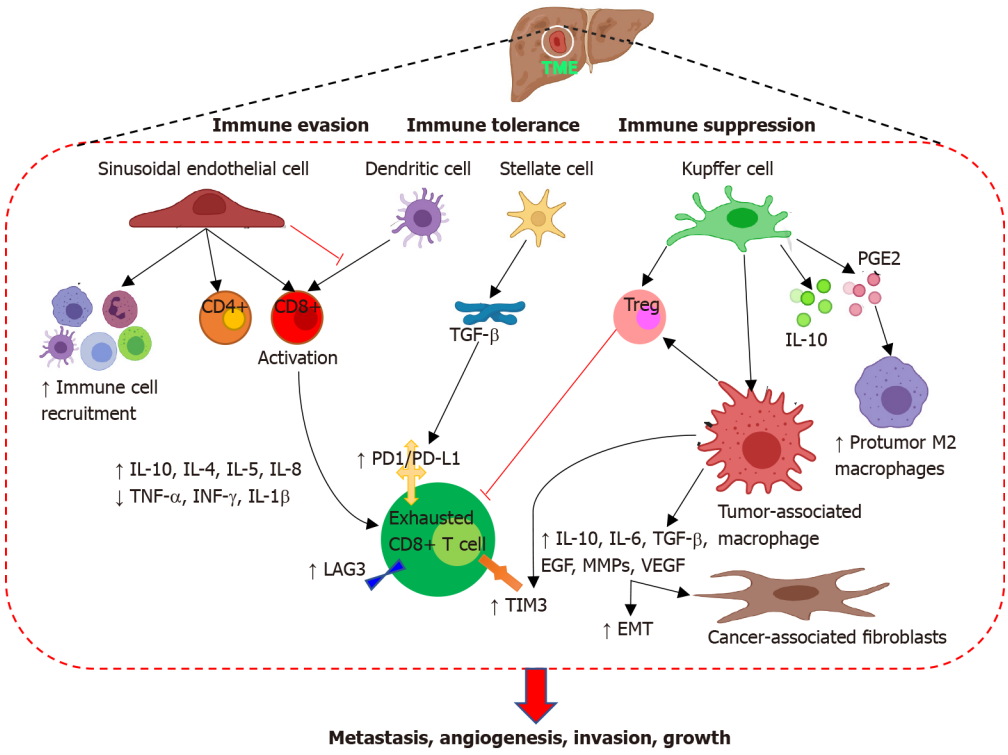

Figure 1 Tumor microenvironment in hepatocellular carcinoma. Tumor microenvironment comprising of liver sinusoidal endothelial cells, liver resident macrophages or Kupffer cells, hepatic stellate cells, hepatic dendritic cells, tumor-associated macrophages, cytokines, fibroblasts, infiltrating immune cells, pro-tumor M2 macrophages, and growth factors mediate immune suppression, immune tolerance, and immune evasion causing increased tumorigenicity with enhanced evasion, angiogenesis, metastasis, tumor growth. IL: Interleukin; TNF-α: Tumor necrosis factor-alpha; TGF-β: Transforming growth factor-beta; IFN-γ: Interferon-gamma; MMPs: Matrix metalloproteinases; PD-1: Programmed cell death protein 1; PD-L1: Programmed death-ligand 1; PGE2: Prostaglandin E2; EGF: Epidermal growth factor; VEGF: Vascular endothelial growth factor; TIM3: T-cell immunoglobulin and mucin-domain-containing molecule-3; LAG3: Lymphocyte-activation gene 3; EMT: Epithelial mesenchymal transition.

integrins (αLβ2, α4β1, α4β7)[1,3,5,12,13]. LSECs prevent immune responses against gut bacterial antigens by inhibiting CD4+ and CD8+ T lymphocytes and reduce the ability of dendritic cells (DCs) to activate T cells. KCs, the non-migratory liver resident macrophages residing in the lumen of liver sinusoids, promote immunological tolerance by increasing the secretion of interleukin (IL)-10 and prostaglandins, removing the gut bacteria, attenuation of CD4+ T lymphocytes, and proliferation of inhibitory CD4+ regulatory T cells (Tregs). The roles of KCs in mediating immune response and immune tolerance, recruiting Tregs and neutrophils, stimulating T cell response to infection, and recruitment and activation of natural killer (NK) cells have been discussed in detail[5]. HSCs, the specialized fibroblasts, are present in the space of Dissé between the parenchymal cells and play an immune sentinel role. HSCs secrete transforming growth factor (TGF)-β which is an immunosuppressive cytokine involved in inflammation, liver regeneration, and liver fibrosis (Figure 1). HDCs being the poor stimulator of effector CD4+ T cells contribute to the tolerogenic microenvironment of the liver. The immune suppressive microenvironment of the liver with downregulation of immune response genes is more evident during the development and progression of HCC and results in a lower tumor immunity during advanced disease. The presence of numerous non-redundant mechanisms of immune-suppression in HCC-tumor microenvironment (TME) synergize with immunotherapy [1,3,12,13]. In addition to immune effector cells LSECs, KCs, HSCs, and HDCs; resident liver lymphocytes including NK cells and innate T-cells play a crucial role in innate immune response against intracellular bacteria, viruses, and parasites. However, a dysfunctional immune response due to higher proportion of CD4+ to CD8+ cells promote immune tolerance and a poor prognosis. A decreased T-cell activation and tumor infiltration due to lower expression of tumor antigens on liver cancer cells results in a less efficient control of tumor growth and worse clinical outcome. A hypofunctional NK cells and insufficiency of tumor-infiltrating lymphocytes (TILs) in controlling tumor growth adds to HCC progression[1,3,5].

Although the immune suppressive microenvironment is important for the self-tolerance in the normal liver, this characteristic of the liver is a major impediment in

developing an effective antitumor immunotherapy. TME helps in escaping the immunological surveillance, growth, and progression of the tumor. The decreased efficacy of antitumor treatment is also mediated by tumor evasion. The presence of cells with immune suppressive functions and higher expression of immune checkpoint molecules characterizing the TME leads to reduced activity of effector antitumor immune response and tumor immune evasion. HCC cancer cells and KCs have a higher expression of programmed death-ligand 1 (PD-L1) and an interaction between programmed cell death protein 1 (PD-1) and PD-L1 on tumor infiltrating lymphocytes and tumor cells mediate T cell exhaustion (Figure 1), tumor-specific T-cell dysfunction and immune evasion[5,14]. Immune evasion and poor prognosis in HCC are also mediated by higher expression of T-cell immunoglobulin and mucin-domain-containing molecule-3 (Tim-3) and lymphocyte-activation gene 3 (LAG-3) on CD8+ T lymphocytes from tumor (Figure 1) and not on T cells from normal liver tissue. TIM3 is also expressed on tumor-associated macrophages (TAM) which are associated with growth, angiogenesis, invasion, and metastasis of HCC[15-20]. Further, an increased expression of immunosuppressive cytokines including IL-4, IL-5, IL-8, and IL-10 and decreased levels of pro-inflammatory cytokines including tumor necrosis factor-α, interferon (IFN)-γ, and IL-1β in TME (Figure 1) contribute to immune dysfunction, immune evasion, an aggressive tumor phenotype, and poor prognosis in HCC patients [3,21,22]. The balance of effectors and immunosuppressive cells in TME as well as the ability of malignant cells to present tumor antigens to APCs are the requisite for an effective antitumor immune response by promoting the cytotoxic T cell infiltration. Increased infiltration of T cells is associated with the expression of non-specific tumor associated antigens (TAAs) and mutated antigens (neoantigens) which might be potential immunological targets because they are derived from mutation in cancer cells. Thus, identifying the immunologically relevant neoantigens present on the tumor cells surface which do not have a homology with wildtype but have a homology with pathogen-derived epitope is warranted for better therapeutics and clinical outcome[1,23].

IMMUNOTHERAPY FOR HCC

The immune-mediated pathogenesis of HCC makes it attractive for immune-based therapies. Immune dysfunction, immunosuppression, immune evasion, the presence of immune checkpoints and neoantigens underlying the pathophysiology of HCC makes immunotherapy a potential therapeutic strategy by targeting different mechanisms involved in the development and progression of HCC; however, there are limitations, and the results of various studies so far are modest. The immunotherapy strategies available for HCC are immune checkpoint inhibitors, cancer vaccines, DC-, NK cells-, cytokine-induced killer (CIK) cell-, and TILs-mediated immunotherapy, chimeric antigen receptor (CAR) T-cell immunotherapy, adoptive T cell transfer therapy, and combinational immunotherapy (Figure 2). Further, targeting molecular mediators including microRNAs, CCL2/CCR2, fibroblast growth factors (FGF), triggering receptor expressed on myeloid cells (TREMs), Wnt signaling, Smads, and TGF-β involved in the pathophysiology of HCC are additional targets[1,3,5,13,20,23-30]. The molecular mechanisms of immunotherapy for HCC based on DC, NK cells, T-cells, and Tregs cells and targeting TREMs, TLRs, folate receptor, chemokine receptor, receptor for advanced glycation end products (RAGE), and microRNAs have been described elsewhere[24]. The current immunotherapy aimed to unmask the immune response of the tumor and to stimulate a different immune response controlling the tumor growth and progression. Of these, immune checkpoint inhibitors involve targeting inhibitory receptors on T cells including PD-1, cytotoxic T-lymphocyte associated antigen 4 (CTLA-4), and immunosuppressive cytokines such as TGF-β; antibodies targeting alpha-fetoprotein (AFP) or glypican-3 (GPC3); and coupling of antibodies with T- or NK cells-mediated therapy to make it more effective[1,3,24]. PD-1/PD-L1 and CTLA-4 play an important role in the suppression of T cell activation by the tumor cells. GPC3, AFP, and heparan sulfate-based immunotherapy has been discussed in the literature[24].

IMMUNE CHECKPOINT INHIBITION

The systemic management of HCC has been revolutionized by the advent of immune checkpoint inhibitors. Immune checkpoint inhibition blocks the negatively regulating

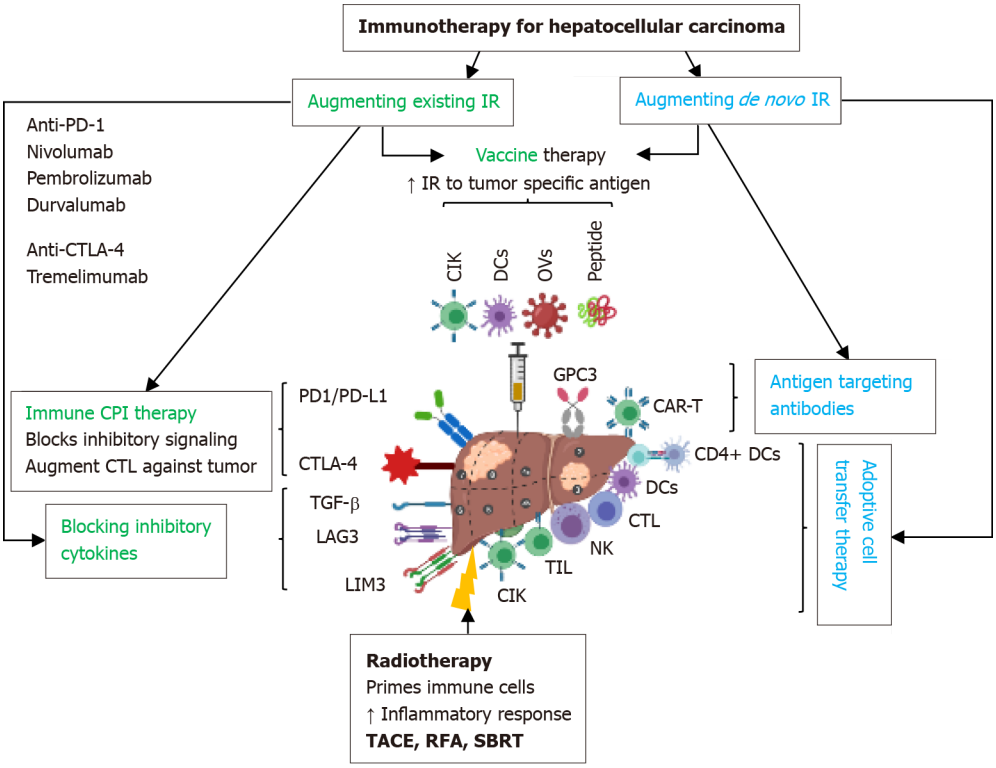

Figure 2 Immunotherapy in hepatocellular carcinoma: Potential strategies and therapeutic targets. IR: Immune response; PD-1: Programmed cell death protein 1; PD-L1: Programmed death-ligand 1; CTLA-4: Cytotoxic T-lymphocyte associated antigen 4; TGF-β: Transforming growth factor-beta; LAG3: Lymphocyte-activation gene 3; CIK: Cytokine-induced killer; TIL: Tumor-infiltrating lymphocyte; NK: Natural killer; DC: Dendritic cell; GPC3: Glypican-3.

signals directly on T cells or on cells interacting with T cells and enhances the anti-tumor immunity. Immune checkpoint inhibitors and therapeutic monoclonal antibodies fine tunes the immune response by blocking the checkpoint proteins from binding with their partner proteins thereby helping the body to recognize and attack cancer cells by T cells leading to death of cancer cells. Immune checkpoint inhibitors are most effective in tumors with high mutagenic load[3,5,13]. Immune checkpoints are mainly expressed on B and T cells, NK cells, DC, TAMs, monocytes, and myeloid-derived suppressor cells (MDSC)[25]. CTLA-4, PD-1, LAG-3, B and T lymphocyte attenuator, and T cell immunoglobulin and mucin-domain containing (TIM-3) are the common immune checkpoints investigated in human cancer and PD-1/PD-L1 and CTLA-4 has become standard of care[31,32]. Immune checkpoint inhibitor therapy using antibodies against PD-1, CTLA-4, PD-L1, and prostatic-acid phosphatase have been shown to be safe and advantageous in treating melanoma, renal cell carcinoma, triple negative breast cancer, urothelial carcinoma, squamous cell carcinomas of the head and neck, prostate carcinoma, Merkel-cell carcinoma, non-small cell lung cancer, AIDS-related Kaposi sarcoma, and hairy cell leukemia[5,25]. PD-1 is expressed on immune cell including CD8+ T cells, CD4+ T cells, B cells, NKs, Tregs, MDSCs, and DCs. Binding of PD-1 with its ligand PD-L1 inhibits the effector T cell response and thus, PD-1 has become an attractive target for immunotherapy. PD-1 inhibitor nivolumab and pembrolizumab have been approved as second line treatment of HCC [33,34] (Table 1). Various phase I, phase II, and phase III clinical trials investigating drugs targeting PD-1 have been summarized in Table 2. Other studies including the ORIENT-32 study (NCT03794440; sintilimab, bevacizumab biosimilar *vs* sorafenib), the RATIONALE-301 study (NCT03412773-phase III trial), the KEYNOTE-240 study (NCT02702401-phase II trial; pembrolizumab *vs* placebo), NCT03713593 (pembrolizumab and lenvatinib *vs* lenvatinib monotherapy), NCT03764293, NCT03434379 (atezolizumab, bevacizumab), NCT03847428 (durvalumab, bevacizumab), NCT0329-8451 (tremelimumab, durvalumab *vs* sorafenib), NCT03755739 (pembrolizumab), NCT02576509 (nivolumab *vs* sorafenib), NCT03062358 (pembrolizumab *vs* placebo), and NCT03764293 (camrelizumab, apatinib) targeting PD-1/PD-L1 with VEGF inhibition have been described and summarized in the literature[3,20]. Of these NCT03298451 involves targeting CTLA-4 (tremelimumab) along with PD-1. Similarly, clinical trials of combination therapies based on PD-1/PD-L1 blockade combined with other agents (immunotherapies, antiangiogenics, targeted agents targeting TGF-β,

Table 1 Immune checkpoint inhibitor therapy for hepatocellular carcinoma

Immune checkpoint inhibitor therapy			
Agent	**Type of study**	**Study details**	**Outcome**
Tremelimumab (anti-CTLA4)[45]	Phase II clinical trial	21 HCC patients infected with hepatitis C virus and not eligible for surgery or locoregional therapy 15 mg/kg IV every 90 d	17.6% patients-partial response; 58.8% patients-stable disease; Time to progression-6.48 mo; Overall survival-8.2 mo; Decreased viral load
TRC105 (carotuximab) antibody to CD105[46]	Phase I/II study	TRC105 (15 mg/kg) every 2 wk given with sorafenib 400 mg twice daily	Tumor ablation utilizing RFA and TACE enhance the efficacy of tremelimumab; Improves intratumoral effector CD8+ T cells infiltration
Nivolumab (anti-PD-1) [47]	CheckMate 040 phase I/II dose-escalation study	182 patients with advanced HCC; Patients naive to or previously treated with sorafenib received 0.1-10 mg/kg and 3 mg/kg once every 2 wk	Durable responses with long-term survival and favorable safety in both sorafenib-naive and -experienced patients; 3.8% complete response, 14.8% partial response, and 62.6% disease control rate
Nivolumab (anti-PD-1) [33]	Phase I/II study NCT01658878	262 HCC patients; HCC patients on sorafenib	1.4% complete response; 18.2% partial response; 83% overall survival at 6 mo
Pembrolizumab (anti-PD-1)[48]	KEYNOTE-224 trial	104 advanced HCC patients on sorafenib	1% complete response; 16% partial response; 54% overall survival at 12 mo
Durvalumab (PD-L1) and tremelimumab (CTLA4)[49]	Phase I/II, open-label, randomized study	For the efficacy of durvalumab combined with tremelimumab in unresectable HCC	No unexpected safety signals with durvalumab and tremelimumab seen in unresectable HCC patients
Tremelimumab (CTLA4)[50]	Phase II trial NCT01853618	32 patients with HCC with HCV; Tremelimumab at 3.5 and 10 mg/kg i.v. every 4 wk for 6 doses, followed by 3-monthly infusions; Combined with subtotal radiofrequency ablation or chemoablation at day 36	No dose-limiting toxicities; Accumulation of intratumoral CD8+ T cells; 26% partial response

CTLA-4: Cytotoxic T lymphocyte protein 4; PD-1: Programmed cell death protein 1; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; RFA: Radiofrequency ablation; TACE: Transarterial chemoembolization.

HSP90, c-met, FGFR4, locoregional therapies including TACE and Y90) for HCC have been discussed in detail[25].

CTLA4 also known as CD152 is a membrane bound protein inhibitory receptor which keeps immune response in check and downregulates immune responses by inhibiting its binding with its ligand CD28. CTLA-4 is upregulated after T-cell activation and antagonize CD80 and CD86 co-stimulatory molecules[13]. CTLA-4 has been an attractive target for the treatment of advanced HCC and some phase I and phase II trials have shown promising results (Table 1), and some are ongoing to evaluate the effects of targeting CTLA-4 (Table 2). As discussed above, TIM-3 plays a crucial role in immune evasion and poor prognosis in HCC, TIM-3 seems to be an important target for immune checkpoint inhibitors. TIM-3 is a transmembrane protein expressed on TILs, Tregs, and CD4+ and CD8+ T-cells and increases the number and activation level of macrophages. TIM3 expression on tumor cells leads to decreased cytotoxic T lymphocytes[13,35]. LAG-3, associated with hypofunctional CD8+ response, is another membrane bound protein which binds with MHC II and suppress T cell activity and cytokine release and upregulates T cell exhaustion in chronic viral infection or cancer[36]. Efficiently augmented proliferation and cytokine production by NY-ESO-1-specific CD8(+) T cells during T-cell priming with dual blockade of LAG-3 and PD-1 in ovarian tumor indicate antitumor function of NY-ESO-1-specific CD8(+) T cells[37] and supports the notion of targeting LAG-3 in HCC as its expression is increased in HCC[1]. Increased expression of TGF-β, a membrane bound protein expressed on Tregs, is associated with suppression of CD4+ T cell response in HCC and promote tumor growth and progression[38]. Targeting PD-1 on CD4+CD69+ Tregs is another potential target for advanced HCC (Table 1). Various completed and ongoing clinical trials investigating the role of immune checkpoint inhibitors have been listed in Tables 1 and 2. Other clinical trials including CheckMate-040, CheckMate-459, Keynote-224, Keynote-524, KeyNote-240, HIMALAYA, IMbrave150, VEGF Liver 100, COSMIC312, LEAP-002 along with their details and outcomes have been summarized in the literature[13]. In addition to this, clinical trials evaluating the immune checkpoint inhibitors nivolumab (ONO-4538, MDX-1106, BMS-936558), pembrolizumab (MK-3475), tislelizumab (BGB-A317), camrelizumab (SHR-1210), and spartalizumab (PDR001) for PD-1; durvalumab (MEDI4736), atezolizumab (MPDL3280A), and avelumab (MSB0010718C) for PD-L1; and tremelimumab (CP

Table 2 Ongoing clinical trials for immune checkpoint inhibitor therapy

Identifier	Type of study	Study design	Status/outcome
NCT02576509 (CheckMate-459)	Global phase III randomized control trial	Comparing nivolumab with sorafenib as first treatment in advanced HCC	Recruitment closed; Results awaited
NCT01658878	Phase I/II dose-escalation, open-label, non-comparative study	Phase 1 to establish the safety of nivolumab at different dose; Phase 2 to compare the efficacy of nivolumab and sorafenib; To study the safety and efficacy of the combination of nivolumab plus ipilimumab and nivolumab plus cabozantinib	Active, not recruiting
NCT03298451	Randomized phase III HIMALAYA trial	To compare the combination of tremelimumab (CTLA-4 inhibitor) and durvalumab (PD-L1 inhibitor) *vs* sorafenib	Recruiting patients
NCT03680508	Phase II trial	To test efficacy of TSR-022 (cobolimab, TIM-3 binding antibody) and TSR-042 (dostarlimab, PD-1 binding antibody) on advanced HCC	Recruiting patients
NCT02947165	Phase I/Ib study	Anti-TGF-β monoclonal antibody NIS793 and PD-1 inhibitor spartalizumab in breast, lung, colorectal, pancreatic, renal, and HCC	Active, not recruiting
NCT03412773	Phase III randomized, open-label, multicenter, global study	To compare the efficacy and safety of tislelizumab *vs* sorafenib in unresectable HCC	Active, not recruiting
NCT03434379 (IMbrave150)[51]	Phase III study	To evaluate the efficacy and safety of atezolizumab in combination with bevacizumab compared with sorafenib in locally advanced or metastatic HCC; To determine overall survival	Atezo + Bevac showed improved survival at 18 mo (52%) with clinically meaningful treatment benefit and safety. The trial confirmed atezo + bevac as a standard of care for previously untreated, unresectable HCC
NCT02702401 (MK-3475-240/KEYNOTE-240)	Phase III study	Pembrolizumab (MK-3475) in advanced HCC treated systemically as a second line therapy; To determine overall survival and progression free survival	Active, not recruiting
NCT03062358 (MK-3475-394/KEYNOTE-394)	Phase III study	To determine the efficacy and safety of pembrolizumab or placebo with best supportive care previously systemically treated HCC	Active, not recruiting
NCT03383458 (CheckMate 9DX)	Phase III study	To investigate if nivolumab will improve recurrence-free survival compared to placebo in HCC undergone complete resection	Active, not recruiting

CTLA-4: Cytotoxic T lymphocyte protein 4; PD-1: Programmed cell death protein 1; HCC: Hepatocellular carcinoma; TGF: Transforming growth factor; TIM3: T cell immunoglobulin and mucin domain-containing protein 3.

675206) and ipilimumab (BMS-734016, MDX-010) for CTLA-4 have also been discussed [30]. The advantage of immune checkpoint inhibitor therapy in augmenting the immune response of liver is due to its safe profile, rates of immune-related toxicity like other tumor type, and without any hepatic dysfunction as supported by the fact that the increase in aspartate aminotransferase and alanine aminotransferase with therapy was not relevant to cause its discontinuation[39,40]. The phase I and phase II clinical trials, which makes the basis for phase III trials, involving immune checkpoint inhibitors nivolumab (anti-PD1), pembrolizumab (anti-PD1), tislelizumab (anti-PD1), durvalumab (anti-PD1), tremelimumab (anti-CTLA4), and camrelizumab (anti-PD1) have been discussed in the literature[30]. Similarly, the clinical trials (NCT01658878, NCT02576509, NCT02702414, NCT02702401, NCT02715531, NCT03434379, NCT01008358, and NCT01853618) with immune checkpoint inhibitors have been summarized[20,29] (Table 2). The results of these studies and outcome of phase I and phase II clinical trials suggest that immune checkpoint inhibitor therapies can provide objective response in advanced HCC. These studies suggest that immunotherapy might be good therapeutic strategies for the treatment of HCC and combinational approach might be even more effective.

Immune check-point inhibitor therapy has been approved as first-line therapy in the cases not suitable for surgery and has been proven beneficial in stabilizing the quality of life, however, only a subset of patients has shown positive outcome and there are reports of tumor progression, worsening of liver function, and poor prognosis in others. The reason behind the equivocal results in clinical trials for immune check-point inhibitor therapy is due to the lack of biomarkers to check the tumor respons-

iveness during therapy[41]. Different strategies to assess the response and cut off values for the biomarkers are another reason for failure of immune check-point inhibitor therapy[42]. Decreased T-cell infiltration, expression of newer or other immune checkpoints, mutation of the immunogenicity of cancer itself, change in gut microbiota, and TME might be other causes for unresponsiveness or failure of the immune checkpoint inhibitors[43]. Limited efficacy or inability of the immune check-point inhibitors to target signaling pathways involved in tumorigenesis, as in case of monotherapy targeting PD-1/PD-L1 but not VEGF-A or Wnt/β-catenin, is another cause for failure or limited response of immune check-point inhibitor therapy[44]. Collectively, these factors are responsible for not a high response rate of immune check-point inhibitors in HCC.

ADOPTIVE CELL TRANSFER

Adoptive cell transfer therapy involves administration of autologous lymphocytes in the HCC patients. Adoptive cell transfer therapy involves infusion of NK cells, CIK cells, TILs, and CAR-T cells. NK cells form nearly 50% of the immune cells in the liver and can kill the cells without any prior activation, thus endowing the defense against infection and tumor development. The cytotoxic role of expanded NK cells on HCC in murine model resulting in reduced tumor growth and improving overall survival[29,52,53] supports the notion of using adoptive cell transfer therapy in HCC. CIK cells are T lymphocytes representing a T cell population which acquire phenotype of NK cells with NK cells surface markers through manipulation with IFN-γ, and IL-2, IL-1, and a monoclonal antibody against the T cell marker CD3 (OKT3) and represent non-MHC-restricted tumor-killing activity and inhibitory effect on tumorigenesis[3,54]. Adoptive cell transfer therapy with CIK cells represents novel immunotherapy and early randomizing trials in HCC patients following surgical resection as adjuvant therapy shows promising results with a significantly reduced risk of recurrence, but without an improvement in overall survival[3]. Improved progression and recurrence free survival has been documented by a systematic review and meta-analysis of CIK cell therapy in HCC[55]. Similarly, studies investigating the efficacy of CIK cell therapy in HCC with the results of no major adverse events after a median follow-up of 14 mo with autologous TILs, benefits of CIK cell treatment, and significant superiority in prolonging the median overall survival, progression free survival, significantly higher disease-free survival rates, and disease control rate in HCC patients has been reviewed [29]. GPC3, a member of the glypican family of heparan sulfate proteoglycans, is tumor specific and important for cell proliferation and its role in the pathogenesis of HCC has been described. Thus, targeting GPC3 and other TAAs or neoantigens with CAR-T cell therapy in HCC seems to be prospective immunotherapeutic option and has been reviewed in the literature[24,29]. The results of various clinical trial and the ongoing clinical trials for adoptive cell therapy has been summarized in Tables 3 and 4, respectively.

Adoptive cell transfer therapy has emerged as a promising therapy in HCC; however, the clinical trials and clinical research is progressing slowly because of various limitations such as inactivity of the infiltrating lymphocytes due to changing TME caused by immunoediting or immunomodulation. Down regulation of MHC class I molecule, cellular heterogeneity of the cells, terminal differentiated cells and short viability of these cells, effectiveness only in in-vitro but limited efficacy *in vivo*, unexpected toxicity (CAR-T), specificity for a target, cytokine storm, presence of neoantigens, presence of suppressive immune cells, evolution of inhibitory ligands, variability in cells processing conditions, defects in antigen processing and presentation, evolving tumor escape mechanisms, and presence of hostile TME associates with the limited success and slow progression of adoptive cell transfer therapy[56-58]. Off-tumor effects are potential concerns related to CAR-T cell therapy and associate with limited efficacy[59]. CAR-T cells function can potentially be altered due to the interaction between CAR-T cells and host TME[58].

VACCINES

Peptide, DCs, whole-cell vaccines, oncolytic viruses, and DNA agents are the most common therapeutic vaccines used to increase immune response to tumor antigen [64]. The common peptide vaccines including AFP, multidrug resistance-associated protein 3 (MRP3), and GPC3 have been proven safe and well-tolerated and clinical

Table 3 Adoptive cell therapy for hepatocellular carcinoma

Adoptive cell transfer			
Agent	**Type of study**	**Study details**	**Outcome**
NK cells stimulated with IL-2[60]	Phase I trial	Patients with liver cirrhosis with HCC undergoing liver transplantation	Upregulation of peripheral NK cell cytotoxicity, no adverse events
CIK cell therapy as adjuvant to RFA[61]	A multicenter, randomized, open label phase III trial	230 HCC patients; CIK cell therapy as adjuvant to RFA, ethanol injection or curative resection	An improvement of 14 mo in recurrence free survival
Autologous TILs[62]	Phase I trial	15 patients with HCC post-resection	Successful expansion of TILs in 88% without any evidence of disease; No serious adverse events
GPC3 CAR-T[63]	Phase I trial	13 Chinese patients with r/r GPC3+ HCC	Feasible and safe for Chinese pts with r/r GPC3+ HCC; Promising antitumor potential when LDC is applied along with GPC3 CAR-T

NK: Natural killer; IL: Interleukin; HCC: Hepatocellular carcinoma; CIK: Cytokine-induced killer; TIL: Tumor-infiltrating lymphocytes; RFA: Radiofrequency ablation; CAR: Chimeric antigen receptor.

trials including UMIN000001395, UMIN000005678, NCT01974661, NCT00554372, and NCT01387555 using these vaccines have been summarized in the literature[29]. As discussed above, the non-specificity of tumor antigen is a major cause of impediment in designing novel therapeutics and tumor vaccines helps in augmenting specific immune responses to tumor antigens. Thus, tumor vaccines seem to be potential therapeutics for advanced HCC, however, disappointing results from previous trials and lack of vaccine efficacy in other tumors cause scarcity of clinical trials of tumor vaccines for HCC. However, this hurdle has been renounced due to the modern techniques of identifying novel targets using RNA/DNA sequencing and bioinformatics[3]. DCs are APCs presenting the TAAs and provide secondary co-stimulation required for the priming of an effective T cell response. Peripheral DCs treated with various factors to activate and mature ex-vivo are reinfused and these primed DCs functions as vaccine *via* inducing recruitment of effector cells and tumor cell lysis[65]. The efficacy of the vaccines can be enhanced by optimizing the TAAs. An effective immune response in the tumor can also be induced by peptide vaccines. GPC3 peptide in increased in HCC and might be a potential target, a study by Wu *et al* [66] showed that GPC3 coupled lymphocytes elicit robust GPC3-specific antibody and cytotoxic T lymphocyte responses in mouse and might be precision therapeutics. A recent phase 1 clinical trial showed that after peptide vaccination in HCC patients, peptide-specific cytotoxic T lymphocytes frequency might be a predictive marker of overall survival[67]. Targeting TAA seems to be effective therapeutics, however, clinical trials showed specific T cell response rate of over 70% while targeting AFP, GPC3, and MRP3 while T cell response rate was below 40% while targeting NY-ESO-1, SSX- 2, MAGE-A, and TERT[68]. The weak efficacy of the vaccines might be due to insufficient immune activation of targeting self/tumor antigen. The use of oncolytic viruses to induce oncolysis is an important strategy in HCC because of the ability of oncolytic virus for selective infection, within tumor replication, and destruction and eradication of tumor cells. The strategies, mechanistic aspects, and the limitations of the oncolytic viruses including New-Castle disease virus (rNDV-18HL, rNDV-IL2-TRAIL, rLaSota/IL2), adenovirus [ZD55-XAF1, QG511-HA-melittin, GOLPH2-regulated GD55, AD55-Mn-SOD, Ad5-HC, Ad5-AFP (IRES), hTERT-Ad, Ad-199T, AdDE1bDVA+2'AP, Adenovirus SP-E1AE1B(D55)-TSLC1(SD55-TSLC1), Adenovirus eSurphSulf1], vaccinia virus [GLV-1h68, GLV-2b372, JX594, Pexa-Vec (JX-594)], recombinant vesicular stomatitis virus (rVSV, VSV with NSC74859), parvovirus (Recombinant H-1 PV), and measles virus (MeV-SCD) have been discussed in detail in the literature[24]. Using oncolytic viruses as vaccines is more recent approach and intra-tumoral injection of viruses is used to selectively replicate in and destroy cancer cells. Pexa-Vec is the most common virus vaccine and is a modified vaccinia poxvirus (JX-594). Various clinical trials have studied the anti-tumor effects of Pexa-Vec on HCC (Table 5) and some trials are ongoing, but results have not been posted and are awaited (Table 6). Changing tumor immunogenicity due to mutation or low tumor immunogenicity limits the response of vaccine therapy in HCC and identification of novel specific tumor epitopes is warranted to improve efficacy of HCC cancer vaccine [69].

Table 4 Clinical trials on adoptive cell transfer therapy

Clinical trials #	Phase	Aim and design	Status
NCT03563170	Phase 1b/2	Combining innate high-affinity natural killer (hank) cell therapy with adenoviral and yeast-based vaccines to induce t-cell responses *vs* sorafenib	Withdrawn
NCT03008343	Phase I/II	Combination of IRE and NK cells immunotherapy *vs* IRE alone	Completed, no result posted
NCT01147380	Phase I	Natural killer cell therapy for hepatoma liver transplantation (MIAMINK); To evaluate feasibility and safety of the adoptive transfer of activated NK cells	Completed; No adverse events reported
NCT02008929	Phase II	To evaluate the safety and efficacy of injecting MG4101 (*ex vivo* expanded allogeneic NK cell) as a secondary treatment after curative liver resection in advanced HCC	Completed; No study results posted
NCT01749865	Phase III	CIK treatment in 200 patients with HCC who underwent radical resection	Completed; No study results posted
NCT02723942	Phase I/II	To evaluate the safety and efficacy of CAR-T cell immunotherapy for GPC3 positive hepatocellular carcinoma	Withdrawn due to revision of local regulations
NCT03198546	Phase I	GPC3 and/or TGF-β targeting CAR-T cells in	Recruiting
NCT03130712	Phase I/II	GPC3-targeted T cells by intratumor injection for advanced HCC (GPC3-CART)	Unknown
NCT02715362	Phase I/II	GPC3 redirected autologous t cells for advanced HCC (GPC3-CART)	Unknown
NCT03013712	Phase I/II	GPC3-targeted T cells by intratumor injection for advanced HCC (GPC3-CART)	Unknown
NCT03349255	Phase I	Autologous ET1402L1-CAR T cells in AFP expressing HCC	Terminated and will study new T-cell construct
NCT02905188	Phase I	To find the biggest dose of GLYCAR T cells that is safe, to see how long they last in the body, to learn what the side effects in GPC3-positive HCC	Recruiting patients; Partial response with no toxicities
NCT03146234	Single arm, open-label pilot study	to determine the safety and efficacy of CAR-GPC3 T cells in patients with relapsed or refractory HCC following cyclophosphamide and fludarabine	Completed; Had a tolerable toxicity profile with no grade 3/4 neurotoxicity; Overall survival 9.1
NCT02395250	Phase I	To evaluate the safety and effectiveness of anti-GPC3 CAR T in patients with relapsed or refractory HCC	Completed, no result posted
NCT03980288	Phase I	4th generation chimeric antigen receptor T cells targeting glypican-3 (CAR-GPC3 T cells) in patients with advanced HCC	Recruiting patients
NCT04121273	Phase I	GPC3-targeted CAR-T cell for treating GPC3 positive advanced HCC	Recruiting patients
NCT03884751	Phase I	Clinical study of chimeric antigen receptor T cells targeting glypican-3 (CAR-GPC3 T cells) in patients with advanced HCC	Recruiting patients
NCT04093648	Phase I	T cells co-expressing a second generation glypican 3-specific chimeric antigen receptor with cytokines interleukin-21 and 15 as immunotherapy for patients with liver cancer (TEGAR)	Withdrawn (the key elements of this study were incorporated into another study)
NCT03013712	Phase I/II	CAR T cells targeting EpCAM positive cancer (CARTEPC); To evaluate the safety and efficacy of chimeric antigen receptor (CAR) T cells targeting EpCAM	Unknown

NK: Natural killer; IL: Interleukin; HCC: Hepatocellular carcinoma; CIK: Cytokine-induced killer; TIL: Tumor-infiltrating lymphocytes; RFA: Radiofrequency ablation; CAR: Chimeric antigen receptor; Adenoviral and Yeast based vaccines: ETBX-011, GI-4000, avelumab, Aldoxorubicin hydrochloride, ETBX-051, ETBX-061, GI-6207, GI-6301, and N-803; IRE: Irreversible electroporation; LDC: Lymphodepleting conditioning; GLYCAR: Glypican 3-specific chimeric antigen receptor expressing T cells for hepatocellular carcinoma; HCC: Hepatocellular carcinoma.

COMBINATION THERAPIES

Since HCC is a multifactorial disease, targeting more than one factor involved in the pathogenesis of HCC seems to be a promising approach. Along with the ongoing clinical trials for immune checkpoint inhibitors targeting PD-1/PD-L1 and CTLA4, the

Table 5 Vaccine therapy for hepatocellular carcinoma

Vaccine	Phase	Study design	Outcome
Autologous dendritic cells (DCs) generated *ex vivo* in the presence of GM-CSF and IL-4 [70]	Phase I	10 patients with unresectable primary liver cancer	Immunization well tolerated without significant toxicity
Mature autologous DCs[71]	Phase II	To investigate the safety and efficacy of intravenous vaccination	Safe and well tolerated with evidence of antitumor efficacy
Ilixadencel (pro-inflammatory allogeneic DCs stimulated by GM-CSF and IL-4)[72]	Phase I trial	17 HCC patients; As monotherapy or in combination with sorafenib to evaluate tolerability	Increased tumor specific CD8+ T cells in peripheral blood (73%); 1 grade 3 adverse event
GPC3 peptide[67]	Open-label, phase I clinical trial	33 patients with advanced HCC; To evaluate safety of GPC3 peptide, immune response, tumor response, time to tumor progression, and overall survival	GPC3 vaccination was well-tolerated; 1 patient partial response; 19 patient stable disease; 2 mo after vaccination; Measurable immune responses and antitumor efficacy
Pexa-Vec (modified poxvirus JX-594)[73]	Randomized phase II dose-finding trial	30 patients with advanced HCC; 3 intra-tumoral injections; To determine the optimal JX-594 dose	Dose related survival benefit; Increased median survival of 14.1 mo compared to 6.7 mo
Pexa-Vec (JX-594)[74]	Phase 2, open-label, randomized dose finding study	Patients with advanced HCC; Intra-tumoral injection 3 times every 2 wk	
Pexa-Vec (pexastimogene devacirepvec) followed by sorafenib[75]	Global, randomized, open-label phase III trial (PHOCUS)	459 patients will be recruited; To evaluate overall survival, time to progression, progression-free survival, overall response rate and disease control rate	Trial completed; 5% adverse events

IL: Interleukin; GM-CSF: Granulocyte-macrophage colony-stimulating factor; HCC: Hepatocellular carcinoma.

Table 6 Ongoing clinical trials on vaccine therapy for hepatocellular carcinoma

Clinical trial #	Phase	Agent/vaccine	Design/aim	Status
NCT01974661	Phase I	COMBIG-DC (ilixadencel)	Is it possible to inject the COMBIG-DC vaccine in a hepatic tumor without getting unacceptable side effects	Completed; No results posted
NCT01821482	Phase II	DC-CIK	To evaluate the efficacy of DC-CIK for HCC	Unknown/not yet recruiting
NCT02638857	Phase I/II	DC precision multiple antigen T cell	To evaluate the safety and efficacy of dendritic cell-precision multiple antigen T cells with TACE in HCC	Unknown/was recruiting
NCT02882659	Phase I	Autologous dendritic killer cell	To evaluate the safety in patients with metastatic solid tumor; To evaluate the maximum tolerated dose	Unknown/was active, not recruiting
NCT03674073	Phase I	Personalized neoantigen-based dendritic cell	A single institution, open-label, multi-arm, pilot study; DC vaccine combined with microwave ablation in HCC	Unknown/was recruiting
NCT03203005	Phase I/II	Cancer vaccine called IMA970A combined with CV8102	To investigate the safety; To check if this combination can trigger an immune response against the tumor in HCC	Completed; No results posted
NCT02562755	Phase III	Pexastimogene devacirepvec (Pexa Vec) and sorafenib	To investigate if the combined treatment increases survival compared to treatment with sorafenib alone in HCC	Completed

DCs: Dendritic cells; CIK: Cytokine-induced killer; HCC: Hepatocellular carcinoma.

recent interest is to design combination therapies. This notion is supported by significantly improved clinical response with the combination of nivolumab with ipilimumab in sorafenib-treated patients with an acceptable safety profile[76]. The combination therapies combining checkpoint inhibitors with other drugs including oncolytic virus/viral vaccines, small molecules, ablative therapies or combining multiple checkpoint inhibitors is the area of interest. The basis of the combination therapies is additive or synergistic effects of the therapy by combining systemic or radiotherapy with immunotherapy[25,28]. Radiotherapy primes the immune cells, increase inflammatory response, and when combined with immunotherapy produce synergistic effect and enhance anti-tumor effects. Various aspects of combination

Table 7 Ongoing clinical trial for combination therapy for hepatocellular carcinoma

Immune checkpoint/vaccine therapy	Radiotherapy/other therapy	Phase	Study design	Status	Trial ID
Ipilimumab	Nivolumab	Phase I/II	To assess the effects of combination treatment with nivolumab and ipilimumab pre-operatively in HCC	Recruiting patients	NCT03682276
Nivolumab	Ipilimumab	Phase I	To compare the overall survival of nivolumab plus ipilimumab *vs* standard of care (sorafenib or lenvatinib) in patients with advanced HCC	Recruiting patients	NCT04039607
Nivolumab	Ipilimumab	Phase II	Nivolumab plus Ipilimumab as neoadjuvant therapy for HCC; To test efficacy, tumor shrinkage, and objective response rate	Recruiting patients	NCT03510871
Nivolumab	Ipilimumab	Phase II	Nivolumab with or without ipilimumab in treating patients with resectable liver cancer		NCT03222076
Nivolumab, ipilimumab	SBRT	Phase I	To determine the safety and tolerability of SBRT followed by nivolumab or ipilimumab in HCC	Active, not recruiting	NCT03203304
Pembrolizumab	Talimogene laherparepvec (genetically modified oncolytic viral therapy)	Phase Ib/II	Multicenter, open-label, basket trial; To evaluate the safety of talimogene laherparepvec injected intra-hepatically into liver tumors alone and in combination with systemic IV administration of pembrolizumab	Recruiting patients	NCT02509507; MK-3475-611/Keynote-611 (MASTERKEY-318)
Nivolumab	Pexa-Vec	Phase I/II	To evaluate the safety and efficacy in HCC	Active, not recruiting	NCT03071094
Modified vaccinia virus ankara vaccine expressing p53	Pembrolizumab	Phase I	To study the side effects of vaccine therapy and in treating patients with solid tumors with metastasis	Active, not recruiting	NCT02432963
GNOS-PV02 (personalized neoantigen DNA vaccine)	Plasma encoded IL-12 (INO-9012) pembrolizumab	Phase I/IIa	A single-arm, open-label, multi-site study of GNOS-PV02 and INO-9012 in combination with pembrolizumab (MK-3475) in histologically or cytologically confirmed HCC	Recruiting patients	NCT04251117
DNAJB1-PRKACA fusion kinase peptide vaccine	Nivolumab and Ipilimumab	Phase I	To study the safety and tolerability of administering a vaccine targeting the DNAJB1-PRKACA fusion kinase, in combination with nivolumab and ipilimumab in unresectable or metastatic fibrolamellar HCC	Recruiting patients	NCT04248569
Durvalumaband tremelimumab	Sorafenib	Phase III	To assess the efficacy and safety of durvalumab plus tremelimumab combination therapy and durvalumab monotherapy *vs* sorafenib in the treatment of patients with no prior systemic therapy for unresectable HCC		NCT03298451
TremelimumabDurvalumab (MEDI4736)	Radiation therapy	Phase II	To test the combination therapy as a possible treatment for HCC or biliary tract cancer	Recruiting patients	NCT03482102
Nivolumab	Y90-radioembolization	Phase II	To evaluate the response rates of Y90 radioembolization in combination with nivolumab in HCC	Recruiting patients	NCT03033446
Ipilimumab	SBRT	Phase I	To find the highest tolerable dose of ipilimumab and SBRT in liver and lung cancer	Completed but no results posted	NCT02239900
Nivolumab	TACE	Phase II (IMMUTACE)	To evaluates the safety and the efficacy of nivolumab in combination with TACE in patients with multinodular, intermediate stage HCC as first line therapy	Active, not recruiting	NCT03572582
Pembrolizumab	TACE	Phase I/II (PETAL)	Open label, single arm, multi-centre study; To determine the safety and tolerability of	Recruiting patients	NCT03397654

			pembrolizumab following TACE		
Durvalumab; Tremelimumab	TACE; RFA; Cryoablation	Phase II	To evaluate the 6-mo progression free survival with combination therapy in patients with HCC	Recruiting patients	NCT02821754
Immune Checkpoint Inhibitor	TACE; SBRT	Phase II; START-FIT	Sequential TACE and SBRT with immunotherapy	Recruiting patients	NCT03817736
Durvalumab	Tremelimumab	Phase II	To evaluate the safety, tolerability, antitumor activity, pharmacokinetics, pharmacodynamics, and immunogenicity of durvalumab or tremelimumab monotherapy, or durvalumab in combination with tremelimumab or bevacizumab in advanced HCC; Initial reports of concerns with safety and efficacy of the combination of durvalumab and tremelimumab in HCC	Active, not recruiting	NCT02519348

SBRT: Stereotactic body radiotherapy; TACE: Trans-arterial chemoembolization; HCC: Hepatocellular carcinoma; RFA: Radiofrequency ablation.

therapies including the primary or acquired resistance to anti-PD-1/PD-L1 therapies in melanoma, increased viral load in patients when tumor started to progress with anti-CTLA-4 therapy ultimately leading to treatment failure, the presence of low mutation rate as a cause of therapy resistance, and strategies to enhance function of effector cells have been discussed[25]. A dose-dependent increase in PD-L1 expression post irradiation in HCC cell lines mediated by IFN-γ-STAT3 signaling pathway support the notion of using combination therapy for advanced HCC[77]. An improved treatment outcome in murine model with HCC, mammary cancer in xenograft murine model, and CT26 murine colon carcinoma xenograft model while combining radiotherapy with PD-1/PD-L1 and CTLA-4 as described in[28] suggests the advantage of combining radiotherapy with immune checkpoint therapy. This rationale is also supported by the results of clinical studies documenting increased PD-1 and PD-L1 expression of T cells and tumor cells[78-80]. Additionally, the combination therapies combining checkpoint inhibitor therapies with other strategies with the mechanism of action and study details of various clinical trials (NCT01658878, NCT02519348, NCT03071094, NCT02572687, NCT03006926, NCT02856425, NCT02942329, NCT02988440, NCT02423343, NCT02859324, NCT03095781, NCT02474537, NCT02325739, NCT03143270, NCT03033446, NCT02837029, NCT03099564) have been summarized[20,25]. Similarly, promising clinical results of combining RT with immunotherapy reported by Chiang *et al*[81] using stereotactic body radiotherapy followed by nivolumab for large unresectable HCC and Y-90-RE and nivolumab bridging therapy prior to partial hepatectomy by[82,83] supports the notion of combination therapy for advanced HCC. The ongoing clinical trials of combination therapies for HCC has been listed in Table 7. Additionally, the clinical trials involving durvalumab + tremelimumab, nivolumab + ipilimumab, atezolizumab + bevacizumab, pembrolizumab + lenvatinib, and SHR-1210 + apatinib have been discussed[30].

CONCLUSION

The limited efficacy of immune-based therapies is due to inherently tolerogenic character of the liver in both healthy and diseased state. Chronic inflammation of liver during the pathogenicity of HCC leads to higher tumor immunogenicity and makes a basis of immunotherapeutic approaches to treat HCC. However, strong intrinsic immune suppressive microenvironment and high immune evasion are major impediment for an effective immune response against tumor with immunogenic approach. Additionally, liver also plays a crucial role in host defense and in the maintenance of self-tolerance, it is important to design personalized immunosuppressive therapies[1]. The intrahepatic immunosuppressive TMEs play a major role in reducing the effects of immunotherapy and thus an effective therapy must be designed to counteract and target factors playing a role in immune evasion and treatment resistance. Additionally, therapeutic regimens which can amplify tumor-specific immunity and counteract immunosuppressive mechanisms might profoundly improve clinical outcomes for HCC patients. The initial results from various clinical trials involving immune

checkpoint inhibitor therapy, adoptive cell transfer therapy, tumor vaccines, and combination therapy are promising but warrant more research in terms of investigating tumor specific antigens and better personalized therapies.

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MINIREVIEWS

Redefining non-alcoholic fatty liver disease to metabolic associated fatty liver disease: Is this plausible?

Jalpa Devi, Aimun Raees, Amna Subhan Butt

ORCID number: Jalpa Devi 0000000187102381; Aimun Raees 00000315107073; Amna Subhan Butt 0000-0002-7311-4055.

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Jalpa Devi, Department of Gastroenterology, Liaquat University of Medical and Health Sciences, Jamshoro 74800, Pakistan

Aimun Raees, Amna Subhan Butt, Department of Gastroenterology, Aga Khan University Hospital, Karachi 74800, Pakistan

Corresponding author: Amna Subhan Butt, FCPS, MBBS, MSc, Associate Professor, Department of Gastroenterology, Aga Khan University Hospital, Stadium Road, Karachi 74800, Pakistan. amna.subhan@aku.edu

Abstract

Recently, a single letter change has taken the world by storm. A group of experts have developed a consensus to upgrade the term non-alcoholic fatty liver disease (NAFLD) to metabolic associated fatty liver disease (MAFLD), suggesting that MAFLD would more accurately reflect not only the disease pathogenesis but would also help in patient stratification for management with NAFLD. However, the difference of opinion exists, which has made the NAFLD *vs* MAFLD debate the current talk of the town. This review will focus on the plausibility and implications of redefining NAFLD as MAFLD.

Key Words: Non-alcoholic fatty liver disease; Metabolic associated fatty liver disease; Fatty liver disease; Non-alcoholic fatty liver; Review

Core Tip: A group of experts have recently developed a consensus towards redefining non-alcoholic fatty liver disease (NAFLD) as metabolic associated fatty liver disease (MAFLD), suggestive of a more accurate differential diagnosis and signifying the exact disease pathogenesis to achieve higher patient stratification and delivery of better care to patients with NAFLD while avoiding stigmatization due to the presence of the word 'alcohol', particularly in regions where alcohol consumption is a taboo for cultural and religious reasons. However, differences in experts' opinions considering the implications of redefining NAFLD as MAFLD still hold strong. Therefore, this review article focuses on the plausibleness and implications of redefining NAFLD as MAFLD.

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INTRODUCTION

The world has seen steady progress in the awareness of non-alcoholic fatty liver disease (NAFLD) owing to its rising prevalence, yet there has been no worthwhile advancement in treatment and management and or breakthrough in the therapeutic field. A great part of this shortcoming has always been linked to the inadequacy of the term NAFLD, as it fails to describe the underlying metabolic factors of the disorder, instead shedding undue light on the etiology which is in actual unrelated to the core pathology, *i.e.*, alcohol. To overcome this problem, many scientists have joined hands and suggested an up-gradation in nomenclature. Hence, the term metabolic associated fatty liver disease (MAFLD) has been under a great uproar for the past couple of years.

NAFLD – AT A GLANCE

NAFLD is a chronic disorder encompassing a spectrum of liver diseases characterized by excessive intrahepatic fat deposition, leading to hepatocyte ballooning injury in the absence of a known cause such as excessive alcohol consumption, viral hepatitis, medications, or a hereditary disorder[1]. It is further classified into NAFL when there is > 5% liver steatosis with no evidence of hepatocyte injury or fibrosis and non-alcoholic steatohepatitis (NASH) when there are > 5% liver steatosis and ballooning of hepatocytes with or without liver fibrosis. NASH may lead to complications such as cirrhosis and hepatocellular carcinoma, and increase liver-related mortality as fibrosis advances. Risk factors associated with fibrosis are old age, diabetes mellitus, obesity, hypertension, and degree of insulin resistance[2].

The nomenclature was introduced in 1980 by a pathologist, Jurgen Ludwig, who used the term NAFLD and NASH to describe the histological findings found in a series of 20 patients[3]. The introduction of the term was based on marked similarities between alcoholic steatohepatitis and NASH. Later, Kleiner *et al*[4] proposed the NAS score to evaluate changes in histological features individually to grade and stage the disease. A decade later, Bedossa *et al*[5] came up with an algorithm to diagnose NASH. However, none of these scores could predict future clinical outcomes in patients with NAFLD.

The prevalence of NAFLD is on the rise in the last three decades in the United States, reaching about 20%-30%, mirroring the steady increase in obesity globally[2,6]. It has become the second most common cause of liver transplantation in Western countries, which is quite alarming[7]. Despite the dangerously rising prevalence, awareness regarding the disease burden is still not up to the mark. This is also reflected by the lack of availability of a non-invasive diagnostic test. The only way to make a definitive diagnosis of NAFLD and assess its severity is a liver biopsy, which is invasive and costly, leading to delays in the final diagnosis[1]. Unfortunately, the therapeutic field has also not seen any breakthrough and to date, there is no FDA-approved pharmacotherapy available. The treatment solely relies on lifestyle modifications with regular exercise and dietary changes with weight loss being the goal of therapy[8]. This requires a good deal of cooperation on part of the patient with a large part of the therapeutic responsibility on the patient himself/herself, making the entire ordeal quite difficult since switching to and long-term sustenance of such a drastic lifestyle is quite arduous. Various agents have been used in addition to lifestyle modification, ranging from pioglitazone and vitamin E (antioxidant) to ursodeoxycholic acid, and while some drugs have been able to reduce liver fat content such as pinositol, vitamin E, and obeticholic acid, none of these had an impact on NASH or fibrosis; on the other hand, adverse effects and long-term safety from the use of these drugs have remained a major concern[9,10].

THE RISE OF MAFLD

Inappropriate nomenclature of any disorder has an overall impact on its perception both by patients and physicians. It may lead to confusion and the development of mistrust between patients and their treating doctors, thus affecting overall outcomes. It is also difficult for patients to understand the magnitude of the problem when the disease terminology is based on a negative term. For instance, establishing a diagnosis of NAFLD requires exclusion of significant alcohol consumption while ironically, there is no accepted cut-off limit for significant alcohol consumption. On the other hand, less alcohol use may also be affiliated with hepatic steatosis and fibrosis progression in NAFLD patients. The current term does not provide any relevant information on what the condition is, rather it represents what it is not!

A significant concern lies in the stigmatization of the disease due to the presence of the word 'alcohol', particularly in regions where alcohol consumption is a taboo for cultural and religious reasons. It exerts a psychological strain on the patient to the existing clinical spectrum of the disease. In such cases, specific inquiries on intake of alcohol may be misinterpreted, adversely affecting the doctor-patient relationship. On the other hand, trivialization is a commonly faced problem with the current terminology as patients rely on quitting alcohol consumption solely to curb the disease progression and are reluctant for any other essentially needed lifestyle or behavioral modifications.

Advancements in the field to curb the existing dilemma are largely affected by the sub-optimal allocation of funding, resulting from a lack of awareness of disease severity and complications. It is expected that a change in nomenclature may be able to deal with the negative consequences, proving to be a catalyst to accelerate funding and health policy action.

Over the years, research has proven that NAFLD is an inadequate term to describe liver disease associated with metabolic dysfunction, as it fails to emphasize the most important and commonly noted etiology of metabolic dysfunction. To address these issues, researchers and societies have become inclined to the idea of changing the terminology. In 2005, Loria *et al* proposed to introduce positive criteria to define NAFLD[11]. Subsequently, in 2018, the European Liver Patient's Association asked for a change in nomenclature owing to the limitations of existing terminology[12]. Eslam *et al*[13] in 2019 suggested updating the term and appealed to consider a more accurate term to define the disease.

The basic reason calling for this substantial shift in terminology was to improve patient awareness and to overcome the challenges in management which seemed to be associated with the term NAFLD. After several modifications in search of the most appropriate terminology, the term MAFLD was coined, as it was most reflective of the most likely associations of the disease. The suggested criteria include the presence of hepatic steatosis radiologically or histologically in obese or overweight individuals. While in lean individuals who are found to have hepatic steatosis, evidence of two or more metabolic risk factors is mandatory as shown in Figure 1. Besides involving a large spectrum of diseases, the term MAFLD also acknowledges the presence of multiple overlapping causes and drivers of the disease, thus rendering it as a hepatic manifestation of a multi-system disease. A recent study illustrated that metabolic syndrome is a more deleterious cardiovascular risk factor than NAFLD, which could represent an epiphenomenon of the metabolic syndrome itself[14]. The change in terminology is not only expected to raise awareness at both patients' and physicians' levels but also gain attention from the funding agencies and other major stakeholders, eventually leading to an acceleration in advancement in the field of diagnosis and disease therapy.

Although the new term MAFLD is expected to deal with several issues posed by the term NALFD, it may have to face challenges of its own. While MAFLD is a flexible term and may encompass a variety of disorders, it is important to note that there is no fixed criterion to describe metabolic dysfunction and there remains some ambiguity regarding a complete definition. Also, genetic disorders associated with NAFLD may remain a separate entity as the term MAFLD fails to include that. Therefore, updating the term NAFLD to another umbrella term that does not adequately describe the underlying driving factors comprehensively, may become counterproductive, as this may need another upgrade following a few decades of advancements.

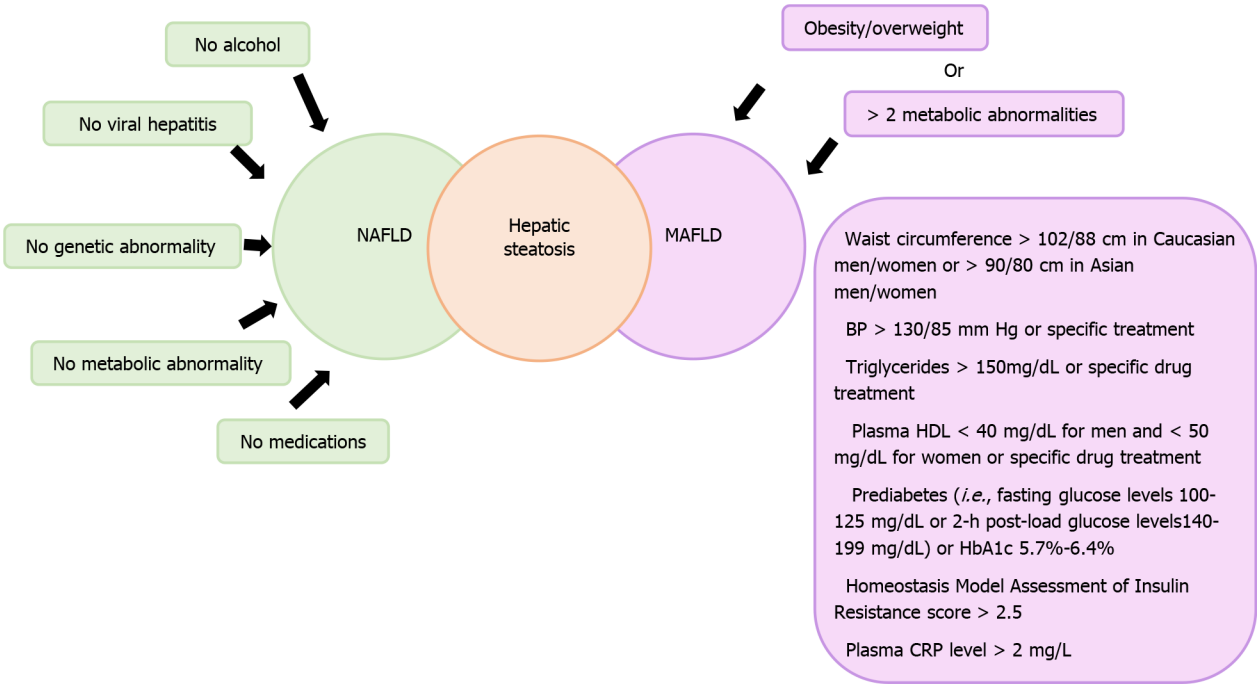

Figure 1 Difference between metabolic associated fatty liver disease and non-alcoholic fatty liver disease criteria.

OPINION *VS* EVIDENCE

Because of the imprecision of the term NAFLD, crucial suggestions have been brought forward by Asian and European researchers to turn it to MAFLD, which can accurately capture the predisposing factor and diminish the exclusion criteria[15]. While upgrading the term may help in improving disease awareness, its impact on other aspects does not seem too promising. For scientific contention, scrutiny and reasoning lie in the evidence-based debate, and recognizing salient characteristics of evidence would be of great value[16,17]. The primary role of metabolic dysfunction makes a pivotal point in the pathophysiology of fatty liver disease[12,13]. Globally, this initiative has been endorsed by the Asian Pacific Association for the Study of the Liver and welcomed by many experts along with patients' organizations[18-25]. On the other hand, a few scholars are skeptical about this change and advocate it as being overhasty[26].

Ever since its proposal, research studies have been carried out to compare the impact of the two terms on the feasibility of diagnosis and overall outcomes of the disease. The very first study by Lin *et al*[27] illustrated that the FIB-4 index and NAFLD fibrosis score were increased in MAFLD criteria and also proposed that the latter is more efficient in diagnosing high-risk patients. An analysis of the recent database (2017-2019) from Japan found that MAFLD was 20% more competent than NAFLD in the diagnosis of fibrosis[28]. Another study by Zheng *et al*[29] reflected that MAFLD diagnostic criteria were more pertinent even in resource-limited countries and was able to recognize more homogenous groups. Xu *et al*[30] validated the high diagnostic capability of the fatty liver index in MAFLD patients as a marker of hepatic steatosis, in particular where the use of ultrasound is narrowed. The MAFLD criteria were found more accurate than older NAFLD criteria when analyzing momentous hepatic fibrosis in the patients with cardiovascular disease[31,32], chronic kidney disease[33], and appraisal of genetic risk factors[34] in fatty liver disease. It also implies that MAFLD may coexist with other liver diseases, for instance, hepatitis B and C[15,35-37], HIV infection[38], Gaucher[39], and coeliac disease[40], hence making it more plausible than NAFLD. Table 1 shows all the studies comparing the two terms [41-44].

Furthermore, the evolution of genome-wide association has led to the recognition of *PNPLA3* and *TM6SF2*[45] genes that dictate the genetic linkage to pathophysiology and support the diagnostic approach of metabolic syndrome. As it is a genetically co-determined metabolic disorder, this terminology aids patients to comprehend the disease and perhaps boost compliance to lifestyle modification. New studies scrutinizing the involvement of the liver in the prognostication of COVID-19 integrated MAFLD criteria[46-48] identified a significant flaw in the MAFLD definition

Table 1 Evidence of non-alcoholic fatty liver disease *vs* metabolic associated fatty liver disease

No.	Ref.	Number of participants	Study type	Outcome measures	Result
1	Lin *et al*[27], 2020	13083	Cross-sectional, cohort from the National Health and Nutrition Examination Surveys III database	Significant fibrosis	Liver enzymes and the non-invasive liver fibrosis scores were significantly higher in MAFLD compared to NAFLD group (P < 0.05)
2	Yamamura *et al*[28], 2020	765	Cross-sectional Japanese cohort	Significant fibrosis	MAFLD (OR = 4.401; 95%CI: 2.144-10.629; P < 0.0001). NAFLD (OR = 1.721; 95%CI: 1.009-2.951; P = 0.0463)
3	Niriella *et al* [31], 2020	2985	A prospective study with 7 years of follow-up	Cardiovascular event CVD (non-fatal + fatal)	Excluded by NAFLD definition but captured by MAFLD definition adjusted OR [8.5 (2.2-32.8)]. Excluded by MAFLD definition but captured by NAFLD definition Adjusted OR [2.0 (0.2-19.2)]
4	Mak *et al* [15], 2020	1134	Cross-sectional chronic hepatitis B and fatty liver	Advanced fibrosis/ cirrhosis	Patients with CHB + MAFLD compared to patients with CHB + NAFLD outside the MAFLD criteria (22.6% *vs* 11.8%, P = 0.043)
5	Zheng *et al* [29], 2021	780	Cohort,(Liver biopsy)	Diagnostic criteria easily applicable even in a resource-limited country	
6	Xu *et al*[30], 2020	35335	Cohort	Fatty liver index as a marker of hepatic steatosis	AUROC of FLI for predicting HS was 0.856 (95%CI: 0.854–0.859) in malesand 0.909 (95%CI: 0.906-0.911) in females, which showed a good diagnostic ability
7	Liu *et al*[41], 2021	361	Cross-sectional study, HIV and fatty liver disease	A positive correlation between LSM and CAP values was found in the MAFLD group	Prevalence of NAFLD (37.67%) and MAFLD (34.90%) (ALT) level (44.44% *vs* 16.17%, P < 0.001) and advanced fibrosis (19.05% *vs* 2.55%, P < 0.001) were significantly higher in the MAFLD group
8	Myers *et al* [42], 2021	920	Population cohort study	The burden of NAFLD and MAFLD associated HCCs increased significantly, driving an increase in HCC incidence, particularly in women.	Proportion of NAFLD-HCC increased more in women (0% to 29%, P = 0.037) than in men (2% to 12%, P = 0.010) while the proportion of MAFLD increased from 21% to 68% in both sexes and 7% to 67% in women (P < 0.001)
9	Guerreiro *et al*[43], 2021	1233	Retrospective cross-sectional study	Differences between NAFLD and MAFLD regarding cardiovascular events	MAFLD and NAFLD, CVR was intermediate/high (36.4 and 25.7%, P = 0.209) and CVD occurred in 20.1 and 12.8% (P = 0.137) of the cases, respectively
10	Ciardullo *et al*[44], 2021	1710	A cross-sectional study of adults recruited in the 2017- 2018 National Health and Nutrition Examination Survey, a representative sample of the general United States population.	Significant fibrosis	The weighted prevalence of NAFLD and MAFLD were similar in the whole population at 37.1% (95% CI 34.0-40.4) and 39.1% (95% CI 36.3-42.1) respectively.

NAFLD: Non-alcoholic fatty liver disease; MAFLD: Metabolic associated fatty liver disease; OR: Odds ratio; CI: Confidence interval; HCC: Hepatocellular carcinoma; CVD: Cardiovascular disease.

similar to that of NAFLD that is the paucity of knowledge about other relevant pathophysiological drivers except for metabolic risk factors, such as genetic components. Concerns are raised that the term MAFLD, shelters a group of large entities, which leads to increased sensitivity at the cost of low specificity. For instance, NAFLD could be misdiagnosed in metabolically healthy individuals unless we rule out alternative or additional reasons. Similar reasoning applies to lean patients having two or more metabolic risk factors[12,18]. This is why a few others suggested the term dysmetabolism-associated fatty liver disease because of its strong association with dysfunctional metabolism leading to NAFLD and its complications[49]. Lately, on the account of AASLD, Younossi *et al*[26] articulated that it is premature to rename the terminology due to various reasons; quoting one as, the term MAFLD may not be enough to clearly describe the genetic and environmental associations of the disease, making it yet another umbrella term requiring upgrading after a few decades once more information on the pathophysiology and natural history of the disorder is available. Inclusion of all significant stakeholders such as regulatory agencies and the

patients' organization is also mandatory in the movement of renaming the disease[26]. Mass screening would only be possible once all the scientific and patient care societies are on the same page.

PROS AND CONS

NAFLD has set its foot in a world full of subspecialties *via* disseminating knowledge in different educational sessions; it yet lacks the channel of screening in the workup of their patients. To increase awareness and to overcome this roadblock, the liver community needs to engage genuinely with non-hepatologist to design a clinical approach based on the metabolic risk factors.

In contrast to viral hepatitis for which[50] the World Health Organization has developed a Global Health Sector Strategy to eliminate it by 2030, no such agendas are programmed to tackle the burden of NAFLD. Nonetheless, it seems to rise in the coming era and has a marked impact on economic and health losses, as it is the third leading cause of hepatocellular carcinoma and the second indication for liver transplantation in the West. To sum up, any terminology with the prefix 'non' may be considered a non-serious issue reducing its overall significance[51,52]. For the same purpose, there are ongoing efforts to eliminate the 'non' by renaming and reframing non-communicable diseases[53-55]. Within this frame of reference, an attempt should be made to instruct policymakers to allocate funding for research to cope with existing inequalities.

NAFLD like other non-communicable diseases, such as diabetes mellitus and obesity, is often referred to as contrition diseases, insinuating that this develops through personal behavioral choices which often lead to shame and accusation for individuals which hinder their desire to seek help. It is universally endorsed that an important part of treatment is a healthy diet and physically active lifestyle, thus essentially requiring the involvement of patients; however, many patients are not even aware of NAFLD as a disease entity[56,57]. Also, a survey conducted in the United States in 2015 indicated that no more than one-fourth of patients enrolled in the study were acquainted with the disease[58]. The National Health and Nutrition Examination Survey conducted from 2013 to 2016, showed that merely 3.5% of the population had awareness of the disease[59]. Similarly, 'Continuum Clinical' illustrated again that 6% of patients had the know-how of their disease. Various other studies likewise supported these results[60]. Primary care physicians also have insufficient sensitivity for establishing a diagnosis of NAFLD and how it should be assessed[61]. Another study depicted incidental diagnosis of NAFLD at the stage of cirrhosis[62]. Research in 2018 showed that despite expanding incidence, a massive number of patients remained underreported[63]. This lack of public health responses to NAFLD may be attributed to the baffled term alcohol in NAFLD which exerts hurdles in understanding and acceptance of the disease, thus damaging the disease response[64]. In many parts of the world, consumption of alcohol is prohibited due to social and religious norms with even more daunting concerns in children where its use is not well established. In such regions, the term imposes potential stigmatization of the disease. The European Liver Patients Association was not satisfied with the term NAFLD and put forward the thoughts to change the name. This NAFLD acronym also creates perplexity and hampers efficient communication that leads to negative repercussions on the physician-patient relationship. The "non" term in NAFLD also underestimates this deleterious health challenge which might be perceived as a trivial matter or as if, one is authorized to consume alcohol. Embracing the latest term MAFLD and related positive diagnostic criteria may resolve many issues regarding confusion, trivialization, and stigmatization related to the term NAFLD.

CONCLUSION

To curtail the serious outcomes associated with fatty liver disease, the previously faced challenges must be identified and tackled effectively. To begin with, owing to the heterogeneity of the disease and dynamic histopathology, no diagnostic biomarker is available as yet. This could be expedited by phenotypic classification for which Non-Invasive Biomarkers of Metabolic Liver Disease and Liver Investigation: Testing Marker Utility in Steatohepatitis work vigorously based on the validated biomarkers. Second, the lack of an FDA-approved drug for the management of the disease is already a huge hurdle, and a change in the disease terminology may hamper the

ongoing drug trials. On the other hand, it was expected that the term MAFLD will aid in the refinement of clinical endpoints for future drug trials. However, for a change of the name, it demands vigilant speculation of societal and medical ramifications and real impact. In the long run, although the advancement is bound to be imperceptible, we must strike into the appropriate pathway to lay down the groundwork for accomplishing the long-term targets.

Nonetheless, being in the modern era, evidence-based medicine, an evolving science, advancement in technology, and personalized medicine will have a positive impact on healthcare. Due to the heterogeneity of the disease, there should be a multidisciplinary approach which not just involves hepatologists but also other specialties like endocrinologists and cardiologists. Notably, the conceptual framework of MAFLD lines up the liver disease with the latest comprehension of metabolic syndrome, obesity, and systemic biology. By doing this, we can adopt the heterogeneity and have many subtyping that can at length lead to a precision of medicine based on system narrative. From this viewpoint, the old term "cryptogenic cirrhosis" will be replaced by MAFLD-related cirrhosis. This name scales down the confusion not just on the etiologic spectrum, but also stigma and revamps physician-patient relationships.

What happens next when MAFLD gains ground? Then we would have to embrace the connection between metabolic disorder and low-grade inflammation, in particular adipose tissue inflammation which might unlock the door to new treatment strategies in the future. Hence, it is also essential to recognize that joint efforts of scientific societies, pharmaceutical industries, and patient associations have led to a marked acceleration in the development of medications currently under trial for NAFLD, which could all go in vain if the nomenclature is to change at this point. Hence, it may be wise to arrange a combined international conference of all the important stakeholders asking for their opinion regarding an upgrade in the nomenclature in addition to deciding an appropriate timing for it.

As it is a nascent idea, the journey can go at a snail's pace as it needs affirmation by clinicians, patients, researchers, and industry worldwide. In addition, this calls for future studies and funding to be escalated to explore the consequences of change in terminology. Unless we all converge to bring a consensus regarding this as well as improving the overall disease outcomes, a mere change in terminology may still not be sufficient. Joint efforts are needed on everyone's part to increase disease awareness, reduce its global burden, and provide effective diagnostic and therapeutic modalities.

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MINIREVIEWS

Stearoyl-CoA desaturase 1: A potential target for non-alcoholic fatty liver disease?-perspective on emerging experimental evidence

Shanmugam Murugaiha Jeyakumar, Ayyalasomayajula Vajreswari

ORCID number: Shanmugam Murugaiha Jeyakumar 0000-0002-4500-8431; Ayyalasomayajula Vajreswari 0000-0002-9465-9994.

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Shanmugam Murugaiha Jeyakumar, Ayyalasomayajula Vajreswari, Division of Lipid Biochemistry, National Institute of Nutrition, Hyderabad 500007, Telangana, India

Shanmugam Murugaiha Jeyakumar, Department of Clinical Pharmacology, National Institute for Research in Tuberculosis, Chennai 600031, Tamil Nadu, India

Corresponding author: Shanmugam Murugaiha Jeyakumar, PhD, Senior Scientist, Department of Clinical Pharmacology, National Institute for Research in Tuberculosis, No. 1 Mayor Sathiyamoorthy Road, Chetpet, Chennai 600031, Tamil Nadu, India. smjkumar@gmail.com

Abstract

Non-alcoholic fatty liver disease (NAFLD) is a progressive disease and one of the leading causes of death. An unnamed disease has become a global epidemic disease of public health concern. This spectrum of diseases manifests itself with initial accumulation of excessive triglycerides (due to *de novo* lipogenesis) in the hepatocytes, leading to simple steatosis. Although its aetiology is multi-factorial, lifestyle changes (diet and physical activity) are considered to be the key thriving factors. In this context, high fructose consumption is associated with an increased risk for developing NAFLD in humans, while high-fructose feeding to experimental animals results in hepatic steatosis and non-alcoholic steatohepatitis, by increasing hepatic lipogenesis. Among several lipogenic genes, the endoplasmic reticulum-bound stearoyl-CoA desaturase 1 (SCD1) is the key determinant of triglycerides biosynthesis pathway, by providing monounsaturated fatty acids, through the incorporation of a double bond at the delta-9 position of saturated fatty acids, specifically, palmitic (C16:0) and stearic (C18:0) acids, yielding palmitoleic (C16:1) and oleic (C18:1) acids, respectively. Various experimental studies involving *SCD1* gene knockout and diet-induced rodent models have demonstrated that SCD1 plays a key role in the development of NAFLD, by modulating hepatic lipogenesis and thus triglyceride accumulation in the liver. Several pharmacological and dietary intervention studies have shown the benefits of inhibiting hepatic SCD1 in the pathogenesis of NAFLD. In this review, we give an overview of SCD1 in NAFLD, based on the current experimental evidence and the translational applicability of SCD1 inhibition in human NAFLD conditions, besides discussing the limitations and way-forward.

Key Words: Steatosis; Lipids; Fatty acids; Vitamin A; Metabolic syndrome; Obesity

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Core Tip: Stearoyl-CoA desaturase 1 (SCD1) is the rate-limiting enzyme of biosynthesis of monounsaturated fatty acids that serve as substrates for *de novo* lipogenesis, thereby increasing the production and accumulation of triglycerides in the liver. The liver-specific inhibition of SCD1 has been shown to attenuate the development of hepatic steatosis and thus non-alcoholic fatty liver disease (NAFLD), as evidenced by experimental studies. The current evidence supports the view that SCD1 is a potential target and the inhibition of this enzyme would certainly help in the control and/or management of NAFLD in humans. However, certain aspects of SCD1 such as its role and regulation need to be addressed in humans to explore its potential translational applicability.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a spectrum of diseases including simple hepatic triglyceride accumulation, otherwise called steatosis, to hepatocellular carcinoma (HCC). Although its aetiology is multi-factorial, lifestyle modifications and genetic susceptibility are considered as the major thriving forces for the development of NAFLD, besides obesity and metabolic syndrome. Like other metabolic diseases, NALFD also contributes to the development of insulin resistance, metabolic syndrome, and obesity. Various external and internal factors influence its progression from simple steatosis to the end-stage disease HCC, which takes several years with an incidence rate ranging between 2.4% to 12.8%. As the aetiology and progression of NAFLD are asymptomatic, diagnosis, control, and management of NAFLD at an early stage are very much challenging. Further, so far no specific therapy to treat the NAFLD has been identified, except weight management therapy[1,2].

Studies from several genetically-engineered rodent models have demonstrated the involvement of numerous genes in the development and/or progression of NAFLD, namely, adiponutrin/patatin-like phospholipase domain-containing protein 3 (*PNPLA3*), caspases 1 and 3, cannabinoid receptor 1, hepcidin, prolyl endopeptidase, stearoyl-CoA desaturase 1 (*SCD1*), and thyroid hormone receptor-α, to name a few[3-10]. Besides, Cole *et al*[11] have described various genetic, drug-induced, and other NAFLD models for drug discovery and their potential use in therapeutics. Previously, Postic and Girard[12] have detailed the role of several genes that are involved in the hepatic *de novo* lipogenesis and their interaction with fatty acid oxidation, triglyceride secretion, and thus hepatic steatosis and its associated complications, including insulin resistance, based on the studies from the genetically engineered mice. In this review, we primarily focus on the role of SCD1 in the development of hepatic steatosis from various experimental models and discuss the potential scope of its inhibition in ameliorating NAFLD, besides highlighting the limitation, especially, the existing translational research gap between the experimental research and its extension to clinical research in the control and/or management of NAFLD.

NAFLD: AN UNNAMED DISEASE TO A GLOBAL EPIDEMIC DISEASE

NAFLD is a spectrum of several related diseases in the absence of alcohol consumption as the etiological origin. The earliest stage in NAFLD is hepatic steatosis/fatty liver, which is characterized by the deposition of triglycerides in the cytoplasmic lipid droplets of hepatocytes. The hepatic steatosis/fatty liver is often self-limiting; however, it can progress to non-alcoholic steatohepatitis (NASH), the condition characterized by the presence of hepatocyte injury (hepatocyte ballooning and cell death), infiltration of immune cells, inflammatory mediators, and activated stellate cells. Due to the vicious cycle of inflammatory insults and stellate cell

activation, NASH progresses to fibrosis and cirrhosis, which can eventually progress to HCC, thus resulting in hepatocellular death[1,2].

In the year 1952, Zelman[13] has reported liver damage in obese humans based on liver function tests and liver biopsy examination. In 1958, Westwater and Fainer[14] have confirmed liver damage in obese patients, as evidenced by abnormal liver function and histology. Adler and Schaffner[15], who have examined a group of 25 overweight patients for the presence of fatty liver, fatty hepatitis, fatty fibrosis, and cirrhosis, based on liver biopsy and function, have reported that an equal frequency of all these pathological conditions. Further, these hepatic pathological changes resembled the liver damage caused by alcohol and post-jejuno-ileal bypass surgery. Ludwig *et al*[16], who have studied liver disease in 20 obese patients in Mayo Clinic, have found similarities between the hepatitis of unknown cause and the alcohol-induced hepatitis with respect to the histological changes, such as fatty changes, lobular hepatitis, and focal necrosis with mixed inflammation. Further, they have coined the term NASH in 1980 for the first time; until then it is known as an unnamed liver disease. The current global prevalence of NAFLD is estimated to be 24%, which has increased from 15% to 25% between 2005 and 2010, and the data from a recent meta-analysis study on the general population have shown a higher prevalence of NAFLD in the Middle East (32%) and South America (31%), followed by United States (24%), while being the lowest in Africa (14%)[17,18]. Further, in Asia, the overall prevalence of NAFLD is estimated to be 29.6%[19]. Undoubtedly, NAFLD is now a global epidemic disease of public health concern and therefore, its control and management are the top research priorities. Further, along with other non-communicable diseases that include obesity, type 2 diabetes, and metabolic syndrome, it contributes to the global disease burden and associated health and economic consequences.

AETIOLOGY AND PATHOGENESIS OF NAFLD

The natural history of NAFLD in terms of its occurrence or causation and pathogenesis is multi-factorial, poorly understood, and further complicated by the involvement of the host's genetics and interactions with lifestyle changes including various environmental factors and other pre-existing co-morbidities and risk factors. Nevertheless, some of the key underlying mechanisms involved in the hepatic triglyceride accumulation are increased hepatic *de novo* lipogenesis, diminished export of triglycerides through lipoproteins, and impaired β-oxidation of free fatty acids[12]. However, the pathogenesis/progression of NAFLD, from fatty liver to hepatocellular death, is explained initially by the two-hit hypothesis. Subsequently, it is substituted by the parallel, multiple-hit hypothesis. Accordingly, the first insult is initiated by the accumulation of lipids, particularly, triglycerides inside the hepatocytes and the development of hepatic insulin resistance. This causes the activation of several cascades of events both at hepatic and extra-hepatic sites, particularly adipose tissue that ultimately leads to the excessive free fatty acid influx, increased lipogenesis, and triglyceride accumulation. Parallelly, this causes a perpetual cycle of multiple insults to the hepatocytes through cellular stress (oxidative and endoplasmic reticulum stress), mitochondrial dysfunction, dysbiosis, inflammatory response, and hypoxia, to name a few and mediated by the interplay between several cell types of hepatic, extra-hepatic and systemic origins[20,21]. Although certain pharmacological agents (lipid-lowering drugs, such as metformin and statins) and weight management therapy are offered, there are no specific drugs to treat NAFLD (except managing the disease conditions) due to high complexity and poor understanding of its pathogenesis[22].

DIETARY CHANGES AND NAFLD

Sugars are naturally occurring sweeteners, and sucrose, fructose, and glucose are the most common sugars in our daily diet. Before the industrial era, the amount of fructose was very low in the human diet and derived mainly from natural resources such as honey, dates, raisins, grapes, raw apples, squeezed apples, persimmons blueberries, and molasses. After industrialization, sweeteners are produced on a large scale from various sources, particularly corn. During this process, starch isolated from corn, is initially hydrolyzed into glucose, and followed by the enzymatic isomerization of the released glucose into fructose. The resultant product/mixture is known as high-fructose corn syrup (HFCS). Relative to sucrose, the usage of HFCS in the food

industries is high, due to its low cost and sweeter taste and also as it stabilizes the texture of processed food better than sucrose. The most widely recognized type of HFCS is HFCS 55, having 55% fructose compared to sucrose which has 50% fructose [23-25]. Although both glucose and fructose are simple carbohydrates, unlike glucose, the absorption and metabolism of fructose are completely different. Moreover, it is more lipogenic than glucose. Therefore, excessive consumption of fructose causes uncontrolled lipogenesis and triglyceride synthesis in the liver, due to the lack of rate-limiting enzyme or metabolic check-point[26]. Hepatic *de novo* lipogenesis is considered to be an important contributing factor in the development of NAFLD[27]. Donnelly *et al*[28] have shown that in the fasted state, 26% of triglyceride and 23% of very-low-density lipoprotein (VLDL)-triglyceride in the liver of NAFLD patients are derived from the *de novo* lipogenesis. In addition, Lambert *et al*[29] have shown that, compared to the control subjects, *de novo* lipogenesis is 3% higher in the NAFLD subjects. Although contradictory findings exist, most of the epidemiological and clinical studies have shown the association between high fructose consumption (majorly in the form of HFCS) and the risk of NAFLD causation and other metabolic complications, including obesity, insulin resistance, and metabolic syndrome[30-34].

SCD1–A REGULATOR OF LIPOGENESIS

SCD1 is an endoplasmic reticulum-bound microsomal enzyme that catalyses the formation of monounsaturated fatty acids (MUFA) from saturated fatty acids (SFA) by incorporating a double bond at the delta-9 position, by involving cytochrome b5, NADPH-dependant cytochrome b5 reductase, and molecular oxygen. Palmitoleic (C16:1) and oleic (C18:1) acids are the SCD1-catalyzed products from their respective substrates palmitic (C16:0) and stearic (C18:0) acids[35,36]. SCD1 is abundantly expressed in adipose tissue and the liver, though different isoforms of SCD have been identified in various species including humans, such as SCD 1-4 in mice, SCD1 and 2 in rats, and SCD1 and 5 in humans. These isoforms display differential expression pattern and tissue specificity, however, the role of some of these isoforms is not fully elucidated. As constituents of cell membranes, MUFA play a crucial role in maintaining membrane fluidity. Therefore, the altered ratio of SFA to MUFA in membranes affects the fluidity, thereby modulating the cellular signalling and physiological functions[36].

SCD1 is the rate-liming enzyme of synthesis of MUFA, which are the major substrates for the synthesis of triglycerides, phospholipids, and cholesteryl and wax esters. Diet-derived and the endogenously (fatty acid biosynthetic pathway) formed palmitic acid (C16:0) and its chain elongation product stearic acid (C18:0) are desaturated by the SCD1 and the newly formed MUFA, *i.e.*, palmitoleic (C16:1) and oleic (C18:1) acids, respectively, are preferably esterified with glycerol-3-phosphate to form lysophosphatidic acid, the first step of triglyceride assembly by the enzyme glycerol-3-phosphate acyltransferase (GPAT). After several enzymatic steps, finally, it results in the formation of triglycerides by the action of diacylglycerol acyltransferase (DGAT) and it is either stored in the liver or assembled into VLDL and exported to extra-hepatic tissues[37]. The SCD is a critical metabolic control enzyme, as its activity determines the fate of fatty acids by diverting them to either oxidation or storage, and hence, modulates the energy homeostasis and thereby obesity. This is evident from the *SCD1* gene knock-out mouse study of Ntambi *et al*[38]. Earlier, a study from our lab has shown that fatty acid desaturation indices (the ratio of product to the substrate; *i.e.* C16:1/C16:0 and C18:1/C18:0) of SCD 1 are associated with body mass index and adiposity in genetically obese rat models[39]. The dysregulated SCD1 is considered to be one of the key mediators in the pathophysiology of several metabolic and/or inflammatory diseases, including obesity, metabolic syndrome, diabetes, NAFLD, cardiovascular diseases, and cancer[40-43]. Importantly, SCD1 is regulated by numerous nutritional (fatty acids, cholesterol, vitamin A, and iron) and hormonal (leptin and thyroid hormone) factors[44-49].

SCD1 AND NAFLD - EXPERIMENTAL EVIDENCE

The very first time, from gene-knockout mouse models (*SCD1*-/- and *SREBP1c*-/-), Miyazaki and colleagues have reported that SCD1 and its enzymatic product oleate (C18:1) are essential for fructose-induced hepatic lipogenesis and triglyceride synthesis through both sterol regulatory element-binding protein 1c (SREBP-1c)-dependent and

independent pathways[50]. A study based on global *SCD1* knock-out mice has demonstrated that SCD1 deficiency resulted in the increased expression of genes involved in the fatty acid oxidation, while decreased the key lipogenic genes, thereby decreasing the triglyceride synthesis and secretion by the liver. Further, *SCD1* gene knockout with leptin deficiency, *i.e.*, in *ob/ob* mice, has resulted in the attenuation of the hepatic triglyceride accumulation and secretion of VLDL. It has been reported that *SCD1* gene knock-out mice display increased hepatic mitochondrial fatty acid oxidation, which is evident from the increased activities of carnitine palmitoyltransferase (CPT), the gate-keeper enzyme of β-oxidation. Further, the authors have reported that the effects are mediated through the activation of the adenosine monophosphate (AMP)-activated protein kinase (a metabolic sensor) due to the deficiency of SCD1. Further, the SCD1 mutation has also led to AMPK activation in *ob/ob* mice[51]. Miyazaki *et al*[52] have shown in a natural homozygous *SCD1* gene mutated asebia mouse model that the absence of SCDI has led to the impaired hepatic synthesis of cholesterol ester and triglycerides. In a liver-specific *SCD1* knock-out mouse model, Miyazaki *et al*[9] have found that these mice are resistant to high-carbohydrate (high sucrose and very low-fat) diet-induced adiposity and hepatic steatosis. In a genetically modified NAFLD mouse model that possesses N-glycosylated cyclic AMP-responsive element-binding protein H (CREBH) (endoplasmic reticulum-anchored transcription factor), there was decreased production of peroxisome proliferator-activated receptor α (PPARα) and activity of SCD1, which in turn resulted in the reduction of hepatic lipid accumulation, lipotoxicity, and inflammation[53]. The study of Flowers *et al*[54] has shown that SCD1 deficiency has diverse effects on lean and obese mice, as evidenced by improved insulin sensitivity in the former, while aggravation of diabetes (due to pancreatic beta-cell loss) in the latter.

Jiang *et al*[55] have demonstrated that the pharmacological inhibition of SCD1 through the anti-sense oligonucleotide has resulted in increased fatty acid oxidation and reduced *de novo* fatty acid synthesis and thus steatosis both in hepatocyte cell line and mouse models. Non-coding ribonucleic acids microRNA-103, 212-5p, and 27a have been shown to suppress the SCD1 in the liver, besides fatty acid synthase (FAS), and thus reduced the diet-induced obesity, hepatic *de novo* lipogenesis, and hepatic lipid accumulation as evidenced by *in vivo* and *in vitro* models[56-58]. Oral administration of a novel SCD-1 inhibitor, N-(2-hydroxy-2-phenylethyl)-6-[4-(2-methylbenzoyl) piperidin-1-yl] pyridazine-3-carboxamide, has been shown to attenuate hepatic lipid accumulation and histological features of NASH, such as hepatocellular degeneration, inflammation, and liver injury in an NASH rat model[59]. Another study using an SCD1 selective inhibitor, 3-[4-(2-chloro-5-fluorophenoxy)-1-piperidinyl]-6-(5-methyl-1,3,4-oxadiazol-2-yl)-pyridazine, has shown a reduction in triglyceride accumulation and promoted liver-specific functions, during the multiple stages of hepatocyte differentiation in human pluripotent stem cells. Further, the authors have observed the MUFA oleate-mediated reversal of SCD1 inhibition. In addition, the authors could find some of these changes due to SCD1 inhibition, during differentiation, in human primary mononuclear cells (hPMN)[60]. Iida and colleagues [61] have discovered a synthetic compound, thiazole-4-acetic acid analogue 48, displaying liver-specific inhibition of SCD1. Further, the investigators have demonstrated the pharmacological effects (such as anti-diabetic and anti-obesity) of hepatic SCD1 inhibition in rodent models of metabolic diseases such as diabetes, obesity, and hepatic steatosis using this analogue. In addition, pre-clinical toxicological evaluation of this compound has displayed no significant adverse events and therefore, the authors have concluded that the compound has a potential therapeutic utility in treating some of the chronic diseases[61]. In Zucker fatty rats (*fa/fa*), oral administration of an SCD1 inhibitor, GSK993, decreased the hepatic lipids, and improved impaired glucose tolerance and insulin sensitivity[62]. Tao *et al*[63] have shown that the intraperitoneal administration of α_2-adrenoceptor agonist dexmedetomidine (DEX) to diet-induced NAFLD mice resulted in the inhibition of hepatic steatosis and improvement of insulin sensitivity and inflammation, associated with a significant reduction in hepatic SCD1 mRNA and protein levels[63]. Previously, Attie *et al*[64] who have assessed the SCD1 and its association with plasma triglycerides have reported an association among hepatic SCD1 activity, fatty acid desaturation index (the ratio of C18:1/C18:0), and plasma triglyceride levels in mice, while a two-fold elevation of desaturation index is associated with a four-fold increase in plasma triglyceride concentration in humans. It has been shown that the SCD1 fatty acid desaturation index (*i.e.* C16:1/C16:0) correlates with fatty liver index of dyslipidaemic individuals, and importantly, the total PUFA were inversely associated with SCD1, thus NAFLD[65]. Qin *et al*[66] have shown that SCD1-mediated lipid desaturation plays a critical role in HCC, by modulating endoplasmic reticulum (ER) stress. Zhou *et*

al[67] who have studied the underlying mechanism of the development of hepatic steatosis have reported that the AMPK activation and lipophagy are the key mediators of SCD1 inhibition-induced amelioration of fatty liver as demonstrated in primary hepatocytes and high-fat diet-fed mice.

Quality and quantity of lipids/fats are known to alter the expression of SCD1 in mice susceptible to diet-induced metabolic diseases, including atherosclerosis, diabetes, obesity, and certain types of cancers[68]. In a study of Sekiya *et al*[69], dietary PUFA-fed *ob/ob* mice displayed SREBP-1-mediated suppression of lipogenic genes, including *SCD1*, and thus reduced hepatic triglyceride contents and liver enzymes, in addition to hyperinsulinemia and hyperglycemia. Mark Brown *et al*[70], who have investigated SCD1 inhibition on metabolic syndrome and atherosclerosis in experimental rat models, found that the SCD1 inhibition protected the mice from developing metabolic syndrome and prevented atherosclerosis synergistically by the treatment with fish oil and anti-sense oligonucleotide-targeted SCD1 suppression[70]. MacDonald *et al*[71] reported that decreased SCD1 activity is associated with improved metabolic syndrome phenotypes, including the reduction in plasma triglycerides, non-high-density lipoprotein (HDL) cholesterol, VLDL triglycerides, hepatic steatosis, fat mass, and insulin resistance induced by a Western diet in a low-density lipoprotein receptor-deficient mouse model. Conjugated linoleic acid (CLA) isomers have been shown to attenuate fructose-induced hepatic lipogenesis, lipid accumulation, and hypertriglyceridemia, through the suppression of lipogenic genes SCD1 and FAS of the liver[72]. Zhu *et al*[73] have reported that metformin, an anti-diabetic drug, ameliorates triglyceride accumulation by inhibiting hepatic SCD1 in the HepG2 cell line. Earlier, a study from our lab showed that SCD1 is a key player in fructose-induced hepatic triglyceride accumulation. However, for the first time, it has been demonstrated that a high fructose diet sans vitamin A failed to induce hepatic steatosis, while replenishment with vitamin A restored the fructose-induced triglyceride accumulation, suggesting that vitamin A is essential for fructose-induced metabolic alterations in the liver, associated with triglyceride metabolism[74]. Overall, targeting the SCD1 by knocking out the gene, dietary factors, and chemical inhibitor results in the reduction of its mRNA or protein or activity and MUFA levels. These events have been associated with improved NAFLD and/or its associated complications eventually, which include hepatic steatosis, NASH, liver injury, hepatocellular degeneration, hypertriglyceridemia, inflammation, hyperinsulinemia, impaired glucose tolerance, insulin sensitivity, hyperglycemia, and obesity. Notably, most of these metabolic complications are characteristic features of NAFLD in humans as well. Therefore, SCDI has significant clinical implications and apparently, SCD1 is a potential target for treating NAFLD in humans. Nevertheless, there are limitations to achieve the translational potential of SCD1 inhibition in clinical situations (Schematic summary is given in Figure 1).

TARGETING SCD1 IS FAR FROM TRANSLATIONAL APPLICABILITY? – CHALLENGES AND WAY-FORWARD

So far experimental evidence from genetic, diet-induced rodent models as well as from supplementation and interventional studies has demonstrated that SCD1 is the central player in lipid metabolism, energy homeostasis, and thus obesity and NAFLD. This has given enormous hope for its clinical utility and driven various pharmaceutical companies to develop potent inhibitors for SCD1. Earlier, Powell[75] has over-viewed several small molecule SCD1 inhibitors (such as piperazinyl pyridazine-based derivatives/analogues, cyclic urea, spirocyclic compounds, bicyclic heteroaromatics, triazole and aryl/heteroaryl linkers, piperidine aryl ketones, aryl diamine, bicyclic aryl diamine linkers, pyrazole and triazole derivatives/analogues, pyridazine-2-one and triazine derivatives to name a few of them) that are patented by pharmaceutical companies during 2009 to 2013 and their potential application in various metabolic diseases, such as obesity, diabetes and cancer. Further, the author has underlined the fact that the safety and efficacy of these inhibitors in humans remain unanswered. Recently, Uto[76] has articulated the current advances in the area of SCD1 inhibitor development and highlighted some of the tissue- or disease-specific SCD1 inhibitors. However, the author has also pointed out the knowledge gap in understanding the role of SCD1 in humans, in addition to the therapeutic applications of these inhibitors in clinical settings[76].

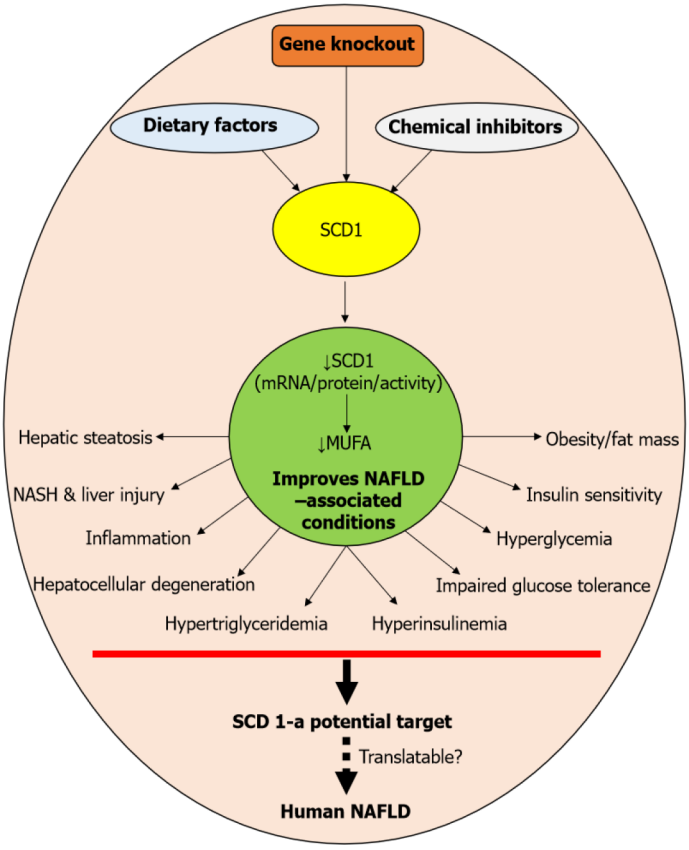

Figure 1 Schematic summary of experimental evidence on stearoyl-CoA desaturase 1 inhibition. SCD1: Stearoyl-CoA desaturase 1; MUFA: Monounsaturated fatty acids; NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis; ↓: Decrease.

Unlike liver-specific inhibition, global SCD1 inhibition or deficiency displays detrimental effect on various organs, particularly, the skin and eyes, and these aspects have been extensively reviewed earlier by Zhang *et al*[77]. However, a comprehensive understanding of other metabolic changes or distortion and the susceptibility to other metabolic insults or dietary and environmental factors due to liver-specific SCD1 inhibition is not even at the experimental stage. It has been shown that hepatic SCD1-deficient mice are susceptible to chemically-induced ulcerative colitis, besides resulting in the elevation of pro-inflammatory responses[78]. Aljohani *et al*[79] have reported that liver-specific SCD1 deficiency increases ER stress by activating the mammalian target of rapamycin complex 1 (mTORC1) in the global *SCD1* knockout mouse model; however, oleate has been shown to deactivate the mTORC1 signalling and dissolute ER stress. SCD1-mediated ER stress in HCC through lipid desaturation has also been reported[66]. Busch *et al*[80] have shown that increased SCD1 and its fatty acid desaturation index have a protective effect on SFA; palmitate-induced pancreatic beta-cell apoptosis and inhibition of SCD1 by CLA have also offered protection against lipotoxic effects of the palmitate. In line with this, previously, a study from our lab has also shown that the suppression of SCD1 and thus the MUFA oleic acid (C18:1) is associated with increased ER stress in the pancreas and hence islet cell apoptosis and decreased pancreatic hormones, namely insulin, glucagon, and C-peptide[81]. Notably, in one of our studies, we have reported that despite a reduction in the liver SCD1, there is no improvement in high fructose diet-induced hepatic steatosis[82]. Therefore, the inhibition of SCD1 may not lead to an improvement in hepatic steatosis, at least in certain conditions.

Since NAFLD is a benign and asymptomatic disease, identifying or diagnosing it at an early stage is very challenging. Importantly, there are no reliable and specific circulatory markers to identify the occurrence and/or classify the stages of NAFLD. Notably, Yamada *et al*[83], who have analysed the liver fatty acid composition and gene expression in patients with NASH, have reported the prevailing differences in these parameters among patients with simple steatosis and NASH. In another important study, Teufel *et al*[84] have reported the significant differences in the expression pattern of several pathway genes associated with NAFLD/NASH between murine models and human liver tissue, along with substantial differences in the

pathogenesis of NAFLD between these two species. So far, the available data have demonstrated the modulatory effect of SCD1 on the initial stage of NAFLD development, particularly, hepatic steatosis and NASH, which are largely derived from experimental studies. Therefore, there is much ambiguity with regard to the inhibition of SCD1, whether it will retard/arrest the progression and/or reverse the conditions of NASH and subsequent stages of NAFLD in humans. Furthermore, the regulatory role of SCD1 in different stages of NAFLD (fibrosis, cirrhosis, and HCC) is poorly understood even in experimental models and more so in humans. In addition, unlike other developed and developing countries, in India, a higher proportion of NAFLD has been reported in lean subjects, whose BMI is < 23 kg/m^2[85]. However, the role of SCD1 in lean NAFLD has not been addressed or defined adequately so far. Lee *et al*[86] have reported sex-specific differential expression of hepatic SCD1 in mice. More importantly, in the recent past, the sexual-dimorphic pathophysiology of NAFLD in humans has also been well received[87-89]. Unlike in rodents, the functions of SCD1 and SCD5 in humans are not well characterized and fully understood. In the NAFLD spectrum, besides the liver, several other players modulate the development and pathogenesis of NAFLD, and particularly the adipose tissue (which abundantly expresses SCD1), through a wide range of secretory adipocytokines[90]. Emerging evidence suggests that the pathogenesis of NAFLD involves an interplay of multiple organs in a system, in addition to environmental factors[90,91]. In such a case, it is unclear whether targeting/inhibiting the hepatic SCD1 alone would yield the desired clinical outcomes in NAFLD? Similarly, several questions are yet to be answered and the knowledge gaps need to be addressed in both experimental and clinical NAFLD. Hopefully, in the coming years, the technological advancements in the life sciences (omics, patient/human-derived organoids, *etc*.) and computational science (*in silico*, AI-based tissue modelling, and tools for prediction, diagnosis, and prognosis) would shed light on some of these grey areas.

CONCLUSION

The endoplasmic reticulum-bound SCD1 enzyme plays a very critical role in the development of NAFLD, by altering the hepatic MUFA concentration. The literature is replete with the reports demonstrating the role of SCD1 in the causation and pathogenesis of NAFLD. Notably, the liver-specific inhibition of SCD1 has been shown to attenuate the development of hepatic steatosis and thus NAFLD in several genetic and diet-induced experimental models, besides supplementation and intervention studies (diet and pharmacological agents). Although these experimental data are encouraging, the role and regulation of SCD1 in the human NAFLD conditions are poorly understood and thus need further research in this direction. Nevertheless, so far, the existing quantum of experimental and some supporting clinical data suggests that the SCD1 is a potential target and infuse a strong hope for translational applicability of SCD1 inhibitors, as a therapeutic option. Certainly, the inhibition of SCD1 would help in the control and/or management of NAFLD in humans.

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MINIREVIEWS

Mitochondrial hepatopathy: Anticipated difficulties in management of fatty acid oxidation defects and urea cycle defects

Aathira Ravindranath, Moinak Sen Sarma

ORCID number: Aathira Ravindranath 0000-0001-9323-7725; Moinak Sen Sarma 0000-0003-2015-4069.

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Aathira Ravindranath, Division of Pediatric Gastroenterology, Institute of Gastrointestinal Sciences, Apollo BGS Hospitals, Mysore 570023, Karnataka, India

Moinak Sen Sarma, Pediatric Gastroenterology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow 226014, Uttar Pradesh, India

Corresponding author: Moinak Sen Sarma, MD, DM, Associate Professor, Pediatric Gastroenterology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Rae Bareli Road, Lucknow 226014, Uttar Pradesh, India. moinaksen@yahoo.com

Abstract

Fatty acid oxidation defects (FAOD) and urea cycle defects (UCD) are among the most common metabolic liver diseases. Management of these disorders is dotted with challenges as the strategies differ based on the type and severity of the defect. In those with FAOD the cornerstone of management is avoiding hypoglycemia which in turn prevents the triggering of fatty acid oxidation. In this review, we discuss the role of carnitine supplementation, dietary interventions, newer therapies like triheptanoin, long-term treatment and approach to positive newborn screening. In UCD the general goal is to avoid excessive protein intake and indigenous protein breakdown. However, one size does not fit all and striking the right balance between avoiding hyperammonemia and preventing deficiencies of essential nutrients is a formidable task. Practical issues during the acute presentation including differential diagnosis of hyperammonemia, dietary dilemmas, the role of liver transplantation, management of the asymptomatic individual and monitoring are described in detail. A multi-disciplinary team consisting of hepatologists, metabolic specialists and dieticians is required for optimum management and improvement in quality of life for these patients.

Key Words: Mitochondrial hepatopathy; Metabolic liver disease; Liver transplantation; Hyperammonemia; Hypoglycemia; Carnitine

Core Tip: Management of fatty acid oxidation defects and urea cycles defects has to be tailored to specific types of defects. Although dietary intervention remains the most

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important pillar of successful outcome, role of definitive and potential medications is assuming renewed significance. Management is challenging due to variations in type and severity of the enzyme defect.

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INTRODUCTION

Mitochondria occupy a unique place in the metabolic milieu which orchestrates complex physiological processes in response to nutrient signals. Beta oxidation of fatty acids and the urea cycle are among the myriad metabolic functions in which mitochondria play a central role. While beta-oxidation involves energy extraction from fats, the urea cycle converts excess nitrogen into urea and both of these processes encompass several steps with multiple enzymes. Defects in any of the steps would result in the accumulation of the intermediate metabolites and deficiency of the downstream products. Appropriate management mandates an in-depth understanding of the biochemistry and the clinical implications of defects in various steps.

FATTY ACID OXIDATION DEFECTS

Epidemiology

Fatty acid oxidation defects (FAOD) are rare metabolic disorders and the true prevalence is difficult to determine as newborn screening cannot detect all the cases especially the milder ones. FAODs were detected in 1:6560 samples by newborn screening with tandem mass spectrometry in a study from Singapore[1]. The incidence of FAOD was determined to be 1:9300 from newborn screening results that included 5256999 samples from Australia, Germany and the United States of America. The incidence varied from 1:3300 in Turkey to 1:217000 in Taiwan[2]. Thus, the incidence of FAOD depends on the geographical region and ethnic background. In a large French study, Medium-chain acyl-CoA dehydrogenase (MCAD) deficiency was found to be the most common (25%) followed by long-chain hydroxyacyl CoA dehydrogenase (LCHAD) deficiency (22%). Very long-chain acyl CoA dehydrogenase (VLCAD) deficiency and carnitine shuttle defects accounted for 19% each[3]. The reported incidence of defects in MCAD is 1:4000 to 1:15000, VLCAD is 1:85000, LCHAD/trifunctional protein (TFP) is 1:250000 and carnitine shuttle defects is 1:750000[4]. Clinical manifestations will present before 2 years of age in 85% of cases and 30% of them will present in the neonatal period although FAOD can present for the first time even in early adulthood[4].

Beta oxidation in brief

Fats are highly concentrated sources of energy that are the ideal reservoir in all the animals as they are non-polar, occupy less space and can be oxidized multiple times to yield more energy compared to carbohydrates and proteins[5]. Though fats undergo alpha and gamma oxidation, beta-oxidation is the most important process by which the energy trapped in fats gets converted to adenosine triphosphate (ATP). Triglycerides are first oxidized by lipoprotein lipase to fatty acids which are then shuttled across the plasma membrane to the cytosol by fatty acid transport proteins. These proteins have acyl CoA synthetase activity which converts fatty acids into acyl CoA after translocation. The carnitine shuttle (Figure 1) aids in transporting acyl CoA esters across the mitochondrial membrane. Carnitine palmitoyl transferase 1 (CPT-1) first converts acyl CoA to acyl carnitine that is shuttled through the mitochondrial membrane by carnitine acyl carnitine translocase. At the inner mitochondrial membrane carnitine palmitoyl transferase 2 (CPT-2) retransforms acyl carnitine to acyl

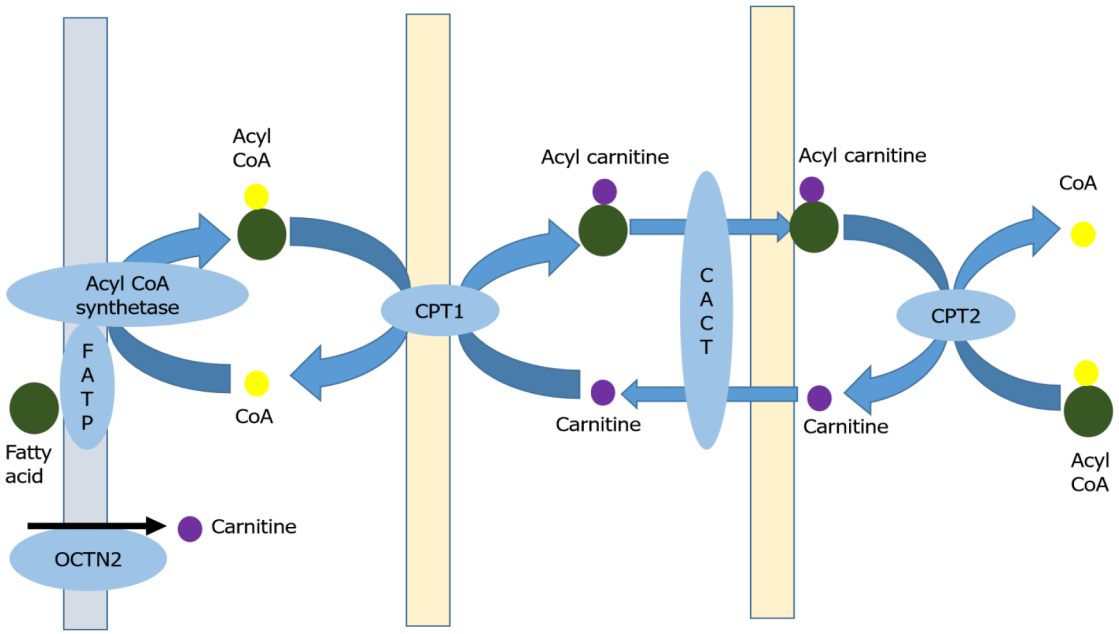

Figure 1 Carnitine shuttle. CoA: Co-enzyme A; FATP: Fatty acid transport protein; OCTN2: Organic cation transporter 2; CPT: carnitine palmitoyltransferase; CACT: Carnitine acyl carnitine transporter.

CoA and free carnitine. Acyl CoA undergoes beta-oxidation (Figure 2) in which by a series of reactions in each cycle two-carbon units are released as acetyl CoA and also nicotinamide adenine dinucleotide (NADH) and Flavin adenine dinucleotide (FADH2) are produced[5]. Acetyl CoA enters the citric acid cycle, NADH and FADH2 enter the electron transport chain. Beta oxidation has four major steps: First, acyl CoA dehydrogenase forms enoyl-CoA on which hydratase will act to generate hydroxyl-acyl CoA. Hydroxy-acyl CoA is dehydrogenated to keto-acyl CoA. Lastly, thiolase will cleave the keto-acyl CoA to acetyl CoA and acyl CoA (with two carbons less). There are three forms of acyl CoA dehydrogenases in humans which are chain length specific-VLCAD, MCAD and Short chain acyl CoA dehydrogenase (SCAD). Short and long chain enoyl CoA hydratases as well as short chain hydroxyl acyl CoA dehydrogenase (SCHAD) and LCHAD are present for metabolizing chain length-specific fatty acids. Similarly, two different thiolases are also present long chain and medium chain[6]. The mitochondrial trifunctional protein has hydratase, hydroxyl acyl CoA dehydrogenase and thiolase activity. Defects in fatty acid oxidation can have effects on multiple organ systems including the liver, muscle, heart, brain and kidneys. Thus, presentations are variable and making a quick and accurate diagnosis is a real challenge.

There are many dilemmas while managing FAODs

With varying clinical presentations in different age groups and multi-system involvement, work-up for FAOD needs to be considered based on detailed knowledge of the manifestations.

Interpreting fatty acid metabolite results based on the severity of the clinical situation and identifying the confounders that can affect the values.

Appropriate dietary management in different age groups.

Asymptomatic FAODs detected on newborn screening mandates a different approach based on the type of FAOD.

Status of the newer therapeutic agents in the overall management.

When not to supplement carnitine and medium-chain triglycerides (MCT).

Challenges in diagnosing FAOD

The common underlying factor in all FAODs is metabolic stress due to fasting, infection or muscular stress. During normalcy, these conditions will stimulate fatty acid oxidation for the ketone body and ATP generation. Defects in any of the enzymes involved in beta-oxidation will result in excess acylcarnitine intermediates accumulation and diversion to omega oxidation that would generate toxic dicarboxylic acids [7]. The probability of detecting abnormal fatty acid metabolites would be maximum during a metabolic crisis and finding a normal profile during a mild episode does not rule out FAOD. Hypoglycemia occurs due to increased glucose utilization and absent

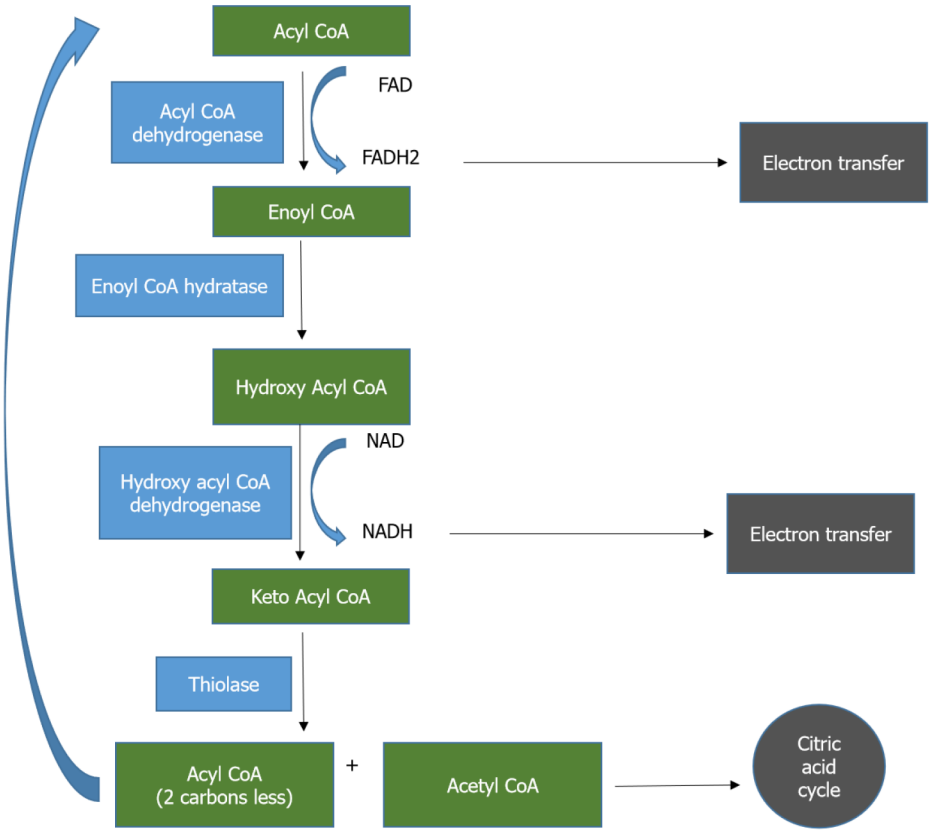

Figure 2 Beta-oxidation of fatty acids. CoA: Co-enzyme A; NAD: Nicotinamide adenine dinucleotide; NADH2: Nicotinamide adenine dinucleotide (reduced); FAD: Flavin adenine dinucleotide; FADH: Flavin adenine dinucleotide (reduced).

gluconeogenesis due to deficiency of acetyl CoA (generated during beta-oxidation) and it is typically hypoketotic. Acetyl CoA is also required for the generation of N-acetyl glutamate (NAG) that activates carbamoyl phosphate synthetase in the urea cycle. As a consequence, conversion of ammonia to urea is impaired which results in hyperammonemia. Since oxidative phosphorylation is impaired in FAOD, pyruvate oxidation takes place leading to lactic acidosis that is more pronounced in long-chain disorders. Acyl CoA accumulation causes lipid peroxidation and steatohepatitis[8].

Neonatal period: The predominant presentation in the first month of life is cardiac arrhythmias, conduction defects, hypotonia, lethargy, coma in the background of facial dysmorphism, hypoketotic hypoglycemia, hyperammonemia, lactic acidosis. Renal dysplasia and brain malformations may also accompany. Sudden death due to arrhythmias is seen in about 7% of cases with a neonatal presentation[9]. Hepatomegaly (93%), increase in transaminases (68%), cholestasis (36%) and liver failure (25%) are seen in the first two months of presentation[3].

Infancy: Hepatic involvement predominates during infancy when children present with Reye-like syndrome, hepatomegaly, hypoketotic hypoglycemia and steatosis. Hepatic failure may be encountered in 10% of infants and rarely cholestasis. Hyperammonemia, lactic acidosis, mild elevation of serum transaminases can be seen. Cardiomyopathy and skeletal myopathy can be seen in about 50%.

Childhood and adolescence: Apart from typical hepatic manifestations, muscular manifestations are frequently seen in older children with episodic muscular pains, rhabdomyolysis and myoglobinuria. Retinitis pigmentosa and peripheral neuropathy are specifically seen in those with LCHAD defects.

Predominant symptoms and biochemical findings in individual FAODs are given in Table 1. Though dividing the predominant manifestation as hepatic, neurological, muscular, cardiac and renal is easy to understand, a given case will have various permutations and combinations of these presentations. Moreover, most of the episodes may be triggered by an infection and differentiating between severe sepsis and FAOD also is a formidable task. The other metabolic diseases that closely resemble FAODs are urea cycle defects (UCD), mitochondrial hepatopathies, organic acidemias and

Table 1 Characteristic findings in clinically significant Fatty acid oxidation defects

Deficiency	Presentation	General biochemical abnormalities	Fatty acid metabolites	Gene	Newborn screen	Treatment
OCTN2	Hepatomegaly; Lethargy; Encephalopathy; Hypotonia	Raised transaminases; Hyperammonemia; Lactic acidosis	Low plasma acyl carnitine and free carnitine, high urine carnitine	*SLC22A5*	Low free carnitine	Carnitine supplementation
CPT-1	Reye-like syndrome; Renal tubular acidosis	Raised transaminases; Acidosis; Hyperammonemia	High free carnitine, low long chain acyl carnitine	*CPT-1A*	Increased free carnitine	Avoid fasting; Uncooked cornstarch; Medium chain triglyceride supplements
CACT	Coma; Cardiomyopathy; Apnoea; Seizures	Raised transaminases; Hyperammonemia; Elevated creatine phosphokinase	Low free carnitine, high long chain acyl carnitine, high urine dicarboxylic acid	*SLC25A20*	Increased long chain acyl carnitine	High carbohydrate low long chain fat diet; Medium chain triglyceride supplements; Carnitine
CPT-2	Encephalopathy; Hypotonia; Myalgia; Myoglobinuria; Neuronal migration defects; Cardiomegaly; Nephromegaly	Raised transaminases; Hyperammonemia; Elevated creatine phosphokinase	Low free carnitine, high long chain acyl carnitine, high urine dicarboxylic acid	*CPT-2*	High long chain acyl carnitine (C16, C16:1, C18, C18:1)	Intravenous glucose; Night-time feeds; High carbohydrate low long chain fat diet; Medium chain triglyceride supplements; Carnitine; Bezafibrate
VLCAD	Hypertrophic cardiomyopathy; Encephalopathy; Exercise induced myolysis	Raised transaminases; Hyperammonemia; Elevated creatine phosphokinase; High lactate	Low free carnitine, High C12, C14:1, C16 acylcarnitines.	*ACADVL*	High C12, C14:1, C16 acylcarnitine	Night-time feeds; High carbohydrate low long chain fat diet; Medium chain triglyceride supplements; Bezafibrate; Triheptanoin
MCAD	Vomiting; Lethargy; Encephalopathy	Hypoketotic hypoglycemia; Raised transaminases, ammonia, lactate, creatine phosphokinase, uric acid, blood urea nitrogen	High medium chain acyl carnitine; Free carnitine may be low or normal. Urine: Dicarboxylic acid, suberyl glycine, hexanoyl glycine	*ACADM*	High C6:0-, C8:0-, C10:0- and C10:1- acylcarnitines	Frequent feeding; Uncooked cornstarch
SCAD[1]	Hypotonia; Seizures; Failure to thrive; Behavioral disorders	Raised transaminases, ammonia, lactate, uric acid, blood urea nitrogen, mild acidosis	High C4 carnitine, ethymalonic acid in urine	*ACADS*	High C4 carnitine	Frequent feeding; Riboflavin; Carnitine
LCHAD	Hypotonia; Hepatomegaly; Rhabdomyolysis; Retinitis pigmentosa; Peripheral neuropathy; HELLP syndrome in mothers	Raised transaminases, ammonia, lactate, creatine phosphokinase	Low free carnitine; High hydroxyacyl carnitine; Hydroxydicarboxylic acid in urine	*HADHA*	High long chain hydroxyacyl carnitine	Intravenous glucose; Night-time feeds; Uncooked cornstarch; Medium chain triglyceride supplement; Docosahexaenoic acid supplement; Triheptanoin

[1]Clinical relevance is still ambiguous.

OCTN2: Organic cation transporter 2; CPT: Carnitine palmitoyltransferase; CACT: Carnitine acyl carnitine transporter; VLCAD: Very long chain acyl CoA dehydrogenase; MCAD: Medium chain acyl CoA dehydrogenase; SCAH: Short chain acyl CoA dehydrogenase; LCHAD: Long chain hydroxyl acyl CoA dehydrogenase.

gluconeogenic disorders. Characteristic hypoketotic hypoglycemia would be the first step to differentiate FAODs from the other disorders. Blood and urine samples for fatty acid metabolite estimation ought to be drawn before carnitine supplementation otherwise the results would be erroneous. Targeted gene panel analysis in children presenting with hypoglycemia can reliably diagnose inborn errors of metabolism including FAOD[10].

Management dilemmas

Since there are no large-scale trials on the management of individual FAODs, treatment recommendations are based on consensus by experts based on retrospective studies. The rarity of the individual FAODs precludes the conduction of randomized controlled trials. The cornerstone of treatment in all FAODs remains the prevention of hypoglycemia especially during times of metabolic stress so that beta-oxidation is not triggered. Recommendations and issues in the management of the discrete disorders

are given below.

Carnitine transport defect

Plasma carnitine levels are very low (< 5 μmol/L) and renal losses are high. L-carnitine supplementation at a dose of 100-400 mg/kg/d in three divided doses remains the mainstay of treatment[11]. Targeting the normal plasma carnitine level of 20 to 50 μmol/L is desirable by titrating the dose, however, large doses of carnitine can cause diarrhea and abdominal discomfort. Regular measurement of plasma carnitine levels during well periods should be done to determine the appropriate oral dosage. Even if plasma carnitine levels normalize with supplementation, intracellular carnitine levels in myocytes do not increase by more than 10% of normal due to the absence of active transport. The accurate plasma carnitine level that can ensure adequate passive diffusion into the myocytes to prevent complications is still unknown[12]. It is imperative to start carnitine supplementation before organ damage for favourable outcomes[13]. During periods of fasting, stress, infection and rigorous exercise, prevention of hypoglycemia by providing intravenous dextrose or uncooked cornstarch are recommended.

Carnitine shuttle defects

In children with CPT-1 deficiency MCT supplementation is essential as MCT can enter the mitochondrial without the carnitine shuttle. In the first year 2-3 g/kg/d of MCT and after infancy, 1-1.25 g/kg/d is recommended[9]. The clinical and biochemical features of CACT and CPT-2 deficiencies will mimic each other that can be confirmed by enzyme assay on cultured fibroblasts or genetic mutation analysis. Diet rich in complex carbohydrates, low in fat with MCT and carnitine supplementation during well periods and intravenous glucose, night-time drip-feeding during crisis time will avoid the accumulation of toxic intermediaries. Carnitine shuttle defects and transport defects are the only conditions in which carnitine supplementation is unambiguous. Emerging therapy in CPT-2 deficiency is bezafibrate which is a peroxisome proliferator-activated receptor (PPAR) alpha agonist. The activated PPAR alpha binds to PPAR responsive areas on the DNA and stimulates gene transcription. Thus, bezafibrate improves fatty oxidation in cells with mild deficiency of CPT-2 and not severe phenotypes with no residual activity[14].

VLCAD deficiency

In the early onset severe VLCAD deficiency characterized by predominant cardiac involvement aggressive treatment with intravenous glucose, MCT-based formula and intensive care need to be provided. The intermediate form of VLCAD deficiency with predominant hepatic manifestations ought to avoid prolonged fasting, continue on MCT supplements (20% of total energy) and restrict long-chain triglycerides (LCT) to 25%–30% of total energy. The myopathic form presents mostly in adolescents with episodic symptoms and they require pre-exercise MCT supplements (0.25 to 0.5g/kg). Administering oral glucose just before exercise may be detrimental as it will block gluconeogenesis. Restriction of exercise is not advocated. Carnitine supplementation in VLCAD deficiency is controversial as it may increase the plasma flux of toxic acyl carnitine intermediates causing cardiac arrhythmias and rhabdomyolysis[15]. This has been proven by studies on cardiomyocytes derived from skin fibroblasts of VLCAD deficient patients. They showed improvement in electrophysiological abnormalities after exposure to etomoxir (inhibitor of CPT-1) which reduced the production of long-chain acyl carnitines[16]. Thus, empirically starting carnitine in a child where FAOD is suspected without ascertaining the individual defect can be harmful. Bezafibrate has also been shown to improve the residual enzyme activity in VLCAD deficient fibroblasts however, in severe forms of VLCAD deficiency with no enzyme activity, bezafibrate is not useful[17]. Attempts to improve exercise tolerance by administering ester form of hydroxybutyrate as the ketone body donor which can be subsequently oxidized has yielded encouraging results in small studies[18]. The outcome of nutritional ketosis on other manifestations and long-term effects is still undetermined.

When VLCAD deficiency is suggested by newborn screening, there are several issues that need to be addressed. First is the accuracy of the test, tandem mass spectrometry is used throughout the world and due to the extreme sensitivity, a large number of heterozygous carriers are detected. Therefore, a positive screen has to be confirmed by enzyme assay in cultured fibroblasts or mutation analysis. The next dilemma is regarding the management of asymptomatic VLCAD deficiency as many of them continue to remain asymptomatic and the need for aggressive therapy is contentious. On the other hand, some may develop myopathic form later[19]. Hence,

in asymptomatic newborns continuation of breastmilk is advised if CPK and transaminases are normal. Whereas high MCT low LCT formula feeds are recommended if CPK or transaminase elevation is present[20].

MCAD deficiency

MCAD deficiency is the most common FAOD and manifests with hepatic and neurological features. Management revolves around avoidance of fasting, taking complex carbohydrates and during periods of crisis intravenous glucose[21]. There is no proven role for carnitine and MCT supplementation. Ammonia scavengers are added in cases with severe hyperammonemia and encephalopathy. Since there are no specific therapies compliance to dietary therapy assumes utmost importance. If detected on newborn screening, breastfeeding can be continued with avoidance of prolongation of feeding intervals. In all patients with FAOD, maximum periods of fasting during periods of wellness are as follows: Neonates-3 h, < 6 mo-4 h, 6 to 12 mo-4 h in the day time and 6 h at night, > 1 year–4 h in the day time and 10 h at night[22].

SCAD deficiency

The clinical significance of SCAD deficiency is questionable because of multiple reasons. Children suspected to have SCAD deficiency had hypotonia, epilepsy and developmental delay however, almost all of them remained asymptomatic at follow-up and some of them were later found to have a different etiology. Newborn screen positive SCAD deficient patients (with elevated C4-carnitine in blood and ethylmalonic acid in the urine) did not develop any abnormality on long-term follow-up[23].

LCHAD deficiency and TFP deficiency

Irrespective of symptoms, if LCHAD or TFP deficiency is detected in the newborn period, mother's milk has to be replaced with a special formula high in MCT and low in LCT. When complementary feeds are introduced fats should account for 25% to 30% of total energy, MCT should contribute to 20% to 25% of energy from fat and essential fatty acid should be about 3% to 4% and LCT should be restricted to the rest. Essential fatty acids ought to be supplemented with walnut, soy or wheatgerm oil at 3.5 g/d (< 4 mo), 5 g/d (4 to 12 mo), 6 g/d (1 to 4 years) and 10 g/d (> 4 years)[22]. Nasogastric feeding can be initiated during mild illness but intravenous glucose is necessary during more severe illnesses. Carnitine supplementation is controversial similar to that in VLCAD defect due to the risk of arrhythmia precipitation.

Docosahexaenoic acid supplementation at 60 mg/d in those < 20 kg and 120 mg/d in > 20 kg have been found to be beneficial in controlling neurological symptoms.

Triheptanoin is a novel therapeutic agent that acts as an anaplerotic metabolite by getting converted to citric acid cycle intermediates for energy production. Triheptanoin is a triglyceride with 7 carbon fatty acids that get metabolized to acetyl CoA and propionyl CoA. When taken at 3 to 4 mL/kg/d it has been shown to improve cardiac functions and reduce metabolic crisis[24]. However, making a universal recommendation based on small case series is precarious. The usefulness of triheptanoin has been demonstrated only in LCHAD and VLCAD deficiencies.

Thus, diagnosing and managing FAODs requires a highly nuanced approach and utmost compliance with dietary advice. Supplemental therapy with other agents has to be done after carefully considering the type of defect and the expected difference in the outcome.

UCD

Epidemiology

The incidence of UCD is difficult to deduce as newborn screening programs routinely screen for only arginosuccinate synthetase (ASS) deficiency and arginosuccinate lyase deficiency. The incidence of these two disorders is 1:117000, however, they account for only 30% of all UCDs. Based on newborn screening and natural history studies the combined incidence of all UCDs is estimated to be about 1 in 35000. Ornithine transcarbamoylase (OTC) deficiency accounts for 57%, carbamoyl phosphate synthetase 1 (CPS-1) deficiency for 8% and the others for 1%-2% of all UCDs[25]. Though the median age at diagnosis of UCD is 362 d, 25% were diagnosed only in adulthood. Those with late-onset symptoms had more delay in diagnosis of the underlying disorder. However, the patients with severe forms of UCDs may have died

before receiving a positive diagnosis. Of 343 UCD cases, 70% were diagnosed after the onset of symptoms, most of whom presented with an acute metabolic crisis[26]. Neonatal onset forms of UCDs have a survival ranging from 75% to 90% whereas late-onset forms have survival of > 90%[27].

Urea cycle in brief

The urea cycle metabolizes ammonia into urea (Figure 3). Ammonia is generated in the body from the breakdown of proteins and also from the gastrointestinal tract by bacterial metabolism. The first and the rate-limiting enzyme of the urea cycle is carbamoyl phosphate synthetase 1 which converts ammonia and bicarbonate into carbamoyl phosphate with the aid of NAG. N-acetyl glutamate synthase (NAGS) produces NAG from acetyl CoA and glutamate. NAG activates CPS-1. The second step of the urea cycle involves the synthesis of citrulline from carbamoyl phosphate and ornithine. The first two steps occur in the mitochondria. Citrulline is transported to the cytoplasm by ornithine transporter 1 (ORNT1). The next step is the production of argininosuccinate from citrulline and aspartate by argininosuccinate synthetase. Aspartate is transported from mitochondria to the cytoplasm by citrin. Arginosuccinate lyase breaks down argininosuccinate into arginine and fumarate. The final step is the hydrolysis of arginine to yield urea and ornithine by arginase 1. Ornithine re-enters the urea cycle by getting transported into the mitochondria by ORNT1 and urea gets excreted in the urine. Hyperammonemia is universal in all UCD and the manifestations are predominantly neurological. Liver involvement has been described in almost all the UCDs and they range from incidentally detected hepatomegaly to fatty liver, neonatal cholestasis, acute liver failure and cirrhosis[28].

Hurdles in diagnosing UCDs

UCDs can present in the neonatal period, childhood and rarely in adulthood for the first time. The disease spectrum varies from severely symptomatic form associated with high mortality to asymptomatic form depending on the carrier state and residual enzyme activity. Hyperammonemia has to be interpreted based on the age of the child as neonates normally have ammonia levels of 110 μmol/L whereas that in adults is 35 μmol/L[29]. When a child presents with encephalopathy and hyperammonemia, the possible causes are acute liver failure of any cause, urea cycle defect, fatty acid oxidation defect, organic acidemia, congenital lactic acidosis, lysinuric protein intolerance (LPI), carbonic anhydrase VA deficiency and portosystemic shunts. Differentiating each of these causes in an acute setting is challenging. UCDs typically do not have acidosis or hypoglycemia, respiratory alkalosis is more common. FAOD, congenital lactic acidosis and organic acidemia will be accompanied by high anion gap acidosis and hypoglycemia. UCDs can present with acute liver failure in 11% to 29% of cases. Cirrhosis also can occur in a proportion of UCDs especially in late-onset forms making the diagnosis extremely difficult for the unsuspecting clinician. Among the various UCDs, the specific defect can be diagnosed based on the profile of the different metabolites as shown in Figure 4. When citrulline is low with low orotic acid, the possibility is CPS-1 deficiency and NAGS deficiency, but if orotic acid is high OTC deficiency is to be considered. With high citrulline, ASS deficiency is the possibility with low argininoscuccinate and arginosuccinate lyase deficiency with high argininosuccinate. Arginase deficiency would have elevated arginine levels. In ornithine translocase deficiency ornithine and homocitrulline levels will be high. In cases with LPI, episodes are triggered by protein-rich meal akin to UCD but in addition, there will be diarrhea, nausea, abdominal pain, hepatosplenomegaly, cytopenia and increased urinary excretion of lysine. In LPI there is defective transport of lysine, ornithine and arginine, hence, there is secondary dysfunction of UCD due to deficiency of arginine and ornithine. Renal, lung, cardiovascular and immunological systems are also affected in LPI[30]. In carbonic anhydrase deficiency, there is impaired production of bicarbonate which is a substrate for CPS-1, propionyl CoA carboxylase, pyruvate carboxylase and 3-methyl crotonyl CoA carboxylase. Hence, apart from hyperammonemia, there will be lactic acidosis, ketonuria, hypoglycemia, respiratory alkalosis and metabolic acidosis[31]. Portosystemic shunts can be diagnosed with ultrasound Doppler or computed tomography. These children would also have hypoglycemia with hyperinsulinemia, high blood galactose levels, hepatopulmonary syndrome[32].

Thus, in a child with hyperammonemia, the initial set of investigations should include liver function test, prothrombin time, abdominal ultrasound with Doppler, arterial blood gas, lactate, blood glucose, urine ketones, plasma amino acids, urine organic acids and acylcarnitine profile. Probable diagnosis can be reached with these investigations which can be further confirmed by enzyme analysis or genetic mutation

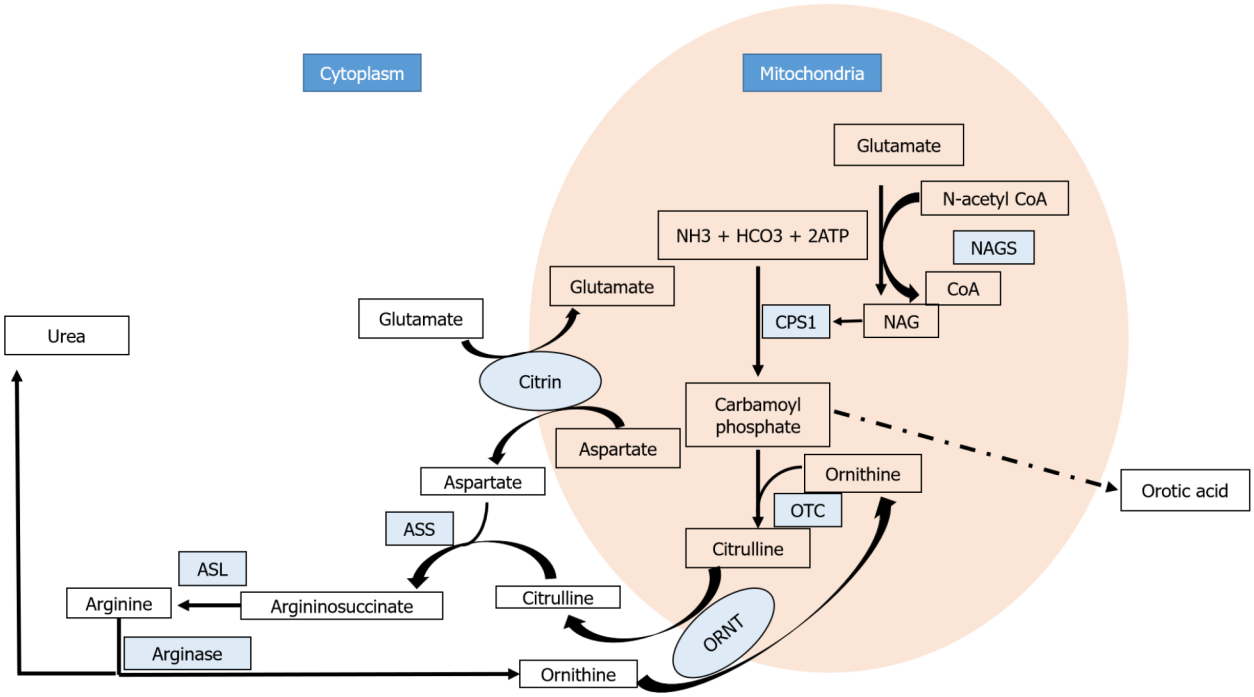

Figure 3 Urea cycle. NAGS: N-acetyl glutamate synthase; NAG: N-acetyl glutamate; CoA: Co-enzyme A; CPS-1: Carbamoyl phosphate synthetase 1; NH3: Ammonia; HCO3: Bicarbonate; ATP: Adenine triphosphate; OTC: Ornithine transcarbamoylase; ORNT: Ornithine transporter; ASS: Argininosuccinate synthetase; ASL: Argininosuccinate lyase.

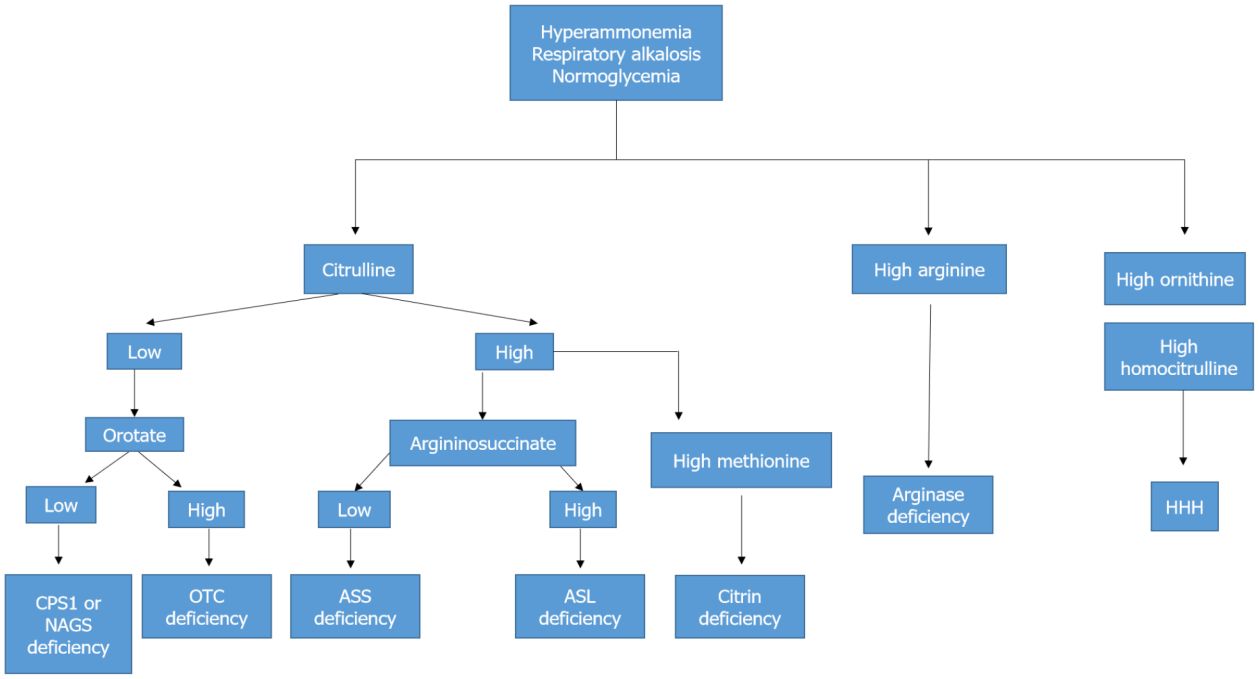

Figure 4 Approach to individual urea cycle defects. NAG: N-acetyl glutamate; CPS-1: Carbamoyl phosphate synthetase 1; OTC: Ornithine transcarbamoylase; ASS: Argininosuccinate synthetase; ASL: Argininosuccinate lyase; HHH: Hyperornithinemia-hyperammonemia-homocitrullinuria syndrome.

analysis.

Management dilemmas

The cornerstone of the management of UCDs is ammonia clearance and reverse catabolism. In the acute phase, intravenous sodium benzoate (250 mg/kg bolus followed by 250-500 mg/kg/d or 5.5 g/m^2/d in those above 20 kg) or sodium phenyl acetate are recommended. For the rapid reduction of ammonia, hemodialysis is

advisable especially if the ammonia level is higher than 500 μmol/L or if there is no reduction after 3 to 6 h of starting ammonia scavengers[33]. Preparedness for dialysis support is mandatory while waiting for a response from ammonia scavengers. Some children may develop relapse after discontinuation of dialysis hence, continuous hemodiafiltration is preferred. In a severely symptomatic neonate with very high ammonia values hemodiafiltration is the only effective option as peritoneal dialysis is less effective, but performing hemodialysis in a neonate is a formidable task and has to be performed in centers with expertise[34,35]. Arginine supplementation (250-400 mg/kg bolus followed by 250 mg/kg/d) has to be given in all UCDs except arginase deficiency. Citrulline supplementation can also increase ammonia excretion through the urea cycle. Oral administration of N-carbamyl glutamate activates CPS-1 similar to NAG. Catabolism reversal is best achieved by the stoppage of protein intake transiently while supplementing carbohydrates and lipids (after ruling out FAOD). However, prolonged discontinuation of proteins would stimulate endogenous protein break-down and hence, 0.1 to 0.3 g/kg/d of proteins have to be restarted from day 2 [36]. Though the approach seems quite simple there are several practical issues that need to be addressed during the acute presentation.

Acute presentation

Ammonia gets metabolized to glutamine in astrocytes in states of normalcy. When ammonia levels are very high the intracellular glutamine concentration in astrocytes increases. The osmotic effect of glutamine contributes to astrocyte swelling and cerebral edema. Fluid administration has to be diligent especially because ammonia scavengers are given in a large volume of fluid. In addition, massive fluid shifts can occur with sodium load when sodium benzoate and sodium phenyl acetate are administered. Hypokalemia and hyperchloremic metabolic acidosis are also known side effects[37]. Thus, close monitoring of electrolytes is recommended. Intravenous glucose infusion aimed at reversing catabolism can result in hyperglycemia which can further worsen cerebral edema. The use of insulin has to be considered in selected situations with close blood sugar monitoring. While the restriction of protein intake in the first 24 h is strongly agreed upon, the use of essential amino acid (EAA) infusion on the first day of decompensation as a nitrogen source to promote anabolism has been used in many centers. Acute hyperammonemia promotes branched-chain amino acid (BCAA) catabolism and ammonia scavengers will also deplete the BCAA pool, hence BCAA supplementation is beneficial[38,39]. Enteral feeding has to be always preferred over the parenteral route whenever possible. Arginine and citrulline promote ureagenesis however, administering arginine without establishing the type of UCD can be dangerous as it is contraindicated in arginase deficiency. Arginine is the precursor of nitric oxide and hence, has to be given with caution if the child has vasodilatation and hypotension. Since ammonia is extremely toxic to the brain irreversible neurological damage can occur. The criteria to be used to change treatment strategy from curative to palliative is still debatable. Presence of hyperammonemia for more than 3 d or if the absolute value of ammonia is more than 1000 μmol/L are regarded as poor prognostic factors for favourable neurological outcomes[33]. Electroencephalogram and magnetic resonance imaging will assist in predicting the potential of recovery. Continuous electroencephalogram monitoring in neonates with hyperammonemia and deep coma can detect clinically unapparent seizures[40]. The decision to continue or withdraw therapy in such situations must be taken after discussion with the family.

Asymptomatic patients

In an asymptomatic neonate with an affected sibling who had a neonatal presentation, glucose infusion ought to be started with 6 hly ammonia measurements. Protein-free feeds and low-dose sodium benzoate (50 mg/kg/d) can be initiated if the baby is asymptomatic after 4 h. If ammonia is consistently < 80 μmol/L, breastfeeds can be introduced after 24 h. If the sibling had a late-onset phenotype, breastfeeding can be given with periodic monitoring of ammonia and symptoms. The relevance of newborn screening in detecting UCDs is still ambiguous. On one hand, the severely affected babies become symptomatic even before the screening results would be available and on the other many positive cases may be detected who may remain asymptomatic throughout. The instability of glutamine and poor accuracy of citrulline levels make them poor markers for screening[41]. Arginine and Arginine succinoacetate levels are used in some countries for newborn screening however, many false-positive cases would add to the burden of further workup, parental stress and close monitoring[42]. The next dilemma is about managing asymptomatic carriers especially in those with OTC deficiency. Females with severe form may become symptomatic in 15% to 20% of

cases. Whether it is prudent to restrict proteins in the initial few years of life is a matter of debate. Relying entirely on genetic studies would be precarious as mosaicism due to lyonization can affect OTC activity. Measuring orotic acid levels after allopurinol administration can detect female carriers with better certainty and it is less dangerous than protein loading tests[43].

Chronic therapy

Long-term management goals revolve around preventing hyperammonemia, ensuring normal growth and development and dealing with periods of anticipated decompensation. Balancing adequate calorie intake with restriction of proteins requires very meticulous planning as the energy required for normal growth in UCD patients is the same as for normal children. The other practical difficulty in achieving optimal protein intake is the food refusal, protein aversions, nausea, vomiting and early satiety in children with UCD that would result in chronic protein deprivation [44]. Defining protein restriction is problematic as the requirement changes with age and state of catabolism. Recommended protein intake in normal children varies from 1.8 g/kg/d during early infancy to 0.8 g/kg/d in later childhood which would again increase during periods of crisis, pregnancy and lactation[45]. When protein intake in children with various UCDs with good metabolic control was assessed it was found that most of them consumed 1 to 1.8 g/kg/d of protein[38]. However, some centers prescribe 0.7 g/kg/d proteins[46]. Severe restriction of proteins will affect growth and cause deficiency of vitamins and minerals as well. EAA deficiency is a real problem due to multiple factors like highly restrictive diet, food aversions and EAA depletion by ammonia scavengers. Of the total protein intake, 20% to 30% can be provided as EAA which can be increased to 50% in disorders such as arginase deficiency. Another unanswered question is whether to administer EAA in addition to natural protein or replace a part of natural protein. BCAA supplementation also has wide variations across centers and the optimal dose is yet to be determined. Maintaining enough protein intake to ensure normal growth, prevent endogenous catabolism and also avoid hyperammonemia is akin to a tight rope walk. An absolute value cannot be recommended as requirements vary with age and clinical situations. Natural proteins, as well as EAA supplements, should be divided and consumed in a day. The last meal should provide 25% of total energy to avoid overnight catabolism.

Sodium benzoate or phenylacetate ought to be given with meals and adequate fluids to reduce mucositis and gastritis. BCAA depletion and hypokalemia can occur with chronic therapy also. Other side effects include loss of taste, unpleasant body odour and menstrual disturbance. When using arginine, plasma levels need to be maintained at 70 to 120 μmol/L. Citrulline is the precursor of arginine and both need not be given together. However, the advantage of one over the other in specific disorders is yet to be understood. Monotherapy with carbamyl glutamate is sufficient for NAGS deficiency hence, it is important to differentiate between CPS-1 and NAGS deficiencies which will mimic each other clinically and biochemically. Therefore, enzyme analysis or genetic testing is mandatory for delineation. Secondary carnitine deficiency can be there in children with UCDs and only in severe deficiency do carnitine supplements need to be given. The role of gut metronidazole or neomycin to reduce ammonia load from the colon is not convincing and is not routinely recommended[33].

Monitoring

Given the problems in the measurement of ammonia levels glutamine has been used in monitoring metabolic control with a target of < 1000 μmol/L. Glutamine has been considered as a portent of hyperammonemia. However, there are many inherent issues with using glutamine as the ideal metabolite for monitoring. Preventing permanent neurological damage is the ultimate objective of treating UCDs. Plasma glutamine levels do not correlate with brain glutamine concentration and may not indicate the actual exposure of the brain to ammonia. The ideal cut-off of glutamine as a measure of metabolic control is also uncertain as many children with glutamine well above 1000 μmol/L have not had decompensation. Measuring the levels of ammonia scavengers in blood has also been tried to avoid toxicity but the varying peaks and nadirs make this an unreliable method[47]. Apart from biochemical monitoring, developmental and behaviour assessment form a major part of routine evaluation. Periodic magnetic resonance imaging is necessary to predict objective outcomes.

Liver transplantation

Liver transplantation has the potential to be curative in UCDs and is the key to

attaining freedom from strict dietary plans. Quality of life and survival improves significantly and since the synthetic functions of the liver are preserved in most cases, long-term outcomes are good. Enzymes of the urea cycle are also expressed in the intestine, kidneys and brain which have physiological functions. Hence, it may be improper to call liver transplantation completely curative. Despite liver transplantation, plasma citrulline levels may be deficient in those with OTC and CPS-1 deficiencies who will require continuing supplementation with citrulline. To achieve maximum benefits from liver transplantation it is crucial to perform it before any irreversible neurological damage occurs. Survival with intact neurological functioning is better with late-onset forms. Thus, in early-onset forms, ideal age for liver transplantation is before 1 year. With the benefits come the challenges of performing transplantation in an infant which demands surgical and critical care expertise[48]. When UCDs present with acute liver failure or rarely with end-stage liver disease liver transplantation becomes the only therapeutic option. The timing of referral for transplantation in those with liver dysfunction (not yet causing liver failure or decompensation) is ambiguous as the UCD patients are also eligible for MELD exception points. The effect of medical management on the overall liver functioning in this situation is unknown. Hepatocyte transplantation and auxiliary partial orthotopic liver transplantation are emerging therapies in UCDs[49].

Evolving therapies for UCDs made a foray into clinical practice with much fanfare as these are ideal disorders for gene therapy[50]. However, fatal inflammatory complications in a subject related to the immunogenicity of the viral vector paused any further experimental therapies for a long period of time[51]. Enzyme replacement therapy in arginase deficiency is not yet a realistic goal in humans[52].

CONCLUSION

Despite making an accurate diagnosis of the disorder management of FAOD and UCD is dotted with ambiguity with respect to the available pharmacological options, strict dietary control and monitoring. We have come a long way in our understanding of metabolic liver diseases but fine-tuning management for optimum results is an ongoing exercise that requires expertise and further research.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Direct-acting antivirals for chronic hepatitis C treatment: The experience of two tertiary university centers in Brazil

Mariana Sandoval Lourenço, Patricia Momoyo Y Zitelli, Marlone Cunha-Silva, Arthur Ivan N Oliveira, Cláudia P Oliveira, Tiago Sevá-Pereira, Flair José Carrilho, Mario G Pessoa, Daniel F Mazo

ORCID number: Mariana Sandoval Lourenço 0000-0001-6749-316X; Patricia Momoyo Y Zitelli 0000-0002-2444-7937; Marlone Cunha-Silva 0000-0002-4424-6606; Arthur Ivan N Oliveira 0000-0002-5226-5823; Cláudia P Oliveira 0000-0002-2848-417x; Tiago Sevá-Pereira 0000-0002-3282-8019; Flair José Carrilho 0000-0002-7682-3105; Mario G Pessoa 0000-0002-0942-7348; Daniel F Mazo 0000-0002-2164-2630.

Author contributions: Lourenço MS and Mazo DF conceived the idea, designed the study, took care of patients, collected and assembled the data, contributed to the data analysis and interpretation and wrote the manuscript; Zitelli PMY, Cunha-Silva M and Oliveira AIN took care of patients, collected and assembled the data; Oliveira CP, Sevá-Pereira T, Carrilho FJ and Pessoa MG critically reviewed the manuscript; all authors approved the final version of the manuscript for publication.

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Mariana Sandoval Lourenço, Marlone Cunha-Silva, Tiago Sevá-Pereira, Daniel F Mazo, Division of Gastroenterology, Department of Internal Medicine, School of Medical Sciences, University of Campinas, Sao Paulo 13083-878, Brazil

Patricia Momoyo Y Zitelli, Arthur Ivan N Oliveira, Cláudia P Oliveira, Flair José Carrilho, Mario G Pessoa, Daniel F Mazo, Division of Clinical Gastroenterology and Hepatology, Department of Gastroenterology, University of São Paulo School of Medicine, Sao Paulo 05403-900, Brazil

Corresponding author: Daniel F Mazo, MD, PhD, Medical Assistant, Professor, Division of Gastroenterology, Department of Internal Medicine, School of Medical Sciences, University of Campinas, Rua Carlos Chagas 420, São Paulo 13083-878, Brazil. dmazo@unicamp.br

Abstract

BACKGROUND

Hepatitis C virus (HCV) treatment has undergone major changes in recent years. Previous interferon-based therapies have been replaced by oral direct-acting antivirals (DAA) regimens, with high sustained virologic response (SVR) rates, and a lower incidence of adverse events (AEs).

AIM

To evaluate the efficacy and safety of DAAs for HCV treatment in subjects from two tertiary university centers in Brazil.

METHODS

This is a multicenter retrospective cohort study of 532 patients with chronic hepatitis C (CHC), undergoing treatment with interferon-free regimens from November 2015 to November 2019. The therapeutic regimen was defined by the current Brazilian guidelines for HCV management at the time of treatment. Demographic, anthropometric, clinical, and laboratory variables were evaluated. SVRs were assessed at 12 to 24 wk after therapy by intention-to-treat (ITT), and modified ITT (m-ITT) analysis. AEs and serious adverse events (SAEs) were registered. In the statistical analysis, a P value of < 0.05 was considered significant.

RESULTS

The mean age was 56.88 years, with 415 (78.5%) being HCV genotype 1, followed by genotype 3 (20.1%). Moreover, 306 (57.5%) subjects had cirrhosis, and a third of

them had decompensated cirrhosis. Sofosbuvir (SOF) plus daclatasvir ± ribavirin was the most frequently used treatment (66.9%), followed by SOF plus simeprevir (21.2%). The overall ITT SVR was 92.6% (493/532), while the m-ITT SVR was 96.8% (493/509). Variables associated with treatment failure *via* ITT evaluation were hepatic encephalopathy (OR: 4.320; 95%CI: 1.920-9.721, $P = 0.0004$), presence of esophageal varices (OR: 2.381; 95%CI: 1.137-4.988, $P = 0.0215$), previous portal hypertensive bleeding (OR: 2.756; 95%CI: 1.173-6.471, $P = 0.02$), higher model for end-stage liver disease scores (OR: 1.143, 95%CI: 1.060–1.233, $P = 0.0005$), lower serum albumin levels (OR: 0.528, 95%CI: 0.322-0.867, $P = 0.0115$), higher serum creatinine (OR: 1.117, 95%CI: 1.056-1.312, $P = 0.0033$), and international normalized ratio (INR) levels (OR: 5.542, 95%CI: 2.023-15.182, $P = 0.0009$). AEs were reported in 41.1% (211/514) of patients, and SAEs in 3.7%. The female gender, higher body mass index, esophageal varices, higher INR values, and longer treatment duration were independently associated with AE occurrence.

CONCLUSION

Treatment with oral DAAs attains a high SVR rate, with fewer SAEs in a real-life cohort of subjects with CHC, from two tertiary university centers in Brazil.

Key Words: Chronic hepatitis C; Antiviral agents; Hepatitis C virus; Sustained virologic response; Liver cirrhosis; Safety

Core Tip: Hepatitis C virus treatment has recently undergone major changes. In this multicenter retrospective cohort study of 532 patients with chronic hepatitis C treated with oral direct-acting antiviral regimens, the overall intention-to-treat (ITT) sustained virologic response (SVR) was 92.6% (493/532), and the modified-ITT SVR was 96.8% (493/509). Advanced liver disease was related to treatment failure. Adverse events (AEs) were reported in 41.1% (211/514) of patients, and serious AEs in 3.7%. The female gender, higher body mass index, presence of esophageal varices, higher international normalized ratio values, and longer treatment were independently linked to AE occurrence.

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INTRODUCTION

Hepatitis C represents a global health problem. It is estimated that there are approximately 71 million people on a global basis who are chronically infected with the hepatitis C virus (HCV), with a prevalence of 1.1%[1]. However, many carriers are unaware of the infection, and do not receive treatment[2]. Despite the rising prevalence of metabolic-dysfunction associated fatty liver disease, HCV is a major cause of cirrhosis, and hepatocellular carcinoma (HCC) worldwide[3]. It is estimated that 0.53% of the total Brazilian population has antibodies against HCV, while in 2019, this virus was the leading cause of death for viral hepatitis in Brazil[4,5].

The main objective of therapy is the eradication of the virus, defined as sustained virologic response (SVR), associated with a reduction of liver inflammation and fibrosis, and the incidence of hepatic decompensation and HCC[6]. In addition, SVR leads to a decrease in mortality from both hepatic and non-hepatic causes[7,8].

HCV treatment has undergone major changes to date[9]. Previous interferon-based therapies with lower SVR rates and several adverse events (AEs)[9] were replaced by oral direct-acting antiviral (DAA) regimens, with SVR rates greater than 90% and a lower incidence of AEs[10-13]. Significant advances in the understanding and

participants.

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management of this disease started over twenty years ago[14]. These early efforts were recognized by the 2020 Nobel Prize in Physiology or Medicine[15].

In Brazil, acquiring and dispensing of all oral DAA regimens for patients with chronic hepatitis C (CHC) was provided through the Sistema Unico de Saude, the national public healthcare system[16]. There are few studies in large centers showing experience with DAAs in patients with chronic HCV infection. So, the aim of this study was to evaluate the efficacy and safety of DAAs for treatment of HCV-infected patients from two tertiary university centers in the southeastern region of the country.

MATERIALS AND METHODS

Study design and patient selection

This is a multicenter retrospective cohort study, carried out at the liver outpatient clinics of the Division of Gastroenterology at the University of Campinas (UNICAMP), and the Department of Gastroenterology at the University of São Paulo School of Medicine (FMUSP) for patients with CHC, who underwent treatment with interferon-free regimens from November 2015 to November 2019.

Inclusion criteria were: (1) age ≥ 18 years and the presence of CHC, defined by HCV RNA positivity through a polymerase chain reaction (PCR) for at least 6 mo, regardless of the HCV genotype; and (2) those treated with oral DAAs. Exclusion criteria were: diagnosis of any other liver disease, human immunodeficiency virus, or hepatitis B virus coinfection, active HCC, liver transplant recipients, previous treatment for HCV with interferon-free regimens, and lack of information on the current HCV treatment.

HCV treatment regimen

The therapeutic regimen was defined by the current Brazilian guidelines for HCV management at the time of treatment[16-18]. HCV therapy was composed of the DAAs: sofosbuvir (SOF), daclatasvir (DCV), simeprevir (SMV), ledipasvir, and the combined regimen ombitasvir plus veruprevir/ritonavir plus dasabuvir. Ribavirin (RBV) was also used. Relevant drug-drug interactions were checked prior to use of DAAs.

Variables evaluated

Demographic and anthropometric variables [age, gender, body mass index (BMI)], presence of comorbidities (arterial hypertension, diabetes mellitus, dyslipidemia, hypothyroidism, psychiatric disorders, previous alcohol use), and laboratory variables were evaluated through computerized medical records. Serum biochemical assessment was conducted before treatment, and 12 to 24 wk after the end of treatment, as per routine clinical practice. Serum HCV-RNA levels were assessed with real-time PCR and the Amplicor HCV Monitor 2.0 test (Abbott Molecular, Des Plaines, IL, United States, detection limit: 12 IU/mL). Viral genotyping was performed with Versant® HCV Genotype 2.0 LiPA test (Imunogenetics, Ghent, Belgium).

The staging of hepatic fibrosis was assessed prior to treatment with histology, according to the Metavir classification, or use of non-invasive methods (transient elastography, APRI, and FIB-4). In patients with cirrhosis, Child-Pugh and model for end-stage liver disease (MELD) scores were also assessed.

HCV treatment efficacy analysis

SVR was defined as undetectable HCV-RNA at 12 or 24 wk following treatment. An intention-to-treat (ITT) analysis was performed, considering patients who abandoned treatment, were lost to follow-up, or did not have complete information about their medical records, seen as virologic failures. A modified intention-to-treat (m-ITT) analysis was carried out, excluding efficacy for patients lost to follow-up, or who discontinued therapy, or any deaths unrelated to treatment or its adverse events.

Safety assessment

The analysis of AE was classified according to the Common Terminology Criteria for Adverse Events[19]. Management of anemia was considered with a drop in hemoglobin (Hb) greater than 3 points or associated symptoms if Hb > 10 g/dL. Anemia was classified into grade 1 (Hb 10-8 g/dL), grade 2 (Hb < 8 g/dL or need for a blood transfusion), grade 3 (risk of death), and grade 4 (death). Serious adverse events (SAEs) were considered: (1) hepatic decompensation; (2) need for hospitalization; (3) need to discontinue treatment; and (4) events resulting in death[20].

Ethical aspects

This study was approved by the Ethics Committee of UNICAMP and Clinics Hospital of FMUSP (Approval No. 2042967 and 2670862, respectively). The protocol was conducted in accord with the ethical guidelines of the 2013 World Medical Association Declaration of Helsinki[21]. Informed consent was waived for participants.

Statistical analysis

To describe the sample according to the variables under study, frequency tables of categorical variables with absolute frequency (*n*) and percentage (%) values, as well as descriptive statistics of numerical variables, with mean and standard deviation were used. To assess the relationship between categorical variables, the Chi-square test and, when necessary, Fisher's exact test were used. For numerical variables, the Mann-Whitney test was utilized. To assess factors related to treatment failure and AEs, univariate and multivariate logistic regression was performed whenever methodologically feasible. The selection of variables in the multivariate logistic regression analysis was done in a stepwise manner. Odds ratio (OR) and 95%CI were calculated. A P value of < 0.05 was considered significant. The Statistical Analysis System (SAS) for Windows software package, version 9.4 (SAS Institute Inc, 2002-2008, Cary, NC, United) was used for statistical analyses by biomedical statisticians from the Statistics Service at the School of Medical Sciences of the University of Campinas.

RESULTS

Baseline characteristics

A total of 532 patients treated with DAAs were included in the study. There was a slight predominance of males, with the mean age of 56.88 years. The mean BMI was 27.01, with most patients having comorbidities, mainly arterial hypertension and diabetes mellitus. There was a predominance of patients with HCV genotype 1 (78.5%), followed by genotype 3 (20.1%). Over 50% of patients were treatment-experienced. Three hundred six (57.5%) patients had cirrhosis, and a third had decompensated liver disease. The main baseline for demographic, clinical, and laboratory characteristics of the study population are shown in Table 1.

HCV therapeutic regimens and efficacy analysis

The combination of SOF plus DCV, associated with RBV, was the most frequently used treatment (66.9%), followed by SOF plus SMV (21.2%). Table 2 shows HCV treatment regimens in the study population. The overall ITT SVR rate was 92.6% (493/532), and the m-ITT SVR rate was 96.8% (493/509). Twenty-three patients were lost to follow-up, with no conclusive SVR data at the end of treatment (Table 3). In the ITT analysis, pretreatment variables related to treatment failure were a higher MELD score (P = 0.001), higher serum levels of aspartate aminotransferase (AST) and international normalized ratio (INR) (P = 0.043 and P = 0.023, respectively), lower serum albumin levels (P = 0.032), and a higher frequency of liver-related complications [hepatic encephalopathy (P = 0.001), esophageal varices (P = 0.018), and previous portal hypertensive bleeding (P = 0.039)], as shown in Table 4.

When assessing m-ITT SVR, the presence of cirrhosis (P = 0.049), higher serum values of AST (P = 0.030), higher MELD scores (P = 0.004), presence of hepatic encephalopathy (P = 0.006), and male gender (P = 0.049) negatively impacted SVR achievement. Genotype 3 HCV patients had numerically lower SVR rates than non-genotype 3 subjects (93% *vs* 97.7%-100%), almost reaching statistical significance (P = 0.055). In the univariate logistic regression analysis, baseline variables associated with treatment failure by ITT evaluation were: hepatic encephalopathy (OR: 4.320; 95%CI: 1.920-9.721, P = 0.0004), presence of esophageal varices (OR: 2.381; 95%CI: 1.137-4.988, P = 0.0215), previous portal hypertensive bleeding (OR: 2.756; 95%CI: 1.173-6.471, P = 0.02), higher MELD scores (OR: 1.143, 95%CI: 1.060–1.233, P = 0.0005), lower serum albumin levels (OR: 0.528, 95%CI: 0.322-0.867, P = 0.0115), higher serum creatinine (OR: 1.117, 95%CI: 1.056-1.312, P = 0.0033), and INR levels (OR: 5.542, 95%CI: 2.023-15.182, P = 0.0009), shown in Table 5. It was not possible to perform multivariate logistic regression analysis, due to the low occurrence of non-responders and missing data.

Safety analysis

AEs were reported in 41.1% (211/514) of patients. The most frequent AE was fatigue,

Table 1 Characteristics of patients with hepatitis C virus (*n* = 532)

	HCV patients (*n* = 532), % (*n*) or mean ± SD
Age (yr)	56.88 ± 11.08
Men/women	51.1% (272)/48.9% (260)
High-blood pressure	44.1% (231/524)
Type 2 diabetes	29.7% (156/526)
BMI (*n* = 442)	27.01 ± 4.99
Dyslipidemia	20.8% (109/524)
Hypothyroidism	16.2% (85/524)
Psychiatric disorder	10.2% (53/521)
Previous alcohol use	19.2% (102/523)
HCV genotype	
1A	37.0% (197)
1B	38.0% (202)
1 non-classified	3.5% (19)
2	0.8% (4)
3	20.1% (107)
4	0.4% (2)
5	0.2% (1)
HCV viral load (log IU/mL)	5.78 ± 0.75
Previous HCV treatment (*n* = 525)	
None	46.3% (243)
Peg-IFN + RBV	46.1% (242)
Peg-IFN + PI	7.6% (40)
Liver fibrosis (Metavir classification)	
F0/F1	8.8% (47)
F2	13.0% (69)
F3	20.7% (110)
F4	57.5% (306)
Liver-related complications (*n* = 306)	
Ascites	30.3% (93)
Esophageal varices	72.0% (221)
Portal hypertensive bleeding	15.6% (48)
Hepatic encephalopathy	14.7% (45)
AST (U/L)	61.41 ± 45.06
ALT (U/L)	61.87 ± 58.29
Total bilirubin (mg/dL)	1.13 ± 1.08
Albumin (g/dL)	4.15 ± 2.64
Platelets (/mm^3)	141.82 ± 77.21
INR	1.18 ± 0.23
Creatinine (mg/dL)	1.31 ± 1.96
Hemoglobin (g/dL)	13.73 ± 1.90
Alpha-fetoprotein (ng/mL)	23.59 ± 103.61

Child-Pugh classification (n = 306)	
A	66.6% (204)
B or C	27.7% (85)
Non-classified	5.7% (17)
Child-Pugh Score (n = 289)	5.94 ± 1.54
MELD Score	10.17 ± 3.95

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BMI: Body mass index; HCV: Hepatitis C virus; INR: International normalized ratio; MELD: Model for end-stage liver disease; Peg-IFN: Pegylated-interferon; PI: Protease inhibitor (boceprevir or telaprevir); RBV: Ribavirin.

Table 2 Hepatitis C virus therapeutic regimens (n = 532)

Features of treatment	% (n)
Regimens	
SOF + DCV + RBV	49.2% (262)
SOF + SMV	21.2% (113)
SOF + DCV	17.7% (94)
SOF + SMV + RBV	8.1% (43)
3D	2.3% (12)
3D + RBV	0.6% (3)
SOF + RBV	0.6% (3)
SOF + LED	0.2% (1)
SOF + LED + RBV	0.2% (1)
Duration	
12 wk	77.2% (411)
24 wk	22.8% (121)
Use of RBV	58.6% (312)
Dose of RBV (mg/kg/day) mean ± SD	12.11 ± 3.01

3D: Veruprevir/ritonavir, ombitasvir and dasabuvir; HCV: Hepatitis C virus; LED: Ledipasvir; RBV: Ribavirin; SMV: Simeprevir; SOF: Sofosbuvir; DCV: Daclatasvir.

present in 140 patients (27.7%), followed by anemia in 87 patients (17.2%) and headache in 47 patients (9.3%). Anemia or a fall in hemoglobin ≥ 2 g/dL points occurred in 87 patients (17.2%). Of these, only one patient did not use RBV, and already had a hemoglobin of 11.2 g/dL before treatment. Of 312 patients who used RBV, 86 (27.5%) had a drop in hemoglobin during treatment, 61 required a dose reduction, and 15 had RBV suspended. Five patients required treatment with erythropoietin, and 4 required blood transfusion. SAE occurred in 20 patients (3.7%), as 14 had decompensation of their liver disease, with 7 hospitalized. Three patients died during the study evaluation.

In the univariate logistic regression analysis, baseline factors associated with the occurrence of AE were female gender (OR: 1.718, 95%CI: 1.205-2.450, P = 0.0028), higher BMI (OR: 1.060, 95%CI: 1.019-1.102, P = 0.0040), presence of cirrhosis (OR: 2.127, 95%CI: 1.476-3.065, P < 0.0001), liver-related complications [ascites (OR: 2.187, 95%CI: 1.352-3.536, P = 0.0014), hepatic encephalopathy (OR: 3.524, 95%CI: 1.762-7.044, P = 0.0004), and the presence of esophageal varices (OR: 2.795, 95%CI: 1.874-4.169, P ≤ 0.0001)], higher MELD (OR: 1.071, 95%CI: 1.019-1.126, P = 0.0073) and Child-Pugh scores (OR: 1.196, 95%CI: 1.014-1.410, P = 0.0332), lower serum albumin (OR: 0.432, 95%CI: 0.314-0.595, P < 0.0001), higher bilirubin (OR: 1.283, 95%CI: 1.052-1.564, P = 0.0138) and INR values (OR: 3.835, 95%CI: 1.539-9.560, P = 0.0039), higher RBV daily dose (OR: 1.249, 95%CI: 1.132-1.379, P < 0.0001), and longer treatment duration (OR:

Table 3 Sustained virologic response rates according to therapeutic regimens, hepatitis C virus genotypes and cirrhosis

SVR	ITT (*n* = 532), % (*n*)	m-ITT (*n* = 509), % (*n*)
Global	92.6% (493/532)	96.8% (493/509)
Genotype		
1	93.7% (392/418)	97.7% (392/401)
2	100% (4/4)	100% (4/4)
3	87.8% (94/107)	93.0% (94/101)
4	100% (2/2)	100% (2/2)
5	100% (1/1)	100% (1/1)
Treatment regimen		
3D ± RBV	100% (15/15)	100% (15/15)
SOF + DCV	86.1% (81/94)	95.2% (81/85)
SOF + DCV + RBV	94.2% (247/262)	97.2% (247/254)
SOF + RBV	100% (3/3)	100% (3/3)
SOF + SMV	92% (104/113)	96.3% (104/108)
SOF + SMV + RBV	95.3% (41/43)	97.6% (41/42)
SOF + LED ± RBV	100% (2/2)	100% (2/2)
Presence of cirrhosis		
No	94.6% (214/226)	98.6% (214/217)
Yes	91.1% (279/306)	95.5% (279/292)

3D: Veruprevir/ritonavir, ombitasvir and dasabuvir; DCV: Daclatasvir; HCV: Hepatitis C virus; ITT: Intention-to-treat; m-ITT: Modified intention-to-treat; LED: Ledipasvir; RBV: Ribavirin; SMV: Simeprevir; SOF: Sofosbuvir; SVR: Sustained virologic response.

1.071, 95%CI: 1.034-1.109, P = 0.0001), as shown in Table 6. Factors independently associated with AE occurrence were female gender (OR: 2.191, 95%CI: 1.145-1.192, P = 0.0178), higher BMI (OR: 1.107, 95%CI: 1.038-1.180, P = 0.0020), presence of esophageal varices (OR: 3.463, 95%CI: 1.688-7.105, P = 0.0007), higher INR values (OR: 3.748, 95%CI: 1.060-13.251, P = 0.0403), and longer treatment duration (OR: 1.062, 95%CI: 1.003-1.125, P = 0.0406).

DISCUSSION

This study evaluated the efficacy and safety of all oral DAAs for CHC treatment in a cohort of 532 patients, followed at two Brazilian tertiary university centers. Most patients were HCV genotype 1, with a slight predominance of males, similar to that found in other studies carried out in our country[22-24].

Our results show the high effectiveness of DAAs in this real-life cohort, reaching global SVR rates of 92.6% in the ITT analysis, and 96.8% in the m-ITT evaluation. However, lower rates of m-ITT SVR were observed in patients with cirrhosis. Advanced liver disease negatively impacts response to treatment, especially in those with decompensated disease, who are considered a more difficult group to treat[25-27]. Although SOF plus DCV was the most common regimen in the present study, it is interesting to cite that other effective treatments have been made available, such as SOF plus velpatasvir, elbasvir plus grazoprevir and glecaprevir plus pibrentasvir, for both naïve and DAA experienced patients[6]. SOF plus velpatasvir plus RBV was effective in patients with decompensated cirrhosis[6]. Treatments with drug combinations might be important to ultimately control the emergence of resistance-associated substitutions, and as rescue therapy for non-responders[6,28].

In the m-ITT SVR analysis, HCV genotype 3 individuals had an almost significant lower SVR rate in comparison to non-genotype 3 patients. Published data show that HCV genotype 3, previously considered an "easy to treat" genotype in the interferon era, with cure rates of up to 70%, turned out to be more challenging in the DAA era,

Table 4 Variables associated with sustained virologic response by intention-to-treat analysis (*n* = 532)

	SVR (*n* = 493), % (n) or mean ± SD	Non-SVR (*n* = 39), % (n) or mean ± SD	*P* value
Age (yr)	56.85 ± 10.96	57.23 ± 12.61	0.811
Men/women	50.1% (247)/49.9% (246)	64.1% (25)/35.9% (14)	0.092
High-blood pressure	44.0% (214/486)	44.7% (17/38)	0.932
Type 2 diabetes	29.9% (146/488)	26.3% (10/38)	0.639
BMI (*n* = 442)	26.97 ± 5.01	27.59 ± 4.65	0.459
Dyslipidemia	20.0% (97/486)	31.6% (12/28)	0.089
Hypothyroidism	15.8% (77/486)	21.1% (8/38)	0.401
Psychiatric disorder	10.1% (49/484)	10.8% (4/37)	0.781
Previous alcohol use	18.6% (90/485)	31.6% (12/38)	0.051
HCV genotype			
1	79.5% (392)	66.7% (26)	0.083
3	19.1% (94)	33.3% (13)	
Other	1.4% (7)	0% (0)	
Previous HCV treatment			
None	45.5% (222)	56.8% (21)	0.409
Peg-IFN + RBV	46.7% (228)	37.8% (14)	
Peg-IFN + PI	7.8% (38)	5.4% (2)	
Liver fibrosis (Metavir classification)			
F0 / F1	8.6% (42)	7.7% (3)	0.218
F2	13.6% (67)	10.3% (4)	
F3	22.2% (109)	10.3% (4)	
F4	55.6% (273)	71.8% (28)	
Presence of cirrhosis			
No	43.4% (214)	30.8% (12)	0.124
Yes	56.6% (279)	69.2% (27)	
Liver-related complications			
Ascites	20.3% (84/413)	25.7% (9/35)	0.451[a]
Esophageal varices	47.8% (197/412)	68.6% (24/35)	0.018[a]
Portal hypertensive bleeding	9.7% (40/412)	22.9% (8/35)	0.039[a]
Hepatic encephalopathy	8.5% (35/413)	28.6% (10/35)	0.001[a]
AST (U/L)	60.41 ± 44.53	74.51 ± 50.36	0.043[a]
ALT (U/L)	60.78 ± 55.75	76.19 ± 84.74	0.434
Total bilirubin (mg/dL)	1.12 ± 1.09	1.24 ± 0.95	0.384
Albumin (g/dL)	4.18 ± 2.73	3.70 ± 0.77	0.032[a]
Platelets (/mm^3)	142.64 ± 78.05	131.10 ± 65.19	0.532
INR	1.17 ± 0.21	1.32 ± 0.40	0.023[a]
Creatinine (mg/dL)	1.23 ± 1.74	2.32 ± 3.77	0.694
Hemoglobin (g/dL)	13.74 ± 1.86	13.65 ± 2.34	0.798
Alpha-fetoprotein (ng/mL)	24.3 ± 107.22	13.20 ± 16.37	0.412
Child-Pugh classification (%)			

A	72.0% (190)	56.0% (14)	0.124
B	25.8% (68)	44.0% (11)	
C	2.3% (6)	0% (0)	
Child-Pugh Score	5.91 ± 1.49	6.28 ± 2.01	0.109
MELD Score	9.98 ± 3.82	12.70 ± 4.81	0.001[a]
Dose of ribavirin (mg/kg/day)	12.11 ± 3.01	12.07 ± 2.98	0.946

[a]*P* value < 0.05. Chi-square test, Fisher's exact test, and Mann-Whitney test. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BMI: Body mass index; HCV: Hepatitis C virus; INR: International normalized ratio; ITT: Intention-to-treat; Peg-IFN: Pegylated-interferon; PI: Protease inhibitor (boceprevir or telaprevir); RBV: Ribavirin; SVR: Sustained virologic response.

with lower SVR rates compared to other HCV genotypes[26,27]. HCV genotype 3 interferes with the metabolism of lipids and glucose, and is associated with an increased risk of progressing to cirrhosis and HCC, which may negatively impact SVR rates[29,30]. SOF plus pegylated-interferon and RBV for 12 wk is also a treatment option in HCV genotype 3 patients[31-33].

In the m-ITT SVR analysis, we observed that HCV therapeutic failure was linked to male gender, higher MELD scores and AST values, presence of cirrhosis and hepatic encephalopathy. In the ITT analysis, lower serum albumin, higher creatinine and INR levels, history of hepatic encephalopathy, esophageal varices, and upper gastrointestinal bleeding were related to lower SVR rates. Several of these factors reflect advanced liver disease. Less severe disease, with lower Child-Pugh scores and serum bilirubin values and higher albumin levels, were associated with a greater chance of achieving SVR[34,35]. A large Spanish cohort of over 3000 patients showed that high values of transient elastography, cirrhosis, serum levels of bilirubin, and albumin values < 3.5 g/dL were significantly associated with therapeutic failure[36]. In Brazil, a 2018 study of 527 patients showed that in those with cirrhosis, the factors associated with SVR were lower MELD scores, higher albumin values, and glomerular filtration rates[37].

AEs were present in 41.1% of the study population, the main ones being fatigue, anemia, and headache; while only 3.7% subjects had SAEs. Our results reinforce the safety of DAAs for the treatment of hepatitis C in the Brazilian population. However, our AE rates were lower than the rates reported in other studies in our country, in which AEs are described in up to 90% of treated patients[38], and SAEs in up to 8.5% of cases[39]. The retrospective study design and possible underreport of AEs on medical charts, could justify the lower AE rate in the present cohort. In addition, more than half of our study population included patients without cirrhosis or with compensated liver disease, in which treatment is safer[39,40]. In our study, the main factors associated with the occurrence of AEs in the univariate logistic regression analysis were female gender, higher BMI, higher Child-Pugh and MELD scores, presence of cirrhosis and its complications, lower values of albumin, higher values of bilirubin and INR, use of ribavirin, and longer treatment. Patients with decompensated cirrhosis, in addition to having lower SVR rates, were also more likely to experience treatment-related AEs, which leads to earlier discontinuation of therapy and, in part, justifies its lower efficacy[41]. In Brazil, a study with 214 patients showed that the factors of HCV treatment discontinuation were advanced age, multiple comorbidities, higher MELD score, higher fibrosis index, and lower hemoglobin[39].

This study has some limitations. Despite the large number of patients, it is a retrospective study, relying on data from medical records. In addition, it was carried out in two public reference centers for hepatology and liver transplantation, which may incur a selection bias for more severe patients. Yet, this study evaluated a large Brazilian cohort of patients with decompensated cirrhosis.

CONCLUSION

In conclusion, in this cohort of patients with CHC followed at two public healthcare facilities in the southeastern region of Brazil, treatment with DAAs proved to be effective, with global SVR rates above 92%, and safe, with a low occurrence of SAEs.

Table 5 Factors associated with failure to achieve sustained virologic response by intention-to-treat analysis

Variable	Univariate analysis		
	OR	95%CI	*P* value
Age	1.003	0.974-1.033	0.8349
Men	1.811	0.814-4.030	0.1458
High-blood pressure	1.029	0.530-1.999	0.9328
Type 2 diabetes	0.837	0.396-1.767	0.6400
BMI	1.024	0.954-1.099	0.5120
Dyslipidemia	1.851	0.902-3.800	0.0934
Hypothyroidism	1.416	0.626-3.206	0.4036
Psychiatric disorder	1.076	0.366-3.165	0.8940
Previous alcohol use	2.026	0.985-4.167	0.0551
Presence of cirrhosis	1.726	0.854-3.486	0.1281
Liver-related complications			
Ascites	1.356	0.612-3.003	0.4526
Esophageal varices	2.381	1.137-4.988	0.0215[a]
Portal hypertensive bleeding	2.756	1.173-6.471	0.0200[a]
Hepatic encephalopathy	4.320	1.920-9.721	0.0004[a]
AST	1.006	1.000-1.012	0.0707
ALT	1.003	0.999-1.007	0.1402
Total bilirubin	1.085	0.846-1.392	0.5198
Albumin	0.528	0.322-0.867	0.0115[a]
Platelets	1.000	1.000-1.000	0.3813
INR	5.542	2.023-15.182	0.0009[a]
Creatinine	1.117	1.056-1.312	0.0033[a]
Hemoglobin	0.975	0.818-1.161	0.7741
Alpha-fetoprotein	0.995	0.980-1.010	0.5055
Child-Pugh score	1.166	0.901-1.511	0.2431
MELD score	1.143	1.060-1.233	0.0005[a]
Ribavirin dose	0.996	0.832-1.192	0.9614
Treatment duration	1.024	0.962-1.090	0.4563

[a]*P* value < 0.05. Univariate logistic regression. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BMI: Body mass index; HCV: Hepatitis C virus; INR: International normalized ratio; MELD: Model for end-stage liver disease; OR: Odds ratio; SVR: Sustained virologic response.

Table 6 Factors associated with the occurrence of adverse events during treatment

Variable	Univariate analysis			Multivariate analysis		
	OR	95%CI	*P* value	OR	95%CI	*P* value
Age	1.011	0.994-1.027	0.2086			
Women	1.718	1.205-2.450	0.0028[a]	2.191	1.145-1.192	0.0178[a]
High-blood pressure	0.838	0.587-1.197	0.3315			
Type 2 diabetes	0.994	0.675-1.461	0.9736			
BMI	1.060	1.019-1.102	0.0040[a]	1.107	1.038-1.180	0.0020[a]
Dyslipidemia	0.714	0.458-1.114	0.1377			
Hypothyroidism	0.971	0.603-1.565	0.9053			
Psychiatric disorder	1.309	0.732-2.340	0.3633			
Previous alcohol use	0.953	0.610-1.488	0.8307			
Presence of cirrhosis	2.127	1.476-3.065	< 0.0001[a]			
Liver-related complications						
Ascites	2.187	1.352-3.536	0.0014[a]			
Esophageal varices	2.795	1.874-4.169	< 0.0001[a]	3.463	1.688-7.105	0.0007[a]
Portal hypertensive bleeding	1.747	0.946-3.228	0.0749			
Hepatic encephalopathy	3.524	1.762-7.044	0.0004[a]			
AST	1.000	0.997-1.004	0.8303			
ALT	0.999	0.996-1.002	0.4848			
Total bilirubin	1.283	1.052-1.564	0.0138[a]			
Albumin	0.432	0.314-0.595	< 0.0001[a]			
INR	3.835	1.539-9.560	0.0039[a]	3.748	1.060-13.251	0.0403[a]
Creatinine	0.934	0.845-1.032	0.1818			
Hemoglobin	0.951	0.866-1.044	0.2910			
Alpha-fetoprotein	0.998	0.993-1.003	0.3656			
Child-Pugh score	1.196	1.014-1.410	0.0332[a]			
MELD score	1.071	1.019-1.126	0.0073[a]			
Ribavirin dose	1.249	1.132-1.379	< 0.0001[a]			
Treatment duration	1.071	1.034-1.109	0.0001[a]	1.062	1.003-1.125	0.0406[a]

[a]*P* value < 0.05. Logistic regression. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BMI: Body mass index; HCV: Hepatitis C virus; INR: International normalized ratio; MELD: Model for end-stage liver disease; OR: Odds ratio.

ARTICLE HIGHLIGHTS

Research background

Hepatitis C represents a global health problem and a major cause of cirrhosis, and hepatocellular carcinoma. Hepatitis C virus (HCV) treatment has undergone major changes in recent years with the advent of direct-acting antivirals (DAA) regimens.

Research motivation

In Brazil, acquiring and dispensing of all oral DAA regimens for patients with chronic hepatitis C (CHC) is provided through the national public healthcare system. However, there are few studies in large centers showing experience with DAAs in patients with chronic HCV infection.

Research objectives
We aimed to evaluate the efficacy and safety of DAAs for HCV treatment in subjects from two tertiary public university centers in the southeastern region of Brazil.

Research methods
We evaluated 532 adult patients with CHC who underwent treatment with interferon-free regimens from November 2015 to November 2019. Demographic, anthropometric, clinical, and laboratory variables were evaluated. Sustained virologic response (SVR) rates were assessed at 12 to 24 wk after therapy by intention-to-treat (ITT), and modified ITT (m-ITT) analysis. Adverse events (AEs) and serious adverse events (SAEs) were registered.

Research results
Sofosbuvir (SOF) plus daclatasvir ± ribavirin was the most frequently used treatment (66.9%), followed by SOF plus simeprevir (21.2%). The overall ITT SVR was 92.6% (493/532), while the m-ITT SVR was 96.8% (493/509). Variables associated with treatment failure *via* ITT evaluation were hepatic encephalopathy, presence of esophageal varices, previous portal hypertensive bleeding, higher model for end-stage liver disease scores, lower serum albumin levels, and higher serum creatinine and international normalized ratio (INR) levels. AEs were reported in 41.1% (211/514) of patients, and SAEs in 3.7%. The female gender, higher body mass index, esophageal varices, higher INR values, and longer treatment duration were independently associated with AE occurrence.

Research conclusions
Treatment with oral DAAs attains a high SVR rate, with fewer SAEs in a real-life cohort of subjects with CHC, from two tertiary university centers in Brazil.

Research perspectives
Long-term follow-up studies of patients after successful HCV eradication are important.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Prognostic factors of survival and a new scoring system for liver resection of colorectal liver metastasis

Kai-Chi Cheng, Ada Sze-Man Yip

ORCID number: Kai-Chi Cheng 0000-0002-6440-7825; Ada Sze-Man Yip 0000-0003-4785-3003.

Author contributions: Cheng KC designed the research study; Cheng KC and Yip ASM performed the research; Yip ASM analyzed the data and wrote the manuscript; all authors have read and approved the final manuscript.

Institutional review board statement: The protocol was approved by the Research Ethics Committee (Kowloon Central/Kowloon East) (Ref: KC/KC-21-0103/ER-1) in accordance with the laws and regulations (including Hong Kong laws), Hospital Authority policy, professional code of conduct, guidance of ICH GCP, and Declaration of Helsinki.

Informed consent statement: This study protocol was reviewed and approved by Hospital Authority Clinical Research Ethics Review Committee, reference number KCC/KEC-2021-0097. Written consent was not required as this is a retrospective study, and all data were retrospective. There was no prospective component to this study (*i.e.* patients were all anonymized, and there was no prospective follow-up). No patient

Kai-Chi Cheng, Ada Sze-Man Yip, Department of Surgery, Kwong Wah Hospital, Hong Kong, China

Corresponding author: Kai-Chi Cheng, FRCS (Ed), Doctor, Department of Surgery, Kwong Wah Hospital, No. 25 Waterloo Road, Kowloon, Hong Kong, China.
thomascheng@hotmail.com

Abstract

BACKGROUND

Hepatic resection has become the preferred treatment of choice for colorectal liver metastasis (CLM) patients.

AIM

To identify the prognostic factors and to formulate a new scoring system for management of CLM.

METHODS

Clinicopathologic and long-term survival data were analyzed to identify the significant predictors of survival by univariate and multivariate analyses with the Cox model. A clinical score was constructed based on the analysis results.

RESULTS

Three factors of worse overall survival were identified in the multivariate analysis. They were number of liver metastases ≥ 5, size of the largest liver lesion ≥ 4 cm, and the presence of nodal metastasis from the primary tumor. These three factors were chosen as criteria for a clinical risk score for overall survival. The clinical score highly correlated with median overall survival and 5-year survival (P = 0.002).

CONCLUSION

Priority over surgical resection should be given to the lowest score groups, and alternative oncological treatment should be considered in patients with the highest score.

Key Words: Colorectal cancer; Liver metastasis; Liver resection; Long-term outcome; Overall survival; Disease-free survival; Prognosis; Score

was contacted for this study. All data were fully anonymized so that they cannot be traced back to an individual in this study.

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Core Tip: Using multivariate analysis with the Cox model, we identified three criteria-number of liver metastases ≥ 5, size of the largest liver lesion ≥ 4 cm, and the presence of nodal metastasis from the primary tumor-for a new clinical scoring system. This new clinical score highly correlated with median overall survival and 5-year survival. We propose to use this score to formulate cancer-specific treatment for the patients. Priority over surgical resection should be given to the lowest score groups, and alternative oncological treatment should be considered in patients with the highest score.

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INTRODUCTION

Colorectal cancer (CRC) is the third leading cause of cancer-related death in developed countries[1]. About half of the cases will develop liver metastasis, and 25% of them will present synchronously[2]. Hepatic resection has become the standard management in selected patients, with a reported 5-year survival rate ranging from 36% to 60% after curative liver resection[2-4]. Yet, this is a heterogeneous group of patients with variable prognoses[2]. As such, many studies have been directed towards the investigation of factors that might influence the recurrence and survival of patients with colorectal liver metastasis (CLM), with a goal to differentiate patients that would best benefit from surgical resection from those who should be directed to palliative care[5-8]. The objectives of the present study were to identify the prognostic factors of survival in patients subjected to resection of CLM and to propose a risk score accordingly, to differentiate these patients.

MATERIALS AND METHODS

Data source and study population

Between June 1999 and June 2020, all resections of CLM in Kwong Wah Hospital were recorded prospectively in the institution's database and retrospectively analyzed. Patients who underwent palliative resection or ablation treatment only were excluded from analysis.

All patients were followed according to a defined protocol including serum carcinoembryonic antigen level, chest X-ray, and computed tomography scan of the abdomen with contrast or ultrasonography of the liver if the patient was contraindicated for contrast injection. Patients were followed every 3 mo for the first 2 years after the operation and every 6 mo afterwards. Patients were actively called back for follow-up if they missed the appointment.

Patient demographics were extracted, including age at resection of liver metastasis and sex. Information on preoperative factors such as the site of the primary tumor, American Joint Committee on Cancer stage of primary tumor, primary tumor nodal stage, extrahepatic metastasis, disease-free interval from CRC resection to development of metastatic liver disease, carcinoembryonic antigen (CEA) level, and administration of systemic chemotherapy before liver resection was recorded. Regional lymph node metastasis of primary tumor was defined as mesenteric lymph node metastasis found histologically after resection of primary CRC. Synchronous metastases were defined as metastases detected by preoperative screening or during resection of the primary tumor or occurring within 6 mo of the initial diagnosis of CRC [9].

Data on operative details including the extent of liver resection (major *vs* minor hepatectomy), concomitant use of ablation and operative approach (laparoscopic *vs* open), volume of blood loss, and requirement of blood transfusion were collected;

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major hepatectomy was defined as a resection of at least three Couinaud liver segments. Perioperative outcomes, including 30-d mortality and complications, were reported. Pathologic details, including number of tumors, size of the largest tumor nodule, and resection margin, were extracted. Positive resection margin was defined as the presence of tumor cells within 1 mm of the transection line.

The primary endpoint was overall survival, which was defined as the time interval between primary surgical treatment of liver metastasis and the date of death or last follow-up. Secondary endpoint was disease-free survival, which was defined as the time interval between primary surgical treatment of liver metastasis and the date of radiological diagnosis of recurrence.

Statistical analysis

Continuous variables are summarized as the median with interquartile range (IQR) and categorical variables as frequencies with percentage. Overall and disease-free survival curves were plotted using Kaplan-Meier estimator. Variables affecting long-term survival were determined using the Cox proportional hazards regression model. In order to formulate a risk score, inclusion of variables into multivariable Cox models was based mainly on preoperative factors with clinical relevance, irrespective of the *P* value in the univariate analysis. This type of variable selection was appropriate because the bivariate selection method wrongly rejects potentially important variables when the relationship between an outcome and a risk factor is confounded by any confounder and when this confounder is not properly controlled[10]. Data were calculated for hazard ratio (HR). Continuous variables were discretized into categorical variables by clinical relevance. A clinical risk score for overall survival was formulated according to factors identified by the multivariate analysis. Statistical significance was defined as *P* value of the Wald test < 0.05. All the statistical analyses were carried out using SPSS software version 26 (IBM Corp., Chicago, IL, United States).

RESULTS

All 98 patients who underwent resection of CLM during the study period were included in this analytic cohort. Median follow-up period was 36 mo (IQR: 17.00-57.75). There were no missing data or patients lost to follow-up. The clinicopathological data are summarized in Table 1. The study population included 62 males (63.3%) and 36 females (36.7%). The median age of patients at liver resection was 65.5 years (IQR: 59-72). The location of the primary colorectal tumor was mostly in the left colon (n = 40, 40.8%) and rectum (n = 32, 32.7%), and 26 patients (26.5%) had a primary right-sided colon cancer. Regional lymph node metastases were present in 62 patients (63.3%). Fifty-nine patients (60.2%) had synchronous hepatic metastasis. Sixteen patients (16.3%) underwent combined liver and colorectal resection, and eleven (68.8%) of them were performed laparoscopically. Only four patients (4.1%) had a synchronous extrahepatic disease; all of them were pulmonary metastases. Two of the pulmonary metastasis patients underwent curative pulmonary metastasectomy. One patient did not have surgery because he was subsequently diagnosed with a brain metastasis before pulmonary resection.

The median operative time was 270 min (IQR: 177.5-376.0). The median length of hospital stay was 7 d (IQR: 6-11). There was no 30-d postoperative mortality. Eight postoperative complications required interventional radiology. Bile leak (n = 4) was the most common cause, followed by intra-abdominal collection (n = 3), and there was one case of drainage of pleural effusion. There were three postoperative endoscopic retrograde cholangiopancreatographies, indicated for bile leakage, with a common bile duct stent inserted. There was one esophagogastroduodenoscopy performed for coffee-ground aspirate from the nasogastric tube, which only showed gastritis. There were three reoperations. One reoperation was due to adhesive intestinal obstruction and the other two because of intra-abdominal sepsis.

Factors affecting survival

The median overall survival of the entire cohort was 45 mo. The 1-, 3-, and 5-year overall survival rates were 93.6%, 65.8%, and 35.5%, respectively. The overall survival curve is shown in Figure 1A. The median disease-free survival was 19 mo. The 1-, 3-, and 5-year disease-free survival rates were 64.4%, 36.8%, and 27.4%, respectively. The disease-free survival curve is shown in Figure 1B. Univariate analyses of factors affecting overall survival and disease-free survival are shown in Tables 2 and 3,

Table 1 Clinicopathological data of patients

Characteristic	Total (*n* = 98)
Age in yr, median (IQR)	65.5 (59-72)
Sex, *n* (%)	
Male	62 (63.3)
Female	36 (36.7)
Location of primary colorectal tumor, *n* (%)	
Right	26 (26.5)
Left	40 (40.8)
Rectum	32 (32.7)
LN involvement in primary tumor, *n* (%)	
Yes	62 (63.3)
No	36 (36.7)
Time of diagnosis of liver metastasis, *n* (%)	
Synchronous	59 (60.2)
Metachronous	
Disease-free interval < 12 mo	9 (9.2)
Disease-free interval ≥ 12 mo	30 (30.6)
Synchronous extrahepatic metastasis, *n* (%)	
Yes	4 (4.1)
No	94 (95.9)
Preoperative CEA level in ng/mL, *n* (%)	
< 200	90 (91.8)
≥ 200	6 (6.1)
Systemic chemotherapy before liver resection, *n* (%)	
Yes	9 (9.2)
No	89 (90.8)
Number of liver metastases, *n* (%)	
< 5 lesions	91 (92.9)
≥ 5 lesions	7 (7.1)
Size of largest liver metastasis, *n* (%)	
< 4 cm	67 (68.4)
≥ 4 cm	28 (28.6)
Surgical margin, *n* (%)	
Positive	19 (19.4)
Negative	78 (79.6)
Concurrent ablation, *n* (%)	
No	90 (91.8)
Yes	8 (8.2)
Operative approach, *n* (%)	
Laparoscopic	57 (58.2)
Open	41 (41.8)
Type of hepatectomy, *n* (%)	

Minor	61 (62.2)
Major	37 (37.8)
Intraoperative blood loss, *n* (%)	
< 500 mL	49 (50.0)
≥ 500 mL	47 (48.0)
Requirement of blood transfusion, *n* (%)	
No	79 (80.6)
Yes	19 (19.4)

CEA: Carcinoembryonic antigen; IQR: Interquartile range; LN: Lymph node.

respectively.

On multivariate analysis, the number of liver metastases ≥ 5 [HR: 2.962, 95% confidence interval (CI): 1.174-7.473, P = 0.022], the size of the largest liver lesion ≥ 4 cm (HR: 2.983, 95%CI: 1.343-6.625, P = 0.007), and the presence of nodal metastasis from the primary tumor (HR: 1.955, 95%CI: 1.031-3.707, P = 0.040) were associated with a worse overall survival (Table 4). On the other hand, the number of liver metastases ≥ 5 (HR: 2.753, 95%CI: 1.052-7.205, P = 0.039) and the presence of nodal metastasis (HR: 2.234, 95%CI: 1.219-4.093, P = 0.009) were associated with a worse disease-free survival on multivariate analysis (Table 5).

Risk score

Three factors–the number of liver metastases ≥ 5, the size of the largest liver lesion ≥ 4 cm, and the presence of nodal metastasis from the primary tumor–were chosen as criteria for a clinical risk score for overall survival. As the HRs of these three factors were similar, for the sake of simplicity, each criterion was assigned 1 point. The total score was compared with overall survival using the log-rank test (Figure 1C). Although the survival of patients with score 0 (5-year survival: 46.8%, median survival of 50 mo) and score 1 was similar (5-year survival: 49.7%, median survival of 49 mo), overall survival clearly separated from those with score 2 (5-year survival: 10.8%, median survival of 33 mo) and score 3 (no 5-year survivors, median survival of 17 mo, P = 0.002).

DISCUSSION

The management of CLM has seen a marked change over the last decade, owing to the advancement of surgical techniques and perioperative treatments[3]. The achievement of curative resection of liver metastasis has transformed the 5-year survival from 11% to a range of 36%-60%[2-4]. The current study demonstrated a 5-year overall survival rate of 35.5%, slightly lower than the reported survival rate. This is probably due to the extended duration of the study period, which could be traced back to as early as 1999, in which management of CLM was less aggressive.

Many studies have investigated the prognostic factors of survival after resection of CLM. The most frequently cited prognostic factors are the number and the largest size of CLM, regional lymph node metastasis of the primary tumor, and preoperative CEA level[2]. Other proposed factors included disease-free interval from the treatment of primary CRC, location of primary CRC, and surgical resection margin[4,11,12]. The present study confirmed that a larger number of liver metastases, a larger size of the liver tumor, and the presence of regional lymph node metastasis of the primary tumor were associated with a poorer long-term survival. Among them, the number of liver lesions and the size of the largest liver tumor had the highest HRs (2.962 and 2.983, respectively).

Our study also identified that the largest tumor size 4 cm was the optimal cutoff value for prognostic purposes. Fong *et al*[5] and Nordlinger *et al*[6] were among the earliest groups of investigators to produce a clinical risk score, which utilized the size of the largest tumor > 5 cm as one of the criteria. This cutoff value has been used in subsequent studies as well[13,14]. Yet, this cutoff value was not universal; other size parameters (*i.e.*, 2 cm, 3 cm, or 4 cm) have been adopted as well[4,15,16]. Hence, size parameter of liver metastasis is a generally accepted risk factor, and our study is

Table 2 Univariate analysis of factors associated with overall survival

Variable	HR	95%CI	*P* value
Age	1.015	0.984-1.047	0.350
Sex			
Male	Ref		
Female	1.259	0.733-1.162	0.405
Location of primary tumor			
Rectum	Ref		
Right	1.542	0.780-3.048	0.213
Left	1.370	0.737-2.545	0.319
Regional LN metastasis			
No	Ref		
Yes	1.444	0.836-2.492	0.187
Time of diagnosis of liver metastasis, %			
Synchronous	Ref		
Metachronous			
Disease-free interval < 12 mo	0.814	0.317-2.094	0.670
Disease-free interval ≥ 12 mo	0.750	0.416-1.352	0.338
Synchronous extrahepatic metastasis			
No	Ref		
Yes	1.884	0.253-14.0	0.536
Preoperative CEA level			
< 200 ng/mL	Ref		
≥ 200 ng/mL	1.104	0.392-3.111	0.851
Systemic chemotherapy before liver resection			
No	Ref		
Yes	1.104	0.439-2.776	0.833
Number of liver metastases			
< 5 lesions	Ref		
≥ 5 lesions	2.506	1.124-5.585	0.025[a]
Size of the largest liver lesion			
< 4 cm	Ref		
≥ 4 cm	1.645	0.934-2.896	0.085
Surgical margin			
Clear	Ref		
Involved	0.965	0.509-1.829	0.912
Concurrent ablation			
No	Ref		
Yes	1.449	0.573-3.663	0.434
Operative approach			
Laparoscopic	Ref		
Open	1.069	0.624-1.832	0.808
Intraoperative blood loss, %			

< 500 mL	Ref		
≥ 500 mL	1.845	0.985-3.457	0.056
Requirement of blood transfusion, %			
No	Ref		
Yes	1.326	0.712-2.472	0.374

[a]$P < 0.05$. CEA: Carcinoembryonic antigen; CI: Confidence interval; HR: Hazard ratio; LN: Lymph node; Ref: Reference.

consistent with previous studies.

The current study evaluated that number of liver metastases 5 was the cutoff value that predicted a negative survival. The number of liver metastases is another frequently reported prognostic factor[2,5,6,13,14,16-18]. Again, there was not a universally accepted cutoff value for the number of liver metastases. However, a Japanese group of researchers analyzed 727 patients who had undergone CLM resections and reported that 4–5 was the most reliable cutoff value (HR: 2.35)[19]. Some studies also demonstrated that solitary liver metastasis had a significantly better prognosis than multiple metastases[16,18,20]. The present study echoed the past studies and was able to demonstrate the prognostic significance of the number of liver metastases.

Our study failed to show that the preoperative CEA level had a significant impact on long-term survival. Half of the published data referred to preoperative CEA level as a poor prognostic factor[2]. One of the possible explanations is that the sample size of the current study was too small to detect a significant result for this factor.

Concerning the surgical approach, past studies suggested that laparoscopic surgery was a favorable alternative to open surgery in selected CLM patients[21,22]. The OSLO-COMET randomized controlled trial, which compared laparoscopic and open parenchyma-sparing liver resection for CLM, concluded that laparoscopic surgery was associated with significantly less postoperative complications[23,24]. Although the evidence of the benefit of laparoscopic surgery on long-term survival is limited, there was a meta-analysis published in 2020 that aimed to evaluate the long-term oncologic outcome of laparoscopic and open liver surgery for CLM patients[25]. The study included 13 propensity-score matched studies and two randomized controlled trials, with a total of 3148 patients. The study concluded that laparoscopic surgery had a restricted mean survival time 8.6 mo longer at 10 years ($P < 0.0001$) and 30.0 mo longer at 15 years ($P < 0.0001$) than the open surgery group. The current study concurred with previous findings of similar survival between laparoscopic and open liver resections. Further research on this subject using a case-matched cohort study would be helpful.

Elderly patients are bound to have less physiological reserve and suffer from more medical comorbidity than younger patients. These factors will cause older patients to be more prone to surgical risks and mortality from other non-cancer related causes. Yet, from our study, liver surgery in elderly patients appeared to be safe, with a comparable outcome to younger patients, and these patients should not be denied surgery due to the sole reason of advanced age[26,27]. As a result of this argument, age should not be used as a criterion in formulating management of CLM.

The first large-scale clinical scoring system was the Nordlinger score, which incorporated preoperative and postoperative factors[6]. Then, Fong *et al*[5] developed a frequently cited clinical score system in 1999. Recently, the Tumor Burden Score was developed based on the concept of the "Metro-Ticket" paradigm and utilized a continuum of liver tumor size and number. This score was developed and validated in studies where most patients received modern neoadjuvant chemotherapy[7]. It is a growing recognition that KRAS and BRAF mutation statuses are important prognostic biochemical markers[28]. Brudvik *et al*[8] and Beamish *et al*[29] created a clinical scoring system specifically examining the impact of KRAS mutational status on survival of CLM patients. Many studies had been conducted to validate these clinical prediction scores[30-32]. A recent study examined the validity of previous clinical risk scoring systems in the contemporary era where chemotherapeutic treatment for CLM patients had significant improvement. It was shown that previous systems were still relevant in modern clinical use[29].

Despite the emergence of numerous clinical scoring systems in keeping with the development of oncological treatment for CLM, the most frequently cited scoring system was still the Fong score due to its incorporation of clinical criteria available for all patients (size, number, nodal status, preoperative CEA level, and disease-free

Table 3 Univariate analysis of factors associated with disease-free survival

Variable	HR	95%CI	*P* value
Age	0.984	0.957-1.012	0.271
Sex			
Male	Ref		
Female	1.000	0.614-1.628	0.999
Location of primary tumor			
Rectum	Ref		
Right	0.892	0.499-1.593	0.698
Left	0.678	0.362-1.271	0.226
Regional LN metastasis			
No	Ref		
Yes	2.324	1.348-4.008	0.002[a]
Synchronous liver metastasis			
No	Ref		
Yes	0.820	0.502-1.342	0.431
Time of diagnosis of liver metastasis, %			
Synchronous	Ref		
Metachronous			
Disease-free interval < 12 mo	1.066	0.452-2.509	0.884
Disease-free interval ≥ 12 mo	0.765	0.446-1.312	0.330
Preoperative CEA level			
< 200 ng/mL	Ref		
≥ 200 ng/mL	1.064	0.426-2.657	0.894
Systemic chemotherapy before liver resection			
No	Ref		
Yes	1.724	0.779-3.818	0.179
Number of liver metastases			
< 5 lesions	Ref		
≥ 5 lesions	3.138	1.409-6.987	0.005[a]
Size of the largest liver lesion			
< 4 cm	Ref		
≥ 4 cm	1.272	0.763-2.121	0.355
Surgical margin			
Clear	Ref		
Involved	1.110	0.616-2.000	0.728
Concurrent ablation			
No	Ref		
Yes	1.705	0.777-3.739	0.183
Operative approach			
Laparoscopic	Ref		
Open	0.785	0.480-1.285	0.336
Intraoperative blood loss, %			

< 500 mL	Ref		
≥ 500 mL	1.305	0.808-2.107	0.276
Requirement of blood transfusion, %			
No	Ref		
Yes	1.037	0.585-1.840	0.900

[a]*P* < 0.05. CEA: Carcinoembryonic antigen; CI: Confidence interval; HR: Hazard ratio; LN: Lymph node; Ref: Reference.

Table 4 Multivariate analysis of factors associated with overall survival

Variable	Adjusted HR	95%CI	*P* value
Age	1.039	0.999-1.080	0.054
Sex			
Male	Ref		
Female	1.874	0.984-3.572	0.056
Location of primary tumor			
Rectum	Ref		
Right	1.180	0.572-2.435	0.654
Left	0.943	0.427-2.084	0.884
Regional LN metastasis			
No	Ref		
Yes	1.955	1.031-3.707	0.040[a]
Time of diagnosis of liver metastasis, %			
Synchronous	Ref		
Metachronous			
Disease-free interval < 12 mo	1.192	0.431-3.295	0.735
Disease-free interval ≥ 12 mo	0.668	0.324-1.378	0.275
Synchronous extrahepatic metastasis			
No	Ref		
Yes	2.454	0.308-19.572	0.397
Preoperative CEA level			
< 200 ng/mL	Ref		
≥ 200 ng/mL	0.495	0.137-1.785	0.282
Systemic chemotherapy before liver resection			
No	Ref		
Yes	1.031	0.363-2.929	0.954
Number of liver metastases			
< 5 lesions	Ref		
≥ 5 lesions	2.962	1.174-7.473	0.022[a]
Size of the largest liver lesion			
< 4 cm	Ref		
≥ 4 cm	2.983	1.343-6.625	0.007[a]
Concurrent ablation			
No	Ref		

Yes	1.241	0.436-3.533	0.685
Operative approach			
Laparoscopic	Ref		
Open	1.655	0.873-3.137	0.123
Requirement of blood transfusion, %			
Yes	Ref		
No	0.681	0.320-1.451	0.320

[a]*P* < 0.05. CEA: Carcinoembryonic antigen; CI: Confidence interval; HR: Hazard ratio; LN: Lymph node; Ref: Reference.

Table 5 Multivariate analysis of factors associated with disease-free survival

Variable	Adjusted HR	95%CI	*P* value
Age	0.988	0.955-1.021	0.467
Sex			
Male	Ref		
Female	1.022	0.579-1.805	0.941
Location of primary tumor			
Rectum	Ref		
Right	1.044	0.538-2.025	0.899
Left	0.635	0.302-1.337	0.232
Regional LN metastasis			
No	Ref		
Yes	2.234	1.219-4.093	0.009[a]
Time of diagnosis of liver metastasis, %			
Synchronous	Ref		
Metachronous			
Disease-free interval < 12 mo	1.392	0.536-3.615	0.496
Disease-free interval ≥ 12 mo	0.846	0.445-1.610	0.611
Synchronous extrahepatic metastasis			
No	Ref		
Yes	9.716	2.034-46.413	0.004[a]
Preoperative CEA level			
< 200 ng/mL	Ref		
≥ 200 ng/mL	0.734	0.238-2.263	0.591
Systemic chemotherapy before liver resection			
No	Ref		
Yes	1.878	0.774-4.557	0.163
Number of liver metastases			
< 5 lesions	Ref		
≥ 5 lesions	2.753	1.052-7.205	0.039[a]
Size of the largest liver lesion			
< 4 cm	Ref		
≥ 4 cm	1.690	0.847-3.374	0.137

Concurrent ablation			
No	Ref		
Yes	0.788	0.267-2.324	0.666
Operative approach			
Laparoscopic	Ref		
Open	1.000	0.572-1.748	1.000
Requirement of blood transfusion, %			
Yes	Ref		
No	0.692	0.342-1.399	0.306

[a]$P < 0.05$. CEA: Carcinoembryonic antigen; CI: Confidence interval; HR: Hazard ratio; LN: Lymph node; Ref: Reference.

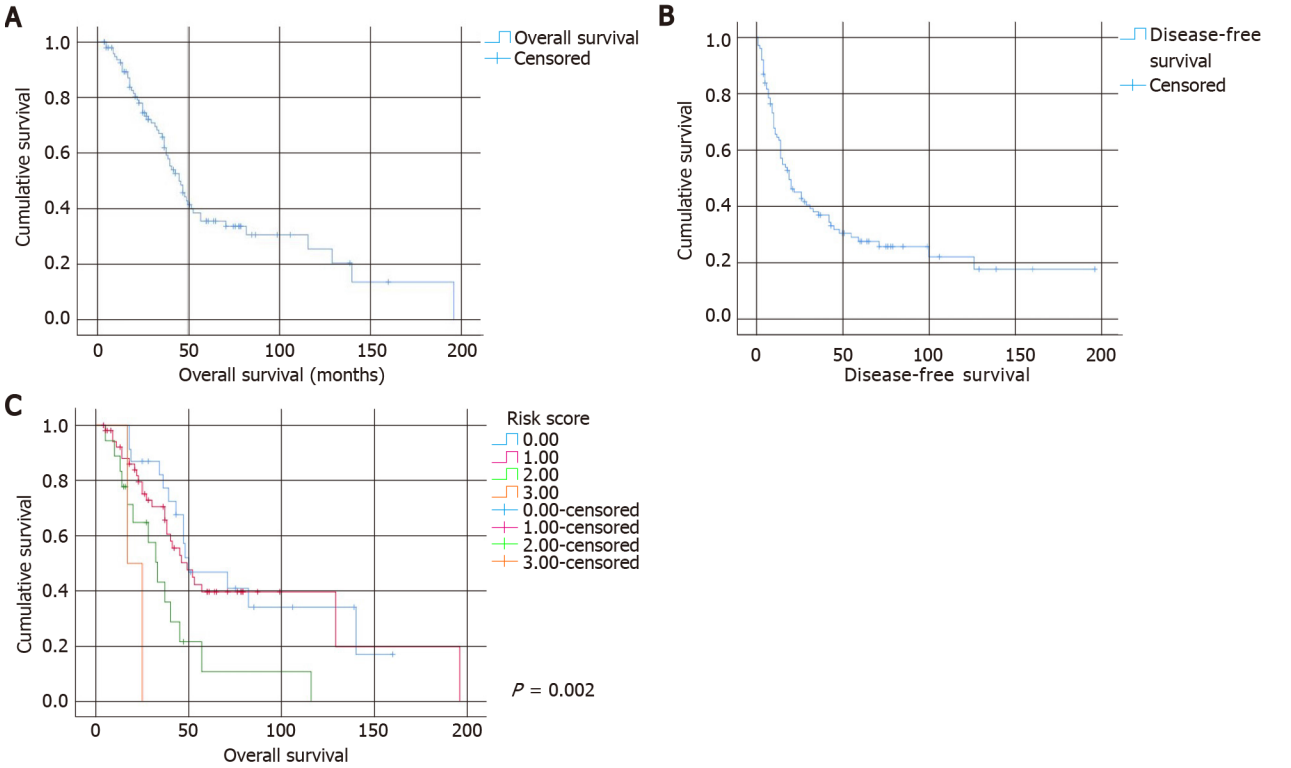

Figure 1 Kaplan-Meier curves. A: Overall survival of patients with colorectal liver metastasis undergoing resection; B: Disease-free survival of patients with colorectal liver metastasis undergoing resection; C: Overall survival of patients with colorectal liver metastasis undergoing resection with difference risk scores.

interval)[5]. This was also applicable to our clinical scoring system, which was basically a simplified version of the Fong score. Apart from its simplicity, the factors of the current scoring system are easily available and are available before resection of the liver tumor (except in cases of synchronous resection). This is of vital importance when clinicians are formulating the cancer-specific treatment for patients. The distinct difference in overall survival between the higher and lower score groups means that we can identify two groups of patients who are the most and the least likely to benefit from surgical treatment. A more reserved attitude should be given to the group of patients with the highest score (score = 3), in which there were no 5-year survivors, and the median survival was 17 mo, which was similar to patients without liver resection (15.5-21.3 mo)[33,34]. With the advancement in chemotherapeutic and radiological treatment, this group of patients may achieve a comparable life expectancy without the need to sustain surgical risks and discomforts. The lowest score groups (score = 0 or 1) are clearly the group of patients that can enjoy the benefit of extension of overall survival as a result of surgical treatment. Grey area existed for the average score (score = 2) group. In this group, additional factors, such as patient premorbid status, should be taken into consideration (Table 6).

Table 6 Risk score

Factor	Score[1]
Number of liver metastases ≥ 5	1
Size of liver metastasis ≥ 4 cm	1
Presence of lymph node metastasis in the primary tumor	1

[1]Score: 0-1, low risk; 2, moderate risk; 3, high risk. Total risk score is the sum of all scores.

Several limitations should be considered when interpreting the results of the current study. The retrospective design may limit its conclusions on associations over time. Second, it is a single-center study involving only a small study population with data recorded over 21 years. Perioperative management, including chemotherapy, changes over time, and consequently survival, may be influenced.

CONCLUSION

Nodal metastasis from the primary tumor, number of liver metastasis, and size of the largest liver tumor have a significant negative impact on overall survival of the patient after resection of CLM. In clinical practice, laparoscopic surgery should be an available option for a selected group of patients due to its potential benefits. When formulating cancer-specific treatment for patients with CLM, we proposed using a simplified clinical scoring system consisting of three significant prognostic factors. Priority over surgical resection should be given to the lowest score groups, and alternative oncological treatment should be considered in the group of patients with the highest score.

ARTICLE HIGHLIGHTS

Research background

Colorectal cancer is the third leading cause of cancer-related death in developed countries. About half of the cases will develop liver metastasis. Hepatic resection has become the standard management in selected patients, with a reported 5-year survival rate ranging from 36% to 60% after curative liver resection.

Research motivation

Patients with colorectal liver metastasis (CLM) are a heterogeneous group, with variable prognoses even after liver resection. As such, many studies have investigated factors that might influence the recurrence and survival of this group of patients, with a hope to differentiate patients that would best benefit from surgical resection from those who should be directed to palliative care.

Research objectives

The objectives of the present study were to identify the prognostic factors of survival in patients subjected to resection of CLM and to propose a risk score accordingly, to differentiate these patients.

Research methods

Between June 1999 and June 2020, all resections of CLM at Kwong Wah Hospital were recorded prospectively in the institution's database and retrospectively analyzed. Variables affecting long-term survival were determined using the Cox proportional hazards regression model. A clinical risk score for overall survival was formulated according to factors identified by multivariate analysis.

Research results

On multivariate analysis, the number of liver metastases ≥ 5 [hazard ratio (HR): 2.962, 95% confidence interval (CI): 1.174-7.473, P = 0.022], the size of the largest liver lesion ≥ 4 cm (HR: 2.983, 95%CI: 1.343-6.625, P = 0.007), and the presence of nodal metastasis

from the primary tumor (HR: 1.955, 95%CI: 1.031-3.707, P = 0.040) were associated with a worse overall survival. These three factors were chosen as criteria for a clinical risk score for overall survival, and the total risk score was compared with overall survival using the log-rank test. Lower total risk score groups had a significantly improved overall survival than the higher total risk score group.

Research conclusions

The newly proposed clinical risk score consisting of three significant prognostic factors (nodal metastasis from the primary tumor, number of liver metastases, and size of the largest liver tumor) is simple and easy to use. Priority over surgical resection should be given to the lowest score groups, and alternative oncological treatment should be considered in the group of patients with the highest score.

Research perspectives

Small study population (98 patients) and retrospective design limit the conclusions on associations over time. Future study with an expanded study population may allow weighting assignment to each component of the clinical risk score for a more accuracy in prognosis prediction. An external validation study is needed for the actual application of this clinical score in clinical use.

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ORIGINAL ARTICLE

Retrospective Study

Short-term outcomes of robotic liver resection: An initial single-institution experience

Manuel Durán, Javier Briceño, Ana Padial, Ferdinando Massimiliano Anelli, Juan Manuel Sánchez-Hidalgo, María Dolores Ayllón, Rafael Calleja-Lozano, Carmen García-Gaitan

ORCID number: Manuel Durán 0000-0003-1161-2195; Javier Briceño 0000-0001-7027-7898; Ana Padial 0000-0002-0608-4175; Ferdinando Massimiliano Anelli 0000-0002-0916-1949; Juan Manuel Sánchez-Hidalgo 0000-0002-0258-0673; María Dolores Ayllón 0000-0003-2493-3756; Rafael Calleja-Lozano 0000-0003-0048-0756; Carmen García-Gaitan 0000-0001-5087-5057.

Author contributions: Durán M and Briceño J have contributed equally to this paper; Briceño J was the principal surgeon in all the procedures, guarantor and designed the study; Durán M, Padial A, Anelli FM, Ayllón MD and García- Gaitán C participated in the acquisition, analysis, and interpretation of the data; Calleja-Lozano R and Durán M did the literature review; Durán M drafted the initial manuscript; Briceno J and Sánchez-Hidalgo JM revised the article critically for important intellectual content.

Institutional review board statement: The study was reviewed and approved by Comité de Ética de la Investigación de Córdoba, Hospital Universitario Reina Sofía, España.

Informed consent statement: All study participants, or their legal

Manuel Durán, Javier Briceño, Ana Padial, Ferdinando Massimiliano Anelli, Juan Manuel Sánchez-Hidalgo, María Dolores Ayllón, Rafael Calleja-Lozano, Unit of Hepatobiliary Surgery and Liver Transplantation, General and Digestive Surgery Department, Reina Sofia University Hospital, Cordoba 14004, Spain

Manuel Durán, Javier Briceño, Ana Padial, Ferdinando Massimiliano Anelli, María Dolores Ayllón, Rafael Calleja-Lozano, GC18 Translational Research in Surgery of Solid Organ Transplantation, Maimonides Biomedical Research Institute, Córdoba 14004, Spain

Juan Manuel Sánchez-Hidalgo, GE09 Research in Peritoneal and Retroperitoneal Oncological Surgery, Maimonides Biomedical Research Institute, Córdoba 14004, Spain

Carmen García-Gaitan, Department of Anesthesiology and Resuscitation, Reina Sofia University Hospital, Cordoba 14004, Spain

Corresponding author: Javier Briceño, MD, PhD, Chairman, Unit of Hepatobiliary Surgery and Liver Transplantation, General and Digestive Surgery Department, Reina Sofia University Hospital, Avda. Menéndez Pidal s/n, Cordoba 14004, Spain. javibriceno@hotmail.com

Abstract

BACKGROUND

Liver surgery has traditionally been characterized by the complexity of its procedures and potentially high rates of morbidity and mortality in inexperienced hands. The robotic approach has gradually been introduced in liver surgery and has increased notably in recent years. However, few centers currently perform robotic liver surgery and experiences in robot-assisted surgical procedures continue to be limited compared to the laparoscopic approach.

AIM

To analyze the outcomes and feasibility of an initial robotic liver program implemented in an experienced laparoscopic hepatobiliary center.

METHODS

A total of forty consecutive patients underwent robotic liver resection (da Vinci Xi, intuitive.com, United States) between June 2019 and January 2021. Patients were prospectively followed and retrospectively reviewed. Clinicopathological characteristics and perioperative and short-term outcomes were analyzed. Data

are expressed as mean and standard deviation. The study was approved by the Institutional Review Board.

RESULTS

The mean age of patients was 59.55 years, of which 18 (45%) were female. The mean body mass index was 29.41 kg/m². Nine patients (22.5%) were cirrhotic. Patients were divided by type of resection as follows: Ten segmentectomies, three wedge resections, ten left lateral sectionectomies, six bisegmentectomies (two V-VI bisegmentectomies and four IVb-V bisegmentectomies), two right anterior sectionectomies, five left hepatectomies and two right hepatectomies. Malignant lesions occurred in twenty-nine (72.5%) of the patients. The mean operative time was 258.11 min and two patients were transfused intraoperatively (5%). Inflow occlusion was used in thirty cases (75%) and the mean total clamping time was 32.62 min. There was a single conversion due to uncontrollable hemorrhage. Major postoperative complications (Clavien–Dindo > IIIb) occurred in three patients (7.5%) and mortality in one (2.5%). No patient required readmission to the hospital. The mean hospital stay was 5.6 d.

CONCLUSION

Although robotic hepatectomy is a safe and feasible procedure with favorable short-term outcomes, it involves a demanding learning curve that requires a high level of training, skill and dexterity.

Key Words: Robotics; Hepatectomy; Minimally invasive surgery; Liver surgery; Da vinci

Core Tip: The number of liver procedures performed laparoscopically remains highly variable, ranging from 10% up to 80% in some centers, and complex hepatectomies are still confined to expert and experienced laparoscopic liver surgeons. The robotic approach is gradually being introduced in liver surgery and has increased notably in recent years, which could compensate for the inherent difficulties of the laparoscopic approach. In this study, we analyzed our single-center data of robotic liver resections using the da Vinci Xi System®.

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INTRODUCTION

Liver surgery has traditionally been characterized by the complexity of its procedures and potentially high rates of morbidity and mortality in inexperienced hands. In recent years, laparoscopic liver surgery has notably increased due to the beneficial outcomes in terms of fewer complications and transfusions, less blood loss and shorter hospital stays compared to open surgery with similar oncologic outcomes[1,2].

The number of liver procedures performed laparoscopically remains highly variable, ranging from 10% up to 80% in some centers[3]. Complex hepatectomies such as major hepatectomies or in posterosuperior segments are still restricted to expert laparoscopic liver surgeons with considerable experience due to their inherent risk, which make them very technically demanding procedures[4-6]. The increase in laparoscopic liver resections (LLR) has been accompanied by the development of parenchymal transection equipment and improved optical systems to compensate for the limitations of this approach and to increase the safety of hepatic resections[7].

The robotic approach is gradually being introduced in liver surgery and could compensate for the inherent difficulties of the laparoscopic approach. However, only some centers have implemented robotic-assisted surgery and the experience continues

guardian, provided informed written consent prior to study enrollment.

Conflict-of-interest statement: There are no conflicts of interest to report.

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to be limited compared to laparoscopy[8]. In this study, we report an initial experience with our first forty robotic liver resections (RLRs) using the da Vinci Xi System© (Intuitive Surgical, Inc., Sunnyvale, CA, United States). The aim of this study was to analyze the outcomes and feasibility of an initial robotic liver program implemented in an experienced laparoscopic hepatobiliary center.

MATERIALS AND METHODS

Following Institutional Review Board approval, we performed a retrospective review on our prospective recorded single-institution data between June 2019 and January 2021, including sixty-three patients who underwent a hepatobiliopancreatic procedure as follows: Nineteen distal pancreatectomies, four bilioenteric reconstructions and forty Liver resections. Forty RLRs were performed by a single hepatic surgeon (Dr. Briceño J) and included in the study. The indication of robotic surgery was established when the patient was considered a suitable candidate for minimally invasive surgical approach. All patients gave their informed consent prior to surgery.

Patient preoperative data included age, sex, body mass index (BMI), American society of anesthesiologists (ASA) classification, comorbidities, previous abdominal surgery and presence of chronic liver disease.

Intraoperative parameters included operative time, blood transfusion, use of inflow occlusion and duration and conversion rate. Operative time was defined as the time from the first incision to closure. Intraoperative complications were defined as an event requiring major deviation from the planned procedure.

The anatomical location of the lesions and surgical resection were defined according to the Brisbane terminology[9]. The difficulty of the liver resections was graded according to the IWATE scoring system as revised in the Morioka consensus conference whereby a score of 0-3 is graded as low difficulty, 4-6 as intermediate difficulty, 7-9 as advanced difficulty and 10-12 as expert difficulty[10].

Postoperative variables were postoperative complications, length of stay, readmission within 30 d and mortality. Postoperative complications were recorded using the Clavien–Dindo classification up to 90 d after surgery[11]. Complications were considered major when Clavien-Dindo > III.

The characteristics, diagnosis, number and size of the lesions were determined by pathological reports. Resection margins were defined as R0 resection when tumor distance from the margin was greater than 1 mm, as R1 resection when tumor distance from the margin was less than 1 mm and as R2 resection upon presence of macroscopic tumor at the margin.

Statistics demographic data and clinical outcomes were analyzed. The descriptive analysis included mean and standard deviation in continuous variables, while categorical and ordinal variables were reported as counts with proportions. A *P*-value of less than 0.05 was considered statistically significant. Statistical analyses were performed using SPSS Statistics version 22.0 (IBM, Armonk, NY, United States).

Surgical technique

After anesthesia, patients are placed in the French position and in the reverse Trendelenburg position (15°) with a slight left tilt (5°). The assistant is located between the patient's legs and the robot is located at the left shoulder. The trocars are placed according to the following surgical requirements: The transection plane, the segments involved and the position of the endostapler. For a right hepatic lobe procedure, a 12 mm camera port is introduced in the right paraumbilical area and the main working ports (first and third robotic arm ports on the left and right, respectively) are placed in the left and right upper quadrant area. The fourth robotic trocar is placed near the left anterior axillary line. For a left hepatic lobe procedure or a left hepatectomy, trocar placement is similar to that described previously; however, the camera port is placed at the umbilicus to visualize the target anatomy. Once the robotic trocars are placed, the da Vinci Xi robotic system (Intuitive.com, United States) is docked. An AirSeal port (SurgiQuest Inc., Milford, CT, United States) is used for bedside assistance. An intracorporeal pringle maneuver using Huang's loop is applied and used when deemed appropriate (Figure 1).

A robotic vessel sealer and bipolar cautery are the main instruments employed for parenchymal transection using the crush-clamp technique. An intraoperative robotic ultrasound device is employed to confirm the location of the tumor and to check the surgical transection line. For anatomic liver resection, indocyanine green may be injected intravenously to visualize hepatic perfusion and the demarcation line by

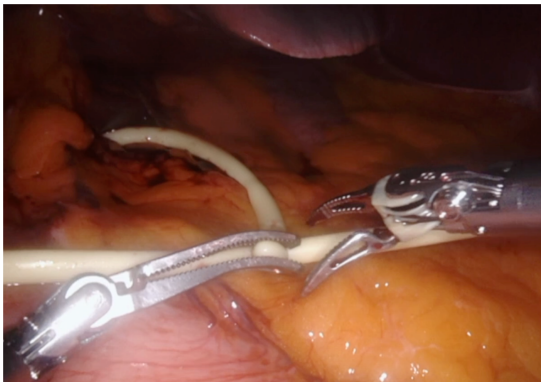

Figure 1 Huang's loop is applied and used when deemed appropriate.

negative staining (Figure 2). Finally, the specimen is removed from the abdominal cavity by means of an extraction bag.

RESULTS

Forty patients underwent RLR for benign or malignant tumors. Overall, the mean age was 59.6 (± 11.8), of which 18 (45%) were female. The mean BMI was 29.4 (± 4.7). Seventeen patients (42.5%) underwent a previous surgery (laparoscopic or "open"). A total of 72.5% (n = 29) of all lesions were malignant (primary lesions n = 23, metastatic lesions n = 6). Five lesions (12.5%) were located in the postero-superior segments (IVa, VII and VIII). The patients' characteristics are summarized in Table 1.

Perioperative outcomes

The type of liver resection, simultaneous combined procedures and outcomes are summarized in Table 2. Nine patients (22.5%) were cirrhotic and another 22.5% had moderate to severe hepatic steatosis. Seven (17.5%) major hepatectomies were performed, of which five were left hepatectomies and two right hepatectomies. Additionally, thirty-three (82.5%) minor hepatectomies were performed: Ten segmentectomies, three wedge resections, ten left lateral sectionectomies, six bisegmentectomies (two V-VI bisegmentectomies, four IVb-V bisegmentectomies), two right posterior sectionectomies and two right anterior sectionectomies. Two patients underwent a simultaneous resection of the primary tumor and the metastatic liver lesions. Of these, one underwent a distal pancreatectomy with splenectomy and anatomic right hepatectomy and the other underwent a low anterior resection with hepatic wedge resection. Only one patient was converted to the "open" approach due to hemorrhage. Based on the IWATE criteria, 3/40 operations were categorized as low difficulty, 19/40 as intermediate, 13/40 as advanced and 5/40 as expert (see Figure 3).

Eight patients developed complications and are summarized in Tables 2 and 3. Major complications (Clavien-Dindo > III) occurred in three patients (7.5%). Postoperative complications included ascites (1), ileus (1), acute renal injury (1) and bile leak (2), for which one patient required endoscopic retrograde cholangiopancreatography. One cirrhotic patient who underwent a right hepatectomy developed post-hepatectomy liver failure, ascites, acute kidney injury and lower gastrointestinal bleeding with no findings at colonoscopy.

The mean operative duration was 247.6 (± 119.2) min. Inflow occlusion was used in 30 cases (75%) and mean total clamping time was 32.6 (± 26.6) min. Two patients were transfused intraoperatively (5%) and vasopressors were used intraoperatively in fourteen cases (35%). The overall mean length of stay was 5.6 (± 6.1) d, while for minor hepatectomies was 4.4 (± 3.6) d and for major hepatectomies was 14 (± 12.6) d.

The pathologic findings are summarized in Table 4. The mean number of lesions was 1.2 (± 0.7), the mean size was 60.6 (± 40.5) and R0 resection was performed in twenty-seven (93%) of malignant cases. Of the forty operations, the most common diagnoses were as follows: Sixteen (40%) hepatocellular carcinoma, six (15%) intrahepatic cholangiocarcinoma, five (12.5%) colorectal metastases and four (10%) giant hemangiomas.

Table 1 Patient demographics and chronic preoperative conditions, *n* (%)

Sex (female)	18 (45)
Age (yr)	59.6 ± 11.8
BMI	29.4 ± 4.7
ASA class	
ASA I	3 (7.5)
ASA II	13 (32.5)
ASA III	24 (60)
Previous abdominal surgery	17 (42.5)
Chronic liver disease	12 (30)
History of type 2 diabetes	14 (35)
History of hypertension	22 (55)
Chronic respiratory disease	7 (17.5)
Chronic cardiac disease	7 (17.5)
Chronic renal disease	2 (5)
Viral infection	
HCV	5 (17.5)
HBV	1 (2.5)
Benign/malignant	11 (27.5)/29 (72.5)

BMI: Body mass index; ASA: American Society of Anesthesiologists; HCV: Hepatitis C virus; HBV: Hepatitis B virus.

Table 2 Type of liver resection

Type of liver resection, *n* (%)		Combined procedures	Cirrhosis	Conversion	Postoperative complications
Wedge resection	3 (7.5)	LAR (1), CH (2)			
Segmentectomy	10 (25)	CH (3)	4	1 (Bleeding)	Ascites (1), colon ischemia (1)
Left lateral sectionectomy	10 (25)	CH (1)	4		AKI (1), POT (1)
Bisegmentectomy V-VI	2 (5)	CH (1)			
Bisegmentectomy IVb-V	4 (10)	CH (3)			
Right posterior sectionectomy	2 (5)	CH (1)			
Right anterior sectionectomy	2 (5)	CH (2)			Ileus (1)
Left hepatectomy	5 (12.5)	RFA (1), CH (4)			Bile leak (1)
Right hepatectomy	2 (5)	PS (1), CH (1)	1		PHLF, ascites, LGIB and AKI (1), Bile leak (1)

LAR: Low anterior resection; CH: Cholecystectomy; RFA: Radiofrequency ablation; PS: Pancreatosplenectomy; AKI: Acute kidney injury; POT: Postoperative red blood cell transfusion; PHLF: Post-hepatectomy liver failure; LGIB: Low gastrointestinal bleeding.

DISCUSSION

The minimally invasive approach in liver surgery has revolutionized the management of patients with liver tumors due to its demonstrated superiority over open surgery in terms of hospital stay, morbidity and blood loss[12,13].

Despite the exponential increase in laparoscopic liver surgery in recent years, the development of this surgical procedure has been a real challenge and even today the majority of hepatectomies performed by laparoscopy are the least complex, while major LLRs are still performed in few expert centers. A recent meta-analysis has demonstrated similar outcomes between laparoscopic and robotic major hepatec-

Table 3 Perioperative and postoperative outcomes

Operative duration (min)	247.6 ± 119.2
Inflow occlusion	30 (75%)
Total clamping time (min)	32.6 ± 26.6
Intraoperative vasopressors use	14 (35%)
Intraoperative blood transfusion	2 (5%)
Postoperative complications	8 (20%)
Clavien–dindo classification	
Grade I	1
Grade II	4
Grade IIIa	2
Grade IIIb	
Grade IV	
Grade V	1
Conversion	1 (2.5%)
Mortality	1 (2.5%)
Length of stay (d)	5.6 ± 6.1
30-d readmission	

Table 4 Pathologic examination

Malignant	***n* (%)**
Primary	
Hepatocellular carcinoma	16 (40)
Intrahepatic cholangiocarcinoma	6 (15)
Gallbladder carcinoma	1 (2.5)
Metastatic	
Colorectal metastases	5 (12.5)
Non-colorectal metastases	1 (2.5)
Benign	
Adenoma	1 (2.5)
Giant hemangioma	4 (10)
Giant focal nodular hyperplasia	3 (7.5)
Hydatid cyst	2 (5)
Simple cyst	1 (2.5)
Number of lesions	1.2 ± 0.7
Tumor size (mm)	60.6 ± 40.5
Surgical margin	
R0	27 (93)
R1	2 (7)

tomies[14]. Most procedures were performed in specialized centers by liver surgeons with great previous expertise on minimally invasive surgery, demonstrating a severe complication rate of 6.7% and 3.6%, respectively, and almost zero risk of death.

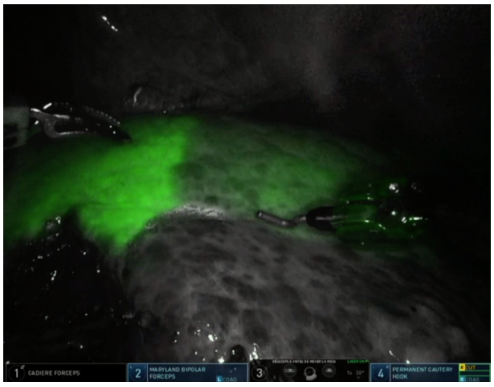

Figure 2 Demarcation line by indole cyanide green negative staining of anatomic segment 3 resections.

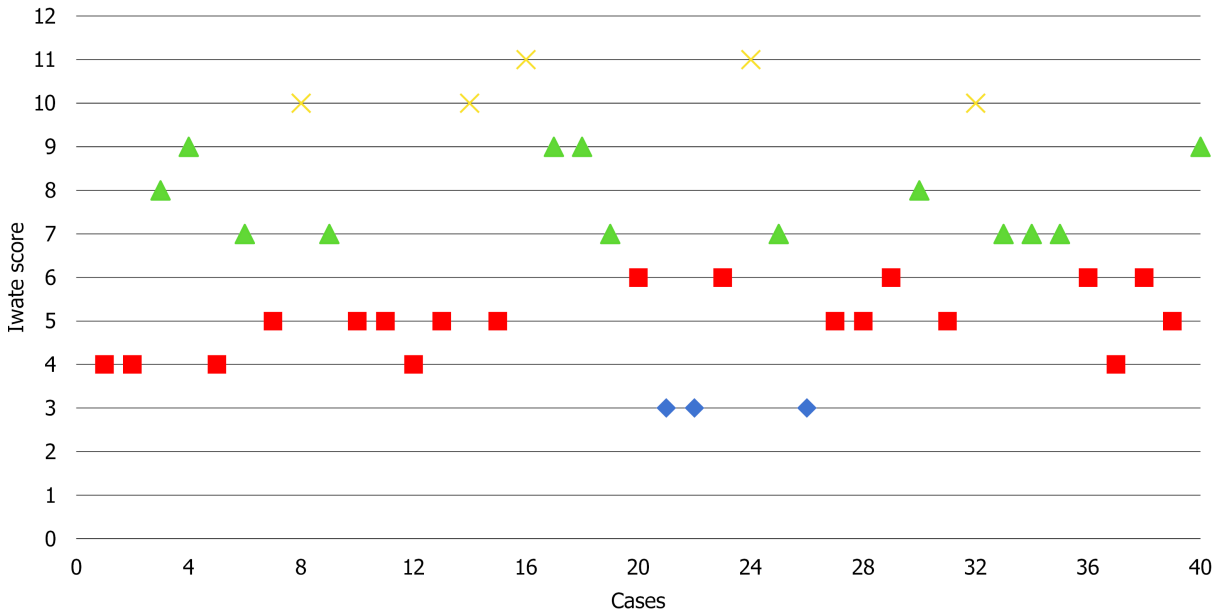

Figure 3 Consecutive case series and degree of difficulty according to the IWATE score. Low: 3 (7.5%) was in blue; Intermediate: 19 (47.5%) was in red; Advanced: 13 (32.5%) was in green; Expert: 5 (12.5%) was in yellow.

Certain aspects associated with the laparoscopic approach, such as unstable cameras, rigid instruments with reduced degrees of freedom, human hand tremors, poor surgeon ergonomics and the difficulty of suturing in hard-to-reach locations constitute a serious limitation for performing complex hepatectomies[15]. Robotic systems have compensated for the limitations inherent in the laparoscopic approach as they use stable, 3D high-definition cameras that eliminate hand tremors and provide 7 degrees of freedom, thus increasing manual dexterity and facilitating liver resections. Moreover, it has been shown that robotic platforms reduce physical workload and are less strenuous for surgeons, so they may reduce musculoskeletal strain and disorders [16,17].

In this study, we present our initial experience in robotic liver surgery. Comparing our experience with those reported by other specialized centers, some have performed a greater number of RLRs, but the study interval is greater[18-25]. In our series, forty RLRs were performed in 18 mo and included major hepatectomies and cirrhotic patients. Most of the patients were overweight, middle-aged men, the majority of whom were ASA class III and had several comorbidities. Resections were mainly performed for malignant lesions, with hepatocellular carcinoma, intrahepatic cholangiocarcinoma and colorectal metastases being the most common diagnoses. Two patients underwent a simultaneous resection of the primary tumor (distal pancreatectomy and low anterior resection) and liver metastasis.

Resections for liver metastases were only performed in the absence of peritoneal carcinomatosis or unresectable extrahepatic disease. All patients with malignant disease except two had negative margins after the resection. Of the total number of

patients, eleven (27.5%) had benign indications for liver resections. Benign lesions were resected because of severe symptoms caused by tumor size, radiological features of malignancy or diagnostic uncertainty despite preoperative biopsies and underwent anatomic resection.

Regarding the type of resection performed, left lateral sectionectomy (25%) and segmentectomy (25%) were the most frequently performed resections. To study the complexity of the RLRs performed, we employed the IWATE score. The effectiveness of the IWATE score as an indicator of operative difficulty in LLR has been demonstrated and several groups have previously considered that its usefulness in laparoscopic liver surgery could be extrapolated to the robotic approach[26,27]. In this study, 45% of the procedures were classified as advanced and expert. These percentages are similar to those of Labadie *et al*[26] (43%) and lower than the data reported by Sucandy *et al*[27] (68.6%). As can be seen in Figure 3 showing the cases performed to date and their degree of difficulty, the 45% of RLRs performed were classified as advanced and expert. In our experience, this is due to two reasons: The advantages of the robotic approach for surgeons and the extensive background of our group in laparoscopic liver surgery, which has provided the surgeons an understanding of the specific features and difficulties of this minimally invasive approach. In the coming years, the IWATE score will likely have to be adapted to robotic-assisted liver surgery, as this approach allows accessing posterior superior segments but shows difficulties associated with the resection of various lesions in different quadrants. RLR may favor the operative feasibility of highly difficult resections reducing the conversion rate and increasing safety. However, it does not translate directly into a postoperative course more favorable than pure laparoscopy, as we are still comparing two minimally invasive approaches[28].

Although the overall complication rate was 20%, major complications (Clavien–Dindo > III) occurred in three patients (7.5%). The postoperative complication rate in our study (20%) is similar to that published in a recent metanalysis by Guan *et al*[29] (19.2%), which included 435 RLRs. Reported conversion rates range from 0%-6%[18,19,21,22,30]. The only conversion to the "open" approach in our series (2.5%) occurred in a cirrhotic patient due to hemorrhage from a right inferior hepatic vein during a segment VI resection which required a cava vein venorrhaphy. The only death in our series occurred in a patient with a previous history of autoimmune vasculopathy who underwent a segment VI segmentectomy. The patient developed pancolonic ischemia at postoperative day 7 requiring intervention and died 18 d after being admitted to the Intensive care unit due to massive intestinal ischemia.

The main limitations to the present study are its single-arm design, the relatively small sample size and the retrospective nature of the analysis despite prospective recording. The lack of financial costs and quality of life analysis prevent a deeper investigation.

CONCLUSION

The results reported in this initial case series reflect perioperative outcomes similar to those published previously which support the safety and feasibility of this approach in liver surgery. Previous experience in minimally invasive liver surgery is necessary to overcome the initial difficulties of the robotic approach and perform complex liver procedures in a short period of time.

ARTICLE HIGHLIGHTS

Research perspectives

Future work is required to clarify the role of the robotic approach in complex hepatectomies.

Research conclusions

The implementation of a liver robotic surgery program is safe and feasible with favorable short-term outcomes.

Research results
Forty consecutive patients underwent robotic liver resection between June 2019 and January 2021. Liver resection included: Ten segmentectomies, three wedge resections, ten left lateral sectionectomies, six bisegmentectomies (two V-VI bisegmentectomies and four IVb-V bisegmentectomies), two right anterior sectionectomies, five left hepatectomies and two right hepatectomies.

Research methods
In this study patients were prospectively followed and retrospectively reviewed. The study was conducted according to STROBE statements.

Research objectives
The authors aimed to analyze the outcomes and feasibility of an initial robotic liver surgery program implemented in an experienced laparoscopic hepatobiliary center.

Research motivation
A robotic liver surgery program has been implemented in our center which has significant previous experience in minimally invasive surgery.

Research background
In recent years, minimal invasive liver surgery has notably increased due to its perioperative and postoperative favorable outcomes.

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ORIGINAL ARTICLE

Retrospective Study

Assessment for the minimal invasiveness of laparoscopic liver resection by interleukin-6 and thrombospondin-1

Takayoshi Kaida, Hiromitsu Hayashi, Hiroki Sato, Shotaro Kinoshita, Takashi Matsumoto, Yuta Shiraishi, Yuki Kitano, Takaaki Higashi, Katsunori Imai, Yo-ichi Yamashita, Hideo Baba

ORCID number: Takayoshi Kaida 0000-0002-9134-6124; Hiromitsu Hayashi 0000-0002-1832-4287; Hiroki Sato 0000-0001-5213-018X; Shotaro Kinoshita 0000-0002-6690-4702; Takashi Matsumoto 0000-0001-6791-6441; Yuta Shiraishi 0000-0002-8064-2163; Yuki Kitano 0000-0001-6292-8670; Takaaki Higashi 0000-0002-1034-8671; Katsunori Imai 0000-0003-0549-1825; Yo-ichi Yamashita 0000-0001-8439-6660; Hideo Baba 0000-0002-3474-2550.

Author contributions: Kaida T and Hayashi H identified the concept and wrote the draft of the article; Sato H, Kinoshita S, Matsumoto T, Siraishi Y, Kitano Y, Higashi T, and Imai K actually treated the patient and collected data; Yamashita Y and Baba Hsupervised article preparation.

Institutional review board statement: This study was retrospective, non-interventional, which approved by the institutional ethics committee of Kumamoto University Hospital (approval No.2052) and was performed in accordance with the Helsinki Declaration of 1975.

Informed consent statement: Written informed consent was obtained from all patients.

Takayoshi Kaida, Hiromitsu Hayashi, Hiroki Sato, Shotaro Kinoshita, Takashi Matsumoto, Yuta Shiraishi, Yuki Kitano, Takaaki Higashi, Katsunori Imai, Yo-ichi Yamashita, Hideo Baba, Gastroenterological Surgery, Graduate School of Life Sciences, Kumamoto University, Kumamoto 860-8556, Japan

Corresponding author: Hiromitsu Hayashi, FACS, MD, PhD, Assistant Professor, Gastroenterological Surgery, Graduate School of Life Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556, Japan. hhayasi@kumamoto-u.ac.jp

Abstract

BACKGROUND

Laparoscopic surgery has been introduced as a minimally invasive technique for the treatment of various field. However, there are few reports that have scientifically investigated the minimally invasive nature of laparoscopic liver resection (LLR).

AIM

To investigate whether LLR is scientifically less invasive than open liver resection.

METHODS

During December 2011 to April 2015, blood samples were obtained from 30 patients who treated with laparoscopic (n = 10, 33%) or open (n = 20, 67%) partial liver resection for liver tumor. The levels of serum interleukin-6 (IL-6) and plasma thrombospondin-1 (TSP-1) were measured using ELISA kit at four time points including preoperative, immediate after operation, postoperative day 1 (POD1) and POD3. Then, we investigated the impact of the operative approaches during partial hepatectomy on the clinical time course including IL-6 and TSP-1.

RESULTS

Serum level of IL-6 on POD1 in laparoscopic hepatectomy was significantly lower than those in open hepatectomy (8.7 *vs* 30.3 pg/mL, respectively) (P = 0.003). Plasma level of TSP-1 on POD3 in laparoscopic hepatectomy was significantly higher than those in open hepatectomy (1704.0 *vs* 548.3 ng/mL, respectively) (P = 0.009), and have already recovered to preoperative level in laparoscopic approach. In patients with higher IL-6 Levels on POD1, plasma level of TSP-1 on POD3 was significantly lower than those in patients with lower IL-6 Levels on POD1.

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Multivariate analysis showed that open approach was the only independent factor related to higher level of IL-6 on POD1 [odds ratio (OR), 7.48; 95% confidence interval (CI): 1.28-63.3; P = 0.02]. Furthermore, the higher level of serum IL-6 on POD1 was significantly associated with lower level of plasm TSP-1 on POD3 (OR, 5.32; 95%CI: 1.08-32.2; P = 0.04) in multivariate analysis.

CONCLUSION

In partial hepatectomy, laparoscopic approach might be minimally invasive surgery with less IL-6 production compared to open approach.

Key Words: Laparoscopic surgery; Liver resection; Hepatectomy; Minimal invasiveness; Interleukin-6; Thrombospondin-1

Core Tip: Laparoscopic liver resection is less invasive than open liver resection and is becoming more popular worldwide. However, reports that have scientifically investigated the minimally invasive nature of laparoscopic surgery remain scarce. In the current study, we scientifically evaluated the minimally invasive nature of laparoscopic surgery using interleukin-6 and thrombospondin-1 as markers of tissue damage.

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INTRODUCTION

Laparoscopic surgery has been introduced as a minimally invasive technique for the treatment of various field such as prostatectomy, hysterectomy, cholecystectomy and gastrectomy[1-5]. Nowadays, robot-assisted surgery is becoming more widespread in the hope of further minimizing invasiveness[6,7]. Laparoscopic surgery is considered to be a minimally invasive technique because of smaller size of skin incision and less bleeding loss than open surgery[8,9]. In fact, laparoscopic surgery results in less morbidity and a much shorter time to discharge compared to open surgery[10]. It is unclear whether laparoscopic technology is really contributing to minimally invasive surgery due to the development of other instruments and the remarkable effect of magnification.

Hepatic resection is the only curative treatment for hepatocellular carcinoma (HCC). It has been reported that laparoscopic hepatectomy is less invasive than open hepatectomy, with less blood loss, fewer postoperative complications, and less hospital stay[9]. However, there are few reports that have scientifically investigated the minimally invasive nature of laparoscopic liver resection (LLR)[11-14].

Surgery induces a systemic stress response and produces various cytokine such as interleukine-6 (IL-6) and IL-10. IL-6 is a proinflammatory cytokine that is produced by many tissues in response to injury. Peak level of L-6 and CRP consistently were associated with the magnitude of operative injury and operative method[15]. Thrombospondin-1 (TSP-1) is expressed during hepatic resection and acts as an inhibitor of liver regeneration[16,17]. It reflects the invasiveness when liver resection. Kuroki *et al*[18] reported that a decrease in TSP-1 after partial hepatectomy was associated with liver damage, and the less invasive the liver, the faster the recovery of TSP-1. Changes in plasma level of TSP-1 after liver resection may reflect surgical invasion.

This study aimed to investigate scientifically whether LLR is less invasive technique than open liver resection (OLR).

MATERIALS AND METHODS

Patients

From December 2011 to April 2015, all patients with tumors in liver (such as HCC, metastatic colorectal cancer, cholangiocellular carcinoma and benign tumor) treated with hepatic resection were enrolled. Patients were prospectively received either OLR or LLR in the department of gastroenterological surgery at hospital of Kumamoto university. The serum samples were collected early in the morning after an overnight fast. Blood samples that used to measure IL-6 and TSP-1 were taken at four time points: immediately before anesthetic induction (preoperative), immediately after closure of the skin incision (postoperative), postoperative day 1 (POD1) and POD3. Thirty patients were treated with partial liver resection and obtained blood samples at all four time points. Blood samples taken up to POD3 were used because previous studies had not found significant changes in TSP-1 Levels after POD5. Ten of 30 patients underwent laparoscopic partial hepatectomy and other 20 patients underwent open partial hepatectomy. All procedures were performed by the same surgical team, and the same surgical and oncological principles were followed in both groups. The patients received similar preoperative and postoperative management.

Patient data were separately collected and analyzed. This study was retrospective, non-interventional, which approved by the institutional ethics committee of Kumamoto University Hospital and was performed in accordance with the Helsinki Declaration of 1975.

Biomarker assays

All serum and plasma biomarker (such as IL-6 and TSP-1) samples were stored at -80 °C until analysis. Serum and plasma samples concentrations were measured by commercially available ELISA kits for IL-6 and TSP-1 (R and D Systems; catalog numbers, D6050 and DTSP10, respectively), according to the manufacturers' instructions.

Estimated operative factors for higher level of IL-6 and lower level of TSP-1

A total of 9 variables were analyzed: age, sex, operative method, the number of tumors, operative time, bleeding loss, the presence of complication, the kind of skin incision, and the presence of liver mobilization. Operative method was divided into OLR and LLR. The presence of complication was defined as more than Clavien-Dindo classification IIIa. The skin incision was divided into reverse L-sharp incision and others. The cut off value of age, operative time, bleeding loss and level of serum IL-6 and plasma TSP-1 was based on the median.

Statistical analysis

All statistical analyses were carried out by using JMP® 14 (SAS Institute Inc., Cary, NC, United States). Continuous variables were compared using Student's *t* tests, and Categorical variables were compared using the Chi-square test. The multivariate analysis to estimate the risk factors was undertaken using the Cox proportional hazard model. The multivariate analysis to be identified clinical factors which related to high level of serum IL-6 on POD1 and lower level of plasma TSP-1 on POD3 was carried out using logistic regression analysis. Continuous variables were converted into two groups at the median. Statistical significance was defined as $P < 0.05$.

RESULTS

Patient characteristics

The median age was 71.0 years with age range from 31 years to 94 years. Among 30 patients, 18 (60.0%) were men and 12 (40%) were women. Two patients were HBs-Ag positive and 12 patients were HCV-Ab positive. The indication for hepatectomy were primary tumor in 20 patients, metastatic liver cancer in 8 patients, and benign tumor in 2 patients. Ten patients underwent LLR and 20 patients underwent OLR. No differences were detected in the clinical data between the LLR group and the OLR group (Table 1). Age, sex, hepatitis B infection, hepatitis C infection, preoperative blood tests (such as white blood cell, platelet, prothrombin time, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and cholinesterase, retention of indocyanine green at 15 min (ICG-R15), Child-Pugh classification and difficulty scoring system were similar. In operative and postoperative data, bleeding loss in LLR

Table 1 Clinical characteristics of study patients

Factors	LLR	OLR	*P*
	n = 10	*n* = 20	
Age, years	74 (31-94)	71 (35-83)	0.91
Sex (male/female)	6/4	12/8	1.0
HBs-Ag (positive/negative)	0/10	2/18	0.20
HCV-Ab (positive/negative)	4/6	8/12	1.0
White blood cell, × 10^3 /uL	5.5 (3.1-6.0)	6.8 (2.5-10.6)	0.23
Platelet, × 10^4 /uL	16.4 (11.2-21.2)	17.5 (8.3-88.0)	0.36
Prothrombin time, %	102 (69-122)	105 (65-126)	0.86
AST, U/L	23 (14-48)	27 (13-175)	0.52
ALT, U/L	23 (10-46)	26 (6-103)	0.31
ALP, U/L	221 (138-375)	243 (134-676)	0.36
ChE, U/L	263 (154-327)	231 (121-357)	0.39
ICG R15, %	10.4 (4.5-24.7)	10.6 (0.9-65.4)	0.84
Child-Pugh classification (A/B)	10/0	18/2	0.20
Difficulty scoring system	4 (2-5)	4 (2-6)	0.44
Operative time, min	290 (126-660)	385 (82-633)	0.88
Bleeding volume, mL	57 (5-750)	528 (116-9527)	0.003
Resected liver volume, g	66.5 (4-206)	77.5 (2-220)	0.76
Longest diameter of the tumor, mm	17 (6-53)	25 (7-55)	0.27
Number of resected lesion	1 (1-2)	2 (1-7)	0.1
Surgical margin, mm	5 (0-55)	1 (0-22)	0.24
Diagnosis(primary/metastasis/benign)	7/2/1	13/6/1	0.77
Postoperative hospital stay, d	10 (6-14)	14 (8-26)	0.003
Complications (no/yes)	10/0	17/3	0.1

No complication means less than IIIa at clavien-dindo classification. LLR: Laparoscopic liver resection; OLR: Open liver resection; HBs-Ag: hepatitis B surface antigen; HCV-Ab: hepatitis C antibody; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; ChE: Cholinesterase; ICG-R15: Retention of indocyanine green at 15 min.

group was significantly less than that in OLR group (P = 0.003). Postoperative hospital stay in LLR group was significantly shorter than that in OLR group (P = 0.003). In operative time, resected liver volume, longest diameter of the tumor, the number of tumors, surgical margin, primary diagnosis and complication, there was no significantly difference between the two groups. The postoperative complications included one case of bile leakage, one case of refractory ascites, and one case of surgical site infection.

Time course in levels of serum IL-6 and plasma TSP-1 according to operative approaches

Time course of serum IL-6 Level and plasma TSP-1 Level before and after liver resection are shown in Table 2. Serum level of IL-6 on POD1 and POD3 in LLR group was significantly lower than those in OLR group (8.7 *vs* 30.3 pg/mL, P = 0.003, 9.3 *vs* 31.7 pg/mL, P = 0.03). There was no significant difference in serum level of IL-6 on pre- and post-operation between the two groups. Plasma level of TSP-1 on POD3 in LLR group was significantly higher than those in OLR group (1704.0 *vs* 548.3 ng/mL, P = 0.009), and have already recovered to preoperative level. There was no significant difference in plasma level of TSP-1 on pre-, post-operation and POD1 between the two groups.

Table 2 Time course of circulating serum level of interleukin-6 and plasma level of thrombospondin-1 before and after laparoscopic and open liver resection

Factors	LLR	OLR	*P*
	n = 10	*n* = 20	
IL-6, pg/mL			
Preoperatively	3.9 (2.6-43.6)	3.7 (2.0-68.8)	0.50
Postoperatively	30.8 (4.9-189.8)	73.6 (4.7-348.8)	0.14
POD1	8.7 (4.8-359.3)	30.3 (10.8-376.3)	0.003
POD3	9.3 (4.1-99.7)	31.7 (7.0-212.3)	0.03
TSP-1, ng/mL			
Preoperatively	1817.1 (141.7-2985.7)	2025.1 (124.6-3815.0)	0.98
Postoperatively	1364.7 (84.1-2459.3)	684.4 (42.5-3580.3)	0.52
POD1	589.1 (139.3-3186.4)	233.6 (36.6-4058.3)	0.12
POD3	1704.0 (665.4-3399.2)	548.3 (30.4-2239.2)	0.009

TSP-1: Thrombospondin-1; IL-6: Interleukin-6; LLR: Laparoscopic liver resection; OLR: Open liver resection; POD: Postoperative days.

Changes in inflammatory and liver functional markers according to operative approaches

At any point in time, the neutrophil ratio in LLR group was not significant differ compared with that in OLR group (Table 3). However, serum level of CRP on POD3, 5 and 7 in LLR group was significantly lower than that in OLR group (2.04 *vs* 5.93 mg/dL, $P = 0.01$, 1.18 *vs* 4.36 mg/dL, $P = 0.01$, 0.65 *vs* 2.77 mg/dL, $P = 0.01$,). In the liver functional biomarker (such as total bilirubin, bile acid and albumin), there was no significant difference between the two groups at any time (for example, pre-operation, POD3, POD5, and POD30).

Factors related to higher level of serum IL-6 in patients who had undergone partial hepatectomy

Among the changes in postoperative serum level of IL-6 over time, the level of IL-6 on POD1 showed the most difference between the two groups. The median serum level of IL-6 on POD1 in 30 patients who had undergone hepatectomy was 17 pg/mL. The higher level of IL-6 group on POD1 was defined as 17 pg/mL or higher and divided into two groups. The statistically significant factors which related to higher level of IL-6 Level on POD1 by univariate analysis are listed in Table 4. The operative method as an operation-related factor and the number of tumors as a tumor-related factor was identified as factors which related to higher level of IL-6 on POD1. Multivariate analysis showed that open approach was the only independent factor related to higher level of IL-6 on POD1 [odds ratio (OR), 7.48; 95% confidence interval (CI): 1.28-63.3; $P = 0.02$].

Relationship between serum level of IL-6 on POD1 and plasma level of TSP-1 on POD3

Table 2 showed that the only plasma level of TSP-1 on POD3 was significant difference between the two groups. The median plasma level of TSP-1 on POD3 in 30 patients who had undergone hepatectomy was 898 ng/mL. The lower level of TSP-1 on POD3 was defined as 898 ng/mL or lower and divided into two groups. The statistically significant factors which related to lower level of TSP-1 on POD3 by univariate analysis are listed in Table 5. The higher level of IL-6 on POD1 and the skin incision (reverse-L sharp *vs* others) was identified as factors which related to lower level of TSP-1 on POD3 (Table 6). Multivariate analysis showed that the higher level of IL-6 than 17.0 pg/mL on POD1 was the only independent factor related to lower level of TSP-1 on POD3 (OR, 5.32; 95%CI: 1.08-32.2; $P = 0.04$). The plasma level of TSP-1 on POD3 in higher IL-6 group was significantly lower than that in lower IL-6 group.

Table 3 Comparison between changes in inflammatory and liver functional markers before and after laparoscopic and open liver resection

Factors	LLR	OLR	*P*
	n = 10	*n* = 20	
Total bilirubin, mg/dL			
Preoperatively	0.8 (0.4-1.4)	0.8 (0.4-2.6)	0.69
POD5	0.7 (0.5-0.9)	0.9 (0.3-5.2)	0.11
POD30	0.7 (0.3-1.1)	0.7 (0.4-2.5)	0.95
Bile acid, μmol/L			
Preoperatively	7.4 (0.8-16.8)	12.4 (0.7-82.1)	0.21
POD3	4.9 (0.6-20.8)	9.9 (1.6-56.7)	0.13
Albumin, g/dL			
Preoperatively	3.8 (3.5-4.5)	3.6 (2.9-4.4)	0.08
POD5	3.0 (2.4-3.3)	3.0 (2.3-3.5)	0.77
POD30	3.9 (3.4-4.6)	3.6 (2.2-4.4)	0.16
Neutrophil, %			
Preoperatively	62.1 (46.7-74.3)	67.3 (28.8-85.2)	0.17
POD1	82.5 (68.1-92.7)	83.1 (70.7 – 92.2)	0.86
POD3	70.9 (58.7-83.7)	76.3 (52.9-86.6)	0.20
POD5	69.2 (46.3-80.4)	67.6 (45.1-80.9)	0.95
POD7	67.3 (49.8-76.7)	69.3 (38.3-77.5)	0.42
CRP, mg/dL			
Preoperatively	0.07 (0.01-2.67)	0.07 (0.01-3.50)	0.57
POD1	0.96 (0.24-6.32)	1.48 (0.38-4.94)	0.28
POD3	2.04 (0.62-9.16)	5.93 (1.12-16.48)	0.01
POD5	1.18 (0.20-5.83)	4.36 (0.44-14.3)	0.01
POD7	0.65 (0.11-1.52)	2.77 (0.31-9.97)	0.01

LLR: Laparoscopic liver resection; OLR: Open liver resection; POD: Postoperative days; CRP: C-reactive protein.

DISCUSSION

From the introduction of laparoscopic cholecystectomy in 1987, the number of laparoscopic procedures quickly increased due to its minimally invasive advantages over laparotomy[19]. Laparoscopic hepatectomy was also reported in 1992 and has been on the rise worldwide year after year[20]. The advantages of laparoscopic surgery include cosmetically attractive scars, less postoperative pain, and shorter hospital stay[21]. On the other hand, conventional laparotomy has good tactile sensation, hepatic mobilization, control of bleeding. LLR has also been reported to be less invasive than laparotomy on the basis of less blood loss, fewer postoperative complications, and shorter hospital stay[22]. Our study showed similar results, with less blood loss and shorter hospital stay in laparoscopic surgery compared to open surgery (57 *vs* 528 mL, $P = 0.003$, 10 *vs* 14 d, $P = 0.003$). In fact, there are only a few reports that have scientifically investigated the minimally invasive nature of laparoscopic hepatectomy [12,23,24].

It has been reported that the degree of surgical trauma is reflected by the extent of systemic response[23]. IL-6 is a known hepatocyte-stimulating cytokine that is easily synthesized and activates the acute immune response in response to infection or tissue damage. IL-6 correlates with the degree of surgical trauma[25]. The present study showed this biochemical parameter of surgical trauma to compare the serum levels of IL-6 in laparoscopic with OLR by using ELISA kit. The serum level of IL-6 was highest

Table 4 Univariate and multivariate analysis of factors that related with higher level of interleukin-6 on postoperative day 1 in 30 patients who has undergone hepatectomy

Factors	Univariate analysis			Multivariate analysis		
	OR	95%CI	*P*	OR	95%CI	*P*
Sex (male/female)	0.80	0.18-3.50	0.77			
Age (≥ 75/< 75, yr)	1.289	0.30-5.57	0.73			
Operative method (OLR/LLR)	9.33	1.74-75.7	0.008	7,48	1.28-63.3	0.02
Number of tumors (single/multiple)	0.21	0.04-0.99	0.048	0.29	0.04-1.64	0.16
Operative time (< 369/≥ 369, min)	2.31	0.54-10.7	0.29			
Bleeding loss (< 443/≥ 443, mL)	3.00	0.70-14.3	0.14			
Complication (yes/no)	0.74	0.20-11.9	0.74			
Skin incision (reverse L-sharp/others)	1.94	0.43-9.62	0.39			
Liver mobilization (yes/no)	1.73	0.31-10.6	0.53			

IL-6: Interleukin-6; OR: Odds ratio; CI: Confidence interval; OLR: Open liver resection; LLR: Laparoscopic liver resection.

Table 5 Univariate and multivariate analysis of factors that related with lower level of thrombospondin-1 on postoperative day 1 in 30 patients who has undergone hepatectomy

Factors	Univariate analysis			Multivariate analysis		
	OR	95% CI	*P*	OR	95% CI	*P*
Sex (male/female)	0.57	0.12-2.48	0.46			
Age (≥ 75/< 75, yr)	1.00	0.23-4.26	1.00			
Higher level of IL-6 on POD1 (≥ 17.0/< 17.0, pg/mL)	5.50	1.22-29.4	0.03	5.32	1.08-32.2	0.04
Operative method (OLR/LLR)	3.50	0.73-20.3	0.29			
Number of tumor (single/multiple)	1.00	0.23-4.39	1.00			
Operative time (< 369/≥ 369, min)	0.33	0.07-1.43	0.14			
Bleeding loss (< 443/≥ 443, ml)	0.44	0.10-1.88	0.27			
Complication (yes/no)	1.63	0.23-14.0	0.62			
Skin incision (reverse L-sharp/others)	4.57	0.97-26.7	0.05	4.38	0.82-29.9	0.09
Liver mobilization (yes/no)	3.24	0.56-26.2	0.19			

TSP-1: Thrombospondin-1; IL-6: Interleukin-6; OR: Odds ratio; CI: Confidence interval; OLR: Open liver resection; LLR: Laparoscopic liver resection.

postoperatively in both groups, but there was no significant difference between the two groups at that time. IL-6 Levels on POD1 and 3 was significantly lower in the LLR group and decreased to preoperative values. In the OLR group, IL-6 Levels on POD1 and 3 decreased from the postoperative level, but the improvement to the preoperative level was not observed and was prolonged. The change in CRP levels showed a peak on POD3 in both groups. Thereafter, it decreased slowly and improved to baseline. CRP levels on POD3, 5, and 7 were significantly lower in the LLR group than in the OLR group. Shenkin *et al*[26] reported that in open cholecystectomy, IL-6 Levels reached a maximum level 1.5-4 h after skin incision and the CRP level was detectable 8-12 h after the skin incision. In this current study, IL-6 and CRP levels showed similar changes over time, and these changes were the same in both the LLR and OLR groups. There was no significant difference in the marker of liver function (such as Total-bilirubin, bile acids, and albumin levels) between the two groups. We showed that laparoscopic surgery was associated with lower IL-6 Levels on the first postoperative day, which was thought to reflect the minimally invasive procedure. In the present study, there was no relationship between operative time or blood loss and the

Table 6 Time course of circulating plasma thrombospondin-1 levels according to serum interleukin-6 levels on postoperative day 1

Factors	Lower IL-6	Higher IL-6	*P*
	n = 14	*n* = 16	
TSP-1, ng/mL			
Preoperatively	2420.6 (223.0-3121.1)	893.9 (124.6-3815.0)	0.088
Postoperatively	1672.7 (42.5-2674.4)	504.8 (53.9-3580.3)	0.067
POD1	447.9 (101.7-3186.4)	246.2 (36.6-4058.3)	0.24
POD3	1432.4 (30.4-3399.2)	548.3 (53.3-3153.2)	0.026

TSP-1: Thrombospondin-1; IL-6: Interleukin-6; POD: Postoperative days.

minimally invasive procedure. There was also no relationship between the skin incision or liver mobilization and the minimally invasive procedure.

The only significant difference in the number of resected lesion was not observed in the multivariate analysis, but was observed in the univariate analysis. The number of resected lesion might be related to surgical invasiveness. Although there was no significant difference in the indications for surgery in this study, there was a tendency for patients with a large number of tumors to choose laparotomy. Complication was not observed in the univariate analysis of the factors that related with higher level of IL-6 on POD1 in 30 patients who has undergone hepatectomy. In present study, complications were not affected higher level of IL-6 on POD1.

TSP-1 is a matricellular protein which can be produced by a variety of cells, particularly by platelets and endothelial cells and can act as a negative regulator of liver regeneration by activating latent transforming growth factor-β1[17,27,28]. Kuroki *et al* [18] showed that the plasma level of TSP-1 decreased to the lowest level on POD1 compared with the pretreatment value, suggesting that a reduced plasma TSP-1 Level is required for a regenerative response in the liver after hepatectomy[16,18]. They expressed that a decrease in TSP-1 after partial hepatectomy was associated with liver damage, and the less invasive it is to the liver, the faster the improvement of TSP-1 Level. In our study, the plasma level of TSP-1 was the lowest on POD1 and showed improvement on POD3. Although the plasma TSP-1 values before and after LLR and OLR showed similar changes, the values on POD3 were significantly higher in LLR than in OLR and improved to the preoperative values. And multivariate analysis of factors associated with low level of plasma TSP-1 on POD3 showed that high level of serum IL-6 on POD1 were significant. Open approach was associated with a greater release of IL-6 on POD1 than laparoscopy approach, and this may contribute to liver regeneration by suppressing the increase in TSP-1 on POD3. This might indicate that laparoscopy is a less invasive procedure than laparotomy. Complication was not observed in the univariate analysis of the factors that related with lower TSP-1 on POD3. In present study, complications were not affected lower level of TSP-1 on POD3.

There are limitations to our research. The number of cases is small and it is not a randomized trial. It needs to be studied in a larger number of cases. However, as far as we could find, there were no reports using IL-6 and TSP-1 to evaluate the degree of invasiveness of the laparoscopic approach. Secondly, this study can't reveal the biological process to explain the relationship between IL-6 and TSP-1. There are no reports on it, and this is a future issue.

CONCLUSION

In conclusion, laparoscopic hepatectomy might be minimally invasive surgery with less IL-6 production compared to open hepatectomy.

ARTICLE HIGHLIGHTS

Research background

There are few reports that have scientifically verified whether laparoscopic surgery is truly minimally invasive in liver resection.

Research motivation

Evaluation of minimally invasive laparoscopic surgery will also be important when robot-assisted surgery becomes more widespread in the future. There are many unclear points, such as whether invasion reflects skin incision size or organ invasion.

Research objectives

We aimed to verify whether the laparoscopic technique contributes to minimally invasive procedures in surgery using biomarkers of interleukin-6 (IL-6) and thrombospondin-1 (TSP-1).

Research methods

This study is a retrospective study. Serum IL-6 and TSP-1 were measured and analyzed by ELISA using blood samples taken before and after surgery. We also evaluated the relationship between the operative approach, the size of the skin incision and the presence of liver mobilization.

Research results

This study demonstrated that laparoscopic liver resection is likely to be scientifically less invasive than open liver resection. The lower IL-6 Level was significantly related to the operative methods. The limitation of this study is that the number of cases is small, so further accumulation and analysis is needed in the future.

Research conclusions

In patients who undergo liver resection, laparoscopic approach that is less invasive than open approach is preferred whenever possible.

Research perspectives

Studies conducted in the future should focus on evaluating whether biomarker such as IL-6 affected not only short-term outcomes but also long-term outcomes and how several biomarker change with robot-assisted techniques.

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ORIGINAL ARTICLE

Retrospective Study

Can the computed tomography texture analysis of colorectal liver metastases predict the response to first-line cytotoxic chemotherapy?

Etienne Rabe, Dania Cioni, Laura Baglietto, Marco Fornili, Michela Gabelloni, Emanuele Neri

ORCID number: Etienne Rabe 0000-0002-9972-5508; Dania Cioni 0000-0002-5120-886X; Laura Baglietto 0000-0002-8193-7529; Marco Fornili 0000-0002-5713-3926; Michela Gabelloni 0000-0002-6668-499X; Emanuele Neri 0000-0001-7950-4559.

Author contributions: Rabe E conceptualized and designed the study; Neri E assisted with the study methodology and supervised the study as Master tutor; Rabe E collected the data, performed the formal image analysis and wrote the original draft; Baglietto L and Fornili M performed the statistical analysis of the data and contributed to the interpretation of the results; Cioni D, Baglietto L, Fornili M, Gabelloni M and Neri E reviewed and revised the manuscript.

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Etienne Rabe, Dania Cioni, Michela Gabelloni, Emanuele Neri, Academic Radiology, Master in Oncologic Imaging, Department of Translational Research, University of Pisa, Pisa 56126, Italy

Etienne Rabe, Bay Radiology-Cancercare Oncology Centre, Bay Radiology, Port Elizabeth 6001, Eastern Cape, South Africa

Laura Baglietto, Marco Fornili, Department of Clinical and Experimental Medicine, University of Pisa, Pisa 56126, Italy

Corresponding author: Etienne Rabe, Academic Radiology, Master in Oncologic Imaging, Department of Translational Research, University of Pisa, Lungarno Antonio Pacinotti Street 43, Pisa 56126, Italy. etienne.rabe@gmail.com

Abstract

BACKGROUND

Artificial intelligence in radiology has the potential to assist with the diagnosis, prognostication and therapeutic response prediction of various cancers. A few studies have reported that texture analysis can be helpful in predicting the response to chemotherapy for colorectal liver metastases, however, the results have varied. Necrotic metastases were not clearly excluded in these studies and in most studies the full range of texture analysis features were not evaluated. This study was designed to determine if the computed tomography (CT) texture analysis results of non-necrotic colorectal liver metastases differ from previous reports. A larger range of texture features were also evaluated to identify potential new biomarkers.

AIM

To identify potential new imaging biomarkers with CT texture analysis which can predict the response to first-line cytotoxic chemotherapy in non-necrotic colorectal liver metastases (CRLMs).

METHODS

Patients who presented with CRLMs from 2012 to 2020 were retrospectively selected on the institutional radiology information system of our private radiology practice. The inclusion criteria were non-necrotic CRLMs with a minimum size of 10 mm (diagnosed on archived 1.25 mm portal venous phase CT

Health Research, published by the Department of Health and the Declaration of Helsinki (last updated October 2013), adopted by the World Medical Association (WMA), which deals with the recommendations guiding doctors in biomedical research involving human participants. Copies of these documents may be obtained upon reasonable request.

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scans) which were treated with standard first-line cytotoxic chemotherapy (FOLFOX, FOLFIRI, FOLFOXIRI, CAPE-OX, CAPE-IRI or capecitabine). The final study cohort consisted of 29 patients. The treatment response of the CRLMs was classified according to the RECIST 1.1 criteria. By means of CT texture analysis, various first and second order texture features were extracted from a single non-necrotic target CRLM in each responding and non-responding patient. Associations between features and response to chemotherapy were assessed by logistic regression models. The prognostic accuracy of selected features was evaluated by using the area under the curve.

RESULTS

There were 15 responders (partial response) and 14 non-responders (7 stable and 7 with progressive disease). The responders presented with a higher number of CRLMs (P = 0.05). In univariable analysis, eight texture features of the responding CRLMs were associated with treatment response, but due to strong correlations among some of the features, only two features, namely minimum histogram gradient intensity and long run low grey level emphasis, were included in the multiple analysis. The area under the receiver operating characteristic curve of the multiple model was 0.80 (95%CI: 0.64 to 0.96), with a sensitivity of 0.73 (95%CI: 0.48 to 0.89) and a specificity of 0.79 (95%CI: 0.52 to 0.92).

CONCLUSION

Eight first and second order texture features, but particularly minimum histogram gradient intensity and long run low grey level emphasis are significantly correlated with treatment response in non-necrotic CRLMs.

Key Words: Colorectal cancer; Liver metastases; Radiomics; Computed tomography texture analysis; Response assessment

Core Tip: Radiomics is a rapidly growing field of radiological research which has the potential to assist with the diagnosis, prognostication and therapeutic response prediction of various cancers and may potentially play an important role in personalized patient care. This retrospective study aimed to identify potential new imaging biomarkers with computed tomography texture analysis which can predict the response to first-line cytotoxic chemotherapy in non-necrotic colorectal liver metastases. Eight first and second order texture features, but particularly minimum histogram gradient intensity and long run low grey level emphasis are significantly correlated with treatment response. These preliminary results need to be validated and confirmed on larger patient cohort studies.

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignant tumors. According to the global burden of cancer worldwide using the GLOBOCAN 2018, it was estimated that colorectal cancer was the fourth most common cancer and second leading cause of cancer related deaths[1].

The liver is the most frequent site of metastatic disease[1] and approximately 20%-25% of the patients with CRC will have synchronous liver metastases at the time of diagnosis and at least another 60% of patients who develop metastatic disease will have metachronous liver-confined metastases[2].

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Unfortunately, approximately half of the patients with colorectal cancer have no treatment response or develop disease progression despite first-line chemotherapy[3]. Since the introduction of targeted therapies (*e.g.*, bevacizumab and cetuximab) there has been an increase in the progression-free and overall survival rates in several clinical studies with the median overall survival exceeding 2 years[4-6].

Oncologists monitor their patients closely with regard to their clinical course, performance status and laboratory tests (for instance liver function tests and tumor marker levels) to determine if their patients with cancer are responding to the chemotherapy or potentially progressing. It will be greatly beneficial to the oncologists if we could identify effective predictive biomarkers on the baseline imaging examination which can estimate the response which can be expected during chemotherapy in order to individualize treatment (precision medicine). These imaging biomarkers may prompt the oncologists to perform earlier follow-up imaging studies to determine whether an alternative chemotherapy treatment should be considered.

The Response Evaluation Criteria in Solid Tumors (RECIST 1.1) is typically and mainly used to assess the response to chemotherapy and measures and classifies the changes in the longest axial tumor diameters[7]. Due to the irregular shapes of tumors these size measurements may, however, not be representative of the true tumor volume. Moreover, the correlation between RECIST and the pathological response is known to be limited[8-9].

Radiomics is a rapidly growing field of radiological research where routine patient scans are converted into mineable quantitative data[10] that can be utilized to decode the tumor phenotype for applications ranging from improved diagnostics to prognostication to therapeutic response prediction[11]. In radiomics, computed tomography (CT) texture analysis quantifies tissue heterogeneity by assessing the distribution of grey-levels, texture coarseness and irregularity within a lesion[12-15]. Studies on different tumors have shown that CT texture analysis has promise in predicting pathological features, overall survival and response to therapy[15-17]. In the last few years a few studies have also reported that texture analysis can be helpful in predicting the response to chemotherapy for colorectal liver metastases (CRLMs)[18-22]. Thus far the CT texture analysis results of responding CRLMs in studies have been heterogeneous which can be secondary to many technical factors. In none of the aforementioned studies were necrotic metastases clearly excluded. The contrast injection protocols were not standardized or defined in all the studies. The CT slice thickness varied between 2 mm and 5 mm in the different studies and some studies combined CT scans with different slice thickness reconstructions for texture analysis. A thicker slice thickness can lead to partial volume effects which can affect the accuracy of the texture analysis results. In most of the studies predominately first order CT texture features were assessed and only a few studies included some second order texture features (predominantly grey level co-occurrence matrix features).

The purpose of this retrospective explorative study is to identify potential new imaging biomarkers by assessing a larger range of first and second order texture features with CT texture analysis which can predict the response to first-line cytotoxic chemotherapy in non-necrotic CRLMs and to compare the results with the findings from previous studies.

MATERIALS AND METHODS

Study design

This retrospective study was approved by the BLINDED Ethics Committee and was conducted in accordance with the ethical standards of the Declaration of Helsinki. Patient informed consent was waived.

The study population was selected in a consecutive retrospective manner by using the ICD-10 codes (International Classification of Diseases and Related Health Problems, 10th revision) for CRC to identify all patients on the institutional radiology information system (RIS) of our private radiology practice for the period of March 2012 to May 2020. All the CT scans were performed at one of the branches of our radiology practice in our demographic region.

The inclusion criteria were histopathological confirmed colorectal cancer with synchronous (diagnosed within 6 mo of primary CRC) or metachronous liver metastases; portal venous phase CT scans with archived slice thickness of 1.25 mm; hepatic metastasis minimum size of 10 mm; one of the following standard first-line chemotherapy regimens: FOLFOX, FOLFIRI, CAPE-OX, CAPE-IRI, FOLFOXIRI or capecitabine.

The exclusion criteria included absent baseline CT scan; poor quality portal venous phase CT scan due to inadequate contrast enhancement or artefacts; hepatic metastasis size less than 10 mm; metastases with clear necrosis or calcifications; fatty liver or other chronic liver pathology; previous chemotherapy; first-line chemotherapy combined with targeted or other therapy; more than 2 mo delay between baseline CT scan and start of chemotherapy; more than 7 mo delay since onset of first line chemotherapy and follow-up CT scans; no follow-up CT scan after chemotherapy; previous liver surgery or surgery/radiofrequency ablation after chemotherapy; mucinous colon carcinoma; history of previous or other coexisting malignancies. The final study cohort consisted of 29 patients.

Data

The following clinical and pathological information was collected from our RIS and patient medical records: patient demographics (age at diagnosis, date of diagnosis, gender); original CRC histology and grade of primary CRC; Kirsten rat sarcoma viral oncogene homolog (KRAS) mutation status (mutant or wild-type), if available; TNM staging of CRC; CEA and CA19-9 tumor marker levels around the time of baseline CT scan; details of first-line chemotherapy.

First-line chemotherapy regimens

All the patients received one of the following cytotoxic chemotherapeutic substances according to the National Comprehensive Cancer Network clinical guidelines in oncology: FOLFOX (intravenous (IV) 5-FU, leucovorin and oxaliplatin), FOLFIRI (IV 5-FU, leucovorin and irinotecan), FOLFOXIRI (IV 5-FU, leucovorin, oxaliplatin, and irinotecan), CAPE-OX (oral capecitabine and oxaliplatin), CAPE-IRI (oral capecitabine and irinotecan) and oral capecitabine. None of the study cases received targeted therapy. Chemotherapy was administered until there was radiological evidence of disease progression according to the RECIST 1.1 criteria.

CT acquisition

The CT examination closest to the date of diagnosis of the liver metastases was selected for the radiomics analysis.

All the scans in the study cohort were performed on three different multidetector CT scanners: GE Lightspeed RT16 (n = 16), GE Optima CT540 (n = 11) and GE Discovery IQ (n = 2) (GE healthcare, Milwaukee, WI). The portal venous phase CT scans were used for the radiomics analysis and were acquired as part of either a four-phase (unenhanced, arterial, portal venous, delayed phases, n = 19), a three-phase (unenhanced, arterial, portal venous, n = 8) or biphasic (unenhanced, portal venous, n = 2) contrast enhanced CT examination. The CT acquisition parameters of the study cohort are summarized in Table 1.

All the patients in the study cohort received intravenously 1.0-1.5 mL/kg of iomeprol 400 mgI/mL (Iomeron 400®, Bracco Diagnostics, Milan, Italy) except for one patient who received intravenously 1.8 mL/kg of ioversol 350 mgI/mL (Optiray PF 350®, Guerbet, Aulnay-sous-Bois, France). Contrast medium was injected at a rate of 2 mL/sec with an automatic power injector and a bolus tracking CT density threshold (SmartPrep®, GE Healthcare) of 100 HU. In the standard CT scan protocol, the portal phase scan is acquired at 80 s. The contrast medium injection was followed by a saline flush of 50-60 mL which was injected at 2 mL/sec.

CRLM segmentation and texture analysis

The texture analysis of the CRLMs was performed with the SOPHiA Radiomics beta-hepatic-metastasis software (version 2.1.7) of SOPHiA GENETICS. The DICOM images of the baseline 1.25 mm portal venous phase scans were used for the texture analysis.

Prior to feature extraction trilinear voxel size normalization (resampling) was performed to normalize the voxel size to 1 mm × 1 mm × 1 mm. A mean ± three standard deviations (3SD) for intensity rescaling was used. For the basic first order intensity-based features there was no discretization applied. Grey level intensity discretization was performed by using 32 grey levels for the discretized intensity-based features as well as for the second order texture features (fixed bin number of 32).

A 3D semi-automatic technique was used to perform the segmentation of a single target CRLM in each patient. Where the segmentation was inaccurate, the contours were manually edited. In a few cases complete manual segmentation of the CRLMs was required. All the segmentations were performed by the principal investigator (general radiologist with 20 years of CT experience). The major hepatic vessels, edge of the liver and the hypervascular rims which can be associated with some CRLMs

Table 1 Computed tomography acquisition parameters in study cohort

CT scanner model	Number of study cases (*n*)	Detector collimation	Spiral pitch factor	Rotation time (s)	Voltage (kVp)	Tube current-time product at level of liver metastases (mAs)	Noise Index	Reconstruction kernel	Slice thickness/reconstruction interval (mm)	Field of view (cm)	Matrix size (pixels)
GE Lightspeed RT16	16	16 × 1.25 mm	1.375:1	0.8 (*n* = 14); 0.9 (*n* = 1); 1.0 (*n* = 1)	120 (*n* = 14); 140 (*n* = 2)	96-300	11.5	SOFT	1.25/1.25	50	512 × 512
GE Optima CT540	11	16 × 1.25 mm	1.375:1 (*n* = 9); 0.938:1 (*n* = 2)	0.7 (*n* = 6); 0.8 (*n* = 4); 0.9 (*n* = 1)	120	109.6-277.2	11.5 -13	SOFT	1.25/1.25	50	512 × 512
GE Discovery IQ	2	16 × 1.25 mm	0.938:1	0.8	120	155.2 and 209.6	11-11.5	SOFT	1.25/1.25	50	512 × 512

CT: Computed tomography.

(rarely encountered on portal venous phase scans) were excluded from the radiomics analysis. No intra- or inter-observer variation was evaluated.

The radiomics features which were calculated and extracted meet the standards and criteria of the Image Biomarker Standardization Initiative (IBSI)[23].

The radiomics features extracted with SOPHiA Radiomics are listed in Supplementary Table S1. The radiomics features include morphological indicators (27 features), statistics (21 features), local intensity indicators (4 features), intensity histogram indicators (24 features), volume intensity histogram indicators (5 features), grey level co-occurrence matrix texture indicators (26 features), grey level run length matrix (GLRLM) texture indicators (16 features), grey level size zone matrix texture indicators (16 features), grey level distance zone matrix texture indicators (16 features), neighborhood grey tone difference matrix texture indicators (3 features) and neighborhood grey level difference (NGLDM) texture indicators (17 features).

Response evaluation

A single target CRLM without clear necrosis or calcification was analyzed in each patient on the baseline and follow-up CT scan. The RECIST 1.1 criteria were used to assess the response to treatment[7]. No non-target liver metastases were included in this study.

The patients in whom the liver metastases demonstrated a complete response (CR) or partial (PR) were classified as responders and the patients with either stable disease (SD) or progressive disease (PD) were classified as non-responders.

Following the technique illustrated by Ahn *et al*[19], a single target CRLM which demonstrated the best PR or CR (not necessarily the largest lesion) was evaluated in each responder. In each non-responder a single target liver metastasis which demonstrated the worst response to treatment (SD or PD) was segmented.

Independent observer

An independent general radiologist (25 years of experience in CT and oncologic imaging) visually confirmed and validated the selected CRLMs and the accuracy of the segmentations of the volumes of interest of target lesions. Where required, further manual editing was performed and a mutual consensus was reached regarding the final segmentations.

Statistical methods

Categorical variables were described by frequencies and percentages and continuous variables by medians and interquartile ranges (IQRs). Associations between exposures and response to chemotherapy were assessed by non-parametric Fisher's exact tests and Kruskal-Wallis tests, for categorical and continuous variables respectively.

In order to limit the influence of extreme values, radiomic features were categorized into tertiles and the corresponding pseudo-continuous variables were calculated assigning 1 to the 1st tertile, 2 to the 2nd tertile and 3 to the 3rd tertile. Logistic regression models were fitted to estimate the associations between clinical response and each pseudo-continuous variable and the likelihood ratio test was applied to assess the significance of the association.

Redundant features were identified and excluded based on analysis of correlations. Features statistically significant in the univariable models were included in the multiple model. The performance of the multiple model in predicting response to therapy was assessed by the area under the receiver operating characteristic (ROC) curve (AUC). The best cut-off of the linear predictor was identified as the point on the ROC curve nearest to the point with sensitivity and specificity both equal to 1; the corresponding sensitivity and specificity were estimated. AUC estimate adjusted for optimism was obtained with a validation procedure based on bootstrap resampling [24]. A nomogram was built from the multiple model.

All the statistical tests were two-sided with a significance level of 0.05. The analyses were conducted with the statistical software *R* version 4.0.2, and its package *rms*.

RESULTS

Patient characteristics

The CT scans of 236 consecutive patients with CRLMs who presented from March 2012 to May 2020 were retrospectively reviewed. Only 29 patients with CRLMs fulfilled all the inclusion criteria (Figure 1).

The demographic, clinical and tumor characteristics of the patient cohort are summarized in Table 2. Fifteen patients were classified as responders (all with PR) and 14 patients were classified as non-responders (7 SD and 7 PD) (Table 3). The median age at diagnosis was 59 years (IQR: 52 to 73) and 62% of participants were male.

Among the patient characteristics, only the number of CRLMs showed a positive correlation with the response group (P = 0.05, Table 2). Only 2 of the responders presented with oligometastases (≤ 5) in comparison with 8 of the non-responders. The responders presented with significantly more extensive CRLMs (> 5 metastases).

Chemotherapy regimens and follow-up periods

The chemotherapy regimens in the response and non-response group are summarized in Table 3. Both groups received between 3 and 12 cycles of chemotherapy between the baseline and follow-up scan, but the median was 8 cycles in the response group and 6 cycles in the non-response group. The FOLFOXIRI regimen was followed by two of the responders, but none of the non-responders. The time interval between the baseline CT scan and the start of chemotherapy varied between 3 and 51 d in the response group (median 18.0 d) and between 6 and 39 d in the non-response group (median 18.5 d). The interval between the baseline and follow-up CT scan varied between 10.3 and 29.0 wk in the response group (median 20.1 wk) and between 10.9 to 28.3 wk in the non-response group (median 15.8 wk).

Radiomic texture features and response to chemotherapy

In univariable analyses eight radiomic features were significantly associated with chemotherapy response (Table 4 and Supplementary Table 1), namely: Minimum histogram gradient intensity (intensity histogram indicator), skewness and discretized skewness (statistics), volume at intensity fraction 10 (volume intensity histogram indicator), three grey level run length indicators (GLRLM, long run low grey level

Table 2 Characteristics of the patients, overall and by response to first-line chemotherapy

	All		Responders		Non-responders		*P* value[a]
	n	%	*n*	%	*n*	%	
Age at diagnosis (yr)[1]	59	(52-73)	60	(52-74)	59	(54-71)	0.79
Sex							1.00
Female	11	(38)	6	(40)	5	(36)	
Male	18	(62)	9	(60)	9	(64)	
Position of colorectal Tumor							0.71
Colon (incl. rectosigmoid)	18	(62)	10	(67)	8	(57)	
Rectum	11	(38)	5	(33)	6	(43)	
T-stage of the primary tumor							1.00
T3	22	(81)	11	(85)	11	(79)	
T4	5	(19)	2	(15)	3	(21)	
Unknown	2		2		0		
N-stage of the primary tumor							0.85
N0	5	(22)	2	(20)	3	(23)	
N1	5	(22)	3	(30)	2	(15)	
N2	13	(57)	5	(50)	8	(62)	
Unknown	6		5		1		
Primary CRC grade							0.60
Moderate	25	(86)	12	(80)	13	(93)	
Poor	4	(14)	3	(20)	1	(7)	
M-stage of the primary tumor							1.00
M0	3	(10)	2	(13)	1	(7)	
M1	26	(90)	13	(87)	13	(93)	
KRAS mutation status							1.00
Wild type	5	(42)	2	(40)	3	(43)	
Mutant	7	(58)	3	(60)	4	(57)	
Unknown	17		10		7		
Extent of metastatic disease							0.71
Liver only	18	(62)	10	(67)	8	(57)	
Liver and extrahepatic	11	(38)	5	(33)	6	(43)	
CRLM timing							
Synchronous	26	(90)	13	(87)	13	(93)	1.00
Metachronous	3	(10)	2	(13)	1	(7)	
Number of metastases							0.05
≤ 5	10	(34)	2	(13)	8	(57)	
6-10	7	(24)	5	(33)	2	(14)	
> 10	12	(41)	8	(53)	4	(29)	
Maximum size of metastases (mm)							0.49
< 30	8	(28)	4	(27)	4	(29)	
30-70	15	(52)	9	(60)	6	(43)	
> 70	6	(21)	2	(13)	4	(29)	

Target liver metastases							
Baseline maximum transverse diameter (cm)[1]	2.7	(2.0-3.3)	2.9	(2.6-3.4)	2.4	(1.8-3.0)	0.14
Baseline lesion volume (cm^3)[1]	7.7	(3.6-12.7)	8.3	(6.2-12.2)	5.2	(3.1-12.3)	0.32
CEA (ng/mL)[1]	107	(10-171)	130	(28-239)	51	(11-136)	0.24
CA19-9 (IU/mL)[1,2]	127	(37-377)	136	(40-327)	59	(21-773)	0.77

[a]Fisher's exact test for categorical variables and Kruskal-Wallis test for continuous variables.
[1]Median (interquartile range).
[2]Number of missing data $n = 12$.
CRC: Colorectal cancer; KRAS: Kirsten rat sarcoma viral oncogene homolog; CRLMs: Colorectal liver metastases; CEA: Carcinoembryonic antigen.

Table 3 Summary of Response Evaluation Criteria in Solid Tumors response and chemotherapy regimes in response and non-response group

	Response group	Non-response group
RECIST response		
CR	0	
PR	15	
SD		7
PD		7
Chemotherapy regimen		
FOLFOX	4	4
FOLFIRI	4	3
FOLFOXIRI	2	0
CAPE-OX	2	3
CAPE-IRI	2	3
Capecitabine	1	1
Number of chemotherapy cycles between baseline and follow-up scan		
Range in cycles (median)	3-12 (8)	3-12 (6)
Time between baseline and follow-up scan		
Range in d (median)	72-203 (141)	76-198(111)

RECIST: Response Evaluation Criteria in Solid Tumors.

emphasis, low grey level run emphasis, short run low grey level emphasis) and low grey level count emphasis (neighboring grey level dependence matrix, NGLDM). Due to strong correlations within two groups of radiomic features (Figure 2), only minimum histogram gradient intensity (tertiles: 21 and 23) and long run low grey level emphasis (tertiles: 0.0086 and 0.0103) were included in the multiple analysis (Table 4).

The AUC of the multiple model was 0.80 (95%CI: 0.64 to 0.96); the best threshold of the linear predictor was 0.42, corresponding to a sensitivity of 0.73 (95%CI: 0.48 to 0.89) and a specificity of 0.79 (95%CI: 0.52 to 0.92). The optimism-adjusted AUC estimate from bootstrap validation was 0.77. Figure 3 shows the prognostic nomogram resulting from the multiple model together with the empirical distributions of the linear predictor from the best model in the two groups. CT images of a few responding and non-responding CRLMs are shown in Figure 4.

DISCUSSION

The aim of this study was to determine if the pre-treatment CT texture analysis of CRLMs can predict the response to first-line cytotoxic chemotherapy with the RECIST

Table 4 Radiomic features associated with response to chemotherapy

	Univariable models			Multiple model		
	OR (95%CI)	*P* value[1]	AUC	OR (95%CI)	*P* value[1]	AUC
Minimum histogram gradient intensity	3.82 (1.26-15.3)	0.02	0.74	3.24 (1.05-12.00)	0.04	0.80
Discretized intensity skewness	0.33 (0.11-0.86)	0.02	0.73			
Skewness	0.33 (0.11-0.86)	0.02	0.73			
Long run low grey level emphasis	3.01 (1.16-9.26)	0.02	0.73	2.84 (0.98-10.09)	0.05	
Low grey level count emphasis	3.01 (1.16-9.26)	0.02	0.73			
Low grey level run emphasis	3.01 (1.16-9.26)	0.02	0.73			
Volume at intensity fraction 10%	0.33 (0.11-0.86)	0.02	0.73			
Short run low grey level emphasis	2.83 (1.08-8.81)	0.03	0.71			

[1]Likelihood ratio test. Results from univariable and multiple logistic regression models. Radiomic features were included as pseudo-continuous tertiles. OR: Odds ratio; CI: Confidence interval; AUC: Area under the receiver operating characteristic curve.

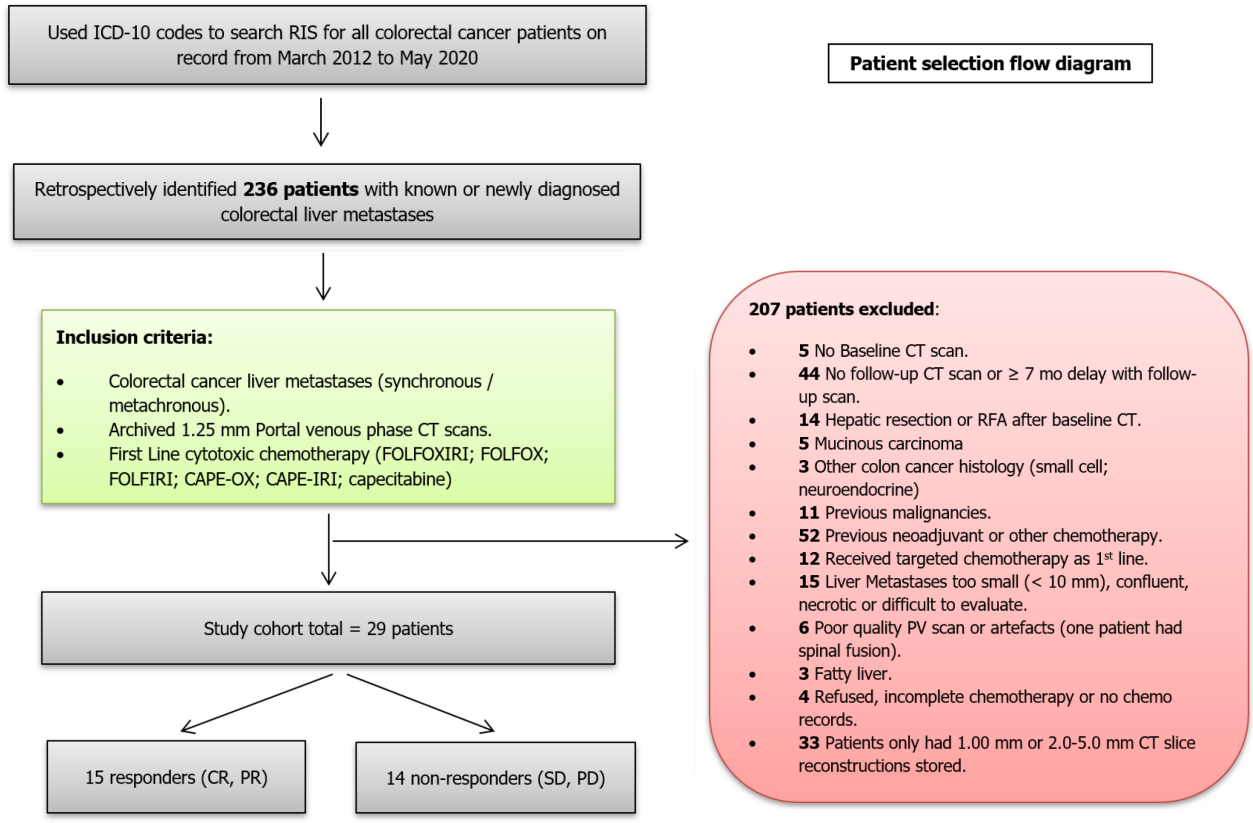

Figure 1 Patient selection flow chart. RIS: Radiology information system; ICD-10: International Classification of Diseases and Related Health Problems, 10th revision; RFA: Radiofrequency ablation; CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease.

1.1 criteria as gold standard. In our study, only the solid soft tissue component of the CRLMs was analyzed with texture analysis and metastases which demonstrated clear necrosis and calcifications were excluded. Compared with other studies, a larger range of first and second order texture features were also analyzed on thin 1.25 mm portal venous phase CT reconstructions.

Our results showed a correlation between the minimum histogram gradient intensity[23], negative skewness[25], discretized intensity skewness, volume at intensity fraction 10, various low grey level GLRLM features (low grey level run emphasis, short run low grey level emphasis, long run low grey level emphasis)[23,26,27] and low grey level count emphasis (NGLDM)[23,28] in responding CRLMs. Except

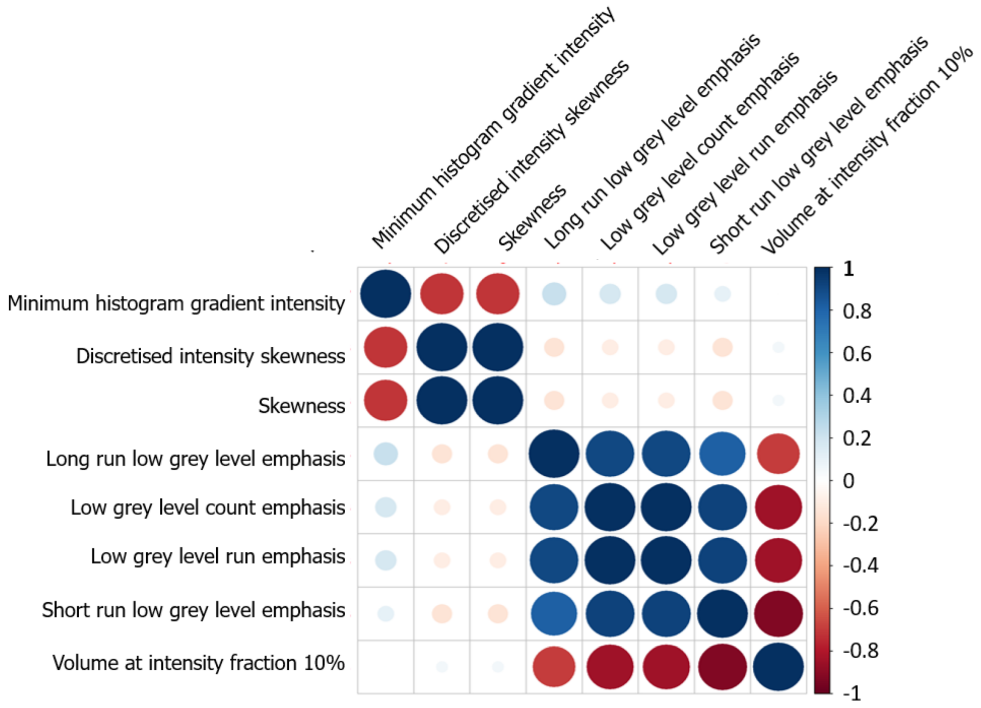

Figure 2 Correlations among radiomic features associated with response. Pearson's correlation between radiomic features tertiles regarded as pseudo-continuous variables.

for skewness, we are not aware that any other studies have reported the predictive first and second order texture features which were associated with response in our study. In the multiple model combining minimum histogram gradient intensity and long run low grey level emphasis the AUC of the multiple model was 0.80 (95%CI: 0.64 to 0.96).

The CRLMs in our study were not biopsied to determine if there are specific histopathological patterns which are correlated with the primary and secondary order textures that were associated with chemotherapy response. Few studies have investigated the correlation between the pathological changes in cancer, texture analysis and various CT density measurements. In general, tumor heterogeneity is associated with higher skewness, higher standard deviation, higher entropy, lower uniformity and higher kurtosis and has been reported to predict a poorer patient prognosis[14,19,29]. Tumor heterogeneity reflects internal variation due to variation in cellularity, hypoxia, distribution of tumor vessels, necrosis, fibrosis, hemorrhage, myxoid changes and other factors[30-32]. Research is suggesting that the CT texture analysis may reflect tumor angiogenesis and hypoxia[31,33] and that tumors with low levels of angiogenesis are more likely to exhibit hypoxia and necrosis[33]. Some studies have demonstrated a correlation between skewness and the presence of an underlying KRAS mutation in CRC. Lubner *et al*[34] reported a negative trend between skewness and KRAS mutations. In the study by Yang *et al*[35] skewness also showed power in predicting the presence of KRAS/NRAS/BRAF mutations in CRC. Unfortunately, the KRAS mutation status was only tested in a limited number of our cases and therefore the texture differences between CRLMs with KRAS wild-type *vs* KRAS mutations were not assessed. Negative skewness may potentially also represent more pronounced low attenuation areas due to small areas of tumoral necrosis, chronic hemorrhage or myxoid change[36] which are not clearly visible to the naked eye. To the best of our knowledge, no studies have investigated the biological correlations of the GLRLM and NGLDM second order texture features.

Patients who received first-line cytotoxic chemotherapy were evaluated in studies by Ahn *et al*[19] and Ravanelli *et al*[37]. Ahn *et al*[19] showed that in the responding CRLMs on cytotoxic chemotherapy two first order histogram features, namely lower skewness in 2D and a narrower standard deviation on the 3D texture analysis, were significantly associated with chemotherapy response. We found no significant correlation between the standard deviation and the prediction of response in our study which can potentially be explained by the fact that we excluded necrotic CRLMs (necrosis will increase the standard deviation) which may be associated with non-responding CRLMs. In the study by Ravanelli *et al*[37] none of the assessed first-order

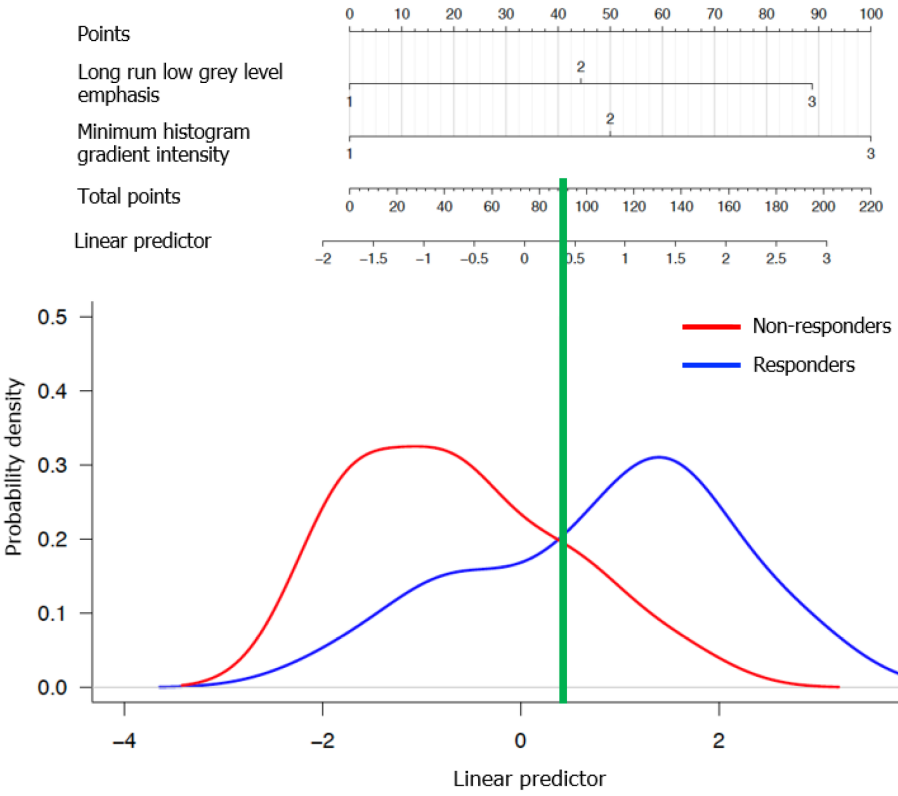

Figure 3 Prognostic nomogram of response to chemotherapy for patients. Interpretation is as follows: for each predictor, determine the corresponding points by drawing a straight line up from the patient's value; sum the points obtained for each predictor and locate the total sum on the upper point line. Identify the corresponding value in the linear predictor scale by drawing a straight line down. Values of linear predictor greater than the threshold (0.42) predict response; values less than the threshold predict no response.

textures could discriminate between the responders and non-responders in the FOLFOX/FOLFIRI group according to the RECIST 1.1 criteria and this is consistent with our findings.

The responding patients in our study presented with more extensive liver metastases. There was no statistically significant difference in the position of the colorectal cancer, in the TNM stage or tumor grade of the primary CRC or in the size (longest diameters according to RECIST criteria) and volumes of the CRLMs between the responders and non-responders. This leads one to assume that the responding CRLMs were probably associated with a more aggressive biological behavior[38].

Although the role of texture analysis is still being investigated it has the potential to impact positively on the therapeutic management of patients with cancer once predictive and prognostic biomarkers have been validated. The correlation between the texture features and the biological, histological, and genetic variables requires further research with histologically validated studies.

This study shows some limitations. The study design is retrospective and included a relatively small cohort of patients. Moreover, some of the CT acquisition parameters [39-43] and the total volume of contrast (mL/kg) injected varied slightly in a few patients.

The selection of the first-line cytotoxic chemotherapy regimen, the number of chemotherapy cycles administered and the time interval between the baseline and follow-up scans varied in the study cohort. Although this may impact on the results, this is reflective of actual clinical oncology practice and it is important to develop radiomics signatures which will have practical applications in clinical practice.

Finally, the accuracy of the segmentations was checked by an independent observer, but the inter- and intra-observer variability was not evaluated. However, a semi-automatic segmentation technique was used which can reduce inter-user variability [44].

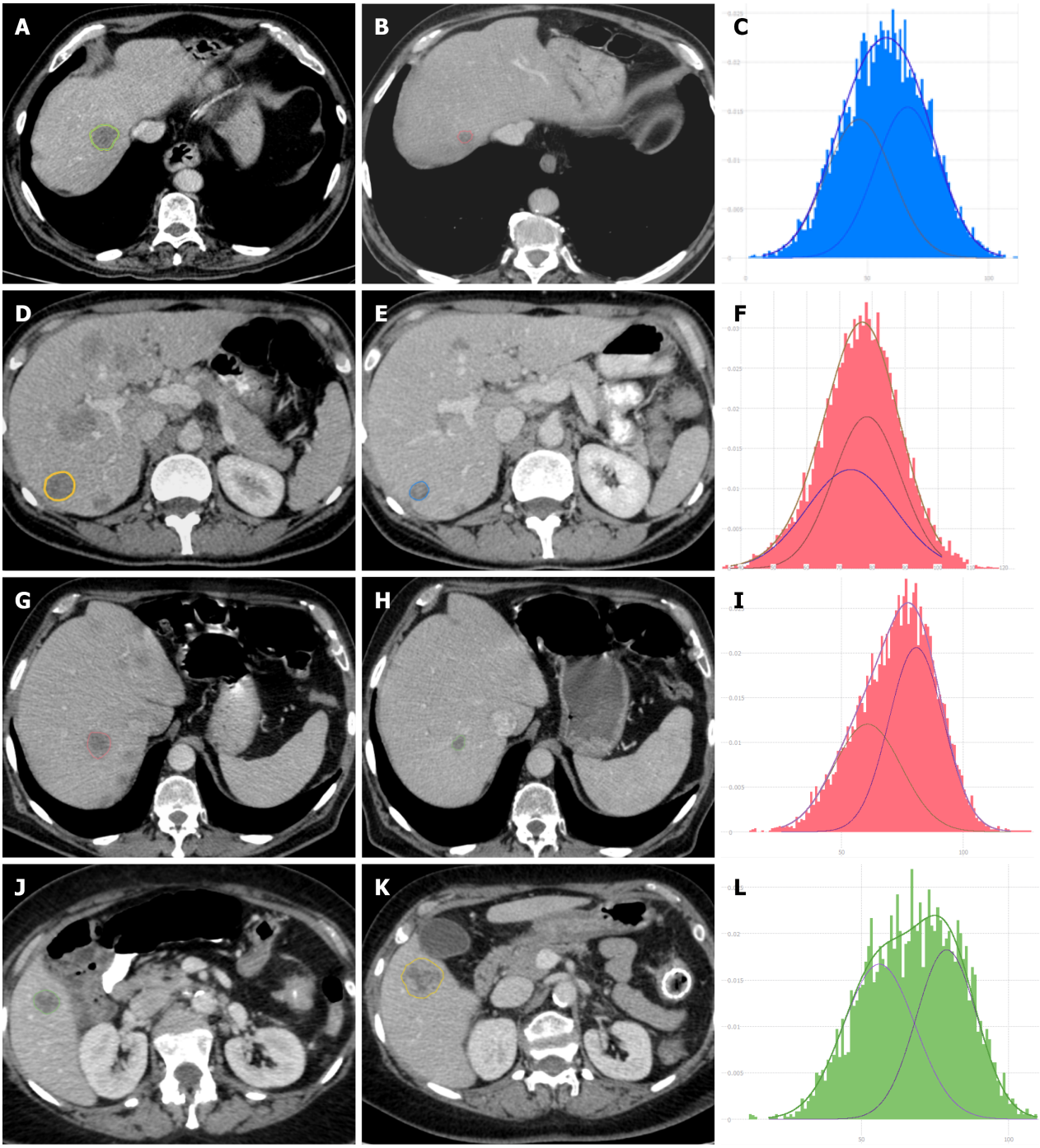

Figure 4 Appearance of typical responding and non-responding liver metastases. A: Responder pre chemotherapy; B: Responder post chemotherapy; C: Responder pre chemotherapy histogram; D: Responder pre chemotherapy; E: Responder post chemotherapy; F: Responder pre chemotherapy histogram; G: Responder pre chemotherapy; H: Responder post chemotherapy; I: Responder pre chemotherapy histogram; J: Non-responder pre chemotherapy with annular carcinoma of transverse colon; K: Non-responder post chemotherapy with metallic stent in the transverse colon; L: Non-responder pre chemotherapy histogram.

CONCLUSION

Our study identified a few new texture features and a promising radiomics signature which are significantly associated with the response of CRLMs to first-line cytotoxic chemotherapy. These preliminary results need to be validated and confirmed on larger patient cohorts. Further investigations are required to determine if the predictive texture features have any prognostic value and are linked to the KRAS mutation status of CRLMs.

ARTICLE HIGHLIGHTS

Research background

Radiomics is a rapidly growing field of radiological research. In radiomics, computed tomography (CT) texture analysis quantifies tissue heterogeneity and has shown promise in predicting pathological features, the overall survival and the response to therapy in oncology. In the last few years a few studies have reported that texture analysis can be helpful in predicting the response to chemotherapy for colorectal liver metastases, but the results have been heterogeneous.

Research motivation

In previously published texture analysis studies on the first-line chemotherapy response of colorectal liver metastases (CRLMs), necrotic CRLMs were not clearly excluded. Thicker CT slice reconstructions were utilized in most studies which could have influenced the radiomics results due to partial voxel artefacts. Limited first and second order texture features were also analyzed in previous studies.

Research objectives

The aim of this study was to identify new predictive imaging biomarkers in patients with non-necrotic CRLMs who received first-line cytotoxic chemotherapy. CT texture analysis was performed on non-necrotic CRLMs utilizing 1.25 mm portal venous phase CT reconstructions. We also assessed a larger range of first and second order texture features.

Research methods

A total of 236 patients with CRLMs who received first-line cytotoxic chemotherapy in our private institution from March 2012 to May 2020 were retrospectively identified on our radiology information system. There were various inclusion and exclusion criteria and the final study cohort consisted of 29 patients. Multiple first and second order texture features were analyzed with the SOPHiA Radiomics software to identify predictive biomarkers in the responding CRLMs.

Research results

Our study identified a few new texture features and a promising radiomics signature which are significantly associated with the response of CRLMs to first-line cytotoxic chemotherapy. In univariable analysis eight texture features of the responding non-necrotic CRLMs were associated with treatment response, but due to strong pairwise correlations among some of the features, only two features namely minimum histogram gradient intensity and long run low grey level emphasis were included in the multiple analyses and final radiomics signature. The results of this study were unique but need to be validated and confirmed on larger patient cohorts.

Research conclusions

Future radiomics studies should attempt to quantify the difference in the texture analysis results of necrotic *vs* non-necrotic CRLMs utilizing different CT slice reconstructions in the same study cohort to compare the predictive value of texture analysis. These factors may partially account for the heterogeneous results which have been reported in the last few years. To allow for the better comparison between radiomics studies we should work towards the standardization of study designs, interscanner differences, acquisition parameters, analysis algorithms, the feature extraction techniques, analysis methodologies and the group of texture features which should be evaluated based on the different types of cancer.

Research perspectives

The preliminary results of our study need to be validated and confirmed on larger patient cohorts. Further investigations are required to determine if the predictive texture features have any prognostic value and are linked to the KRAS mutation status of CRLMs. Standardization of radiomics studies is required to compare the texture analysis results of different studies.

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ORIGINAL ARTICLE

Retrospective Study

Correlation of hepatitis B surface antigen expression with clinicopathological and biochemical parameters in liver biopsies: A comprehensive study

Anil Alpsoy, Haydar Adanir, Zeynep Bayramoglu, Gulsum Ozlem Elpek

ORCID number: Anil Alpsoy 0000-0003-4978-7652; Haydar Adanir 0000-0003-1899-5846; Zeynep Bayramoglu 0000-0001-7075-8819; Gulsum Ozlem Elpek 0000-0002-1237-5454.

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Conflict-of-interest statement:

Anil Alpsoy, Zeynep Bayramoglu, Gulsum Ozlem Elpek, Department of Pathology, Akdeniz University, Medical School, Antalya 07070, Turkey

Haydar Adanir, Department of Gastroenterology, Akdeniz University, Medical School, Antalya 07070, Turkey

Corresponding author: Gulsum Ozlem Elpek, MD, Professor, Research Assistant Professor, Department of Pathology, Akdeniz University, Medical School, Dumlupınar Bulvarı, Antalya 07070, Turkey. elpek@akdeniz.edu.tr

Abstract

BACKGROUND

Chronic viral B hepatitis (CHB) is a potentially life-threatening liver disease that may progress to liver failure and cirrhosis. Currently, although combinations of different laboratory methods are used in the follow-up and treatment of CHB, the failure of these procedures in some cases has led to the necessity of developing new approaches. In CHB, the intrahepatic expression pattern of viral antigens, including hepatitis B surface antigen (HBsAg), is related to different phases of inflammation. However, many studies have focused on the intracytoplasmic properties of HBsAg staining, and HBsAg positivity in liver tissue has not been evaluated by objective quantitative methods.

AIM

To investigate the relationship of image analysis-based quantitative HBsAg expression and its staining patterns with clinicopathological factors and treatment in CHB.

METHODS

A total of 140 liver biopsies from treatment-naïve cases with CHB infection were included in this study. Following diagnosis, all patients were treated with entecavir (0.5 mg) and followed up at three-month intervals. The percentage of immunohistochemical HBsAg (p-HBsAg) expression in the liver was determined in whole tissue sections of biopsies from each case by image analysis. The immunohistochemical staining pattern was also evaluated separately according to 3 different previously defined classifications.

There is no conflict of interest.

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RESULTS
A positive correlation between p-HBsAg and serum levels of hepatitis B virus (HBV) DNA and HBsAg was observed ($P < 0.001$). The p-HBsAg value was significantly higher in younger patients than in older patients. When the groups were categorized according to the hepatitis B e antigen (HBeAg) status in HBeAg-positive cases, p-HBsAg was correlated with HBV DNA, hepatitis activity index (HAI) and fibrosis scores ($P < 0.001$). In this group, p-HBsAg and HBsAg expression patterns were also correlated with the viral response (VR) and the serological response (SR) ($P < 0.001$). Multivariate analysis revealed that p-HBsAg was an independent predictor of either VR or SR ($P < 0.001$). In HBeAg-negative patients, although HBsAg expression patterns were correlated with both HAI and fibrosis, no relationship was observed among p-HBsAg, clinicopathological factors and VR.

CONCLUSION
In pretreatment liver biopsies, the immunohistochemical determination of HBsAg expression by quantitative methods, beyond its distribution within the cell, may be a good predictor of the treatment response, especially in HBeAg-positive cases.

Key Words: Hepatitis B; Hepatitis B surface antigens; Hepatitis B e antigens; Fibrosis; Immunohistochemistry; Image analysis

Core Tip: This report describes a study that investigated image analysis-based quantitative hepatitis B surface antigen (HBsAg) expression and its different staining patterns in liver biopsies from patients with chronic viral B hepatitis (CHB) and correlated them with clinicopathological factors and treatment. Our findings confirmed the association of cytoplasmic HBsAg staining patterns with disease activity. Besides, the determination of immunohistochemical HBsAg expression by image analysis may be an important predictor of the response to therapy, especially in hepatitis B e antigen-positive cases. Accordingly, evaluation of the percentage of HBsAg expression by objective methods in liver tissues from treatment-naïve CHB patients might provide a useful tool in the follow-up and treatment of this disease.

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INTRODUCTION

Despite advances in its prevention, diagnosis and treatment, chronic viral B hepatitis (CHB) continues to be an important worldwide health problem with high morbidity and mortality due to its dramatic consequences, such as cirrhosis and hepatocellular carcinoma[1]. In recent years, researchers have suggested that the combination of various laboratory methods with histopathological findings may allow the assessment of the prognosis and treatment of patients with CHB[2-4]. High liver biopsy activity scores, low viremia and high serum alanine aminotransferase (ALT) levels are associated with hepatitis B e antigen (HBeAg) seroconversion, while a decrease in serum hepatitis B surface antigen (HBsAg) levels is among the factors associated with an effective response to therapy[5-7]. Unfortunately, the presence of these factors is not always adequate to determine the progression of the disease and the efficacy of treatment. The treatment response is not sufficient in some patients with these factors, which contrasts with the adequate response in other patients without these factors, suggesting that other viral and host-related parameters may also be involved in the progression and therapy response.

Recent studies have also provided evidence that in patients with CHB, the expression of viral antigens and their expression patterns detected by immunohistochemistry may be related to clinicopathological factors and might have prognostic implications for the disease process. The expression of hepatitis B core antigen (HBcAg) and its expression patterns were found to be associated with serum HBeAg, viral replication and the hepatitis activity index (HAI)[8-11]. In addition, the results of some studies have suggested the potential use of HBcAg expression as a marker of treatment response[12-14].

Regarding HBsAg, the results of many different studies investigating the relationships between the intrahepatic expression of this antigen revealed that differences in the intracytoplasmic HBsAg staining pattern were related to viremia and active inflammation[14-18]. In these investigations, HBsAg expression was evaluated semiquantitatively, and different classifications were used for the HBsAg staining pattern. On the other hand, HBsAg, beyond being the main diagnostic marker of the disease, is a very important tool in the follow-up of the course and treatment of CHB, since a decrease in its level reflects the low replicative phase[19-21]. Therefore, more objective methods are needed to determine whether the immunohistochemical expression of HBsAg in pretreatment-performed liver biopsies can be useful in monitoring the prognosis and treatment of CHB.

Therefore, this study was undertaken to investigate the relationship between image analysis-based quantitation of HBsAg expression and corresponding staining patterns with clinicopathological factors and treatment.

MATERIALS AND METHODS

Study design

This retrospective study was conducted in accordance with the Declaration of Helsinki. Approval for this study was obtained from the Akdeniz University Faculty of Medicine Clinical Research Ethics Committee (Date: January 28, 2015; Approval number: 2015.01.211.016). All patients provided written informed consent for participation in the study.

The study group consisted of 140 patients who were followed up and treated after the diagnosis of CHB at Akdeniz University Medical School between 2015 and 2020. The inclusion criteria were the presence of positive serum HBsAg for at least 6 mo and the absence of coinfection with human immunodeficiency, hepatitis delta and hepatitis C viruses. None of these patients had end-stage liver failure, immunosuppressive therapy, malignancy, autoimmune hepatitis or alcoholic hepatitis.

Clinical and laboratory parameters were recorded from all cases on the day of liver biopsy. These included the age of the patients, sex, HBV DNA, HBsAg ALT, INR, and platelet count. Forty-nine cases with elevated ALT levels underwent antiviral therapy. Ninety-one patients with low ALT levels received this treatment based on the increase in ALT levels in the first three months of follow-up. Accordingly, all patients were treated with entecavir (0.5 mg) within three months after the liver biopsies and followed up at three-month intervals. An HBV DNA level below 20 IU/mL detected by real-time polymerase chain reaction (PCR) was defined as the viral response (VR). HBeAg seroclearance and a decrease in serum HBsAg level during follow-up > 0.5 log10 IU/mL were defined as the serological response (SR) to treatment[22].

Laboratory analysis

Serum HBsAg, HBeAg, antibodies against HBsAg (anti-HBs) and HBeAg were tested by using commercial kits (Abbott Laboratories, Abbott Park, IL, United States). Serum HBV-DNA was extracted from 200 μL of serum by a QIAmp DNA Blood Mini Kit (QIAGEN Inc., Valencia, CA, United States) and quantified by a real-time PCR amplification assay using a LightCycler (Roche Diagnostics, Basel, Switzerland). The detection sensitivity was 20 IU/mL[23]. Serum HBsAg was quantified with the ARCHITECT HBsAg assay (Abbott Laboratories) with a dynamic range from 0.05 IU/mL to 250 IU/mL.

Histopathological evaluation

Microscopic evaluation was performed on serial sections stained with hematoxylin and eosin from paraffin-embedded 10% formalin-fixed liver tissues. The HAI and fibrosis stages were determined according to the Ishak system[24]. HAI grading consists of the sum of necroinflammatory scores, including portal inflammation, periportal interface hepatitis, confluent necrosis and focal lytic necrosis, and each was

given a score from 0 to 4-6 with a maximal HAI grade of 18. The fibrosis staging ranged from 0 to 6.

Immunohistochemistry

Immunohistochemical staining for HBsAg was performed by using a primary antibody against HBsAg (mouse monoclonal antibody, clone 3E7, Dako, Carpinteria, CA, United States). Staining was performed automatically with a BenchMark XT (Ventana Medical Systems, Tucson, AZ, United States) in the Department of Pathology, Akdeniz University Medical School.

The quantification of HBsAg

Image analysis was performed using a SAMBA 2005 image processor (Alcatel-TITN, Grenoble, France). This system consists of a Leitz Diaplan Microscope connected to a personal computer through a Sony color camera and a data translation frame grabber board. For detection of the percentage of HBsAg immunostaining, the system was calibrated for a 20X objective. In each area, antigen immunoreactivity was measured in the entire tissue section. The results obtained were expressed as the percentage of the ratio of the HBsAg (p-HBsAg) immunoreactive area to the total scanned area.

The immunohistochemical staining pattern

The immunohistochemical staining pattern was evaluated separately according to 3 different previously defined classifications[15,18].

Staining pattern 1: The staining was classified according to the expression type and distribution into five major patterns. Patterns A and B were characterized by diffuse cytoplasmic staining in discrete hepatocytes (Figure 1A and B). While the intensity of staining was stronger in pattern A, it was faint in pattern B. In pattern C, cytoplasmic HBsAg was similar to that in pattern B but was distributed in clusters of hepatocytes (Figure 1C). Pattern D included globular and spotty cytoplasmic staining in either discrete or clustered hepatocytes (Figure 1D). Pattern E (marginal HBsAg) was characterized by submembranous staining of HBsAg beneath the cell membranes in the hepatocyte groups (Figure 1E).

Staining pattern 2: The expression of HBsAg was categorized according to its intracytoplasmic expression: 1: Diffuse (Figure 1A-C); 2: Globular (Figure 1D); and 3: Submembranous (Figure 1E).

Staining pattern 3: In this classification, HBs staining was categorized according to the presence or absence of membranous staining (Figure 1F).

Statistical analysis

Statistical analysis was performed using SPSS version 25.0 (IBM Corp., Armonk, NY, United States). Nominal and ordinal data are expressed as frequencies (percentages), and continuous variables are expressed as the mean (standard error). The χ^2 test was employed to examine categorical data. Continuous variables between the groups and their relationship with the clinicopathological parameters were investigated by *t* tests. Univariate analysis, including response to therapy, was estimated with the Kaplan-Meier method. The log-rank test was employed for comparisons of response rates. A Cox proportional hazards regression model was applied for multivariate analysis. Spearman's correlation test was used to determine relationships between p-HBsAg and the other continuous variables. The threshold *P* value accepted for statistical significance was < 0.05.

RESULTS

The mean age of the patients in the whole group was 42.79 ± 11.53 years, and the male to female ratio was 1.25 (Table 1). There was a positive correlation between p-HBsAg and the serum levels of HBV DNA and HBsAg (r: 0.490, r: 0.468 $P < 0.001$, respectively). When all cases were grouped according to mean age, the p-HBsAg value was significantly higher in younger patients than in older patients (48.47 ± 16.06 *vs* 31.72 ± 12.89 $P < 0.01$). According to their HBeAg status, 86 cases were HBeAg positive (61.5%), and 54 cases were HBeAg negative (38.5%). Other findings of the cases are presented in Table 1. Briefly, HBeAg-positive patients were younger than HBeAg-negative patients, and serum HBV DNA and HBsAg levels were higher in this group

Table 1 Basic clinical, histopathological and immunohistochemical findings according to hepatitis B e antigen status

Parameters	Total	HBeAg positive	HBeAg negative
n	140	86	54
Age (yr)	42.75 ± 11.53	40.30 ± 11.03	46.74 ± 11.29[a]
Gender			
Male	78	48 (61.5)	30 (38.5)
Female	62	38 (61.3)	24 (38.7)
HBV DNA (log_{10}IU/mL)	7.27 ± 2.17	7.67 ± 2.39	6.65 ± 1.58[a]
HBsAg (log_{10}IU/mL)	3.85 ± 1.04	4.01 ± 1.07	3.61 ± 0.94[b]
ALT IU/L	7.27 ± 2.17	155.80 ± 114.32	134.54 ± 98.71
ALT < 200 IU/L	91	52 (51.7)	39 (42.9)
ALT ≥ 200 IU/L	49	34 (69.4)	15 (30.6)
INR	1.08 ± 0.21	1.04 ± 0.14	1.15 ± 0.27[b]
Platelets (× 10^3/μL)	226.4 ± 73.43	224.54 ± 70.67	227.59 ± 75.53
HAI score ≥ 9	61	38 (62.3)	23 (37.7)
HAI score < 9	79	48 (60.8)	31 (39.2)
Fibrosis ≥ 4	53	33 (62.3)	20 (37.7)
Fibrosis < 4	87	53 (60.9)	34 (39.1)
p-HBsAg	41.05 ± 22.78	47.2 ± 13.98	31.26 ± 13.98[a]
HBsAg SP1			
A	33	17 (51.5)	16 (48.5)
B	24	10 (41.7)	14 (58.3)
C	21	16 (76.2)	5 (23.8)
D	16	10 (62.5)	6 (37.5)
E	46	33 (71.7)	13 (28.3)
HBsAg SP2			
Diffuse	59	29 (49.2)	30 (50.8)
Globular	34	24 (70.6)	10 (29.4)
Submembranous	47	33 (70.2)	14 (29.8)
Membranous expression			
Present	53	33 (63.3)	20 (37.7)
Absent	87	53 (60.9)	34 (39.1)

Data are presented as mean ± SD, number (%).
[a]$P < 0.05$.
[b]$P < 0.01$.
p-HbsAg: Percentage of hepatitis B surface antigen expression; SP: Staining pattern; ALT: Alanine aminotransferase; INR: International normalized ratio, HAI: Histologic activity index; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus.

($P < 0.05$). On the other hand, the INR levels were found to be lower than those in the HBeAg-negative group. The average p-HBsAg level was significantly higher in the HBeAg-negative group than in the HBeAg-positive group. There was no difference in the HBsAg staining patterns between the two groups.

The relationship between viremia and histopathological findings in cases with positive HBeAg are summarized in Table 2. The average p-HBsAg level was found to be significantly higher in patients with higher HBV DNA values than in those with low HBV DNA levels. In contrast, an inverse relationship was noted between p-HBsAg and either HAI or the fibrosis score. Regarding staining pattern 1 (SP1), the number of cases with pattern A was higher among cases with higher HBV DNA levels.

Table 2 The correlation of clinicopathologic factors and hepatitis B surface antigen expression patterns with viral replication in hepatitis B e antigen positive cases

	HBV DNA		HAI		Fibrosis	
Parameters	**< 7.67**	**≥ 7.67**	**< 9**	**≥ 9**	**< 4**	**≥ 4**
n	32	54	48	38	53	33
Age (yr)	48.28 ± 9.6	35.57 ± 8.93[b]	37.79 ± 10.83	43.47 ± 10.59[a]	36.06 ± 9.39	47.12 ± 10.11[b]
Gender						
Male	23 (71.9)	25 (46.3)[a]	23 (47.9)	25 (65.8)	27 (50.9)	21 (63.6)
Female	9 (28.1)	29 (53.7)	25 (52.1)	13 (34.2)	26 (49.1)	12 (36.4)
HBV DNA (log_{10}IU/mL)	-	-	8.36 ± 2.26	6.79 ± 2.30[a]	8.15 ± 2.32	6.89 ± 2.34
HBsAg (log_{10}IU/mL)	3.02 ± 0.91	4.59 ± 0.64[b]	4.28 ± 0.91	3.66 ± 1.61[a]	4.19 ± 1.01	3.07 ± 1.10[a]
ALT IU/L	106.97 ± 73.55	184.74 ± 124.85[a]	148.97 ± 61.46	121.46 ± 50.84	167.74 ± 72.1	136.64 ± 56.2
ALT< 200 IU/L	26 (81.2)	26 (48.1)[a]	34 (70.8)	18 (47.4)[a]	32 (60.4)	20 (60.6)
ALT ≥ 200 IU/L	6 (18.8)	28 (51.9)	14 (29.2)	20 (52.6)	21 (39.6)	13 (39.4)
INR	1.03 ± 0.08	1.05 ± 0.16	1.03 ± 0.10	1.06 ± 0.17	1.04 ± 0.16	1.04 ± 0.09
Platelets (× 10^3/μL)	217.59 ± 66.85	233.98 ± 80.60	226 ± 74.74	228 ± 77.51	224.53 ± 74.72	232.61 ± 77.82
HAI score ≥ 9	22 (68.8)	16 (29.6)[b]	-	-	17 (32.1)	21 (63.6)[b]
HAI score < 9	10 (31.2)	38 (70.4)	-	-	36 (67.9)	12 (36.4)
Fibrosis ≥ 4	19 (59.4)	14 (25.9)[a]	12 (25)	21 (55.3)[b]	-	-
Fibrosis < 4	13 (40.6)	40 (74.1)	36 (75)	17 (44.7)	-	-
p-HBsAg	29.92 ± 10.84	57.44 ± 25.50[b]	53.53 ± 25.12	39.21 ± 22.85[a]	54.49 ± 26.02	35.49 ± 18.34[b]
HBsAg SP1						
A	2 (6.2)	15 (27.8)[a]	11 (22.9	6 (15.8)[a]	17 (32.1)	0[b]
B	4 (12.5)	6 (11.1)	1 (2.1)	9 (23.7)	6 (11.3)	4 (12.1)
C	6 (18.8)	10 (18.5)	12 (25)	4 (10.5)	14 (26.4)	2 (6.1)
D	4 (12.5)	6 (11.1)	7 (14.6)	3 (7.9)	8 (15.1)	2 (6.1)
E	16 (50)	17 (31.5)	17 (35.4)	16 (42.1)	8 (15.1)	25 (75.8)
HBsAg SP2						
Diffuse	8 (25)	21 (38.9)	12 (25)	17 (44.7)[a]	23 (43.4)	6 (18.2)[b]
Globular	8 (25)	16 (29.6)	19 (39.6)	5 (13.1)	22 (41.5)	2 (6.1)
Submembranous	16 (50)	17 (31.5)	17 (35.4)	16 (42.1)	8 (15.1)	25 (75.8)
Membranous expression						
Present	5 (15.6)	28 (51.9)[b]	21 (43.8)	12 (31.6)	29 (54.7)	4 (12.1)[b]
Absent	27 (84.4)	26 (48.1)	27 (56.3)	26 (68.4)	24 (45.3)	29 (87.9)

Data are presented as mean ± SD, number (%).
[a]$P < 0.05$.
[b]$P < 0.01$.
p-HBsAg: Percentage of hepatitis B surface antigen expression; SP: Staining pattern; ALT: Alanine aminotransferase; INR: International normalized ratio; HAI: Histologic activity index; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus.

Their number decreased among cases with HAI ≥ 9, and it was not observed among cases with fibrosis. When staining pattern 2 (SP2) was analyzed, it was noted that the number of cases with diffuse staining was higher in the HAI ≥ 9 group. In contrast, among the cases with high HAI and fibrosis scores, the frequency of pattern E was observed in 42% and 75% of the cases, respectively.

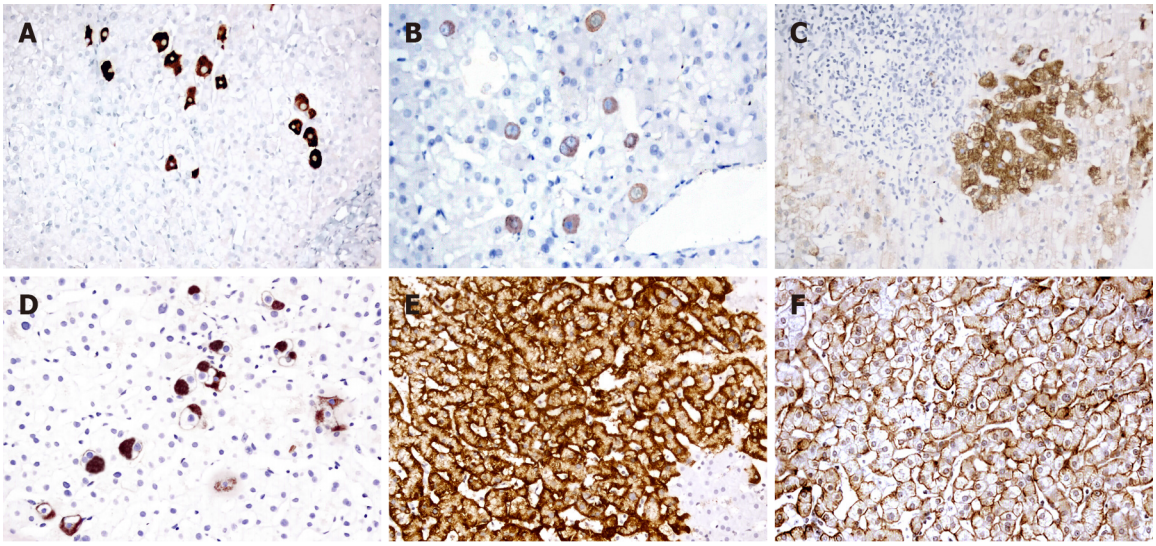

Figure 1 Different staining patterns of hepatitis B surface antigen in liver tissues. A: Dense cytoplasmic staining in many discrete hepatocytes; B: Diffuse but faint cytoplasmic staining in discrete hepatocytes; C: Diffuse faint cytoplasmic staining in a group of hepatocytes near an inflamed portal area; D: Globular and spotty staining in discrete and in a small group of hepatocytes; E: Submembranous staining in a large group of cells; F: Membranous expression of hepatitis B surface antigen. Original magnifications A and C × 200, B, D, E and F × 400, counter-stained with Mayer's hematoxylin.

The relationship between viremia and histopathological findings in cases with negative HBeAg is presented in Table 3. In this group, no correlation was observed between either the p-HBsAg or the membranous staining pattern and HBV DNA, HAI, or fibrosis. In the evaluation of patients according to SP1, in cases with HAI ≥ 9, the number of patients with pattern A was lower than that of patients with HAI < 9. In the latter, pattern E was rarely observed (12% of cases). SP2 was found to be related to HBV DNA levels, and diffuse staining was more frequent in cases with a higher level of HBV DNA. However, globular staining was rarely observed in these cases.

Patients were treated for a median duration of 28 mo (range, 4 mo to 62 mo). VR was achieved in 54 of 86 HBeAg-positive patients (66.7%) and in all 54 HBeAg-negative patients (100%), with a median time to VR of 16 and 9 mo, respectively. Factors associated with VR are shown in Table 4. In HBeAg-positive patients, the p-HBsAg and HBsAg expression patterns were correlated with VR, along with age, fibrosis, HVDNA and HBsAg levels in the log-rank test ($P < 0.05$). VR was observed in 53.7% of the patients with a high p-HBsAg. Similarly, pattern A and the presence of membranous expression were frequently observed in patients with VR. In multivariate analysis, the sole independent factor associated with VR was p-HBsAg ($P < 0.01$) (Figure 2A and Table 5). Forty-seven (54.6%) of 86 HBeAg-positive patients exhibited HBeAg SR, with a median time of 22 mo (range, 4 mo to 60 mo). Univariate analysis revealed that the p-HBsAg and HBsAg expression patterns were correlated with SR together with HBsAg levels and ALT > 200 ($P < 0.05$) (Table 4). Multivariate analysis demonstrated that p-HBsAg and the HAI score were independent factors related to SR (Figure 2B and C and Table 5). On the other hand, in the HBeAg-negative group, the most valuable determinant of VR was found to be the HAI score (Figure 2D and Table 5).

DISCUSSION

The few previous studies that evaluated HBsAg expression by semiquantitative methods have shown that serum HBsAg levels are correlated with the number of intrahepatic HBsAg-positive cells[19,20]. This observation was interpreted as serum and hepatic HBsAg being interrelated. On the other hand, in our study, we determined the percentage of intrahepatic HBsAg expression in the whole tissue section of each case by image analysis. Our results showed that in HBeAg-positive cases, p-HBsAg was associated not only with serum HBsAg but also with serum HBV DNA levels, indicating that intrahepatic HBsAg in liver tissues is closely related to viremia. Recently, Su *et al*[25] investigated HBsAg expression in the liver and its relationship with both serum HbsAg and interferon therapy using morphometric methods in treatment-naïve HbeAg-positive patients. Parallel to the findings of the

Table 3 The correlation of clinicopathologic factors and hepatitis B surface antigen expression patterns with viral replication in hepatitis B e antigen negative cases

	HBV DNA		HAI		Fibrosis	
Parameters	**< 6.65**	**≥ 6.65**	**< 9**	**≥ 9**	**< 4**	**≥ 4**
n	23	31	31	23	34	20
Age (yr)	48.13 ± 10.34	45.71 ± 12.02	46.52 ± 11.73	47.04 ± 10.92	45.82 ± 11.63	48.30 ± 10.81
Gender						
Male	10 (43.5)	20 (64.5)	19 (61.3)	11 (47.8)	23 (67.6)	7 (35)[a]
Female	13 (56.5)	11 (35.5)	12 (38.7)	12 (52.2)	11 (32.4)	13 (65)
HBV DNA (log_{10}IU/mL)	-	-	4.97 ± 1.49	7.56 ± 1.21[a]	4.16 ± 0.97	3.98 ± 0.93
HBsAg (log_{10}IU/mL)	3.19 ± 0.84	3.92 ± 0.90	3.30 ± 0.8	3.86 ± 0.91[a]	3.46 ± 0.95	4.03 ± 0.98
ALT IU/L	96.09 ± 33.55	163.06 ± 50.85[a]	102.55 ± 41.36	177.65 ± 80.65	126.18 ± 64.87	148.75 ± 58.6
ALT < 200 IU/L	21 (91.3)	18 (58.1)[b]	26 (83.9)	13 (56.5)[a]	25 (73.5)	14 (70)
ALT ≥ 200 IU/L	2 (8.7)	13 (41.9)	5 (16.1)	10 (43.5)	9 (26.5)	6 (30)
INR	1.13 ± 0.27	1.17 ± 0.28	1.15 ± 0.3	1.16 ± 0.24	1.22 ± 0.32	1.04 ± 0.10[b]
Platelets (× 10^3/μL)	234.91 ± 80.6	216.31 ± 61.29	237.67 ± 73.34	206.64 ± 73.34	218.21 ± 71.85	236.5 ± 68.79
HAI score ≥ 9	2 (8.7)	21 (67.7)[b]	-	-	14 (26.5)	9 (70)[b]
HAI score < 9	21 (91.3)	10 (32.3)	-	-	20 (73.5)	11 (30)
Fibrosis ≥ 4	4 (17.4)	16 (51.6)[a]	6 (19.4)	14 (60.9)[b]	-	-
Fibrosis < 4	19 (82.6)	15 (48.4)	25 (80.6)	9 (39.1)	-	-
p-HBsAg	30.39 ± 14.18	31.91 ± 14.04	28.68 ± 11.16	34.75 ± 16.71	33.21 ± 15.32	27.96 ± 10.93
HBsAg SP1						
A	6 (26.1)	10 (32.3)	13 (41.9)	3 (13)[a]	13 (38.2)	3 (15)
B	5 (21.7)	9 (29)	5 (16.1)	9 (39.1)	7 (20.6)	7 (35)
C	4 (17.4)	1 (3.2)	4 (12.9)	1 (4.3)	5 (14.7)	0
D	5 (21.7)	1 (3.2)	5 (16.1)	1 (4.3)	3 (8.8)	3 (15)
E	3 (13)	10 (32.3)	4 (12.9)	9 (39.1)	6 (17.6)	7 (35)
HBsAg SP2						
Diffuse	11(47.8)	19 (61.3)[b]	18 (58.1)	12 (52.2)[a]	20 (58.8)	10 (50)
Globular	9 (39.1)	2 (3.2)	9 (29)	1 (4.3)	7 (20.6)	3 (15)
Submembranous	3 (13.1)	10 (31.5)	4 (12.9)	10 (43.5)	7 (20.6)	7 (35)
Membranous expression						
Present	7 (30.4)	13 (41.9)	10 (32.3)	10 (43.5)	14 (41.2)	6 (30)
Absent	16 (69.6)	18 (58.1)	21 (67.7)	13 (56.5)	20 (58.8)	14 (70)

Data are presented as mean ± SD, number (%).
[a]$P < 0.05$.
[b]$P < 0.01$.
p-HbsAg: Percentage of hepatitis B surface antigen expression; SP: Staining pattern; ALT: Alanine aminotransferase; INR: International normalized ratio; HAI: Histologic activity index; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus.

present study, they observed a strong correlation between hepatic HbsAg expression and serum HbsAg levels. In addition, the relationship between HbsAg level and HbeAg loss after interferon therapy observed in this study suggested that the HbsAg expression level may be a useful marker to determine the response to therapy.

Therefore, in our study, we also aimed to elucidate the relationship between p-HbsAg levels and the treatment response in patients receiving entecavir treatment together with other HbsAg expression patterns. In the HbeAg-positive group, VR and

Table 4 The relationship between clinicopathological factors and hepatitis B surface antigen expression patterns with viral response and serological response

	HBeAg positive				HBeAg negative
	VR		SR		VR
Parameters	Absent	Present	Absent	Present	Present
Age (yr)					
< Mean	11 (34.4)	39 (72.2)[b]	19 (48.7)	31 (66)[b]	24 (44.4)
≥ Mean	21 (65.6)	15 (27.8)	20 (51.43)	16 (34)	30 (55.6)
Gender					
Male	21 (34.4)	27 (50)	24 (61.5)	24 (48.9)	30 (55.6)
Female	11 (34.4)	27 (50)	15 (38.5)	23 (51.1)	24 (44.4)
HBV DNA					
Low	17 (53.1)	39 (72.2)[a]	16 (41)	16 (34)	23 (42.6)
High	15 (45.9)	15 (27.8)	23 (59)	31 (66)	31 (57.4)
HBsAg					
Low	21 (65.6)	15 (27.8)[a]	21 (53.8)	15 (31.9)[b]	30 (55.6)
High	11 (34.4)	39 (72.2)	18 (46.2)	32 (68.1)	24 (44.4)
ALT					
< 200 IU/L	23 (71.9)	29 (53.7)	29 (74.4)	23 (48.9)[a]	39 (72.2)
≥ 200 IU/L	9 (28.1)	25 (46.3)	10 (25.6)	24 (51.1)	15 (27.8)
INR					
Low	15 (46.9)	26 (48.1)	19 (48.7)	22 (46.8)	39 (72.2)
High	17 (53.1)	28 (51.9)	20 (51.3)	25 (53.2)	15 (27.8)
Platelets					
Low	15 (46.9)	31 (57.4)	20 (51.3)	26 (55.3)	31 (57.4)
High	17 (53.1	23 (42.6)	19 (48.7)	21 (44.7)	23 (42.6)
HAI score ≥ 9	13 (40.6)	25 (46.3)	11 (25)	28 (59.6)[a]	23 (42.6)
HAI score < 9	19 (59.4)	29 (53.7)	28 (75)	19 (40.4)	31 (57.4)
Fibrosis ≥ 4	17 (53.1)	16 (29.6)[a]	17 (43.6)	16 (34)	20 (37)
Fibrosis < 4	15 (46.9)	10 (70.4)	22 (56.4)	31 (66)	34 (63)
p-HbsAg					
Low	26 (81.3)	25 (46.3)[b]	29 (74.4)	24 (51.1)[b]	21 (38.9)
High	6 (18.8)	29 (53.7)	10 (25.6)	23 (48.9)	33 (61.1)
HBsAg SP1					
A	1 (3.1)	16 (29.6)[b]	2 (5.1)	15 (31.9)[b]	16 (29.6)
B	0	10 (18.5)	0	10 (21.3)	14 (25.9)
C	6 (18.8)	10 (18.5)	11 (28.2)	5 (10.6)	5 (9.3)
D	6 (18.8)	4 (7.4)	6 (15.4)	4 (8.5)	6 (11.1)
E	19 (59.4)	14 (25.9)	20 (51.3)	13 (27.7)	13 (24.1)
HBsAg SP2					
Diffuse	1 (3.1)	28 (58.1)[b]	2 (5.1)	2 (57.4)[b]	30 (55.6)
Globular	12 (37.5)	12 (22.2)	17 (43.6)	7 (14.9)	10 (18.5)
Submembranous	19 (59.4)	14 (25.9)	20 (51.3)	13 (27.7)	14 (25.9)

Membranous expression					
Absent	27 (84.4)	26 (48.1)[b]	29 (74.4)	24 (51.1)[b]	21 (38.9)
Present	5 (15.6)	28 (51.9)	10 (25.6)	23 (48.9)	33 (61.1)

[a]$P < 0.05$.
[b]$P < 0.01$.
VR: Viral response; SR: Serological response; p-HbsAg: Percentage of hepatitis B surface antigen expression; SP: Staining pattern; ALT: Alanine aminotransferase; INR: International normalized ratio; HAI: Histologic activity index; HBsAg: Hepatitis B surface antigen; HBeAg: Hepatitis B e antigen; HBV: Hepatitis B virus.

Table 5 The association of clinicopathological factors and hepatitis B surface antigen expression patterns with viral response and serological response

	HBeAg positive (n = 86)						HBeAg negative (n = 54)	
Parameters	**VR**			**SR**			**VR**	
	HR (95%CI)	***P* value[1]**	***P* value[2]**	**HR (95%CI)**	***P* value[1]**	***P* value[2]**	**HR (95%CI)**	***P* value[2]**
Age (yr)	0.685 (0.228-2.055)	0.001	0.449	0.892 (0.301-2.644)	0.007	0.837	0.936 (0.460-1.904)	0.855
Gender: male *vs* female	0.581 (0.301-1.121)	0.72	0.105	0.516 (0.251-1.062)	0.79	0.072	2.410 (0.986-5.985)	0.054
HBV DNA (log_{10} IU/mL)	0.782 (0.275-2.224)	0.003	0.645	0.707 (0.246-2.029)	0.40	0.519	0.620 (0.255-1.508)	0.292
HBsAg (log_{10}IU/mL)	1.931 (0.757-4.923)	0.001	0.168	2.252 (0.878-5.773)	0.001	0.091	0.700 (0.294-1.669)	0.422
ALT ≥ 200 IU/L *vs* < 200 IU/L	0.992 (0.476-2.068)	0.90	0.982	1.037 (0.436-2.471)	0.018	0.934	0.854 (0.372-1.962)	0.710
INR	0.539 (0.271-1.072)	0.751	0.078	0.493 (0.222-1.092)	0.901	0.081	1.815 (0.791-4.161)	0.159
Platelets (× 10^3/μL)	0.619 (0.309-1.240)	0.194	0.176	0.538 (0.243-1.189)	0.389	0.125	0.925 (0.452-1.892)	0.831
HAI score ≥ 9 *vs* < 9	1.566 (0.692-3.546)	0.282	0.282	2.881 (1.170-7.094)	0.132	0.021	6.876 (2.219-21.309)	0.001
Fibrosis ≥ 4 *vs* < 4	1.245 (0.517-2.999)	0.021	0.625	1.079 (0.427-2.730)	0.092	0.872	0.833 (0.326-2.125)	0.702
p-HBsAg	3.178 (1.582-6.387)	0.001	0.001	3.990 (1.843-8.638)	0.001	0.001	1.089 (0.575-2.063)	0.793
SP1	0.899 (0.495-1.634)	0.001	0.727	3.760 (1.742-7.861)	0.001	0.531	0.959 (0.443-2.167)	0.990
SP2	0.727 (0.240-2.200)	0.001	0.573	0.721 (0.216-2.410)	0.001	0.595	0.996 (0.552-1.798)	0.990
Membranous expression	1.085 (0.513-2.294)	0.001	0.831	1.082 (0.472-2.482)	0.001	0.952	0.692 (0.246-1.944)	0.485

[1]Univariate analysis.
[2]Cox regression analysis.
VR: Viral response; SR: Serological response; HR: Hazard ratio; CI: Confidence interval; p-HbsAg: Percentage of hepatitis B surface antigen expression; SP: Staining pattern; ALT: Alanine aminotransferase; INR: International normalized ratio; HAI: histologic activity index; HBsAg: Hepatitis B surface antigen; HBeAg: Hepatitis B e antigen.

SR were observed more frequently in cases with pretreatment values of p-HbsAg levels above the group average. In the HbeAg-positive group, the pretreatment values of p-HbsAg were strongly related to either VR or SR, suggesting the clinical significance of p-HbsAg in the management of HbeAg-positive CHB patients with antiviral treatment. Moreover, multivariate analysis revealed that in this group, p-HbsAg was an independent factor associated with VR and SR, warranting further investigation to evaluate the potential role of p-HbsAg as a tool to predict the response to therapy.

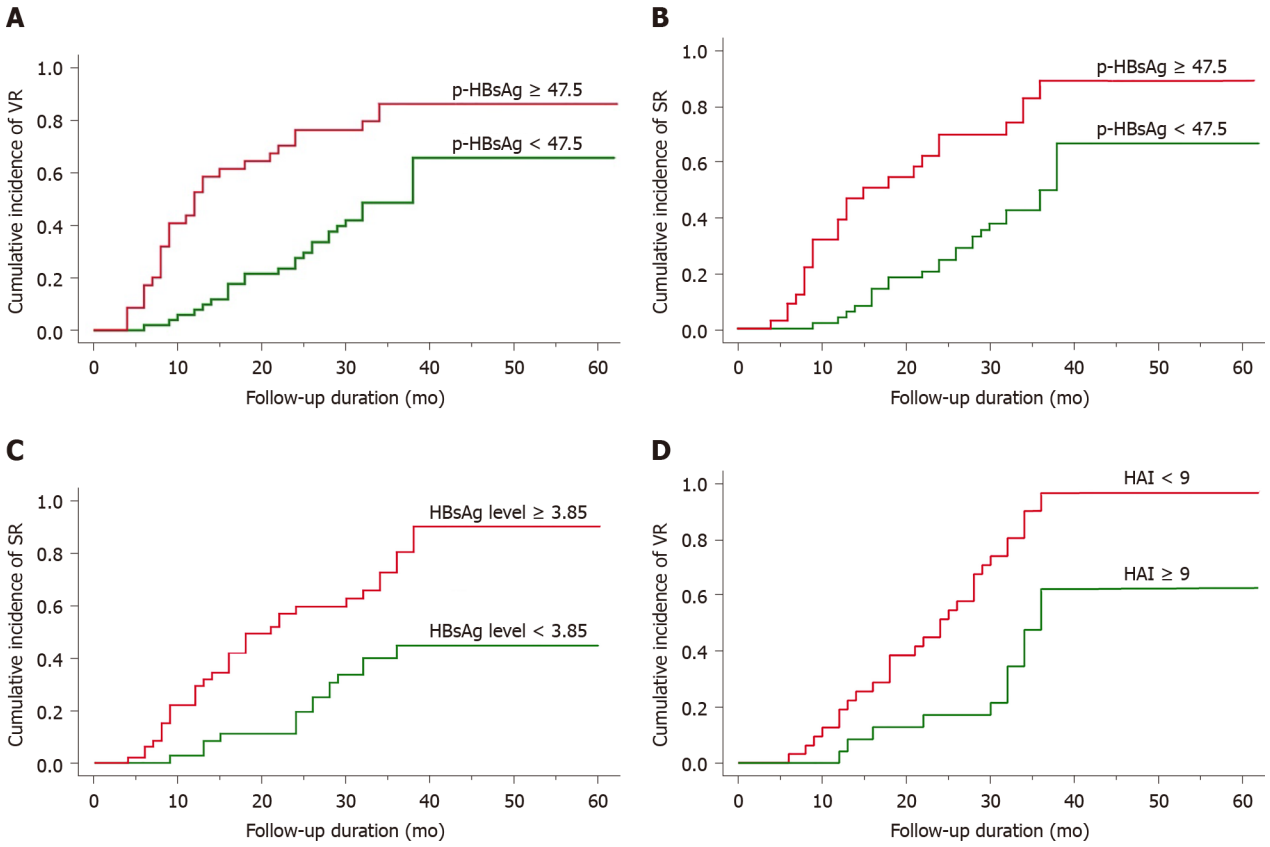

Figure 2 Kaplan-Meier analysis in hepatitis B surface antigen positive and hepatitis B e antigen negative cases ($P < 0.01$). A: Cumulative incidence of viral response in hepatitis B e antigen (HBeAg) positive patients is more frequently observed in cases with higher percentage of immunohistochemical hepatitis B surface antigen (p-HBsAg) expression; B-D: In the same group, serological response is also significantly related to p-HBsAg expression (B) and HBsAg levels (C). However, in HBeAg negative in patients, VR is closely related to hepatitis activity index (D). p-HBsAg: Percentage of immunohistochemical hepatitis B surface antigen; HBsAg: Hepatitis B surface antigen; HAI: Hepatitis activity index; VR: Viral response; SR: Serological response.

Another interesting result of this study is the presence of an inverse relationship between p-HbsAg and HAI and fibrosis scores in the HbeAg-positive group. Parallel to this finding, in cases with high HAI and/or fibrosis scores, the serum HBV DNA and HbsAg levels were significantly lower, suggesting that in the liver, the replacement of hepatocytes by both increased inflammatory infiltration and increased connective tissue can lead to this situation.

Our study demonstrated that HbsAg expression patterns, evaluated by various immunohistochemical methods, have different relationships with clinicopathological parameters and treatment responses in patients with CHB. Many years ago, following the detection of ground-glass hepatocytes by immunohistochemical methods in the liver tissues of patients with anti-Hbe positivity in the serum and a low HBV DNA level, the relationship of HbsAg expression patterns in hepatocytes with clinicopathological factors was investigated.Some studies have shown that patterns A and B, as opposed to those of C and D, are more common in younger subjects[15,18]. However, no similar age-related difference finding in HbsAg staining patterns was observed in other studies[16]. In our study, most of the patients with patterns A and B were younger than those with patterns C and D.

When the relationship of these staining patterns with disease activity and viremia has been investigated in recent studies, most of the data indicate that patterns A and B are associated with viremia[15,18,21,26,27]. In parallel with this finding, in our study, these patterns were associated with high HBV DNA and HAI in the HbeAg-positive group and were rarely observed in cases with high fibrosis scores, supporting the hypothesis that both are associated with active disease. Interestingly, the frequent detection of pattern B in the HbeAg-negative group, especially in cases with high HAI scores, also points to a relationship with active disease in these cases[15]. While the preliminary results indicate that the marginal staining pattern (pattern E) may be a determinant of inactive virus replication, other studies concluded that pattern C may be a marker of this situation[15,28]. In our cases, pattern E was more frequent in cases with low levels of HBV DNA and more frequent in cases with high fibrosis scores in

the HbeAg-positive group, supporting data about its relationship with inactive disease reported by previous studies. Similar to recent findings in HbeAg-positive cases, membranous expression was absent in 84% and 87% of patients with lower HBV DNA values and fibrosis scores, respectively[18]. These findings suggest that this pattern of staining reflects active liver disease.

In an elegant study, Yim *et al*[26] investigated the relationship between these staining patterns and antiviral treatment response in patients with CHB. Diffuse staining patterns were associated with SR in HBeAg-positive patients. However, multivariate analysis revealed that this was not an independent predictor. In our study, although HBsAg expression patterns were associated with VR and SR in univariate analysis, they were not found to be independent factors. In contrast, p-HBsAg was a good predictor of the response to therapy in HBeAg-positive cases. This finding emphasizes that the determination of HBsAg expression by quantitative methods, beyond its distribution within the cell, may be an objective marker that can be used during the course and treatment of the disease.

In recent years, there has been growing interest in developing various noninvasive methods for monitoring the course and treatment of CHB. On the other hand, although it is an expensive and invasive method, liver biopsy remains the gold standard in determining liver injury and fibrosis during the follow-up of the disease. However, beyond these parameters, our results emphasize that the HBsAg expression levels measured in pretreatment liver biopsies *via* quantitative methods may be a useful marker for determining the treatment response.

CONCLUSION

This study demonstrated that HBsAg expression patterns, evaluated by various immunohistochemical methods, have different relationships with clinicopathological parameters and the treatment response in patients with CHB. Although the expression pattern of HBsAg differs significantly according to the disease status, it shows lower clinical significance in predicting the response to therapy than the percentage of HBsAg expression. Therefore, the quantitative evaluation of HBsAg expression in pretreatment-naïve liver biopsies of patients with CHB may provide a prediction of the response to antiviral therapy, especially in cases with HBeAg positivity. However, our findings should be supported by additional large studies.

ARTICLE HIGHLIGHTS

Research background

The combination of various laboratory methods with histopathological findings is not always adequate to predict the progression and treatment response in patients with chronic viral B hepatitis (CHB). Therefore, efforts are being made to determine new parameters that may be useful in the follow-up of the disease.

Research motivation

It has been reported that differences in intracytoplasmic hepatitis B surface antigen (HBsAg) staining patterns are associated with viremia and active inflammation in CHB. However, HBsAg expression was evaluated semiquantitatively in these studies, and different classifications were used for the HBsAg staining pattern.

Research objectives

To investigate the relationship between image analysis-based quantitation of HBsAg expression and staining patterns with clinicopathological factors and treatment.

Research methods

The study group consisted of 140 patients who were followed up and treated after the diagnosis of CHB. All cases were treated with entecavir. The evaluation of immunohistochemical HBsAg staining was performed in whole tissue sections by image analysis and expressed as a percentage of HBsAg staining (p-HBsAg). The staining pattern was also evaluated separately according to 3 different previously defined classifications. The correlations among immunohistochemical and clinicopathological findings were analyzed by χ^2 and *t*-tests. The log-rank test was employed for comparisons of

response rates. A Cox proportional hazards regression model was applied for multivariate analysis. Spearman's correlation test was used to determine relationships between p-HBsAg and the other continuous variables.

Research results

There was a positive correlation between p-HBsAg and the serum levels of hepatitis B virus (HBV) DNA and HBsAg. The p-HBsAg was significantly higher in younger patients than in older patients, and the mean p-HBsAg level was significantly higher in the HBeAg-negative group than in the HBeAg-positive group. In the HBeAg-positive group, the average p-HBsAg level was significantly higher in patients with higher HBV DNA values than in those with low HBV DNA levels. Furthermore, an inverse relationship was noted between p-HBsAg and either hepatitis activity index (HAI) or the fibrosis score. Regarding staining pattern, diffuse cytoplasmic staining in many discrete hepatocytes was higher among cases with higher HBV DNA levels, and their number decreased among patients with HAI ≥ 9. However, submembranous staining was more frequent in subjects with higher HAI and fibrosis scores. In the same group, the p-HBsAg and HBsAg expression patterns were correlated with the viral response (VR) and serological response (SR). Multivariate analysis revealed that p-HBsAg was an independent factor associated with VR and SR. In the HBeAg-negative group, no correlation was observed between either the p-HbsAg or the membranous staining pattern and HBV DNA, HAI, or fibrosis. Diffuse staining was more frequent in cases with a higher level of HBV DNA. On the other hand, globular staining was rarely observed in these cases. In this group, the most valuable determinant of VR was the HAI score.

Research conclusions

We have demonstrated that the expression pattern of HBsAg shows lower clinical significance in predicting the response to therapy than the percentage of HBsAg expression. Therefore, the quantitative evaluation of HBsAg expression in pretreatment-naïve liver biopsies of patients with CHB may predict the response to antiviral therapy, especially in cases with HBeAg positivity.

Research perspectives

The evaluation of HBsAg expression by image analysis in pretreatment-naïve liver biopsies may be a valuable tool to predict the response to antiviral therapy. However, our findings should be supported by additional large-scaled studies.

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ORIGINAL ARTICLE

Observational Study

COVID-19 emergency: Changes in quality of life perception in patients with chronic liver disease-An Italian single-centre study

Alessandra Zannella, Silvia Fanella, Massimo Marignani, Paola Begini

ORCID number: Alessandra Zannella 0000-0001-5300-0459; Silvia Fanella 0000-0003-1900-918X; Massimo Marignani 0000-0002-8912-9972; Paola Begini 0000-0001-7286-0278.

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Alessandra Zannella, Silvia Fanella, Massimo Marignani, Paola Begini, Department of Gastroenterology, Sant'Andrea Hospital, Rome 00189, Italy

Corresponding author: Silvia Fanella, MD, Doctor, Department of Gastroenterology, Sant'Andrea Hospital, Via di Grottarossa 1089, Rome 00189, Italy. silviafanella91@gmail.com

Abstract

BACKGROUND

In December 2019, the coronavirus disease-2019 (COVID-19) emerged and rapidly spread worldwide, becoming a global health threat and having a tremendous impact on the quality of life (QOL) of individuals.

AIM

To evaluate the awareness of patients with chronic liver disease (CLD) regarding the COVID-19 emergency and how it impacted on their QOL.

METHODS

Patients with an established diagnosis of CLD (cirrhosis, autoimmune hepatitis, primary biliary cholangitis, and primary sclerosing cholangitis) who had been evaluated at our Outpatient Liver Disease Clinic during the 6-mo period preceding the start of Italian lockdown (March 8, 2020) were enrolled. Participants were asked to complete a two-part questionnaire, administered by telephone according to governmental restrictions: The first section assessed patients' basic knowledge regarding COVID-19, and the second evaluated the impact of the COVID-19 emergency on their QOL. We used the Italian version of the CLD questionnaire (CLDQ-I). With the aim of evaluating possible changes in the QOL items addressed, the questionnaire was administered to patients at the time of telephone contact with the specific request to recall their QOL perceptions during two different time points. In detail, patients were asked to recall these perceptions first during time 0 (t0), a period comprising the 2 wk preceding the date of ministerial lockdown decree (from February 23 to March 7, 2020); then, in the course of the same phone call, they were asked to recall the same items as experienced throughout time 1 (t1), the second predetermined time frame encompassing the 2 wk (from April 6 to April 19) preceding our telephone contact and questionnaire administration. All data are expressed as number (%), and continuous variables are reported as the median (interquartile range). The data were compared using the Wilcoxon paired non-parametric test.

report.

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RESULTS
A total of 111 patients were enrolled, of whom 81 completed the questionnaire. Forty-nine had liver cirrhosis, and all of them had compensated disease; 32 patients had autoimmune liver disease. The majority (93.8%) of patients were aware of COVID-19 transmission modalities and on how to recognize the most common alarm symptoms (93.8%). Five of 32 (15.6%) patients with autoimmune liver disease reported having had the need to receive more information about the way to manage their liver disease therapy during lockdown and nine (28.2%) thought about modifying their therapy without consulting their liver disease specialist. About the impact on QOL, all CLDQ-I total scores were significantly worsened during time t1 as compared to time t0.

CONCLUSION
The COVID-19 epidemic has had a significant impact on the QOL of our population of patients, despite a good knowledge of preventive measure and means of virus transmission.

Key Words: COVID-19; Liver disease; Quality of life; Liver cirrhosis; Chronic liver disease questionnaire

Core Tip: Although negative mental health outcomes in the Italian general population during coronavirus disease-2019 (COVID-19) lockdown have already been reported, our study was among one of the first investigating the impact of the COVID-19 pandemic on patients with chronic liver disease and addressing the subpopulation of patients with autoimmune chronic hepatitis.

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INTRODUCTION

The World Health Organization defines health as "a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity"[1]: This is the multidimensional target which healthcare professionals pursue. The coronavirus disease-2019 (COVID-19) pandemic strongly impacted on this definition at many levels, affecting both health and psychological well-being[1]. In Italy, in response to this worldwide emergency, measures were enforced, aimed at the reduction of inappropriate access to healthcare centres, which were considered as environments at a potential risk of crowding and viral infection spread. Outpatient visits were thus limited to emergencies or to supply life-saving medications, considering that even if all individuals are susceptible to COVID-19, those with underlying conditions, such as patients with chronic liver disease (CLD) or reduced immune functions, have been recognized to be at an increased risk to experience a more severe disease course and increased mortality rates[2].

As a result, all patients affected by CLD experienced a substantial change in their usual routine care. As emerged from the survey by the Italian Association for the Study of the Liver[3], the reorganization of the health-care system, which followed the pandemic phase, has severely affected the management of liver diseases in Italy.

It is well known from previous studies that patients suffering from CLD, especially cirrhosis, have been shown to experience a significantly impaired health related quality of life (HRQOL) when compared to patients without liver disease[4]. These issues encompass mental impairment and determine limitations which affect patients' ability to perform normal daily activities. Collecting information on HRQOL has been shown to be helpful in assessing the multidimensional impact of liver disease on

patients' well-being. The issue of quality of life (QOL) among patients with chronic diseases during the COVID-19 pandemic has been the subject of clinical studies in different countries and clinical settings. During the pandemic and because of it, the presence of a chronic health problem has generally been shown to represent per se a risk factor for a worsening in the QOL. In a study from Singapore, analyzing QOL among patients with cardiovascular disease, a significant worsening of mental health related issues was described[5]. In another study from Poland, conducted among stage III and IV oncology patients undergoing chemotherapy, a questionnaire was administered including questions about QOL during the COVID-19 pandemic: The study showed a significant reduction in cognitive ($P < 0.0001$) and social ($P < 0.0001$) functioning, as well as worsening of symptoms such as fatigue, insomnia, and appetite [6]. In the study by Falcone *et al*[7] patients with thyroid cancer during the COVID-19 pandemic followed in a single endocrine cancer centre in Italy received two questionnaires to evaluate changes in QOL. Some patients were contacted by phone and answered questions during a single call. Others, instead, had access to the questionnaires by links provided by mail. The research team produced *ex novo* the first questionnaire, which was developed *ad hoc* to explore and measure the emotional impact of the rapid spread of the COVID-19 pandemic given the absence in the literature of a similar instrument, while the second questionnaire was a validated Italian translation of the European Organization for Research and Treatment of Cancer Quality of Life Questionnaire. In that study, HRQOL appeared to be affected by the COVID-19 pandemic among this cohort of patients, regardless of their disease severity or current health-care needs[7]. Nevertheless, diverging results have been described among patients with inflammatory bowel disease (IBD), both by Azzam *et al*[8] and Algahtani *et al*[9] in two different studies from Saudi Arabia. In the latter, as explored and analyzed by means of a questionnaire submitted through various online communication channels including email, organizational portals, and social media platforms (WhatsApp, Twitter, and Facebook), HRQOL appeared to be unaffected by the COVID-19 pandemic. In that study, evaluating HRQOL among patients with IBD pre- and post-COVID-19 pandemic using the IBD-disk questionnaire in a Saudi Arabia tertiary care IBD centre, HRQOL appeared to be unaffected by the COVID-19 pandemic among this cohort of patients. These results suggest that different diseases, patients' samples, and investigative methods adopted might account for the diverging results observed in these latter IBD studies.

Given the scarcity of HRQOL data among patients with CLD during the COVID-19 pandemic, the present study aimed to assess patients' grade of awareness about the current global emergency, and how this event impacted on their interaction with the healthcare system and planned follow-up programmes. We also explored patients' grade of awareness about the COVID-19 emergency, assessing the effect of mandatory social limitations on their QOL.

MATERIALS AND METHODS

Patients

All outpatients with an established diagnosis of CLD [cirrhosis and autoimmune liver diseases (ALD), *i.e.* autoimmune hepatitis, primary biliary cholangitis, and primary sclerosing cholangitis], ≥ 18 years of age, who had been attending our hepatology outpatient clinic (Sant'Andrea University Hospital, "Sapienza" University of Rome) during the 6 mo prior to introduction of the social lockdown strategies (March 8, 2020) were enrolled in this study. As part of the suggested telemedicine clinical approach, and in order to maintain contact with our CLD patients, we asked participants to complete a questionnaire on their awareness regarding the COVID-19 emergency, and how it had impacted on their QOL. Patients with either language or cognitive difficulties, and those unable to understand or complete the questionnaire were excluded. Considering the mandatory action of government-imposed lockdown strategy[10], and that the healthcare strategy of our department was switched from face-to-face to telehealth as requested by our Hospital upon Regional order, the questionnaire was completed by means of telephonic interviews.

The study was conducted according to Sant'Andrea Hospital directives following Latium Region order (No. 3405/2020 and 4888/2020, Regione.Lazio.Ufficiale UO54467/U0218196, 11/03/2020). All patients included in the study had already provided written consent to the use of their clinical data for research purposes at the time of their first hospital visit; verbal consent was also obtained at the beginning of the telephone interview. Patients were carefully informed about the purpose of our

survey, and given the option to opt-out in case of refusal. We collected clinical information regarding age and aetiology of liver disease. The Child-Pugh score was calculated for each patient with liver cirrhosis based on laboratory tests no older than 3 mo.

The study protocol complied with the ethical guidelines of the 1975 Declaration of Helsinki (6th revision, 2008), and was approved by our institution's human research committee.

Questionnaire

A two-part questionnaire was used, and is fully available in Table 1.

The first part was specifically developed by our liver team and aimed to: (1) Investigate patients' awareness about the COVID-19 emergency and explore how this event had impacted on their contact with the healthcare system and their planned hepatological follow-up programmes; (2) Investigate whether patients had used personal protective equipment (PPE) (*i.e.* surgical masks and gloves), and applied preventive/protection measures such as social distancing and adequate hygiene strategies (*e.g.* frequent hand washing), as indicated by the Italian Government following the advice of the Italian National Institute of Health; (3) Assess the basic knowledge of COVID-19, including its means of transmission, patients' capacity to recognize alarm symptoms, and awareness of being at higher risk to develop a more severe clinical form since affected by liver disease; and (4) Explore whether patients had encountered difficulties in contacting their general physician and/or their liver diseases specialist. Adherence to the performance of liver ultrasound testing scheduled during the study period was also assessed among patients with either liver cirrhosis or advanced liver fibrosis. Three questions (Q) of this first section of the questionnaire (Q11 to 13) were addressed only to patients with ALD, in particular to investigate if, during the lockdown period, they had thought of independently modifying their therapy.

As suggested in the recent publication by the Food and Agriculture Organization of the United Nations, regarding the development of questionnaires to be used as instruments to investigate the impact of COVID-19 pandemic, even if in different settings, the questionnaire was designed to be easily conducted in the same manner each time[11].

We have thus formulated closed-ended questions to make answering easier. We developed simple and clear questions in order to avoid misunderstandings, and asked the simplest questions initially and the most difficult at the end, so that the interviewees could feel more comfortable.

The response to each question was scored as either positive (yes) or negative (no).

This section was pre-tested and validated on a smaller-scale target group of 25 inpatients [15 (60.0%) men, mean age 64 years], affected by CLD and admitted to our hospital ward during March 2020, to verify its full comprehensibility and practical applicability as a tool to be administered over the phone. The results obtained in this test group were not included in the final analysis.

The second section was aimed at evaluating the impact of the COVID-19 emergency on the QOL of study patients, using the Italian version of the CLD questionnaire (CLDQ-I)[12]. With the aim of evaluating possible changes in the QOL items addressed, the questionnaire was administered to patients at the time of telephone contact with the specific request to recall their QOL perceptions during two different time periods. In detail, patients were asked to recall these perceptions first during time 0 (t0), a period comprising the 2 wk preceding the date of ministerial lockdown decree (from February 23 to March 7, 2020); then, in the course of the same phone call, they were asked to recall the same items as experienced throughout time 1 (t1), the second predetermined time frame encompassing the 2 wk (from April 6 to April 19) preceding our telephone contact and questionnaire administration. We formulated a total score that included each question regarding the same domain of interest for time t0 and time t1.

Total scores are summarized as main text tables. Data summarizing single scores regarding each item of all single domains are presented as tables in the supplementary data section (Supplementary Tables 1-12).

Statistical analysis

All data are expressed as number (%), and continuous variables are reported as the median ± interquartile range (IR). The data were compared using the Wilcoxon paired non-parametric test. A value of $P < 0.05$ was considered statistically significant. All data analyses was reviewed by our biomedical statistician. Statistical analyses were performed using MedCalc Statistical Software version 19.0.4.

Table 1 Questionnaire

Questions
First part
1 Are you aware that the novel coronavirus is transmitted to people?
2 Are you aware that symptoms including fever, cough, and breathing difficulties are signs of alarm?
3 Are you aware that people with chronic liver disease are at higher risk of developing a severe form of the disease?
4 Are you aware that when you go out, you always have to use personal protective equipment such as masks and gloves?
5 Do you always respect prevention and protection measures such as social distancing?
6 Do you use adequate hygiene measures such as frequently washing your hands?
7 During this period, did you need to contact your general physician for your liver disease?
8 If you answered yes, did you have difficulty in contacting him/her?
9 During this period, did you need to contact a gastroenterologist specialist for your liver disease?
10 If you answered yes, did you have difficulty in contacting him/her?
11 Have you received more detailed information about the management of your therapy?
12 Do you know that immunosuppressed patients are more at risk of getting severe acute respiratory syndrome coronavirus-2 infection?
13 Have you thought about modifying your immunosuppressive therapy on your own?
14 Did you manage to perform the six-month follow-up ultrasound for hepatocellular carcinoma surveillance?
Second part
1 How much of the time during the last two weeks have you been troubled by a feeling of abdominal bloating?
2 How much of the time have you been tired or fatigued during the last two weeks?
3 How much of the time during the last two weeks have you experienced bodily pain?
4 How often during the last two weeks have you felt sleepy during the day?
5 How much of the time during the last two weeks have you experienced abdominal pain?
6 How much of the time during the last two weeks has shortness of breath been a problem for you in your daily activities?
7 How much of the time during the last two weeks have you not been able to eat as much as you would like?
8 How much of the time in the last two weeks have you been bothered by having decreased strength?
9 How often during the last two weeks have you had trouble lifting or carrying heavy objects?
10 How often during the last two weeks have you felt anxious?
11 How often during the last two weeks have you felt a decreased level of energy?
12 How much of the time during the last two weeks have you felt unhappy?
13 How often during the last two weeks have you felt drowsy?
14 How much of the time during the last two weeks have you been bothered by a limitation of your diet?
15 How often during the last two weeks have you been irritable?
16 How much of the time during the last two weeks have you had difficulty sleeping at night?
17 How much of the time during the last two weeks have you been troubled by a feeling of abdominal discomfort?
18 How much of the time during the last two weeks have you been worried about the impact your liver disease has on your family?
19 How much of the time during the last two weeks have you had mood swings?
20 How much of the time during the last two weeks have you been unable to fall asleep at night?
21 How often during the last two weeks have you had muscle cramps?
22 How much of the time during the last two weeks have you been worried that your symptoms will develop into major problems?
23 How much of the time during the last two weeks have you had a dry mouth?
24 How much of the time during the last two weeks have you felt depressed?
25 How much of the time during the last two weeks have you been worried about your condition getting worse?

26	How much of the time during the last two weeks have you had problems concentrating?
27	How much of the time have you been troubled by itching during the last two weeks?
28	How much of the time during the last two weeks have you been worried about never feeling any better?

RESULTS

Demographic and clinical characteristics

One hundred and eleven [54 males (48.6%) and 57 females (51.4%), average age 66.5 years] consecutive patients affected by CLD fulfilled the inclusion criteria (Figure 1). Out of these, 81 (73%) finally participated in the study as shown in the flowchart (Figure 1), and were equally distributed by gender [43 males (53%) and 38 females (47%); mean age was 65.6 ± 11.8 years]. Forty-nine (60.5%) patients had liver cirrhosis, and all of them had compensated disease (≤ 7). Thirty-two (39.5%) patients had ALD; of them, 13 had autoimmune hepatitis, 17 primary biliary cholangitis, and two primary sclerosing cholangitis. In the population with ALD, eight had liver cirrhosis. Clinical characteristics of the study patients are summarized in Table 2**.**

Patients' awareness about the COVID-19 emergency

Most of the patients (76/81 93.8%) reported being aware of both COVID-19 transmission modalities (Q1), and of its most common alarm symptoms (76/81 93.8%) (Q2).

Twelve (15%) of the 81 patients reported feeling the need to contact their family physicians during lockdown, and 11 of them (11/12, 91.6%) encountered difficulties in reaching them (Q7-8). The majority of these patients (10/12, 83%) had liver cirrhosis.

Questions 11, 12, and 13 were only addressed to the 32 patients with ALD, as they focused specifically on the impact of the infection among these patients. Examining the responses to Q11 to 13, asked only to the 32 patients with ALD, we observed that five (15.6%) of them reported having had the need to receive more information on how to manage their liver disease therapy during lockdown (Q11), and nine (28.2%) thought about modifying their therapy without consulting their liver disease specialist.

Finally, regarding the last question (Q14), out of 57 patients with liver cirrhosis, 49 (49/57, 85.9%) had a follow-up liver ultrasound scheduled for hepatocellular carcinoma (HCC) surveillance during the lockdown period; although most of them (29/49, 60%) were able to perform it, adhering to the scheduled timing, 40% (20/49) could not. The complete panel of questions and relative answers are summarized in Table 3.

CLDQ-I questionnaire

All the 81 patients completing the first part of the questionnaire also fully completed the following CLDQ section. First, we collected the data of all patients enrolled. Patients with autoimmune CLD were then examined as a subgroup.

In each group, all CLDQ-I total scores were significantly worsened during time t1 as compared to time t0. Global data for all patients enrolled and for those with ALD are summarized and expressed as total scores in Tables 4 and 5.

Significant worsening in all answers regarding abdominal symptoms and systemic symptoms between the two-time frames ($P < 0.0001$; $P < 0.0001$) was observed (Table 4).

The second domain assessed perception of fatigue and total score regarding these questions, and a significant worsening was noted ($P < 0.0001$) (Table 4).

The fifth domain evaluated emotional function (total score, Table 4).

To provide a deeper insight on this latter domain, we listed single scores of each item composing it (single scores, Supplementary Table 5).

In detail, when observing single scores, a significant worsening of anxiety ($P < 0.0001$), and an increased perception of irritability ($P < 0.0001$) and depression ($P < 0.0001$) were detected. Mood swings were also significantly frequent ($P < 0.0001$), as well as the tendency to feel unhappy ($P = 0.0066$), and the difficulties in sleeping ($P = 0.0002$) and in falling asleep at night ($P = 0.0001$). The ability to concentrate was also perceived as being worsened ($P = 0.0010$) (Supplementary Table 5).

The sixth and last domains analysed the state of worry (total score, Table 4).

The items in this section identified a significant increase in worries about the impact of their liver disease on their family ($P \leq 0.0001$) and a worsening of their symptoms ($P < 0.0001$).

Table 2 Clinical characteristics of patients

Variable	*n* = 81
Sex, *n* (%)	
Male	38 (47)
Female	43 (53)
Aetiology, *n* (%)	
Cirrhosis	49
HBV	7 (8.6)
HCV	22 (27.2)
Cirrhosis Alcoholic-related	9 (11.1)
Cirrhosis HBV + alcoholic-related	1 (1.2)
Cirrhosis HCV + alcoholic-related	1 (1.2)
Cirrhosis Metabolic-related	9 (11.1)
AIH	8 (9.9)
Cirrhosis AIH-related	5 (6.2)
PBC	14 (17.3)
Cirrhosis PBC-related	3 (3.7)
PSC	2 (2.5)
Child-Pugh score, *n* (%)	
A5	46 (80.7)
A6	6 (10.5)
B7	5 (8.8)

HBV: Hepatitis B virus; HCV: Hepatitis C virus; AIH: Autoimmune hepatitis; PBC: Primary biliary cirrhosis; PSC: Primary sclerosing cholangitis.

The results also showed an increase in the time spent thinking about the possibility of a worsening of their condition ($P < 0.0001$) or of never getting better again ($P = 0.0008$) (Supplementary Table 6). Dataset analyzed in this study is included in the Supplementary Table 6.

Patients with ALD

Findings among patients with ALD differed minimally from those observed in the non-autoimmune group. In the former group, a worsening of total score regarding abdominal ($P = 0.0045$) and systemic symptoms ($P < 0.0004$) was observed (total score, Table 5).

In detail, perception of shortness of breath, itching, and the tendency to feel unhappy, which significantly increased during the second time period in the non-ALD group ($P = 0.0004$, 0.0259, and 0.0027, respectively), did not differ in the ALD subgroup ($P = 0.7695$, 0.0840, and 0.3054, respectively), suggesting, however, a role of sample size in determining such numeric differences.

Data regarding the autoimmune subgroup are available in the Supplementary Tables 7-12.

DISCUSSION

Although negative mental health outcomes in the Italian general population during COVID-19 lockdown have already been reported[13,14], our study is original since it investigated the impact of the COVID-19 pandemic on patients with CLD, in addition addressing the subpopulation of patients with ALD.

The presence of CLD and cirrhosis, with the latter being a condition acknowledged to be associated with immune deregulation[15], have been proven to be significant comorbidities, identifying these liver disease patients as running an increased risk of

Table 3 Questionnaire and answers

Questions	Patients' answer "YES" number (%)	Patients' answer "NO" number (%)
1 Are you aware that the novel coronavirus is transmitted to people?	76 (93.8)	5 (6.2)
2 Are you aware that symptoms including fever, cough, and breathing difficulties are signs of alarm?	76 (93.8)	5 (6.2)
3 Are you aware that people with chronic liver disease are at higher risk of developing a severe form of the disease?	69 (85)	12 (15)
4 Are you aware that when you go out, you always have to use personal protective equipment such as masks and gloves?	77 (95)	4 (5)
5 Do you always respect prevention and protection measures such as social distancing?	60 (74)	21 (26)
6 Do you use adequate hygiene measures such as frequently washing your hands?	60 (74)	21 (26)
7 During this period, did you need to contact your general physician for your liver disease?	12 (15%)	69 (85)
8 If you answered yes, did you have difficulty in contacting him/her?	11 (91.7)[1]	1 (8.3)
9 During this period, did you need to contact a Gastroenterologist specialist for your liver disease?	12 (15%)	69 (85)
10 If you answered yes, did you have difficulty in contacting him/her?	8 (67%)[2]	4 (33.3)
11 Have you received more detailed information about the management of your therapy?	5 (15.6)	27 (84.4)
12 Do you know that immunosuppressed patients are more at risk of getting severe acute respiratory syndrome coronavirus 2-infection?	29 (90.6%)	3 (9.4%)
13 Have you thought about modifying your immunosuppressive therapy on your own?	9 (28.2%)	23 (71.8%)
14 Did you manage to perform the six-month follow-up ultrasound for hepatocellular carcinoma surveillance?	29 (60%)[3]	20 (40%)

[1]Data refer to the number of patients answering positively to question 7.
[2]Data refer to the number of patients answering positively to question 9.
[3]Data refer to the number of patients who were scheduled to undergo liver ultrasound during lockdown timeframe.
Data are expressed as number (%).

Table 4 Total chronic liver disease questionnaire pooled scores for each single quality of life perception domain in all patients enrolled in the study

Items	Median, t0	Range interquartile, t0	Median, t1	Range interquartile, t1	*P* value
Abdominal symptoms: 1, 5, 17	1	1-2	1	1-2	[a]$P < 0.0001$
Fatigue: 2, 4, 8, 11, 13	2	1-2	2	1-2	[b]$P < 0.0001$
Systemic symptoms: 3, 6, 21, 23, 27	1	1-2	1	1-2	[c]$P < 0.0001$
Activity: 7, 9, 14	2	1-5	3	1-5	[d]$P = 0.0245$
Emotional function: 10, 12, 15, 16, 19, 20, 24, 26	2	1-2	2	1-3	[e]$P < 0.0001$
Worry: 18, 22, 25, 28	2	1-2	2	1-3	[f]$P < 0.0001$

more severe outcomes and complications in case of COVID-19[2,16]. A recent meta-analysis, published by Kovalic *et al*[17], showed that patients with CLD do not have an increased risk of contracting COVID-19 but, in case of infection, are more likely to experience a severe/critical COVID-19 related disease, with mortality rates higher than those without CLD.

Probably due to the capillary spread of information (media broadcast *via* television, internet, newspaper, *etc*.), and to the enforcement of government measures aimed at controlling spread of the pandemic, we observed that most of the patients evaluated in our study were aware of the main routes and means of transmission and of the most relevant alarm symptoms of COVID-19. The patients reported to correctly apply PPE, respect social distancing, and frequently wash their hands, in compliance with the governmental recommendations. Furthermore, it should be noted that most of the

Table 5 Total chronic liver disease questionnaire pooled scores for each quality of life perception domain in patients with autoimmune liver disease enrolled in the study

Items	Median t0	Range interquartile t0	Median t1	Range interquartile t1	*P* value
Abdominal symptoms: 1, 5, 17	1	1-1	1	1-2	[a]$P = 0.0045$
Fatigue: 2, 4, 8, 11, 13	1	1-2	1	1-2	[b]$P < 0.0001$
Systemic symptoms: 3, 6, 21, 23, 27	1	1-2	1	1-2	[c]$P < 0.0004$
Activity: 7, 9, 14	2	1-5	2	1-5	[d]$P = 0.0938$
Emotional function: 10, 12, 15, 16, 19, 20, 24, 26	1	1-2	2	1-3	[e]$P < 0.0001$
Worry: 18, 22, 25, 28	1	1-2	2	1-3	[f]$P < 0.0001$

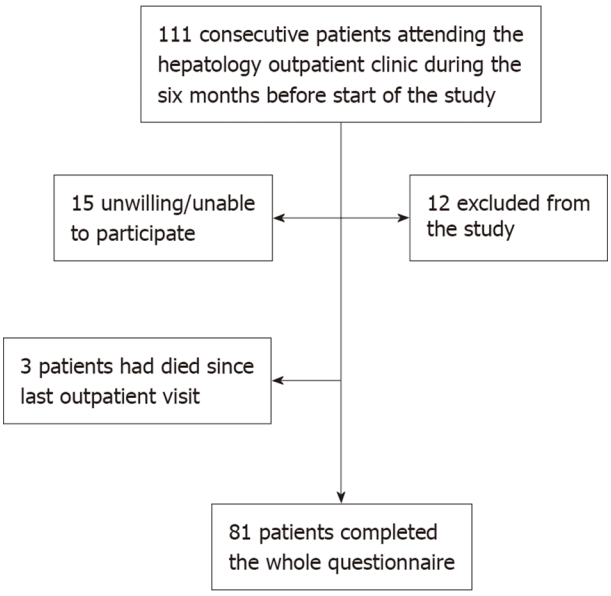

Figure 1 Flow chart of patient enrolment.

patients were aware of an increased risk of developing a more severe clinical form of COVID-19, because of their liver diseases. Our observation confirms how a correct and responsible communication campaign is able to effectively reach special populations including patients with specific medical problems, and to increase their awareness of the risks of emergent and unpredictable situations such as a pandemic, fostering the adoption of appropriate preventive strategies and promoting infection containment.

With regard to contacts with the health-care system, the rapid spread of the pandemic has posed critical challenges for public health, research, and medical communities[10]. Since the early stages of the COVID-19 outbreak, outpatient visits were reduced to avoid nosocomial contact and limit contaminations. Government agencies and scientific societies[3] supported a general indication to manage patients remotely, in particular for patients with CLD and cirrhosis, even if available resources at the beginning of the pandemic were not prepared for this switch. A telemedicine approach was adopted where feasible, contacting patients by phone. However, this approach is clearly incapable of reaching all patients in need of assistance, as suggested by the not negligible rate of patients (27%) unwilling/unable to participate in our alternative clinical contact. Nevertheless, among those who participated in the study, we observed how the pandemic had a significant impact on their daily activities and feelings and in particular on their scheduled face-to-face clinical controls. This observation is even more relevant when considering patients with autoimmune diseases. Indeed, only less than 1/3 of our patients with ALD (28%) had thought of modifying their treatment on their own. Therefore, most patients acted according to the indications provided by major immunology and liver disease scientific societies[3, 18] which suggest maintaining treatments, fearing reactivation more than immunosuppression, especially in a historical period in which exacerbations had to be absolutely

avoided in order to limit access to hospital facilities[2].

It is, however, to be underlined that, regarding overall adherence to treatment among patients with ALD, studies indicate that these patients usually display an overall adherence of 80% or more to prescribed medications[19,20]. Nevertheless, these studies also show how altered/depressed mood might interfere with adherence to medical treatments. Sockalingam *et al*[20] noted that in patients with ALD, overall treatment adherence was more than 80%, and that subjects with greater adherence were the less anxious and depressed. Accordingly, a recent review indicates that the co-presence of anxiety or depression, regardless of the stage of liver disease, significantly reduces QOL and may be associated with non-adherence to prescribed therapy regimens among patents with ALD[21]. Thus, early recognition of "more fragile" patients might increase adherence to treatments, and in the context of a policy aimed at maintaining and boosting physician-patient relationship, telemedicine contact in times of social distancing might help reaching out and support uncertain patients in making the correct decisions[22]. Their specialists should reassure patients during these difficult social health emergencies to maintain a solid and realistic vision of their disease in order to avoid developing altered perceptions of its potential consequences, and dangerous drifts such as those regarding chronic medical treatments. It can be thus speculated that providing continuous contact with the patients, even with a limited telephone approach, would help them maintain a better QOL and correctly follow medical prescriptions.

The difficulties in contacting the liver specialist, and to attend the biannual follow-up abdominal ultrasound, were a clear example of how the lockdown strategies impacted on the routine clinical follow-up programme of these patients. For instance, a possible impact on the future percentages of delayed incident HCC diagnoses might be predicted, considering the observed 40% of missed follow-up ultrasound testings.

Even more relevant, our study underlines how most of the participants have had negative mental health-related lifestyle changes. This suggests that the sudden and unforeseen availability of void time resulted in patients having more time to think about their diseases and related consequences, a negative subject with psychological consequences further magnified by social isolation and causing an inevitable increase in terms of generalized concern. This hypothesis is supported by the data resulting from the evaluation of patients' emotional status, in which the specific items show how the state of anxiety worsened, along with the perception of a more frequent sensation of irritability and depression. Significant was also the patients' heightened concern about the impact that their liver disease could have on their familial relationships and the increased time spent in thinking about a worsening of their health condition and the possibility of not getting better.

It is also interesting to note that these significant changes were observed in a population that was proven to be aware of the emergency and correctly informed on how to cope with it. This suggests that informative campaigns should not only aim at providing up-to-date information, but should also be integrated by conveying positive messages when possible. Younossi *et al*[4] observed that decompensated cirrhosis was associated with a variety of symptoms that impact profoundly and negatively on their HRQL. Our cohort of patients with CLD, in addition to non-cirrhotics, included only patients with compensated liver cirrhosis (CHILD A-B7), a condition in which disease has a limited impact on QOL. Therefore, our data suggests that the differences in QOL observed through this study are more likely to be a consequence of the radical social changes caused by the COVID-19 pandemic, rather than a worsening of the liver disease status.

We acknowledge that the small number of patients enrolled and the potential suggestion effect of our questionnaire in the format that we have adopted might represent a limitation of our study. We were aware of potential suggestion effect of this part of the questionnaire as it was formulated; however, considering the peculiar and at times awkward modality of contact (telephone call) for which it was developed, which demands for a short, simple, and clear questions and answers format, grouped by category, we decided to opt for this potentially criticisable but straightforward and explicit format. Nevertheless, the novelty constituted by a global viral pandemic find researchers with limited dedicated instruments to study the event, thus we believe that these potential limitations are to be considered as an expected element of the studies addressing QOL.

CONCLUSION

In conclusion, the rapid outbreak of the pandemic worldwide has had a significant, worsening impact on the QOL of patients with CLD, especially on those psychological areas involving states of worry/concern, sadness, and anxiety. The slight, at times marginally statistically significant, differences in the QOL questionnaire observed in the ALD and non-ALD groups should be interpreted considering the small sample size of the former group, globally suggesting an overall similar effect of lockdown on the general well-being of the two subgroups.

Even if our experience is limited to patients with compensated liver cirrhosis and ALD managed on our outpatient's service, we believe that the utmost observation emerging from our study is the importance of maintaining a contact with patients, guaranteeing them a continuity of care. The spread of telemedicine with video call, telephone, e-mail, fax made it possible to maintain a contact with them without the risk of infectious exposure inherent to the face-to face approach. This novel approach to patient-physician contact, as recommended by the Italian Society of Hepatology, has to be institutionalized by the hospital; however, *ad hoc* reimbursement codes are to be developed as well as dedicated booking agendas. This method can replace a face-to-face visit in many hepatological conditions such as compensated cirrhosis or in patients with ALD who have reached durable medical control of their disease, since in a scenario of substantial clinical stability, it is not necessary to carry out a physical examination of the patient but only to collect, even indirectly, clinical-laboratory parameters easily communicated by the patient himself. For patients with a more severe disease, or during the course of HCC follow-up, especially if a liver lesion is detected, we opted for a face-to face approach considering it to be remain the preferred follow-up modality; however, reorganization of resources favoring remote clinical evaluations over face-to-face visits in stable patients, helped in reallocating clinical resources and allowed for the identification of dedicated time slots and spaces to perform ultrasound and HCC follow-up visits. It would also be desirable to integrate general practitioners in this novel, telemedicine-based, patient-physician network.

Even if the results of this study might not be exhaustive, due to the small number of patients tested and the limited set of issues evaluated, they provide relevant insights and raise several issues that deserve to be expanded in future studies. Reaching out to patients and investing time in listening to their health concerns will contribute to limiting and containing the state of anxiety related to their liver diseases. This will also probably contribute to the optimization of healthcare resources, reducing unnecessary accesses to emergency departments, and the related risk of infection exposure.

ARTICLE HIGHLIGHTS

Research background

In December 2019, the coronavirus disease-2019 (COVID-19) emerged and rapidly spread worldwide, becoming a global health threat and having a tremendous impact on the quality of life (QOL) of individuals. In Italy, measures were enforced aimed at the reduction of inappropriate access to healthcare centres, which were considered as environments at a potential risk of crowding and viral infection spread.

Research motivation

All patients affected by chronic liver disease (CLD) experienced a substantial change in their usual routine care. As emerged from the survey by the Italian Association for the Study of the Liver, the reorganization of the health-care system, which followed the pandemic phase, has severely affected the management of liver diseases in Italy.

Research objectives

We wanted to evaluate the awareness of patients with CLD regarding the COVID-19 emergency and how it impacted on their QOL, and on their interaction with the healthcare system and planned follow-up programmes. Furthermore, we assessed whether for patients with autoimmune liver diseases the lack of contact with the specialist weighed on the management of their therapy.

Research methods

Participants were asked to complete a two-part questionnaire, administered by telephone according to governmental restrictions: The first section assessed patients'

basic knowledge regarding COVID-19; the second evaluated the impact of the COVID-19 emergency on their QOL. We used the Italian version of the CLD questionnaire (CLDQ-I). With the aim of evaluating possible changes in the QOL items addressed, the questionnaire was administered to patients at the time of telephone contact with the specific request to recall their QOL perceptions during two different time periods.

Research results

The majority (93.8%) of patients were aware of COVID-19 transmission modalities and on how to recognize the most common alarm symptoms (93.8%). Five (15.6%) of 32 patients with autoimmune liver disease reported having had the need to receive more information about the way to manage their liver disease therapy during lockdown and nine (28.2%) thought about modifying their therapy without consulting their liver disease specialist. About the impact on QOL, all CLDQ-I total scores were significantly worsened during time t1 as compared to time t0.

Research conclusions

The rapid outbreak of the pandemic worldwide has had a significant, worsening impact on the QOL of patients with CLD, especially on those psychological areas involving states of worry/concern, sadness, and anxiety. We believe that the utmost observation emerging from of our study is the importance of maintaining a contact with patients, guaranteeing them a continuity of care. The spread of telemedicine with video call, telephone, e-mail, and fax made it possible to maintain a contact with them without the risk of infectious exposure inherent to the face-to-face approach.

Research perspectives

The telemedicine has to be institutionalized by the hospital; however, *ad hoc* reimbursement codes are to be developed as well as dedicated booking agendas. This method can replace a face-to-face visit in many hepatological conditions such as compensated cirrhosis or in patients with ALD who have reached durable medical control of their disease, since in a scenario of substantial clinical stability, it is not necessary to carry out a physical examination of the patient but only to collect, even indirectly, clinical-laboratory parameters easily communicated by the patient himself.

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CASE REPORT

Acute liver failure secondary to acute antibody mediated rejection after compatible liver transplant: A case report

Todd J Robinson, James B Hendele, Idoia Gimferrer, Nicolae Leca, Scott W Biggins, Jorge D Reyes, Lena Sibulesky

ORCID number: Todd J Robinson 0000-0001-5230-8435; James B Hendele 0000-0003-1477-9003; Idoia Gimferrer 0000-0002-5535-352X; Nicolae Leca 0000-0003-3279-2930; Scott W Biggins 0000-0002-3081-4668; Jorge D Reyes 0000-0003-3065-8674; Lena Sibulesky 0000-0001-5435-737X.

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Todd J Robinson, Department of Surgery, Virginia Mason, Seattle, WA 98101, United States

James B Hendele, Jorge D Reyes, Lena Sibulesky, Department of Surgery, University of Washington Medical Center, Seattle, WA 98195, United States

Idoia Gimferrer, Bloodworks Northwest, Seattle, WA 98104, United States

Nicolae Leca, Department of Nephrology, University of Washington Medical Center, Seattle, WA 98195, United States

Scott W Biggins, Department of Gastroenterology and Hepatology, University of Washington Medical Center, Seattle, WA 98195, United States

Corresponding author: Lena Sibulesky, MD, Associate Professor, Surgeon, Department of Surgery, University of Washington Medical Center, UWMC 1959 NE Pacific St, Box 356410 Seattle, Seattle, WA 98195, United States. lenasi@uw.edu

Abstract

BACKGROUND

The liver has traditionally been regarded as resistant to antibody-mediated rejection (AMR). AMR in liver transplants is a field in its infancy compared to kidney and lung transplants. In our case we present a patient with alpha-1-antitrypsin disease who underwent ABO compatible liver transplant complicated by acute liver failure (ALF) with evidence of antibody mediated rejection on allograft biopsy and elevated serum donor-specific antibodies (DSA). This case highlights the need for further investigations and heightened awareness for timely diagnosis.

CASE SUMMARY

A 56 year-old woman with alpha-1-antitrypsin disease underwent ABO compatible liver transplant from a deceased donor. The recipient MELD at the time of transplant was 28. The flow cytometric crossmatches were noted to be positive for T and B lymphocytes. The patient had an uneventful recovery postoperatively. Starting on postoperative day 5 the patient developed fevers, elevated liver function tests, distributive shock, renal failure, and hepatic encephalopathy. She went into ALF with evidence of antibody mediated rejection with portal inflammation, bile duct injury, endothelitis, and extensive centrizonal necrosis, and C4d staining on allograft biopsy and elevated DSA. Despite various

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interventions including plasmapheresis and immunomodulating therapy, she continued to deteriorate. She was relisted and successfully underwent liver retransplantation.

CONCLUSION
This very rare case highlights AMR as the cause of ALF following liver transplant requiring retransplantation.

Key Words: Liver transplant; Acute antibody mediated rejection; Acute liver failure; Donor specific antibody; Liver rejection; Case report

Core Tip: The liver has traditionally been regarded as resistant to antibody-mediated rejection (AMR). AMR in liver transplants is a field in its infancy compared to kidney and lung transplants. We present a case of a 56 year-old woman with alpha-1-antitrypsin disease who underwent ABO compatible liver transplant. The flow cytometric crossmatches were noted to be positive for T and B lymphocytes. After initial posttransplant recovery she progressively developed acute liver failure with evidence of antibody mediated rejection with portal inflammation, bile duct injury, endothelitis, and extensive centrizonal necrosis, and C4d staining on allograft biopsy and elevated donor-specific antibodies. Despite various interventions including plasmapheresis and immunomodulating therapy, she required retranpslantation.

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INTRODUCTION

Acute antibody mediated rejection after liver transplantation is a rare phenomenon. antibody-mediated rejection (AMR) is a well-known phenomenon in ABO incompatible liver transplantation, and there is a growing body of literature demonstrating the presence of rejection in ABO compatible, crossmatch positive liver transplantation. Medical treatments including plasmapheresis and immune modulating medications have been successful in halting rejection[1]. Here we present a case of acute AMR after ABO compatible, crossmatch positive liver transplantation resulting in acute liver failure (ALF) and rapid clinical deterioration requiring re-transplantation.

CASE PRESENTATION

Chief complaints

A 56-year-old woman with a history of decompensated cirrhosis secondary to alpha-1-antitrypsin deficiency (ZZ phenotype) presented for liver transplantation.

History of present illness

The patient developed refractory ascites requiring repeated large-volume paracentesis and spontaneous bacterial peritonitis.

History of past illness

The patient's past medical history was remarkable for systemic lupus erythematosus mostly manifesting with arthralgia, hair loss, multiple miscarriages, and one successful pregnancy.

Physical examination

The patient's temperature was 36.5 °C, heart rate was 60 bpm, respiratory rate was 14 breath/min, blood pressure was 100/60 mmHg and oxygen saturation on room air was 100%. Her abdomen was distended with ascites. She was not encephalopathic.

Laboratory examinations

Laboratory values were sodium 125 meq/L, creatinine 0.95 mg/dL, aspartate aminotransferase (AST) 96 U/L, alanine aminotransferase (ALT) 59 U/L, alkaline phosphatase 263 IU/L, total bilirubin 6.1 mg /dL, albumin 2.6 g/dL, international normalized ratio (INR) 1.8, platelets 60 10^3/mL.

Imaging examination

A computed tomography (CT) of the abdomen and pelvis demonstrated cirrhosis and multiple varices in the abdomen. There was no evidence of malignant liver lesions. Vasculature was patent. There was moderate ascites.

FINAL DIAGNOSIS

The patient was diagnosed with decompensated cirrhosis with the MELD NA score of 28.

TREATMENT

The patient underwent orthotopic liver transplantation from a 55-year-old deceased female donor (cause of brain death was an intracranial hemorrhage). Both recipient and donor were blood type A. Serologic studies revealed the recipient was cytomegalovirus (CMV), Epstein-Barr virus (EBV), and hepatitis C and B negative. Similar testing on the donor revealed CMV seronegativity and EBV seropositivity. The flow cytometric crossmatches were noted to be positive for T and B lymphocytes with the median channel shift (MCS) of 11 and 96, respectively. At transplant, donor-specific antibodie (DSA) against human leukocyte antigen (HLA) Class 1 were B51 at 700 mean fluorescent intensity (MFI), and HLA Class 2 DR04 at 2700 MFI, DR53 at 21,200 MFI, DQ07 at 13, 100 MFI, DQ08 at 12, 900 MFI (Figure 1A and Table 1).

The transplant operation was 5 h. Blood loss was 1500 cc. Intraoperative transfusions were: 6 units of packed red cells, 6 units of FFP, 2 units of cryoprecipitate, and one unit of platelets. The patient had an uneventful recovery postoperatively in the intensive care unit (ICU). She was extubated on POD 0 and was transferred from ICU to an acute surgery care unit on POD 1. *Per* our protocol her immunosuppression regiment included an induction course of antithymocyte globulin (ATG, 1.5 mg/kg × 3 d) with methylprednisolone taper (1 gm intraoperatively, followed by 500 mg, 250 mg, and 125 mg). This was followed by a maintenance immunosuppression with tacrolimus twice daily monotherapy starting on POD 4 with the goal trough level of 8-10 ng/mL. She achieved a tacrolimus trough level of 11.5 ng/mL on POD 7. Antimicrobial prophylaxis included trimethoprim-sulfamethoxazole, valgancyclovir, and fluconazole. An immediate postoperative Doppler liver ultrasound (US) and a routine POD 4 US demonstrated patent vasculature with adequate flow with normal velocities. Lactate normalized to 1 on POD 1. On POD 5 AST was 119 U/L, ALT 305 U/L, alkaline phosphatase 79 U/L, and total bilirubin 1.3 mg /dL, INR 1.4 (Figure 2). On POD 5 the patient developed a fever to 40.5 °C and was started on empiric antibiotic therapy with intravenous vancomycin and piperacillin-tazobactam.

A CT of the abdomen and pelvis was performed on POD 7 which did not demonstrate any evidence of intraabdominal abscess or other pathology. An US of the allograft was performed on POD 8 which was again unremarkable.

Between POD 8 and 10 the patient began experiencing intermittent episodes of hypotension. Echocardiography demonstrated a left ventricular ejection fraction of 66% and pulmonary arterial hypertension with pressure of 45 mmHg. The patient developed acute kidney injury with a creatinine of 1.8 mg/dL in a setting of a supratherapeutic tacrolimus levels close to 12 ng/mL. Mycophenolate mofetil was added to her immunosuppression maintenance regimen for renal sparing with the goal to decrease the target tacrolimus trough level of 5 ng/mL.

She worsened acutely clinically with persistent hypotension, volume overload, and grade 2 encephalopathy on POD 11 and was transferred to the ICU. Allograft US

Table 1 Donor specific antibody levels after 1st transplant

	A11	A34	B51	B64	C8	C15	DR4	DR11	DR52	DR53	DQ7	DQ8	DP4
Pre-transplant	0	0	700	0	0	0	2700	0	0	21200	13100	12900	0
POD 12	3300	12100	5300	15500	11200	8800	23100	0	0	19600	14300	14400	8200
POD 14	1600	6100	3200	10600	6200	5100	20200	0	0	20200	12900	14200	6500
C1q	A11	A34	B51	B64	C8	C15	DR4	DR11	DR52	DR53	DQ7	DQ8	DP4
Pre-transplant	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	40300	Neg	Neg	Neg
POD 14	Neg	Neg	400	9700	Neg	Neg	17800	Neg	Neg	41800	42300	42540	Neg

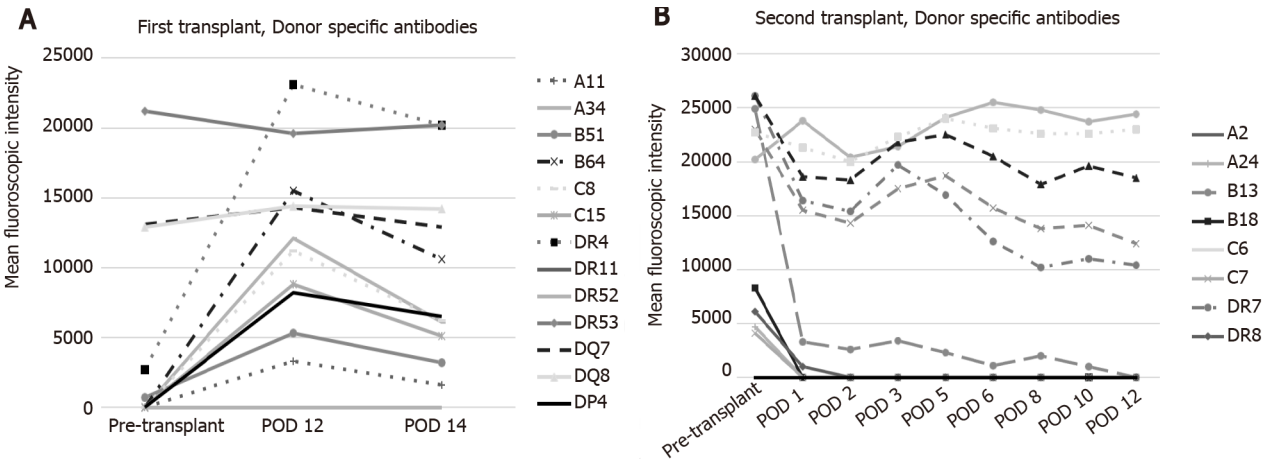

Figure 1 Donor specific antibodies after liver transplant. A: After 1st liver transplant; B: After 2nd liver transplant.

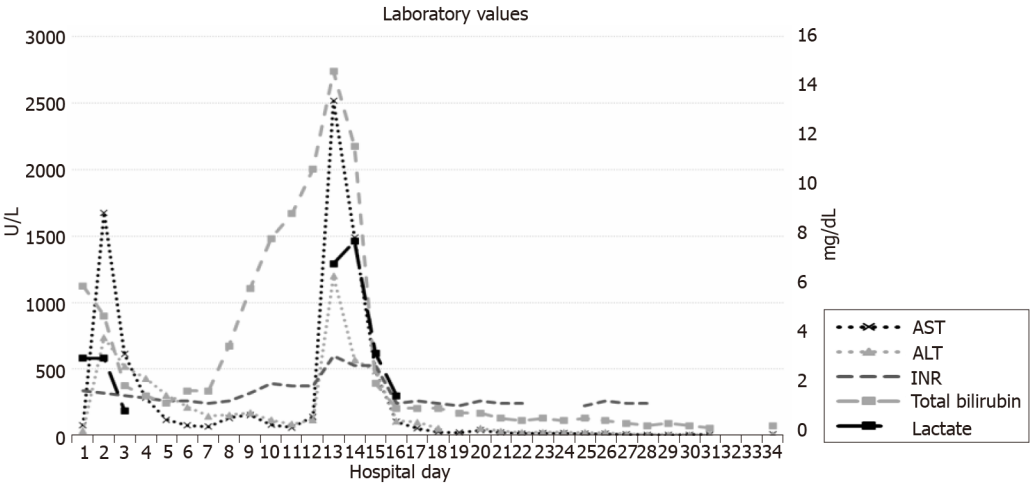

Figure 2 Trend of pertinent laboratory values during clinical course. Please note, the patient received intermittent administration of fresh frozen plasma. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; INR: International normalized ratio.

demonstrated new low bidirectional flow in left, right, and main portal veins, making it difficult to exclude portal vein thrombosis, but CT scan with contrast confirmed patent portal and hepatic artery inflow. DSAs were rechecked and were noted to be even more elevated (Figure 1), and the patient underwent plasmapheresis on POD 12. A biopsy of the liver was also performed which demonstrated portal inflammation, bile duct injury, endothelitis, and extensive centrizonal necrosis (> 40%) with positive stain for C4d, consistent with acute AMR. This would score as C4d: "3." and the h-score of "2." based on the Banff Working Group scoring criteria. On POD 13 she became oliguric and hemodialysis was initiated.

She underwent plasmapheresis followed by a dose of eculizumab as well as ATG. Given her rapid decompensation, multiorgan failure, and evidence of ALF (INR > 1.5, altered mental status, < 26 wk from onset)[2] she was listed for repeat liver transplant. Sample from POD 14 was retrospectively tested for C1q binding and was strongly positive for class I and class II DSA.

A liver became available and she underwent a second orthotopic liver transplantation on POD 14. The donor was a 27-year-old male of standard risk, donation after brain death (trauma), blood type O, CMV+, EBV+, hepatitis C negative and hepatitis B surface antigen negative. The donor liver had conventional anatomy and the transplant was uncomplicated. The flow cytometric crossmatch was positive also with MCS of 89 and 106 for T and B cells, respectively. DSA were HLA-A24, B13, B18, Cw07, DR07, DR08, DR53, DQ02, DQ04, and DP03, being also C1q positive B13, B18, DR7, DR53, DQ2, DQ4 and DP3 (Figure 1B and Table 2). The explanted liver allograft again demonstrated hepatic parenchyma with extensive centrizonal necrosis (approximately 40%), portal inflammation, and positive C4d staining.

She underwent induction with ATG and methylprednisolone. Her maintenance immunosuppression included tacrolimus and mycophenolate mofetil. She was treated with plasmapheresis and intravenous immune globulin G (IVIG) in the immediate post-operative period as well as rituximab (POD 3 and 19 after second transplant). Liver biopsy on POD 8 demonstrated mild endothelitis, prompting additional treatment with IVIG and plasmapheresis as well as bortezomib (POD 7 and 9 after her second transplant).

Her post-transplant course was complicated by vancomycin resistant enterococcal infection of the wound and ascites that was adequately treated with daptomycin. Immune globulin G (IgG) against donor class I HLA were quickly reduced after the second liver transplant (POD 1) but class II HLA antibodies remained high in post-transplant monitoring.

OUTCOME AND FOLLOW-UP

The patient recovered well from the second transplant and was discharged home on hospital day 30. Her maintenance immunosuppression consisted of tacrolimus, rapamycin, and prednisone. She is currently over 2 years after transplant with normal liver transplant and native kidney function, still with DSA class II being positive at moderate levels with DR53 at 5100.

DISCUSSION

The liver has traditionally been regarded as resistant to AMR. Various reasons for this have been postulated, including dual blood supply, large vascular bed resulting in a diluted effect of circulating DSA, and secretion by kuppfer cells of HLA that neutralize DSA[3]. Rejection after liver transplantation could be cellular, humoral or mixed[1]. Rejection can be sub-classified according to the timing of onset. There are three types of AMR: Hyperacute, acute, or chronic[4]. In particular, acute AMR in the setting of ABO-compatible liver transplantation is an exceedingly rare phenomenon and the true incidence is unknown. A large French series of 1788 liver transplant patients reported an acute AMR rate of 0.56%[5]. Another smaller study reported up to a 3.6% rate of AMR[6]. Although there is an increasing body of literature on the topic, most of this is in the form of case reports. While there are assays performed prior to transplantation that can provide information about preformed antibodies, cytotoxic potential of these antibodies, as well as the presence of reactive T- and B-lymphocytes, the clinical significance of alloantibody is not fully understood[7]. At present testing pre-transplant for preformed DSA is not a standard practice for all liver transplant centers. Both HLA and non-HLA antibodies can cause rejection, but it is important to note that not all HLA antibodies are pathogenic[8]. Class I HLA with or without class II HLA are associated with acute AMR while class II HLA, specifically HLA-DQ, are associated with worse outcomes in chronic rejection[9]. DSA and resulting complement fixation have been found to be markers for AMR, both acute and chronic [10-12]. Risk factors for DSA development as well as the detrimental effects on the allograft have been identified, including higher MELD score, re-transplantation, use of cyclosporine, lower immunosuppression, variability in the level of tacrolimus, and non-adherence to immunosuppression therapies, female donor, and recipient/donor gender mismatch[9]. In 2016, the Banff Working Group published diagnostic criteria

Table 2 Donor specific antibody levels after 2nd liver transplant

	A2	A24	B13	B18	C6	C7	DR7	DR8	DR53	DQ2	DQ4	DP3	DP4
Pre-transplant	0	4700	24900	8300	0	4100	26100	6100	20200	23000	26100	22700	0
POD 1	0	0	3300	0	0	0	16400	1000	23800	15500	18600	21300	0
POD 2	0	0	2600	0	0	0	15400	0	20400	14300	18300	20000	0
POD 3	0	0	3400	0	0	0	19700	0	21400	17500	21800	22300	0
POD 5	0	0	2300	0	0	0	16900	0	24100	18700	22500	24000	0
POD 6	0	0	1100	0	0	0	12600	0	25500	15700	20500	23100	0
POD 8	0	0	2000	0	0	0	10200	0	24800	13800	17900	22600	0
POD 10	0	0	1000	0	0	0	11000	0	23700	14100	19600	22600	0
POD 12	0	0	0	0	0	0	10400	0	24400	12400	18500	23000	0
C1q	A2	A24	B13	B18	C6	C7	DR7	DR8	DR53	DQ2	DQ4	DP3	DP4
Pre-tx	Neg	Neg	5200	3500	Neg	Neg	36300	Neg	40800	35400	36500	39200	Neg

for acute AMR which include histopathologic pattern of injury consistent with acute AMR, positive serum DSA, diffuse microvascular deposition of C4d, and reasonable attempts made to exclude other causes of allograft failure[13]. Recently Halle-Smith *et al*[14] described two cases of AMR presenting with graft dysfunction and being associate with lactic acidosis, hypoglycemia, and eosinophilia in both blood and liver biopsies[14]. Baliellas *et al*[15] described AMR manifesting as a sinusoidal obstruction syndrome with the patient presenting with pleural effusion, ascites found to have venulitis and diffuse C4d staining in the central veins on liver biopsy[15]. Vascular thromboses both venous and arterial in a setting of elevated DSAs presumably related to AMR have also been described in the literature[16,17]. All these criteria will hopefully lead to increased diagnostic sensitivity which will in turn lead to greater understanding of the true incidence and risk factors, improved treatment options, and potentially changes in practice in crossmatch positive liver transplantation.

Treatment of acute AMR has been described mostly in case reports and is of variable efficacy. Regimens have been based on advances made in ABO incompatible liver transplantation and include plasmapheresis, intravenous immune globulin, rituximab, and basiliximab[18]. Case reports have demonstrated successful rescue from AMR with the use of regimens of mycophenolate mofetil and plasmapheresis, IVIG, corticosteroids, ATG, rituximab, bortezomib, or a combination of the above[19-22].

Our sensitized female patient with pre transplant DSAs developed ALF a week after successful deceased donor liver transplant in a setting of robust immunosuppression exhibiting graft dysfunction, hepatic encephalopathy, rising lactate, and renal failure requiring dialysis with definitive evidence of AMR. She was treated with salvage eculizumab, plasmapheresis, and IVIG. Her graft biopsy was impressive for over 40% hepatocyte necrosis with C4d staining without evidence of eosinophilia. As a result of minimal improvement despite aggressive therapy, the decision was made to pursue re-transplantation. Her explant allograft biopsy confirmed similar findings. After her second liver transplant, she underwent induction with ATG and methylprednisolone and was kept on tacrolimus and mycophenolate mofetil. After induction was complete, DSA levels were followed (Figure 1B and Table 1) and plasmapheresis and IVIG therapy was instituted along with rituximab and bortezomib. Even though she has normal liver function tests, because of the presence of HLA class II Ab, future liver biopsies are contemplated.

CONCLUSION

In conclusion, we believe our case is one of the rare cases in the literature that describes AMR resulting in ALF treated with re-transplantation. We hypothesize that even though the DSA levels were higher after the second liver transplant, the class I Ab became negative immediately after the second transplant, while class I Ab associated with C1q positivity dramatically increased after the first liver transplant leading to irreversible liver injury requiring re-transplantation. We advocate for closer

monitoring of DSA post liver transplantation to further elucidate their effect on liver transplant outcomes.

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LETTER TO THE EDITOR

Vitamin D supplementation for autoimmune hepatitis: A need for further investigation

Consolato M Sergi

ORCID number: Consolato M Sergi 0000-0002-2779-7879.

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Consolato M Sergi, Anatomic Pathology Division, Children's Hospital of Eastern Ontario (CHEO), University of Ottawa, Ottawa K1H 8L1, ON, Canada

Consolato M Sergi, Department of Laboratory Medicine and Pathology, University of Alberta, Edmonton T6G 2B7, AB, Canada

Corresponding author: Consolato M Sergi, MD, PhD, Chief, Anatomic Pathology Division, Pediatric Pathologist, Children's Hospital of Eastern Ontario (CHEO), 401 Smyth Road, Ottawa K1H 8L1, ON, Canada. csergi@cheo.on.ca

Abstract

Autoimmune hepatitis is a chronic liver disease harboring an autoimmune basis and progressive character. Despite still obscurity in etiology and pathogenesis, some evidence supports the importance of sustaining the immune system. Vitamin D is a lipo-soluble vitamin, which has been identified as decreased in our body. It is often due to the daily habit change and decrease of individual sun exposure due to the increase of the ultraviolet-induced potential melanocytic transformation. Here, we emphasize the importance of vitamin D supplementation in patients affected with liver disease.

Key Words: Vitamin D; Autoimmune hepatitis; Supplementation; Immunostimulants; Liver

Core Tip: Autoimmune hepatitis (AIH) is a chronic liver disease with an autoimmune basis. It can progress quite dramatically. The immune system may play a role in determining how this disease progresses. Vitamin D is key in supporting the immune system with or without vitamin deficiency. Here the vitamin D supplementation in patients affected with AIH is emphasized.

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TO THE EDITOR

We read with interest the article of Fan *et al*[1] on the pathogenesis of autoimmune hepatitis (AIH) in one of the most recent issues of the journal *World Journal of Hepatology*[1]. The authors summarize the evidence on this complex disease with evidence of the last five years. Although several autoimmune conditions may involve the liver, the three most common autoimmune hepatopathies are AIH, primary sclerosing cholangitis, and primary biliary cholangitis. These diseases may occur *seriatim* or as part of "overlap" syndromes. AIH is a chronic progressive liver disease whose, unfortunately, etiopathogenesis is still obscure. AIH has conventional features of autoimmune disorders, including antibodies generated by an organism in response to an element of its own tissues (autoantibodies), association with a complex of genes on chromosome 6 in humans (human leukocyte antigen genes or haplotypes) which encode proteins located on the cell surface and responsible for the regulation of the immune system, and a T-cell mediated necroinflammatory process. Histologically, a prominent inflammatory infiltrate consisting of both lymphocytes and plasma cells is seen. These inflammatory cells form clusters with accentuation at the edges of the portal tracts with striking periportal interface inflammatory activity at places (Figure 1). Genetic susceptibility, immunologic dysregulations, and environmental factors may play a role. In particular, viral infection and drugs may trigger a set of dysfunctions of the immune system. Currently, almost all patients require steroid hormones plus immunosuppressive maintenance therapy independently of age or genetic predisposition. However, long-term medication adverse effects, complications, and relapse after drug withdrawal are pretty variable. It has been suggested that we may need to do more in improving the satisfaction of our patients[2].

Vitamin D can be successful in numerous diseases[3-5], and it is crucial to emphasize the role of vitamin D in AIH. After the turn of the last century, the interest in vitamin D increased enormously. Rickets has accompanied human civilization for centuries in both old and new continents. Still, despite supplementation of this vitamin in our diet, hypovitaminosis and child neglect cases have been diagnosed[6]. Population surveys have indicated that important sectors of childhood and pregnant women will be affected by vitamin D inadequacy in their life. The change of the habits with indoor living, inadequate sunlight exposure, vitamin-deficient diets, and high rates of nutrition allergy are likely playing a significant role in growing the inadequacy of vitamin supplementation for some people worldwide.

Vitamin D is fat-soluble. It plays a leading role in calcium homeostasis[7,8]. Vitamin D is tightly linked to immunity. It plays a remarkable role in inflammatory and cancer pathways[5,9,10]. The immune system's cellular components possess vitamin D receptors (VDRs). These receptors can metabolize the active form of vitamin D or calcitriol, which is also labelled 1,25-dihydroxy vitamin D, and abbreviated as $1,25(OH)_2D$. The storage form of vitamin D is 25-hydroxyvitamin D or 25(OH)D. It can be transformed by activated T and B lymphocytes to $1,25(OH)_2D$ *in vitro*. In addition, $1,25(OH)_2D$ acts on immunological cells in an obvious autocrine or paracrine fashion. This aspect has been considered crucial for several infections, as suggested in COVID-19 (coronavirus disease-2019), the infection caused by the severe acute respiratory syndrome–related coronavirus 2 (SARS-CoV-2), despite controversial data[11-13].

Peripheral blood mononuclear cells harbor VDR, supporting vitamin D's notable role in regulating the immune system. Other than activity on the cells of the immune system, vitamin D increases the absorption of calcium from the small intestine and participates in cilia movements[14]. Nicolaysen *et al*[15] and Haavaldsen *et al*[16,17] also observed that animals, which were kept for determined set of the experiments on a low calcium diet, exhibited much greater calcium absorption competence than experimental animals kept on a diet with an adequate amount of calcium. Calmodulin, an intermediate calcium-binding messenger protein expressed in all eukaryotic cells, is localized in the hamster's ciliated cells. Indeed, calcium is prominent for cilia's bioenergetic activity and the bile canaliculus[18-21].

Vitamin D is crucial in maintaining innate immunity[22-29]. Various studies have demonstrated that it also behaves as an essential regulator of immnunologic system and host defense. It includes exquisitely respiratory host defense. Transforming growth factor-beta 1 (TGF-β1) decreases the host defense of epithelial cells by altering the vitamin D-reconciled expression of host defense peptides and proteins[30]. When primary CD4+ T cells from healthy donors were obtained and cultured in specific and well detailed Th17-polarizing requirements, vitamin D lowered the expression of Th17 markers. Subsequently, there was also a decrease in the secretion of proinflammatory cytokines. It involved interleukin 17A (IL-17A) and interferon-gamma (IFN-γ) predominantly. It induced an expansion of the CD4+ T cell subset expressing the

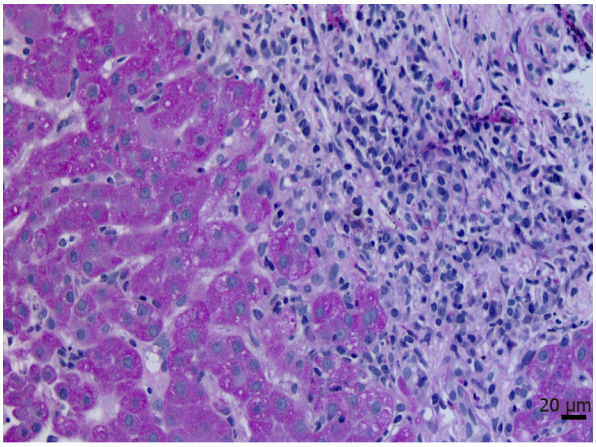

Figure 1 Autoimmune hepatitis. The portal tract shows a prominent inflammatory infiltrate consisting of numerous lymphocytes and plasma cells with interface inflammatory activity (upper right corner). The hepatocytes are highlighted using periodic acid Shiff (PAS) stain due to the accumulation of glycogen (lower left corner) (PAS stain, × 200 original magnification, bar = 20 micrometers).

highest levels of CD25 cells. It also upregulated their expression of CTLA-4 (cytotoxic T-lymphocyte-associated protein 4) and Foxp3 (forkhead box P3) regulatory markers. It seems that vitamin D supplements regulate the microbiome by increasing the abundance of beneficial bacterial strains.

Moreover, vitamin D improves glucocorticoids[31,32], increases the production of glutathione[33], and inhibits hepatic stellate cells[34]. Vitamin D may influence the histological severity of AIH and advanced liver fibrosis, and the need for liver transplantation. A recent systematic review summarized the importance of vitamin D supplementation for AIH[31]. A substantial scientific background supports the use of vitamin D in AIH, showing that circulating concentrations of 25-hydroxyvitamin D are less than 30 ng/mL or 75 nmol/L (1 nmol/L = 0.4 ng/mL 25-hydroxyvitamin D, and 1 ng/mL = 2.5 nmol/L 25-hydroxyvitamin D) in more than four fifths (81%) of Turkish patients with AIH, matched to about two fifths (44%) of healthy individuals[35]. These patients have clearly demonstrated a higher frequency rate of non-response to steroids (glucocorticoid therapy) than patients exhibiting no identifiable vitamin D deficiency (P = 0.04). In AIH, severe interface inflammatory activity of the liver or interface hepatitis, higher stages of hepatic fibrosis, and therapy failure have been linked with 25-hydroxyvitamin D3 levels in serum of less than 10 ng/mL. Several international hepatologists have suggested that vitamin D deficiency should be proposed as a prognostic biomarker in AIH[35].

Most people are satisfactorily protected against vitamin D deficiency, but others may show a level of susceptibility. In addition, some individuals may develop such deficiency, which may target several critical functions of the body. Therefore, the National Institutes for Health recommends supplementation of vitamin D for some categories. They include breastfed infants, individuals living in cold climatic conditions, or subjects working night shifts, adults harboring a body mass index of 30 or over, and individuals who underwent gastric bypass surgery, suffer from inflammatory bowel disease, or cover continuously their skin for religious reasons. Infants should receive 400 international units (IU) of vitamin D *per* day until weaning. It equals 10 micrograms (mcg or μg) considering that the mass that gives 1 IU is dependent on the rate or potency of the compound and varies obviously from compound to compound depending on what is being measured. In the case of vitamin D, 1 IU is the biological equivalent of 0.025 mcg ergocalciferol or cholecalciferol. After infancy and up to the 70th year of age, the dosage of vitamin D supplementation should be 600 IU (15 mcg). Individuals older than 70 years should receive 800 IU (20 mcg) of vitamin D. Skin exposure for 5–30 min at least twice *per* week may be sufficient for most individuals. However, underlying conditions may still require an increase in vitamin D supplementation.

In conclusion, vitamin D is not a new miraculous drug but a compound discovered more than half a century ago. It has immunoregulatory, anti-inflammatory, anti-oxidative, and anti-fibrotic effects, affecting remarkably the occurrence and outcome of immune-mediated diseases. Vitamin D may influence the histological severity of AIH, liver fibrosis, and the need for liver transplantation. Thus, the supplementation of this vitamin in these patients is potentially critical in reducing the interface hepatitis. There are still several unexplored fields for vitamin D, and the study of how genetic variants

of *VDR* genes can affect the susceptibility of individuals to chronic autoimmune liver diseases should be at the forefront of the developments in hepatology. Still, independently from the *VDR* haplotype, it seems that patients affected with AIH will benefit from vitamin D supplementation, and an advocacy for its use may be critical in internal medicine.

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LETTER TO THE EDITOR

Current highlights on solid pseudopapillary neoplasm of the pancreas

Simone Sibio, Sara Di Carlo

ORCID number: Simone Sibio 0000-0002-5694-951X; Sara Di Carlo 0000-0001-6519-991X.

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Simone Sibio, Department of Surgery Pietro Valdoni, Sapienza University of Rome, Umberto I Hospital, Rome 00161, Italy

Sara Di Carlo, Minimally Invasive Surgery Unit, Tor Vergata Hospital, Tor Vergata University of Rome, Rome 00133, Italy

Corresponding author: Simone Sibio, MD, PhD, Associate Professor, Surgical Oncologist, Department of Surgery Pietro Valdoni, Sapienza University of Rome, Umberto I Hospital, Viale del Policlinico 155, Rome 00161, Italy. simone.sibio@uniroma1.it

Abstract

Solid pseudopapillary neoplasm of the pancreas is a low-grade malignant tumor that predominantly affects young women in their third and fourth decade. Etiology and risk factors are unknown. Clinical symptoms are aspecific and most commonly due to mass effect. Diagnosis is made by computed tomography scan or magnetic resonance imaging and histological characterization is obtained by endoscopic ultrasound-guided fine needle biopsy. Microscopically, these lesions are composed by both solid and pseudopapillary structures with necrotic and hemorrhagic areas. Occasionally, the biological behavior is aggressive with tumor recurrence and distant metastasis. Usually, curative R0 surgical resection is the best option able to provide long term survival even in advanced disease. Unresectable disease is the main predictor of poor prognosis. Chemotherapy and radiotherapy regimens are not well standardized. However, they could be effective in reducing tumor size as neoadjuvant treatment or disease control in palliative setting. Although complete surgical resection provides a cure rate of > 95%, considering young age of the patients and morbidity associated to pancreatic surgery, further studies are needed to better investigate risk factors and responsiveness to hormones in order to allow early diagnosis and follow up strategies that could avoid unnecessary surgery in less aggressive disease.

Key Words: Pseudopapillary neoplasm; Pancreatic tumor; Pancreaticoduodenectomy; Distal pancreatectomy; Pancreas; Surgery

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Core Tip: This letter aims to underline the utmost importance of early diagnosis and standardization of treatment for a subset of rare pancreatic malignant tumors that affect young women and have good prognosis when curative surgery is performed. However, little is known about clinical behavior and hormonal responsiveness of such diseases and treatment option availability is still scarce for advanced, recurrent and metastatic disease so further investigation is claimed.

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TO THE EDITOR

We read with great interest the review written by Omiyale[1] which outlines the clinical and pathological features of solid pseudopapillary neoplasm of the pancreas (SPN) including the epidemiology, molecular pathology, cytology, differential diagnosis, treatment and prognosis.

As already reported by the aforementioned author, this is a rare tumor of uncertain histogenesis, known with several names or as Frantz tumor (after the name of who first described it) which accounts for 0.3% to 2.7% of all pancreatic neoplasms[2]. The author underlines the predominant incidence rate in young women.

Available data on this tumor behavior and prognosis are scarce and reported experiences are based on small number of patients or single case reports[3-7] even in high volume referral centers for pancreatic diseases. Nevertheless, no certain risk factors nor relationships with functional endocrine syndromes have been identified. SPN shows a bimodal incidence in women with two peaks at 28 years and 64 years and a unimodal behavior in men at 64 years[8]. Furthermore, recent studies described larger masses and more aggressive disease in men and post-menopausal women, suggesting an estrogen dependent behavior of these tumors[9,10]. These findings deserve further investigation in order to find out other possible non-surgical treatment options. It is very interesting to highlight that SPN are low-grade malignant tumors with an excellent overall prognosis and a curative rate of > 95% following complete surgical resection. It is worth emphasizing that although 10% to 15% of SPN have an aggressive behavior, the disease-free survival and overall survival are much better compared to other pancreatic tumors as long as R0 resection is achieved. Hao *et al*[11] in their review and metanalysis on a sample of 59 patients with aggressive SPN (one of the most consistent experiences available in literature) described a 5-year disease-free survival rate of 26.8% and a 5- and 10-year overall survival rates of 71.1% and 65.5%, respectively, with a recurrence or metastatic rate of up to 69.5%. This leads to the conclusion that about one third of patients affected by aggressive SPN will die of this disease. These consistent rates emphasize the outstanding importance of standardization of diagnostic tools and treatment procedures in order to guarantee early diagnosis and best therapy options to this small but challenging subset of patients[11]. Since the disease is often asymptomatic and symptoms are aspecific (mainly abdominal pain and distension) due to mass compression, the identification of homogeneous parameters able to predict an aggressive behavior is one of the major concerns in all the published studies. In fact, as reported by the author, diagnosis is mainly accidental and relies upon computed tomography (CT) scan and endoscopic ultrasound with fine needle biopsy for histological characterization. Often, SPN appears as a large (mean size 7.2 cm)[4] and heterogeneous mass (composed by both solid and cystic portions with fibrous septs, necrotic and hemorrhagic areas). A differential diagnosis of other exocrine or neuroendocrine pancreatic tumors can be challenging but crucial given the differences in clinical and prognostic behavior as well as treatment options. The tail of the pancreas seems to be the most frequent site of presentation although bifocal lesions have been sometimes reported. Based on these findings, Flores *et al*[8] proposed to classify SPN as follows: Unifocal SPN, referred to single lesions, bifocal SPN when there are two lesions and multifocal SPN when they are three[8].

Some authors emphasized the role of positron emission tomography-CT scan to better predict the aggressive pattern: An elevated standard uptake value seems to be correlated to higher Ki-67 expression (> 3%)[12,13] that is sometimes reported to be a sign of aggressiveness[14,15]. However, while the role of aberrant Beta catenin expression is well known, the real prognostic meaning of Ki-67 expression is still not confirmed by the literature[16].

Histogenesis remains unclear and, although no specific immunochemistry pattern has been identified, most lesions show loss of positivity for E-cadherin and positivity for β-catenin, vimentin, alpha-1-antitrypsin, alpha-1-antitrypsin, CD10, CD117 and progesterone receptors. These characteristics may be added to the clinical, imaging and histological findings to provide diagnosis[8].

Curative resection, as conservative as possible, with both open or laparoscopic approach, is the best treatment option[17-19] providing long term overall and disease-free survival even in node positive patients[20]. Surgical planning is crucial and the classification proposed by Flores *et al*[8] could be useful in this matter[8].

Although overall and disease-free survival is good even in locally advanced and metastatic patients after curative (*i.e.* R0) resection, unresectable disease remain the most important predictor of poor survival in all experiences[11]. Given the low-grade malignancy of these tumors and the prognostic efficacy of surgery, non-surgical therapies have been scarcely investigated and no standardized protocol exists for this subset of patients. Some studies suggested the use of various drugs, in monotherapy or combinations, such as cisplatin, 5-fluorouracil, gemcitabine with uncertain results in recurrent, unresectable or metastatic disease[21,22]. Radiotherapy has been reported to reduce lesion size in little case series[21,23,24]. Despite this evidence, no standardized chemotherapy or radiotherapy regimen has been identified for unresectable, metastatic or recurrent patients. From this point of view, investigation into the possible estrogen-depending behavior of SPNs could perhaps open the way to new non-surgical treatment strategies.

Finally, the author reported a cure rate of > 95% following curative resection for these tumors even for advanced, recurrent and metastatic disease. However, since some other experiences described worsen overall and have a low disease-free survival and high recurrence rates[10], considering the young age of the patients and the relevant morbidity associated to pancreatic surgery, we strongly think that further studies are needed to better understand etiology, risk factors and hormonal relationships of this disease. This could improve early diagnosis, standardization of medical regimens thus limiting treatment invasiveness and it will help to identify patients with less aggressive disease who could benefit just from a strict follow up.

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