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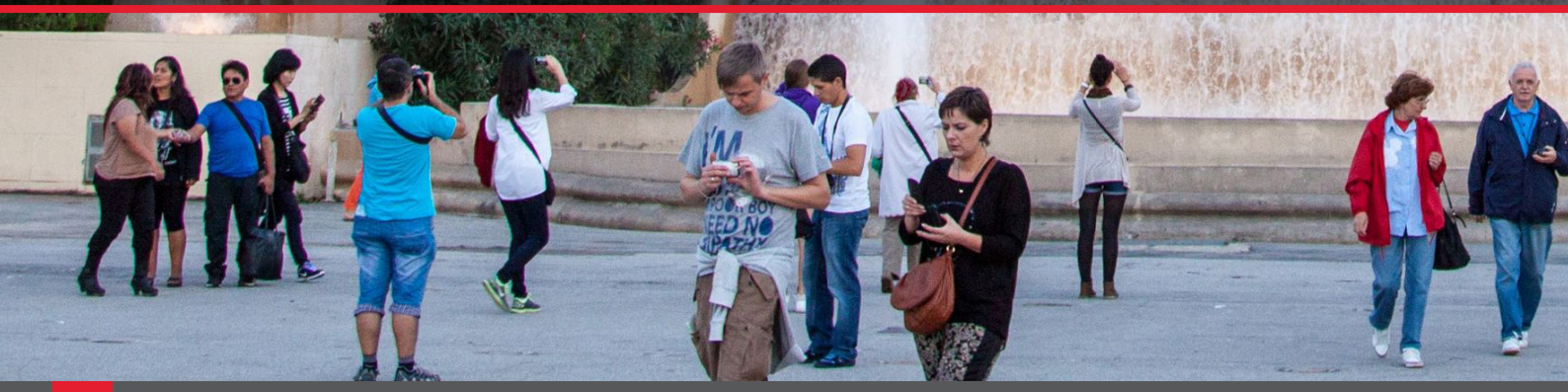
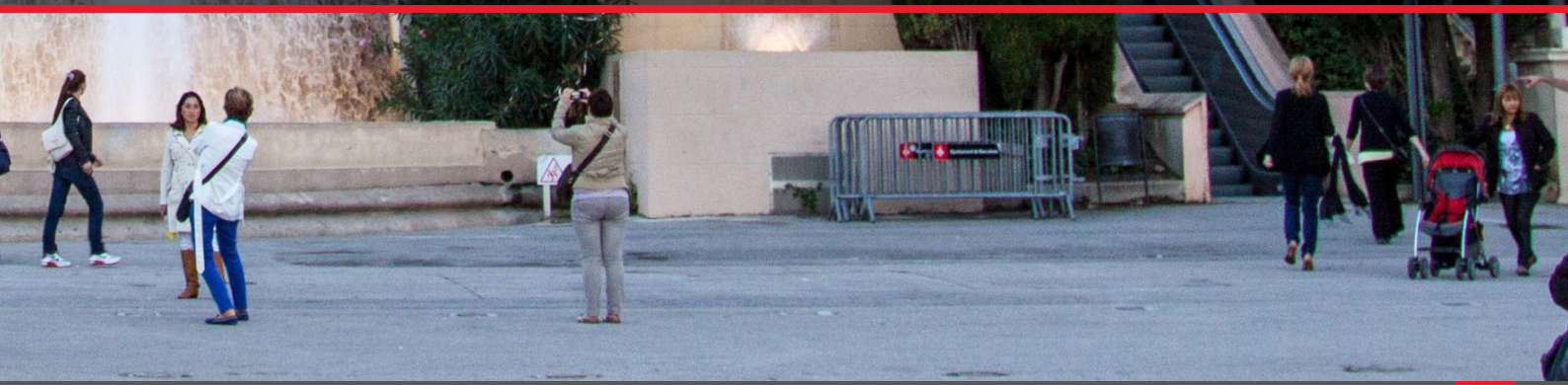


HEPATOLOGY



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Welcome

Welcome to the latest edition of *EMJ Hepatology*, which provides a collection of articles on the latest hepatology research and news from the International Liver Congress (ILC) 2016. This issue includes a comprehensive review of the entire congress from the biggest news stories to fascinating abstracts, interviews with our esteemed editorial board, and many peer-reviewed articles, all for your perusal.

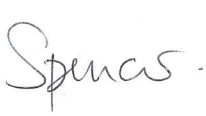
The ILC returned bigger and better than ever in 2016 in the bustling city of Barcelona, Spain. We were astounded at the quality of work presented and the progression that has been made over the past year. We bring you our pick of the biggest advances from the congress and a selection of abstracts from the most influential presentations.

The news we bring from the congress includes stories relating to the most innovative research in all areas of liver disease, along with technological developments; we also cover the awards that were presented this year. Our abstract reviews come straight from the presenters at ILC and focus on the developments and discussions that emerged from the event. These reviews look at a broad range of topics, from long-term studies to future perspectives, and are not to be missed.

As always there are a number of cutting-edge peer-reviewed articles. The review by Martinez-Gili et al. seeks to enclose the main metabolic and signalling connections between lipotoxic lipid species, such as free fatty acids and sphingolipids, and how their homeostasis is disrupted in non-alcoholic fatty liver disease. Fengler et al. look at modelling different liver diseases in murine models, evaluating gender, genetic, and dietary influences.

We have a pair of articles that consider the multiple facets of hepatocellular carcinoma. In our editor's pick, Quagliata deliberates the various classifications from clinical to laboratory tests in addition to Kennedy's assessment of the available non-surgical treatment options, accounting for changes in survival and quality of life. Kgatle and Setshedi discuss the progression of the immunopathogenesis of chronic hepatitis B from early acute inflammation to late-stage malignant transformation, finishing with an anticipatory glance at possible future directions for research and treatment.

We hope this edition provides you with some fascinating insights in to the most up-to-date research in hepatology as well as information that you can incorporate into your daily practice. We are continuing to see many major developments in hepatology and hope to see more as the year continues.



Spencer Gore

Director, European Medical Journal

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Front cover and contents photograph: Barcelona, Spain, home of ILC 2016.

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Foreword

Prof Markus Peck-Radosavljevic

Professor of Medicine and Chairman, Department of Gastroenterology and Hepatology, Endocrinology and Nephrology, Klinikum Klagenfurt am Wörthersee, Klagenfurt, Austria.

Dear Colleagues,

I would like to welcome you to this new issue of the *European Medical Journal Hepatology*, which brings you coverage of the latest research and updates in this field. It has been another record-breaking year for the European Association for the Study of the Liver (EASL), which this year featured another very interesting International Liver Congress (ILC) in Barcelona, Spain. Scientifically, the meeting has been somewhat transformed owing to the dramatic advances in the field of hepatology, and this is displayed in the congress review section of this edition.

It was good to see the renewed interest in gaining a better understanding of hepatitis B virus (HBV) biology and the translation of these new insights into potential clinically practicable treatment approaches. Renewed interest in the immunopathogenesis of HBV and, as a consequence, the application of immunotherapy to the elimination of HBV-infected hepatocytes might usher in a new area of HBV treatments, where we can really dream of complete elimination of the virus from chronically infected patients.

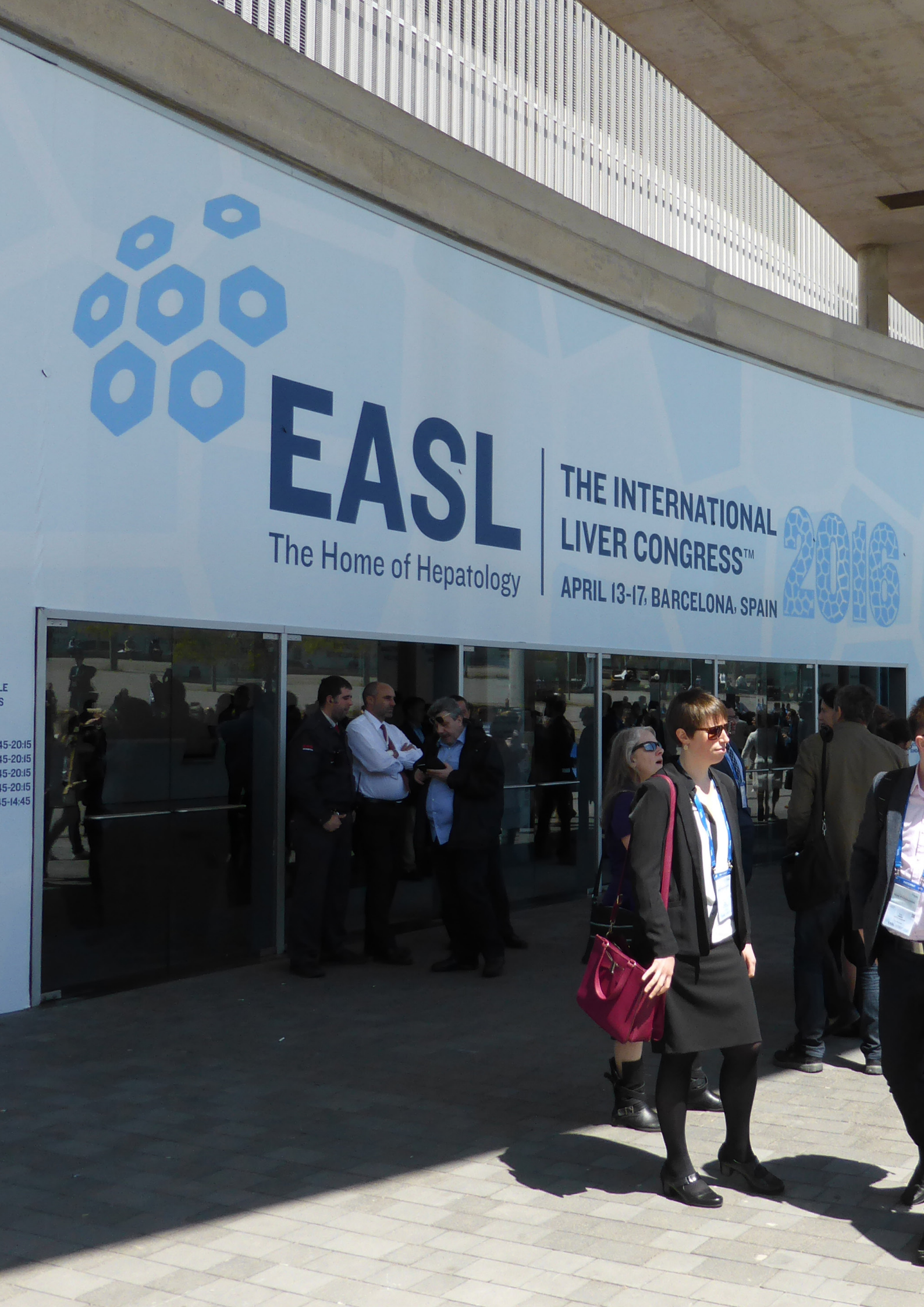
It is particularly satisfying to see that many of the new developments are a result of immense efforts in basic liver disease research undertaken by many members of the EASL scientific community worldwide. Despite the rapid transformation in the field of hepatitis C, liver research is alive and kicking more than ever; it is also expanding globally, as witnessed by the number of international delegates at ILC and the ever growing scientific contribution from countries who are relatively new to the liver disease research table.

Thus I am happy to present to you the latest edition of *EMJ Hepatology*, which provides details of everything that happened during ILC 2016, as well as a number of peer-reviewed papers covering a diverse range of topics such as hepatocellular carcinoma and non-alcoholic fatty liver disease. I also invite you to attend the next ILC in April 2017 in Amsterdam, Netherlands, and become part of our ever-growing community of physicians and researchers dedicated to the advancement of knowledge on liver disease and how to tackle it.

Yours sincerely,

Markus Peck-Radosavljevic

Professor of Medicine and Chairman, Department of Gastroenterology and Hepatology, Endocrinology and Nephrology, Klinikum Klagenfurt am Wörthersee, Klagenfurt, Austria; Fellow of the Austrian College of Physicians; Member of the American Association for the Study of Liver Disease (AASLD), the European Association for the Study of the Liver (EASL), the Austrian Transplant Association, the Austrian Society for Infectious Diseases and Tropical Medicine (OEGIT), the Austrian Association for Gastroenterology and Hepatology, and the Austrian Society for Internal Medicine (ÖGIM); Past Secretary-General of EASL.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13TH-17TH APRIL 2016

Welcome to the *European Medical Journal* review of the 51st Annual Meeting of the International Liver Congress

The historic architecture of Barcelona, Spain, was the backdrop to this year's annual ILC congress, marking its 51st birthday. The city of Barcelona welcomes around 8 million visitors every year, making it the third most visited city in Europe, and a perfect location for this prestigious event.

This year's intake of visitors will include all 10,064 attendees of ILC 2016 from all over the globe, who descended on the city for 5 days of presentations, discussions, and debate on all aspects of hepatology practice and research. Nearly 3,000 abstracts were received and 1,580 were presented at the congress, with over 150 presented orally. This year saw more topics and posters presented, and more grants and fellowships awarded than ever before. Secretary General Dr Laurent Castera, University of Paris-XII, Clichy, France, talked of the growing presence of the European Association for the Study of the Liver (EASL) in European hepatology practice, saying: "We also started 50 years ago, as a small society, with less than 60 members, we are now more than 4,000 members from 109 countries!" He went on to announce the latest EASL clinical practice guidelines in non-alcoholic fatty liver disease, hepatitis C, and benign liver tumours.

As in previous years, awards were given in recognition of the outstanding contributions of individuals to liver disease care and research and this year there were three exceptional recipients. The first was Antonio Craxi, University of Palermo, Palermo, Italy, who has contributed extensively to research into hepatitis. Secondly, Roberto Groszmann, Yale School of Medicine, New Haven, Connecticut, USA, was acknowledged for his work in cirrhosis and portal hypertension. Last but certainly not least there was Jordi Bruix, University of Barcelona, Barcelona, Spain, who focusses on hepatic oncology.

Now in its second year, the Young Investigator Award seeks to acknowledge those who, whilst still in training, have already made a significant contribution to the international liver community. This year the award was given to two researchers. Firstly, to Jordi Gracia-Sancho, Barcelona, Spain, who presented an abstract on the maintenance of the hepatocyte phenotype *in vitro* using a superior microfluidic bioreactor to co-culture primary liver sinusoidal endothelial cells to produce a

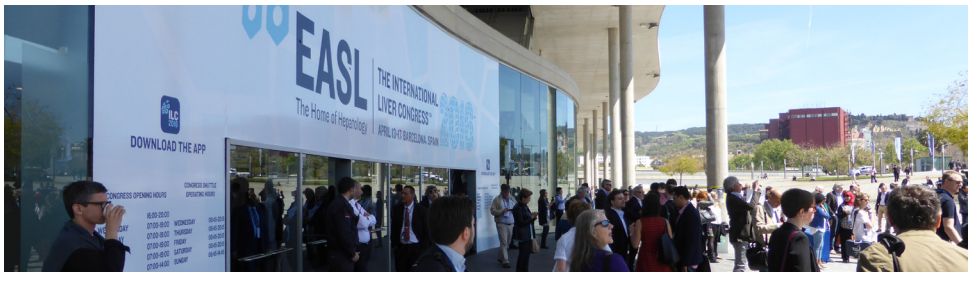
better phenotypic model than conventional methods. Veronika Lukacs-Kornek, Saarland University Medical Center, Homburg, Germany, received the second Young Investigator Award and presented an abstract that looked at the innate immunity contribution from toll-like receptor 4 stimulation on progenitor cell subset expansion during liver injury, finding that changes in genetic expression and oval cell activation occur.

> “We also started 50 years ago, as a small society, with less than 60 members, we are now more than 4,000 members from 109 countries!”

There were also seven fellowships awarded to physician scientist, post-doctoral, and entry-level researchers. Three registry grants were awarded to: Jesus Banales, Biodonostia Institute, San Sebastián, Spain, for a registry collecting data on cholangiocarcinoma across Europe; Ken Simpson, University of Edinburgh, Edinburgh, UK, for a study into the epidemiology, management, and outcome of acute liver failure; and to Deirdre Anne Kelly, University of Birmingham, Birmingham, UK, for a study on long-term graft outcomes.

As always the standard of the work presented at the congress was exemplary and provided a unique opportunity to experience research highlights from across Europe. Amongst the presentations and topics on display were potential new treatment options for common hepatological conditions, such as hepatitis C and non-alcoholic fatty liver disease, which are sure to change the treatment landscape in the future.

In the following pages, you will find a review of some of the most significant research from the congress and a number of summaries of selected presentations that we hope will excite and refresh your perspective on the field.



New Research Highlights the Potential for a Combined HIV and Hepatitis C Virus Vaccine

PRIME-BOOSTING, a sequential immunisation technique often compared to jump starting the immune system, has been shown to be compatible with co-administration of vectors encoding HIV and hepatitis C virus (HCV) antigens. This has highlighted the potential for a promising new combination vaccine against HIV and HCV.

HIV/HCV co-infection is a global issue, particularly in Europe where prevalence is extremely high and rising. With an estimated 2.3 million co-infected individuals identified worldwide, it currently represents the leading cause of non-AIDS death in co-infected individuals.

In an ILC press release dated 13th April 2016, Prof Lucy Dorrell, Principal Investigator, University of Oxford, Oxford, UK, explained: "While we have drugs to treat both HIV and HCV, these are out of reach for many and do not prevent re-infection."

In the Phase I study healthy volunteers (N=32) were randomised in to one of three experimental groups. Volunteers received an investigational vaccination delivered intramuscularly at Weeks 0 and 8; volunteers in Group One received HCV only vaccines and Group Two volunteers received HIV only vaccines. HIV and HCV vaccinations were co-administered to Group Three volunteers, allowing observation of changes in immune response compared with either vaccine alone.

> "Knowing that it may be possible to vaccinate a single individual against both diseases opens up huge possibilities for rolling back epidemics of disease and co-infection."

Immune response to vaccination was measured using a simple blood test which demonstrated a significant difference in HIV and HCV-specific T cells in the blood samples; where the peak means of 608.5 and 785 spot-forming units per million peripheral blood mononuclear cells (SFU/106 PBMC), respectively, were boosted to 4260 and 3760 SFU/106 PBMC, respectively. No impairment of the magnitude and breadth of either T cell responses were observed in Group Three compared to Groups One and Two.

Prof Ellie Barnes, Research Fellow, University of Oxford, Oxford, UK, states: "Knowing

that it may be possible to vaccinate a single individual against both diseases opens up huge possibilities for rolling back epidemics of disease and co-infection."

Hepatitis Prevalence Amongst Refugee Populations in Europe

HEPATITIS B is likely to increase in prevalence in Europe due to the high rates of the condition amongst new refugee populations, providing potential challenges to healthcare systems across Europe.

The number of refugees and asylum seekers entering the European Union has increased in the last year, with more than 1 million asylum applications in Germany alone. Many amongst these populations have left unstable and dangerous states, where healthcare systems are presently limited or non-existent.

A study tested 793 patients from all age groups for serological markers of hepatitis B (hepatitis B surface antigen [HBsAg] and hepatitis B core antibody [anti-HBc]), and liver enzymes (alanine transaminase [ALT], aspartate transaminase [AST], bilirubin, gamma glutamyl transpeptidase, alkaline phosphatase). The cohort was composed of patients living in refugee reception centres in Northern Germany in August 2015.

HBsAg was present in 2.3% of patients and anti-HBC in 14%, which indicates higher levels of hepatitis B infection than the German control group, but is consistent with other migrant groups working in Germany. Elevated ALT and AST were found in 15.9% and 5.8% of patients, respectively. Sixty-two percent had no immunity to hepatitis B and only 18.6% had been vaccinated. The prevalence of HBsAg was higher in males (2.5%) and in middle-aged and older patients (3.1%). Males were also more likely to have anti-HBC then females (14.5% and 13.5%, respectively). The highest reported levels were seen in patients >50 years (38%).

This data shows that Europe faces increased challenges in national and refugee health as Dr Philipp Solbach, Department of Gastroenterology, Hepatology, and Endocrinology, Hannover Medical School, Hannover, Germany, explained in an ILC press release dated 16th April 2016: "The prevalence of the data we have recorded, alongside decreased levels of immunity and non-immunisation, reveal the true extent of the public health challenge that Europe is facing with regard to hepatitis B."

Four Out of Five Can Expect 20-Year Survival After Childhood Liver Transplant

SURVIVAL following a childhood liver transplant can now be estimated at 20 years for approximately four out of five patients, according to new data. A recent study looked at the medical records of children who received a liver transplant over a period of 5 years, with a mean follow-up of 22 years, in order to shed light on long-term outcomes.

"Until now there has been no good answer as to how long children could be expected to live after liver transplantation," commented Dr Josefina Martinelli, Paediatric Liver Unit, AP-HP, Hospital Bicêtre, University Hospital South Paris, Paris, France, in an ILC press release dated 14th April 2016. "While each child receiving a transplant is unique and every procedure is different, this study provides robust evidence on the average expected survival rates, an important consideration for the parents of children who undergo this complicated procedure."

10,064 attendees

In the study, the medical records of 128 consecutive children who received cadaveric transplantation (whole liver n=47, partial n=77, split n=4) in Bicêtre Hospital, Paris, France from 1988–1993 at a median age of 2.5 years were retrospectively analysed.

> While each child receiving a transplant is unique and every procedure is different, this study provides robust evidence on the average expected survival rates, an important consideration for the parents of children who undergo this complicated procedure.

The team found that patient survival rates recorded at 5, 10, 15, and 20 years were 84%, 82%, 80%, and 79%, while graft survival rates were 73%, 72%, 67%, and 65%, respectively. They also discovered that the most common complications following surgery were infection (59%) as well as acute (44%) and chronic (37%) rejection. Chronic kidney disease Stage ≥2 was present in one-third of patients, however a total of 100 of the original 128 children survived ≥20 years following transplantation.

"This study is evidence of the great progress the medical community is making as we continue to learn more about how the body deals with transplanted organs," commented Prof Laurent Castera, Department of Hepatology, Hôpital Beaujon, AP-HP, University of Paris-VII, Clichy, France.

Sofosbuvir and Ledipasvir may be Effective in Shorter Treatment Courses

SHORTER durations of treatment for hepatitis C virus (HCV) may soon be possible. Prof Heiner Wedemeyer and colleagues, Department of Gastroenterology, Hepatology and Endocrinology, Hannover Medical School, Hannover, Germany, studied the use of direct-acting antiviral treatments ledipasvir-sofosbuvir (LDV-SOF), focussing on whether treatment time could be reduced, a change which would also reduce the cost of therapy as well as the side effects. Acute HCV clears spontaneously in 10–50% of infected individuals, however early diagnosis rarely occurs in those who do not clear the infection, leading to serious liver damage.

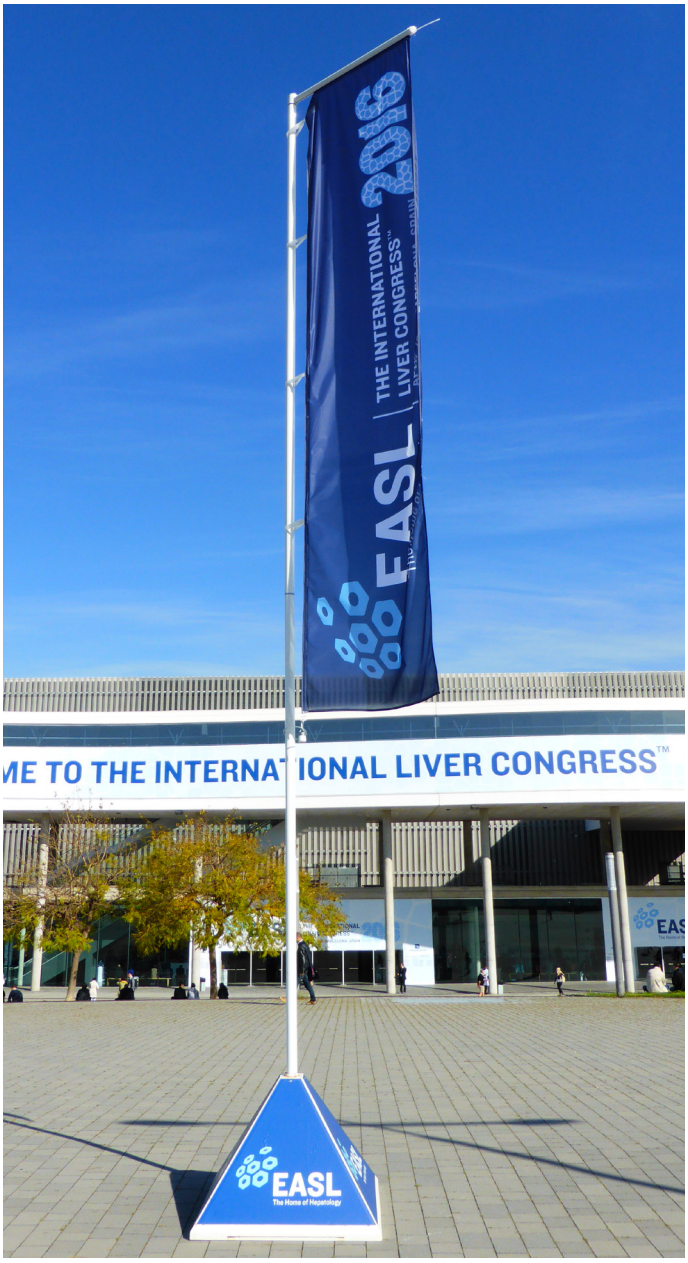
There are a number of treatment regimens available for HCV, one of which is LDV-SOF. These regimens are normally prescribed for a 12-week period, following which patients are tested for a sustained virological response (SVR) to confirm the efficacy of the treatment. This 12-week treatment course currently results in a SVR in 95% of patients.

The study included 20 patients infected with HCV of various aetiologies: sexual transmission (n=11), medical procedures/needle stick (n=5), drug use (n=1), nail treatment complications (n=1), and unspecified (n=2). All 20 patients were given a 6-week course of LDV-SOF without ribavirin and 100% of these achieved SVR at the 12-week follow-up with no

detectable HCV. Fatigue was a side effect in 30% of the participants.

In an ILC press release dated 16th April 2016, Prof Wedemeyer summarised, "our research demonstrates that not only is the combination of sofosbuvir and ledipasvir safe, well tolerated, and effective in acute HCV genotype 1 patients who have severe liver disease with very high liver enzymes, but a shorter treatment duration does not appear to hinder efficacy."

These findings are encouraging and exciting for both patients and healthcare institutions as they may potentially reduce healthcare costs and the incidence of side effects that can occur during treatment. The findings now need to be validated in further studies with larger cohorts beyond this initial pilot study.



Community-Based Treatment Providers Boost Fight Against Hepatitis C

INNOVATIVE community-based treatment strategies, presented at ILC 2016, have provided successful treatment of hepatitis C virus (HCV)-infection to a range of patient subgroups in various non-specialist contexts, opening the doors to the therapeutic administration of hepatitis C treatment outside of the specialist setting.

HCV patients represent a large, global cohort in need of therapeutic treatment; providing this care in a safe and efficient manner is problematic as there currently exists a far smaller proportion of experienced specialists than can cater to the 130–150 million people across the world living with chronic HCV.

Responding to the constricted nature of current HCV care, a multicentre, open label, Phase IV clinical trial explored the outcomes of a standardised treatment for chronic HCV-infected patients across providers; specialists, primary care physicians, and nurse practitioners were given 3 hours of guideline training, after which the direct-acting antivirals, ledipasvir and sofosbuvir, were administered at community health centres across the USA. The cohort (N=304) included patients with genotype 1a (72%), co-infected with HIV and HCV (24%), and with cirrhosis (20%); 18% of the total cohort were treatment-experienced.

> "This research has the potential to be a genuine game changer in the way we look at HCV treatment across the board..."

The results were promising; 93.8% of patients achieved a sustained virological response at 12 weeks (SVR12). Regardless of the particular treatment provider, SVR12 was achieved in >90% of the cohort, suggesting no significant difference in efficiency. "We know we have too few experienced specialists treating HCV, and this is severely hampering our ability to eradicate this disease once and for all. This research has the potential to be a genuine game changer in the way we look at

HCV treatment across the board, and could provide the opportunity to increase access to care and treatment to many regions of the world," asserted Prof Tom Hemming Karlsen, Department of Transplantation Medicine, Division of Cancer medicine, Surgery, and Transplantation, Oslo University Hospital Rikshospitalet, Oslo, Norway, in an ILC press release dated 13th April 2016.

Breastfeeding and Healthy Pre-Pregnancy BMI Rates Reduce Risk of NAFLD in Teenagers

BREASTFEEDING and maternal pre-pregnancy body mass index (BMI) rates significantly affect the chances of children developing non-alcoholic fatty liver disease (NAFLD) in their teenage years, according to a study presented at ILC 2016.

The results showed that healthy pre-pregnancy BMI levels and the exclusive breastfeeding of a child for at least 6 months can each reduce the risk of the onset of NAFLD in infants; a disease that can lead to scarring (fibrosis) of the liver and cirrhosis, a potentially life-threatening condition.

The research was conducted in light of the rising prevalence of NAFLD in children and adolescents. Factors that have been associated with this rise include excessive childhood weight gain.

The team used questionnaires, direct interviews, physical examinations, and blood tests to obtain data on maternal pregnancy, birth, childhood, and adolescent characteristics. Liver ultrasounds were carried out in 1,170 17-year-old participants to assess NAFLD status.

Over 15% (n=179) of the teenagers studied had NAFLD. A pre-pregnancy BMI within the normal range reduced the chances of developing this condition by half (odds ratio [OR]: 0.49, 95% confidence interval [CI]: 0.33–0.72, p=0.001). Those who were exclusively breastfed for ≥6 months had a one-third reduced risk of developing adolescent NAFLD compared with teenagers who had been breastfed for <6 months (OR: 0.66, 95% CI: 0.56–0.95, p=0.03). However, breastfeeding >9 months did not have any additional impact on the chances of NAFLD developing during adolescence (OR: 0.73, 95% CI: 0.46–1.16, p=0.18).

"Our results demonstrate the grave impact maternal factors can have on the risk of developing liver disease in adolescence," commented Dr Oyekoya Ayonrinde, Clinical Senior Lecturer for Medicine and Pharmacology, University of Western Australia, Perth, Australia, in an ILC press release dated 13th April 2016. These findings demonstrate the need for proper nutrition and the benefit of exclusive and extended breastfeeding during infancy.

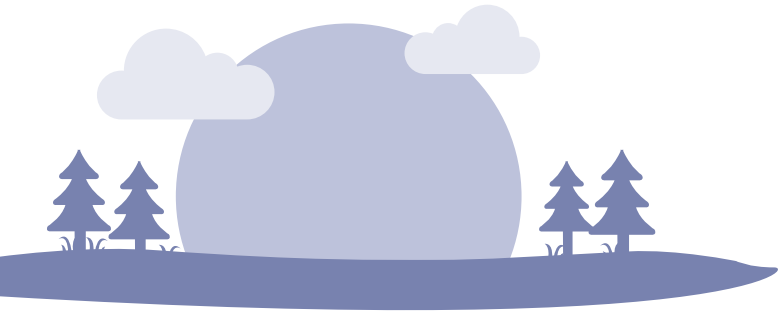
Waist Over Weight in Non-Alcoholic Liver Disease

WAIST circumference may be more important than weight or BMI in the development of non-alcoholic fatty liver disease (NAFLD) and the severe related complications, a recent study presented at ILC 2016 has shown.

Accumulation of fat around the liver can lead to cirrhosis, which results in impaired liver function. NAFLD is strongly linked with obesity and has a prevalence of up to 80% in obese patients. However, 16% of people with a normal body weight have NAFLD; this is known as 'lean' NAFLD and is linked with diabetes and hypertension. In an ILC press release dated 16th April 2016, Dr Rosa Lombardi, Unit of Internal Medicine, Policlinico Hospital, University of Milan, Milan, Italy, commented that "while NAFLD is commonly associated with obesity, research has highlighted that a percentage of patients are not obese."

In this study of 323 patients with biopsy-confirmed NAFLD, the participants were divided in to groups based on waist circumference, abdominal fat, and BMI (<25 kg/m^2 defined as lean-NAFLD). When considering those with a larger waist circumference, the study found that a waistline of >35 inches in women and a waistline of >40 inches in men was more strongly associated with metabolic syndrome (p=0.0001), carotid plaques (p=0.03), and significant liver fibrosis (p=0.03) compared with obese patients with NAFLD. This was consistent even in those with lean-NAFLD (who were a normal weight but had a larger waist circumference).

5 days of presentations, discussions, and debates



Dr Rosa Lombardi explained the novelty of these results: "This is the first study to show that patients with lean-NAFLD who have increased levels of waist fat can in fact be at greater risk than obese patients with NAFLD."

Further research is needed to analyse the mechanisms by which this may occur and the best method for detecting an individual's risk of developing the disease or the allied metabolic, cardiovascular, and tissue complications.

Capsid Assembly Inhibitor Provides Novel Treatment for Hepatitis B

A FIRST-IN-CLASS drug named NVR 3-778 has been confirmed as a novel treatment for chronic hepatitis B following promising results from a study presented at ILC 2016.

An estimated 14 million people are chronically infected with the hepatitis B virus (HBV) in the World Health Organization (WHO) European region. Many treatment options are available and effective at suppressing the virus but they rarely clear the condition permanently.

NVR 3-778 is a viral capsid assembly inhibitor, which modulates the core protein function. This Phase Ib trial included a cohort of 64 treatment-naïve HBV-infected patients. The patients received treatment for a period of 28 days. They were separated into six dosing groups: three receiving 100, 200, or 400 mg of NVR 3-778 daily; two groups were treated with NVR 3-778 600 mg twice daily, either with or without pegylated interferon (PEG-INF), and a final group received PEG-INF combined with placebo.

A dose-related reduction in HBV DNA was observed, the largest of which occurred in the 600 mg plus PEG-INF group (1.97 log IU/mL). Patients who received NVR 3-778 alone had a 1.72 log IU/mL decrease in the 600 mg twice daily group; the control group had a decline of 1.06 log IU/mL. All groups tolerated the drug well and there were no discontinuations; most adverse events were mild and not ascribed to NVR 3-778. Researchers also noted early reductions in hepatitis B eantigen levels, which were greatest in the NVR 3-788 group.

While further research is required, these results are a step towards improved treatment as Prof Man-Fung Yuen, Department of Gastroenterology and Hepatology, Queen Mary Hospital, University of Hong Kong, Hong Kong, commented in an ILC press release dated 16th April 2016: "It is promising to see that the combination of NVR 3-778 with PEG-INF produces responses that are greater than those seen with either monotherapy."

Long-Term Use of NUCS Therapy Increases Risk of Colorectal and Cervical Cancers

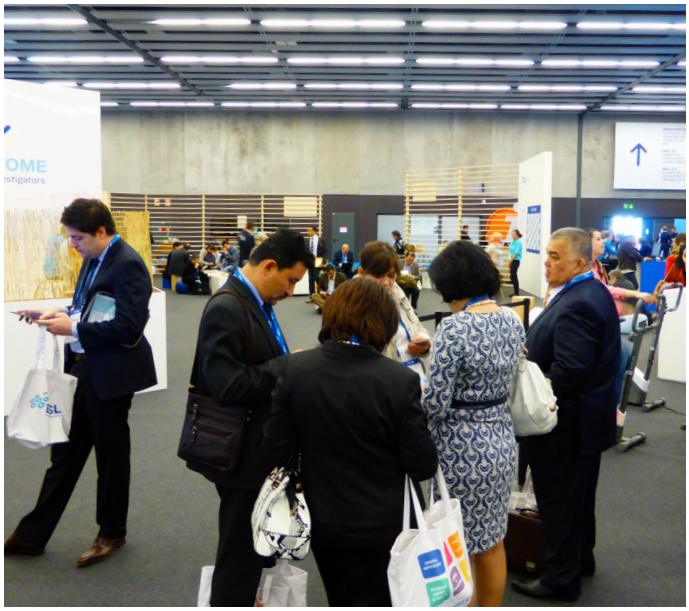
PROLONGED treatment with nucleos(t)ide analogues (NUCS) in patients with chronic hepatitis B virus (HBV) infection has been statistically linked to an increased risk of developing colorectal and cervical cancer.

The burden of chronic HBV infection is felt worldwide, and there is constant demand for the development of new approaches to management and prevention. NUCS inhibit viral reproduction and are thus often used as a therapeutic strategy for patients presenting with chronic HBV. Despite their efficacy in suppressing the spread of viral infections, long-term administration of NUCS can cause serious adverse events in chronic HBV patients.

A recent study, presented at ILC 2016, investigated potential associations between the treatment and various malignancies. The primary outcome in this study was incident malignancies with the exception of hepatocellular carcinoma. A large cohort of 45,299 chronic HBV patients, 16.16% of whom had received NUCS treatment (n=7,323), were followed for up to 7 years, measuring the relative risk of the primary outcome.

At median follow-up (4.4 years), malignancies were found in 2.1% and 5.7% of patients in the NUCS-naïve and NUCS-treated cohorts, respectively. Though, when examined at a higher resolution, the analysis revealed a specific risk pattern for HBV patients treated with NUCS. "Although our analysis showed that NUCS treatment does not increase overall incidence of liver, lung, breast, and urinary/renal malignancies, it did reveal that patients with HBV on this treatment had a higher risk of developing colorectal and cervical cancers," clarified Prof Grace Wong, Department of Medicine and Therapeutics, The Chinese University of Hong Kong, Hong Kong, China, in an ILC press release dated 15th April 2016.

The statistical risk of developing cervical and colorectal cancers was demonstrated as significant, with adjusted hazard ratios of 4.41 and 2.17; 95% confidence intervals of 1.01–19.34 and 1.08–4.36; and p-values of 0.049 and 0.029, respectively. As a result, the long-term safety of NUCS treatment for HBV patients has been called into question.



High Rates of Liver Cancer Recurrence in Hepatitis C Patients Taking Antiviral Drugs

PATIENTS who have successfully battled hepatocellular carcinoma (HCC) in the past have a high rate of recurrence if they are taking direct-acting antiviral treatments (DAAs) to treat hepatitis C, according to data presented at ILC 2016.

Liver cancer, of which HCC is the most common form, accounts for 662,000 deaths worldwide. The vast majority of HCC cases occur in patients with chronic liver disease, and approximately 80–90% have cirrhosis; most within the remaining 10% have moderate-to-advanced fibrosis.

Researchers from Italy analysed the medical records of 344 HIV-negative patients with hepatitis C virus-related cirrhosis who did not have active HCC. The patients had received one of the following DAA combinations: sofosbuvir and simeprevir (34%); 3D combination, which comprises ABT-450 with ritonavir, ombitasvir, dasabuvir, and ribavirin (22%); sofosbuvir and ribavirin (17%); sofosbuvir and daclatasvir (16%); and sofosbuvir and ledipasvir (10%). To assess the rates of HCC occurrence in these patients, baseline enhanced-ultrasonography and MRI/CT-scans were compared with those taken during the 6-month post-treatment follow-up.

A sustained virological response was achieved in 89% of patients at 12 weeks post-treatment. At the same time, active HCC was found

in 7.6% of all patients (n=26) with no history of HCC at 24 weeks post-treatment. Most significantly, 29% of the patients who had a previous history of HCC experienced a recurrence.

While these findings need to be investigated further, the results suggest that HCV patients taking DAAs will require a greater level of scrutiny for signs of HCC onset in the future, and could be used to develop management strategies for high-risk patients.

"Even in a relatively short observation period, we have shown that high recurrence rates of HCC can occur in HCV patients taking DAAs," said Dr Federica Buonfiglioli, Department of Medical and Surgical Sciences, University of Bologna, Bologna, Italy, in an ILC press release dated 13th April 2016.

Coffee Consumption Could Help Fight Non-Alcoholic Fatty Liver Disease

SYMPTOMS of non-alcoholic fatty liver (NAFLD) could be alleviated by the consumption of coffee, researchers have discovered. In a study conducted on three groups of mice, scientists found that mice that consumed a daily dose of coffee alongside a high-fat diet displayed an improvement in several key markers of the disease as well as gaining less weight than those mice that did not consume any coffee.

> "Previous studies have confirmed how coffee can reverse the damage of NAFLD but this is the first to demonstrate that it can influence the permeability of the intestine."

Researchers analysed three different groups of mice over a 12-week period. Group One mice were fed a standard diet, Group Two a high-fat diet, and Group Three was given a high-fat diet plus a decaffeinated coffee solution equivalent to six cups of espresso coffee for a 70 kg person.

The results of the study showed significantly reversed levels of cholesterol ($p<0.001$), alanine aminotransferase ($p<0.05$), steatosis ($p<0.001$), and ballooning degeneration ($p<0.028$) in the third group. The scientists also showed how coffee protects against NAFLD by raising levels of zonulin (ZO)-1, which reduces the permeability of the gut; increased gut permeability is believed to be a factor in liver injury and the advancement of NAFLD.

"Previous studies have confirmed how coffee can reverse the damage of NAFLD but this is the first to demonstrate that it can influence the permeability of the intestine," stated Dr Vincenzo Lembo, University of Naples Federico II, Naples, Italy.

"Italy is famous for its coffee and this Italian study has reinforced our knowledge on the link between it and NAFLD. Although not suggesting that we should consume greater levels of coffee, the study offers insights that can help future research into, and understanding of, the therapeutic role coffee can play in combating NAFLD," commented Prof Laurent Castera, Department of Hepatology, Hôpital Beaujon, AP-HP, University of Paris-VII, Paris, France, in an ILC press release dated 13th April 2016.

Scoring System Defines Non-Alcoholic Fatty Liver Disease Mortality Risk

ACCURATE prediction of a patient's risk of death from non-alcoholic fatty liver disease (NAFLD) has been enabled through the development of a new scoring system, named 'SAF', which combines three individual measures of liver function; as announced by a research team at ILC 2016.

NAFLD is the most common form of liver disease worldwide; closely associated with obesity and diabetes, the accumulation of fat in the liver causes inflammation and cirrhosis, leading to decreased liver function. Although an increased risk of death has long been associated with NAFLD, this study, conducted over a long-term follow-up period, offers a technique for individual patient risk stratification.

The SAF scoring system combines data on liver steatosis, activity, and fibrosis from a simple questionnaire. Its efficacy was tested via a statistical study of 139 biopsy-proven NAFLD patients, which used a Cox regression model, adjusted for body mass index and the presence of Type 2 diabetes, to explore patient survival over an average of 26 years (standard deviation: 6.1, range: 1.7–40.8 years). At baseline, 69 patients presented with a severe form of NAFLD, whilst 35 were classified as exhibiting mild or moderate disease. Over the follow-up period 70 patients died, 59% of whom were originally defined as presenting with severe NAFLD.

"We suspected that steatosis, activity, and fibrosis were important to overall risk but we wanted to validate their impact on mortality over a long-term follow-up period through a validated and simple scoring system," explained lead author Dr Hannes Hagström, Unit for Inflammation, Gastroenterology and Rheumatology, Department of Medicine, Karolinska University Hospital, Stockholm, Sweden, in an ILC press release dated 14th April 2016. The Cox regression model allows predictions of treatment effect on survival as Dr Hagström confirmed: "This new analysis is vital in showing the link between severe NAFLD and mortality, which is an important measure given that this is the most common liver disease worldwide."

Algorithm Predicts End-Stage Liver Disease Treatment Outcomes

CALCULATING the potential failure of treatment for end-stage liver disease (ESLD) has recently become an algorithmic reality, thanks to a recent study of cirrhosis patients.

A topical issue in medicine today is pragmatic decision-making regarding treatment strategy for individuals. "When patients are very ill, physicians must ensure that our concern for the patient should not result in the recommendation of treatment that will be of no benefit," said Dr Katrine Lindvig, Research Assistant, Medical Gastroenterology, University of South Denmark, Odense, Denmark, in an ILC press release dated 15th April 2016.

A new predictive algorithm, presented at ILC 2016, combines a measure of pre-morbid liver function with the Acute-on-Chronic Liver Failure (ACLF) grade. ACLF can be differentiated from decompensated cirrhosis in a number of ways; patients with ACLF are typically younger, present with more

alcohol-related than hepatitis C virus-induced scarring of the liver, and have increased rates of organ failure and mortality. ACLF is not uncommon, occurring in 31% of patients with cirrhosis and acute complications, who are hospitalised, also being their most common cause of death.

In this regard, physicians must decide whether or not an ESLD patient will survive and benefit from intensive care unit (ICU) treatment, a choice that, as previously mentioned, can prove difficult. To develop a predictive algorithm, the researchers used scales such as Child–Pugh, Model for End-Stage Liver Disease, and CLIF-SOFA-score data, pertaining to severity of disease, urgency of, and success of transplant, respectively, from 354 hospitalised cirrhosis patients. The algorithm isolated two groups within the cohort: those likely to benefit and survive and those unlikely to benefit or survive ICU treatment, and these outcomes were accurately predicted in 96% of patients (odds ratio: 4.7; 95% confidence interval: 2.50–9.05).

"We now have well validated data that allows us to more accurately predict who is likely to benefit from treatment compared with previous measures," concluded Dr Lindvig.

A New Potential Treatment Option for Primary Sclerosing Cholangitis

A NOVEL treatment, known as norursodeoxycholic acid, may soon become available for primary sclerosing cholangitis (PSC), a condition for which there are currently no therapeutic options; according to a study presented at ILC 2016.

In PSC, liver tissue can become damaged, as a build-up of bile acids, and consequent blockage of the bile ducts, leads to accumulation of bile in the liver. In the EU, approximately 6.3 per 100,000 people are affected, with symptoms ranging from itching and fatigue to cirrhosis, hypertension, liver failure, and in the later stages, liver cancer. As such, there is a dire need for efficacious treatments.

Recently a multicentre, randomised, double-blind, Phase II trial evaluated the use of norursodeoxycholic acid in PSC, enrolling 159 patients with elevated serum alkaline phosphatase (which is increased in the presence of liver disease). Patients were randomised into four groups receiving 500, 1,000, or 1,500 mg of norursodeoxycholic acid, or placebo, administered for 12 weeks with a 4-week follow-up with any treatment.

Efficacy was measured using the primary endpoint: mean relative change in serum alkaline phosphatase between baseline and end of treatment in the intention-to-treat groups. There was a reduction in serum alkaline phosphatase in all groups, with a 12.3% reduction in the 500 mg group ($p=0.0029$), 17.3% in the 1,000 mg group ($p=0.0003$), and 26% in the 1,500 mg group ($p<0.0001$).

> "When patients are very ill, physicians must ensure that our concern for the patient should not result in the recommendation of treatment that will be of no benefit."

Adverse event rates were similar across all the groups; pruritus occurred at low frequencies. Prof Michael Trauner, Lead Author, Division of Gastroenterology and Hepatology, Medical University Vienna, Vienna, Austria, commented in an ILC press release dated 16th April 2016: "Our study demonstrates that norursodeoxycholic acid could be a viable treatment option for patients with this chronic and debilitating condition."

This treatment could provide relief for the ~82,000 patients in Europe who suffer from the condition. A larger scale Phase III trial is required to examine the safety and efficacy in larger cohorts.

New Triple-Combination Drug Shows Promise for Treating Genotype 1 Hepatitis C

A COMBINATION of sofosbuvir, velpatasvir, and the investigational drug GS-9857 with or without ribavirin has been shown to be safe and effective in the treatment of hepatitis C patients whose prior treatment with direct-acting antivirals (DAAs) failed. In a recent study, the drug combination resulted in high rates of sustained

virological response 12 weeks after treatment (SVR12) in genotype 1 hepatitis C patients who had previously received and failed treatment with DAAs.

In the study, a total of 49 patients were randomised to treatment, of whom 65% were male and 88% had hepatitis C virus genotype 1a. Forty-one percent of the patients examined had previously received an NS5A inhibitor, and 47% of patients had previously received at least two classes of DAA. The primary endpoint of the study was SVR12.

SVR12 was achieved in 100% of patients who took sofosbuvir, velpatasvir, and GS-9857 without ribavirin, and in 96% of patients who received ribavirin.

The triple combination proved to be generally safe and well-tolerated. The researchers recorded only one serious adverse event while two patients discontinued treatment with ribavirin due to adverse effects. Fatigue and anaemia were the most frequent adverse effects; however, they were only observed in patients that received ribavirin.

"This new combination of treatments could add to our arsenal of therapies for patients with hepatitis C, a disease which could eventually be eradicated. In the hard-to-treat patient population who had previously failed on existing treatment regimens, the combination with GS-9857 could provide these people with another hope," commented Prof Tom Hemming Karlsen, Department of Transplantation Medicine, Division of Cancer medicine, Surgery, and Transplantation, Oslo University Hospital Rikshospitalet, Oslo, Norway, in an ILC press release dated 14th April 2016.

Genotype 3 Hepatitis C: Is ABT the Answer?

HOPE for the difficult-to-treat genotype 3 hepatitis C patient group has been found in experimental drugs ABT-493 and ABT-530. Whilst progress has been made in the treatment of genotype 1 using direct-acting antiviral therapies, there remains much room for improvement in the treatment of genotype 3 patients.

The current therapy for genotype 3 is sofosbuvir, a nucleotide polymerase inhibitor, combined with weight-based ribavirin (RBV) for 24 weeks. Evidence supporting the use of this treatment originates from the Valence study where high sustained virological response (SVR) rates (~92%) were seen in treatment-naïve patients, however treatment-experienced patients achieved lower SVR rates (60%).

New research highlights the potential of this new therapeutic treatment via a Phase II study that enrolled 24 cirrhotic, treatment-naïve patients to two study arms. These two groups took oral ABT-493 and ABT-530 with and without daily RBV, achieving SVR in 100% of patients after 12 weeks. In a third trial arm, including 29 non-cirrhotic, genotype 3 patients, 97% of patients achieved SVR after only 8 weeks. None of the patients experienced virological failure, although some side effects were observed, including headache and fatigue.

Prof Paul Kwo, Department of Gastroenterology, Indiana University School of Medicine, Indianapolis, Indiana, USA, commented in an ILC press release dated 16th April 2016: "We are pleased to see the efficacy of this two direct-acting antiviral investigational, pan-genotypic regimen has been validated for treatment-naïve hepatitis C genotypic 3 patients, with 100% of cirrhotic patients treated for 12 weeks and 97% of non-cirrhotic patients treated for 8 weeks achieving SVR at 12 weeks post-treatment."

Although clinical use of these regimens continues to be assessed across different cohorts, these findings represent a promising alternative treatment for patients worldwide. Dr Kwo explained: "Clinical trials are ongoing to evaluate the safety and efficacy of the investigational treatment, and we are now focussing on a larger cohort of HCV genotype 3 patients, including treatment-experienced patients."

> "We are pleased to see the efficacy of this two direct-acting antiviral investigational, pan-genotypic regimen has been validated for treatment-naïve hepatitis C genotypic 3 patients..."

Immunotherapy Offers Effective Treatment for Hepatitis D Patients

CHRONIC hepatitis delta (D) infection can be ameliorated using biologic therapy, a study presented at ILC 2016 has recently confirmed.

"There has been significant debate over whether there are long-term benefits to patients with hepatitis D receiving antiviral treatment," explained Dr Anika Wranke, Fellow of Hannover Medical School, Hannover, Germany, in an ILC press release dated 15th April 2016. Hepatitis D is a severe condition that emerges from hepatitis B disease progression. The dual infection worsens patient outcomes, and a recent study was interested in exploring the efficacy of interferon alpha (IFN-α) therapy in comparison to nucleos(t)ide analogues (NUCS) treatment, a common option for treating hepatitis B.

Out of 136 chronic hepatitis D patients chosen for the study, 40% presented clinical endpoints at baseline. These included ascites (fluid accumulation causing abdominal swelling), oesophageal bleeding (enlarged veins that bleed in the oesophagus), encephalitis (brain inflammation), (hepatocellular carcinoma), liver transplant, or death. The cohort was followed for 6 months, with a median follow-up of 5 years, and the study compared the frequency of these clinical endpoints in patients receiving IFN-α-therapies (n=52) to those who received NUCS or no treatment. Within the IFN-α-based therapy subgroup, 35% achieved a sustained suppression of hepatitis D, and clinical endpoints were less frequent than in other subgroups. The study therefore highlighted the significance of the European Association for the Study of the Liver (EASL) guidelines, which recommend pegylated interferon therapy as the only effective treatment for hepatitis D.

"Our study demonstrates that the long-term outcomes for patients with severe hepatitis D, who have limited treatment options, could be improved with a widely available medication," asserted Dr Wranke. Such progress is very promising for hepatitis D patients, and future research will aim to further improve immunotherapies for the treatment of this dual infection.

Nearly 3,000 abstracts were received

Genetic Markers Highlight Increased Risk of Alcoholic Hepatitis

MUTATIONS in two genes have been linked with an increased susceptibility to severe alcoholic hepatitis according to a study presented at ILC 2016.

Severe alcoholic hepatitis is a serious syndrome with a high rate of mortality; globally, hepatic cirrhosis due to alcohol-related liver disease (ARLD) claimed almost 500,000 lives in the year 2010, at a male to female ratio of roughly 2:1. Within this global pattern, the World Health Organization (WHO) has deemed Europe the region with the heaviest drinking. The lack of a specific treatment strategy and the <50% chance of survival by 5 years which characterise this syndrome are thus concerning.

In light of these observations, a two-stage genome-wide association study (GWAS) aimed to explore genetic risk associations with the syndrome, which is just one of the complex range of ARLD manifestations. "Given that the spectrum of ARLD varies widely, with the majority of patients being asymptomatic, we were interested to find out why a small proportion of these people go on to develop severe alcoholic hepatitis," stated Dr Stephen Atkinson, Department of Surgery and Cancer, Imperial College, London, UK, in an ILC press release dated 14th April 2016.

By rapidly scanning for markers across comparative genome sets of patients with severe alcoholic hepatitis (n=860) and alcohol-dependent subjects without liver disease (n=1191), the study isolated a variant of the *PNPLA3* gene, which was

significantly associated with an increased risk of developing severe alcoholic hepatitis. Furthermore, a specific mutation of the *SLC38A4* gene was found to be another indicator of risk.

This research is just one example of the potential use of genetics on the development of robust treatment strategies for common yet complex diseases. "This first analysis of data means that we may now be able to use genetic profiles to identify people who are at increased risk of developing severe alcoholic hepatitis," concluded Dr Atkinson.

Livers Infected with Hepatitis C Prove Effective in Transplant Patients

MORTALITY in hepatitis C virus (HCV) patients receiving a HCV-positive liver transplant does not significantly differ from those receiving a healthy liver, a long-term study has revealed.

Liver organs for transplant are in high demand, with over 20,000 people on the waiting list across Europe and the USA. From 2013–2014 the demand increased by 12% in the UK alone, and figures from the USA suggest that around 16% of patients will die whilst waiting. Healthy donor livers are hard come by, and in response to the growing number of HCV cases, the use of HCV-positive donor livers has also increased; from 1995–2013 their use has tripled in the USA.

> "As highly effective treatments for HCV are available for transplanted patients, the future of these patients is bright."

Using the Scientific Registry of Transplant Recipients, data on the medium to long-term outcomes of 33,668 adult transplant patients receiving both HCV-positive and HCV-negative livers were compared. Spanning from 1995–2013, the recent study examined both long-term graft loss and mortality in this cohort, with 5.7% of patients receiving a HCV-positive liver. In a promising turn for transplant waiting list patients, the results demonstrated no difference in the amount of time to post-transplant death between those receiving an HCV-infected or healthy liver.

"Our study clearly shows that people with HCV who received HCV-positive livers had the same medium to long-term outcomes as people that received healthy livers. As highly effective treatments for HCV are available for transplanted patients, the future of these patients is bright," explained lead author of the study Prof Zobair Younossi, Chairman of Department of Medicine, Inova Fairfax Hospital, Falls Church, Virginia, USA, in an ILC press release dated 14th April 2016.

In light of the rapidly increasing number of people with HCV, this study has demonstrated the potential for greater use of available HCV-positive organs and post-transplant HCV treatment.

Current Controversies in the Treatment of Hepatitis C/HIV Co-infected Patients

THE INCREASINGLY high number of individuals co-infected with HIV and hepatitis C virus (HCV) is an issue worldwide and the complex drug interactions between direct-acting antiviral (DAA) and antiretroviral medications remain a concern.

In research, conflicting results are common. This was exemplified by two recent studies; a Spanish, prospective, multi-cohort study demonstrated that HIV negatively impacts rates of response to DAAs in co-infected patients, whereas a real-word, retrospective

US veterans' health administration study found no statistically significant impact of HIV co-infection on achievement of a sustained virological response at 12 weeks (SVR12).

Dr Karin Neukam, Unit of Infectious diseases and Microbiology, University Hospital of Valme, Seville, Spain, commented of the Spanish trial: "Our study demonstrates the impact of HIV co-infection on the effectiveness of DAA-based treatment. We must keep a close eye on co-infected patients to ensure that they receive the treatment they need." The study of 1,276 patients found that the efficacy primary outcome, SVR12, was less likely to be met in HIV/HCV co-infected patients; the SVR12 rate was 11% lower in patients treated using interferon-based DAAs and 6% lower in those treated with interferon-free DAAs compared with HCV only patients.

Conversely, in the US real-world retrospective study (N=408 patients, most of whom had HCV genotype 1), SVR12 rates exceeding 88% were observed post-treatment. Patients were treated with a combination of therapies (either simeprevir/sofosbuvir, ledipasvir/sofosbuvir, or ombitasvir/paritaprevir/ritonavir/dasabuvir) and researchers performed logistic regression analysis, controlling for patient demographics, disease severity, and other comorbidities.

51st birthday

"We know that these patients are at increased risk of liver disease progression from their HCV status, and these data suggest the co-infected patient group could benefit from treatment," stated Mr Justin McGinnis, University of South Carolina, Columbia, South Carolina, USA, in an ILC press release dated 13th April 2016.

These contradictory results indicate an ongoing need for the study of HCV treatment in HIV co-infected patients.

> "We must keep a close eye on co-infected patients to ensure that they receive the treatment they need."

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VTL-308 Clinical Trial Overview: www.AAHStudy.com

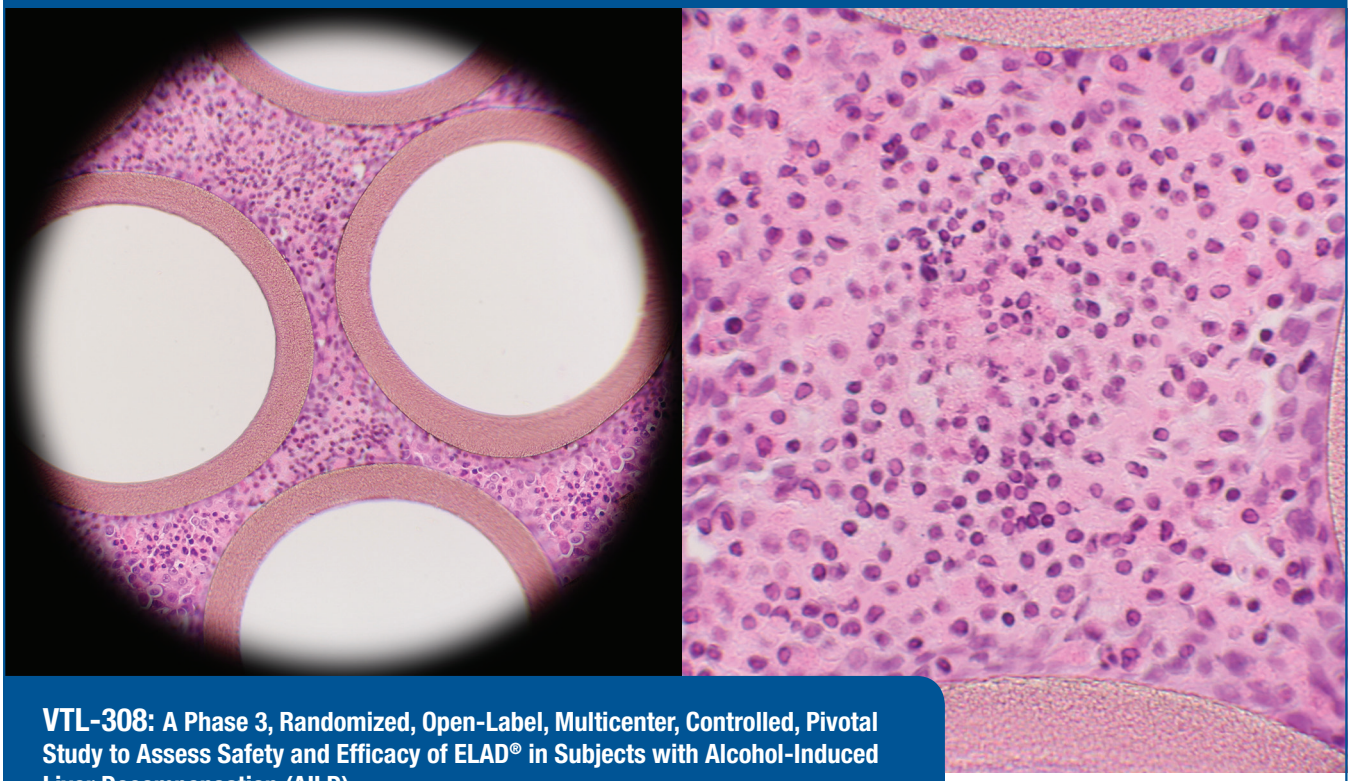

Image: ELAD cartridge cross-section (40X). C3A cells surrounding the filter membrane.

VTL-308: A Phase 3, Randomized, Open-Label, Multicenter, Controlled, Pivotal Study to Assess Safety and Efficacy of ELAD® in Subjects with Alcohol-Induced Liver Decompensation (AILD)

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- MELD score <30
- Maddrey score ≥32
- Association (6 weeks or less) of alcohol use and hospital admission for this episode of AILD

*Although subjects may meet the criteria above, they may not qualify for VTL-308. Please visit www.clinicaltrials.gov for full inclusion/exclusion criteria and for more information about participation.

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Editorial Board Interviews

Kenneth Simpson

Senior Lecturer in Hepatology, University of Edinburgh; Honorary Consultant Physician, Scottish Liver Transplant Unit, Royal Infirmary of Edinburgh, Edinburgh, UK.

Q: Why did you originally decide to pursue a career in the field of hepatology?

A: Perhaps I am lucky in many respects as I decided what I wanted to do as a specialist career in medicine when I was a medical student. When I was a young and impressionable medical student I was taught by Prof Ian Bouchier and Prof Alfred Cushieiri. Both were inspiring teachers and leaders in medical and surgical gastroenterology (especially the liver). I then spent some time at the Medical Research Council (MRC) Clinical Research Centre in Northwick Park with Prof Tim Peters, investigating alcohol related fatty liver, followed by further training at King's College, London. By then I was a committed hepatologist!

Q: To what extent has our understanding of the causes and mechanisms of liver disease increased since you began research in this area?

A: From my answer to the first question you will have gathered that I have been interested in liver disease for a long time! Just around the time that I was investigating alcohol related fatty liver in Northwick Park, the understanding of the non-alcohol related fatty liver was being recognised and we now understand so much more about this condition. Hepatitis C was discovered just as I transferred to Kings College; it is truly amazing to see how we have gone from post-transfusion nonA-nonB hepatitis to now being able to cure the hepatitis C virus (HCV) infection in this time. These are just a couple of research areas but there are so many more, including therapies for our patients with liver disease. We have been able to organise ourselves and produce well powered clinical trials, and hepatology is now a well organised subspecialty within gastroenterology.

Q: What have been the most important breakthroughs in terms of treatments for liver disease in the last 5 years?

A: The development of all oral therapy for the HCV is currently the most exciting development, but at the same time raises the challenge of how best to use these drugs in specific populations and target them to give the best return for cost.

Q: What particular challenges does the healthcare service in Scotland face in regard to liver disease? What needs to change to improve patient care in your view?

A: It is interesting that deaths from liver disease appear to be falling, but admissions keep on rising. The reasons for these observations are not clear. I think what we need to try and develop are applicable and realistic alternatives to hospital admission. If we could also reduce alcohol consumption in our population, it would make a big difference.

Q: Have you noticed any trends in the type and prevalence of conditions that you treat in recent years? If so, what are the reasons for this?

A: Alcohol related liver disease is a huge burden; it is affecting more individuals, younger patients, and more women than when I first started managing cases of liver disease. The HCV was discovered and non-alcoholic fatty liver disease

(NAFLD) did not exist, so both medical advances and increased adverse lifestyle issues have played a part in these changes.

Q: In your opinion, how much of an issue is the hepatitis E virus in Scotland and the rest of Europe? Are extra research and funding required to tackle the problem?

A: The hepatitis E virus (HEV) is another interesting development. A virus that we have recognised for years has 're-emerged' as a significant clinical problem for us in Scotland and other parts of the developed world. Currently we are still learning just how much of a problem the HEV might be; it certainly appears to be bigger than we first thought. This is due both to increased testing and also a genuine increase in incidence. More research and funding are needed to try and clarify these issues.

Q: How fulfilling do you find your position as a Senior Lecturer in Hepatology at the University of Edinburgh? What aspect of this role makes you most proud?

A: My role is a very fulfilling one. I am able to manage difficult and complex clinical cases, research liver disorders, and help to train postgraduate and undergraduate students. There is not one specific area that I would be most proud of, but I do particularly like helping out school students trying to get into medical school. To achieve a place in medical school is such a fantastic achievement for them and to help them out in a small way is really very satisfying.

Q: What is your assessment of the state of medical education in the UK, generally? How does it compare with other parts of Europe?

A: I am not very experienced in Europe wide medical education. Clearly in the UK, medical education is undergoing a very big change. There are so many competing pressures. Different medical schools provide their students with different experiences and so hopefully there is something for everyone.

Q: How important is the annual EASL ILC congress to hepatologists such as yourself? What do you think are the most important things to get out of these meetings?

A: The ILC is a really important event for hepatologists, not only from Europe but around the world. It has been amazing to watch how these meetings have expanded so much over the years. The ILC is a great way to hear about all the up-to-date clinical and basic research in liver disease, and network with old friends.

Q: What advice do you have for young medical students who may wish to begin a career in hepatology?

A: It is a great specialty, which is still developing in our clinical and scientific understanding. They will never be bored!

Ahmed Elsharkawy

Consultant Hepatologist, Liver Transplant Unit, University Hospitals Birmingham, Birmingham, UK; Secretary of the British Viral Hepatitis Group.

Q: When did you first become interested in hepatology? What were the main factors that influenced your decision to work in the field?

A: I first became interested as a medical student doing an intercalated BSc in the liver unit at Southampton University. I was fascinated by the pathophysiology of liver failure and how there are intricate links between the liver and virtually every other organ in the body.

> "The lack of donors for liver transplantation is always an issue..."

Q: How much of an issue is the lack of donors for liver transplantation at the moment, particularly in the UK? What is your opinion on the recent change to opt-out donation in Wales, and what more could the UK do to ensure a greater number of donors in the future?

A: The lack of donors for liver transplantation is always an issue especially as in Birmingham we have a waiting list mortality of between 15–20%. This is the reason I am a strong advocate for live-related liver donation and am in fact the leading hepatologist for donor work-ups in my institution. I am in favour of the recent opt-out in Wales although I wait to see what difference it will make to donation rates. I think the biggest change may come from recent machine perfusion trials. If the results are as promising as our experience suggests these will revolutionise organ donation.

Q: To what extent has the management of liver transplant patients improved in recent years? What further steps could be taken to improve this process in your view?

A: There have been a lot of advances in the management of liver transplant patients over recent years. It is amazing that although the quality of organ donors we use has gone down our outcomes have been maintained if not improved slightly. I still feel that we over-immunosuppress our liver transplant patients and if I had one wish for the future it would be that we could have the tools to scientifically and accurately risk stratify which patients could do with little or no immunosuppression and which need more. We are transplanting more and more patients in Birmingham and last year we achieved the milestone of 250 adult and paediatric transplants in 2015.

> “There are still large variations in the quality of liver services in the UK although with the growth of Liver Quest I would hope that quality will continue to improve, akin to what happened with endoscopy services in the UK.”

Q: What are the main factors that require patients to undergo liver transplantation at the Liver Transplant Unit, University Hospitals Birmingham, Birmingham, UK, where you act as a Consultant Hepatologist?

A: Our main indications for transplant are alcoholic liver disease, hepatitis C related liver disease, hepatocellular cancer, and primary sclerosing cholangitis.

Q: How far has our understanding of the mechanisms and factors for acute liver failure developed since you first began work in this area?

A: Unfortunately not as much as it should have done. Funding is very difficult in this field and the rarity of the condition means that it is only with truly collaborative work that things can improve. Dr Kenneth Simpson has recently been successful in obtaining a small grant from EASL that will hopefully help start a true pan-European collaboration.

Q: Please explain a little about your role as secretary of the British Viral Hepatitis Group. What have been the main accomplishments of this body since you have been a member and held this position?

A: I am involved in organising the three annual meetings and have also organised and chaired the first national School of Viral Hepatitis in November 2015. The British Viral Hepatitis Group has grown in size and goes from strength to strength with an established paediatric and maternal subgroup, and a recently established pharmacist group.

Q: What is your assessment of the quality of treatment for liver related conditions in the UK? How does this compare to other parts of Europe in your view?

A: There are still large variations in the quality of liver services in the UK although with the growth of Liver Quest (led by my colleague Dr James Ferguson and the Royal College of Physicians) I would hope that quality will continue to improve, akin to what happened with endoscopy services in the UK. Personally, I think the best liver services in the UK can compete with any other European

countries. We lag behind in some areas such as accessibility to novel hepatitis C therapies; this is, however, improving.

Q: What are going to be the biggest challenges facing hepatologists over the next 5 years? What can hepatologists do to tackle such issues?

A: The most immediate challenges in the UK will be how to maintain and improve services in the face of wider financial pressures in the National Health Service. Also, we need to screen and diagnose all those hepatitis C patients that we do not know about in order to prevent more people dying of hepatitis C virus related liver failure. Finally, we need to continue campaigning for minimal alcohol unit pricing.

Q: What has been the proudest achievement of your career to date? And what specific goals do you have in the future in relation to your work in hepatology?

A: To be appointed a consultant in one of the most prestigious units in the UK was a very proud day for me. I would love to improve our understanding of cirrhotic sarcopaenia and how to reverse this, as well as setting up a regional outreach hepatitis C service.

Q: What advice do you have for young hepatologists who are about to start their career in the field?

A: Well done for choosing a great career. Enjoy it. The opportunities in the field are immense. Always persevere and never let a bad day, week, or even month in the lab or clinic reduce your enthusiasm or love of your job. We are privileged to do what we do.

WELCOME TO THE REAL WORLD: HEPATITIS C VIRUS CLINICAL TRIALS TO PATIENT REALITIES

Summary of presentations at a satellite symposium that took place on 14th April 2016 as a part of the International Liver Congress (ILC) 2016 organised by the European Association for the Study of the Liver (EASL) in Barcelona, Spain

Chairpersons

Heiner Wedemeyer,[1] Ira Jacobson[2,3]

Speakers

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Disclosure: Professor Wedemeyer has received honoraria for consulting or speaking from Abbott, AbbVie, Biolex, BMS, Boehringer Ingelheim, Gilead, ITS, JJ/Janssen-Cilag, Medgenics, Merck/Schering-Plough, Novartis, Roche, Roche Diagnostics, Siemens, Transgene, and ViiV. He has also received research grants from Abbott, BMS, Gilead, Merck, Novartis, Roche, Roche Diagnostics, and Siemens. Professor Brown has received grants and consulting honoraria from AbbVie, Gilead, BMS, Janssen, and Merck. Professor Craxi has received honoraria as a speaker or a member of speakers' bureau, or as an advisor or consultant, or has received grants for clinical research from Abbott, AbbVie, Achillion, Bayer, BMS, Boehringer Ingelheim, Gilead, JJ/Janssen-Cilag, Merck/Schering-Plough, Novartis, Roche, and Roche Diagnostics. Professor Jacobson has received grant/research support from AbbVie, BMS, Gilead, Merck, and Janssen. He is also a member of speakers' bureaux for AbbVie, BMS, Gilead, Janssen, and Intercept, and has received consultant/advisor honoraria from AbbVie, BMS, Gilead, Janssen, Merck, Trek, and Intercept.

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MEETING SUMMARY

The symposium addressed the efficacy and safety of compounds currently available for treatment of hepatitis C virus (HCV) and chronic kidney disease (CKD) in North American and European countries, comparing data from trials and clinical practice. Prof Wedemeyer opened the meeting with a discussion of real-world experiences, with a focus on HCV genotypes (GTs) and resistance-associated variants (RAV). Prof Brown concentrated on trial and real-world data from patients with advanced liver disease, while Prof Craxí's presentation focussed on chronic kidney conditions and infection. Prof Jacobson led the question and answer session and summarised the discussions.

Real-World Experience: What Have We Learned Overall?

Professor Heiner Wedemeyer

Although randomised controlled trials (RCTs) are considered the gold standard for evidence on clinical efficacy and safety, they cannot address all clinical questions and some essential data gaps can be obtained in the real-world setting. In contrast to RCTs, evaluation of real-world cohorts takes place in a clinical practice setting where patients are not randomised and data capture may or may not be protocol-driven. Data on patient baseline characteristics, treatment efficacy, along with patient safety and tolerability is either captured prospectively or analysed retrospectively. RCTs typically involve limited numbers of patients with extensive inclusion and exclusion criteria, while real-world cohorts present a larger, more diverse spectrum of patients with fewer restrictions. RCTs are typically shorter in duration and more expensive, whereas the clinical setting evaluates long-term efficacy and safety, and costs less per patient.[1,2]

There is a rich heritage of collecting data from the real world on anti-HCV treatments, and over the past 3 years many real-world studies of first and second-generation direct-acting antiviral (DAA) treatments have been published in different countries, answering key questions from Phase III trials. The European Association for the Study of the Liver (EASL) International Liver Congress (ILC) 2016 was of particular interest as data from many real-world cohorts covering different regions and countries were presented for the first time.

Globally, GT1b is the most common subtype of HCV, accounting for 20–25% of all infections. It is the most common GT in Europe, while GT1a prevails in America.[3] Phase III RCTs of 8, 12, 16, and 24-week regimens that are approved or soon to be approved have shown high rates of sustained virological response (SVR) for treatment of GT1-infected patients.[4-9]

HCV TARGET represents a consortium of academic and community medical centres in North America, Germany, and Israel, conducting a longitudinal, observational study of HCV treatment that included simeprevir (SMV)/sofosbuvir (SOF); daclatasvir (DCV)/SOF; ledipasvir (LDV)/SOF; and also ombitasvir (OBV)/paritaprevir (PTV) and dasabuvir (DSV) regimens. A recent analysis within TARGET showed similar results to Phase III trials where most regimens produced uniformly high SVR rates in this real-world cohort (48% in SOF+ribavirin [RBV] regimen, 93–97% for the remaining regimens). Adverse events and discontinuations were infrequent, and the majority were moderate or mild in severity. The patient population in this study comprised a cohort of GT1b-infected patients that started treatment with interferon (IFN)-free regimens prior to August 2015, where 56% of patients had cirrhosis and 59% were treatment-experienced.[10]

The ABACUS study gave real-life SVR rates in patients with compensated cirrhosis of GT1a: 93%, and GT1b: 96% (intention-to-treat population), which were comparable to those reported in Phase III studies (TURQUOISE-II [GT1a: 95%; GT1b: 99%] and III [GT1b: 100%]).[11,12] This Italian national compassionate use programme for early access to HCV therapy for patients recruited >1,000 participants from 176 sites across Italy.[13] The primary efficacy for this analysis is based on data from 762 GT1b-infected patients with compensated cirrhosis. The majority of these patients were treatment-experienced and received OBV/PTV/ritonavir (r)+DSV+RBV for 24 (GT1a) or 12 weeks (GT1b).

In Spain, a retrospective, multicentre, non-randomised, prospective data analysis of 1,635 patients again showed high SVR data after treatment with OBV+PTV/r+DSV+RBV. Adverse events were reported in only 1.8% of the patients treated with this '3D' regimen. Hepatic decompensation was reported in seven patients; six patients died, three of these cases attributable to liver failure. In this patient population, almost 50% of participants had liver cirrhosis (Child–Pugh [CP] A and CP-B); in addition, severe adverse events (SAEs) were reported in 72 patients.[14]

In France, real-world data from the observational ANRS CO22 HEPATHER cohort gave optimal SVR rates for GT1a-infected patients with SMV+SOF for 24 weeks, while SVR rates for GT1b-infected patients were optimal with SMV+SOF+RBV for 12 weeks.[15] Overall, the response rates were in line with Phase III data. This study included 15,000 patients with HCV with an 8-year follow-up across 32 centres. There were 552 patients with GT1 or GT4 treated with SMV+SOF±RBV for 12 or 24 weeks; >70% of patients had cirrhosis and were treatment-experienced.

Overall, the real-world data confirm the efficacy and safety of the regimens used for treatment of

GT1. For GT4, however, there were limited Phase III data. Infection with GT4 is a real problem in immigrant populations in various countries, and it is also rising in Southern Europe with a prevalence of ≤18%.[16] Efficacy data in the real-world cohort may be more important with GT4 than other HCV subtypes due to the enormous variability within the subtype.

High SVR rates with good safety profiles were achieved in GT4-infected patients across PEARL-1 (OBV+PTV/r+RBV), open-label (LDV/SOF), and NEUTRINO (IFN/RBV+SOF) trials, as well as the pooled Phase II/III analysis (elbasvir [EBR]/grazoprevir [GZR]±RBV). However, all these cohorts had <100 patients per trial.[17-20] Data from three real-world studies have included GT4-infected patients from France, Egypt, and Qatar. In the Qatar study, 90% (28 out of 31) of patients with compensated cirrhosis and 100% (4 out of 4) with CP-B cirrhosis had undetectable viral load at Week 2 of treatment with OBV/PTV/r±RBV.[21]

As previously mentioned, real-world SVR rates for GT1 or GT4-infected patients (N=552) analysed in the French ANRS cohort were optimal with SMV+SOF+RBV for 12 or 24 weeks.[15] In Egypt, 547 GT4-infected patients were treated with SOF+pegylated IFN/RBV (12 weeks); all patients had advanced fibrosis, while 52% had cirrhosis and 32% were treatment-experienced. An SVR rate of <80% was observed in the treatment-experienced group, which may have been associated with old age, male gender, higher BMI, and cirrhosis.[22]

Another important topic is the global prevalence of RAV of HCV. Non-structural protein 5A (NS5A) RAV are prevalent worldwide and, as Phase III trials have shown, these variants can be detected in patients who have never been exposed to an antiviral drug before. Sensitive assays show that in most regions about 25% of patients already have naturally occurring substitutions in the NS5A genome, which may lead to a lower susceptibility to NS5A inhibitors. While many patients with RAV on DAA treatment showed high SVR rates, others with NS5A RAV had decreased virological response to some of the regimens (e.g. LDV/SOF±RBV[23] or EBR/GZR+RBV).[9] If these RAV are induced by treatment failure, they may be particularly long lasting. In contrast to RAV induced by protease inhibitors, the RAV induced by NS5A inhibitors do not disappear (except in a small proportion) over time. This effect has been shown for the LDV/SOF regimen where NS5A RAV were detectable up to 2 years after treatment.[24] The HCV TARGET trial and another study in Japan showed that baseline RAV influenced SVR rates.[25,26] Due to the limited data available, it remains to be seen whether, despite high response rates in controlled conditions, RAV may become a sudden problem in the real world of suboptimal regimens and settings.

Real-World Experience: What Have We Learned About Advanced Liver Disease?

Professor Robert Brown

Liver disease, such as cirrhosis, is more common and develops more rapidly in HCV-positive patients than HCV-negative patients.[27] Survival of patients with compensated cirrhosis is significantly longer than in patients with decompensated cirrhosis. About 7% of patients per year will transition from compensated cirrhosis Stage 1 to Stage 2, while between 7% and 10% will move from compensated to rapid decompensation stage. Over 50% of decompensated patients will die each year, and there is currently no US Food and Drug Administration (FDA) or Medical Devices Agency (MDA) approved medication to improve their chances of survival. If the patients do not improve and recompensate, they will have no long-term benefit, unless their condition is linked to transplantation.[28]

Following successful clinical trials, DAA treatments are now available for patients with HCV and advanced liver disease. Most randomised trial data, such as from TURQUOISE-II, focusses on compensated or CP-A cirrhosis and shows high efficacy and safety for all 3D regimens with RBV. In the TURQUOISE-III safety trial (N=60), RBV was removed for GT1b patients with cirrhosis, yet high SVR rates with no decompensating events were still achieved.[12] Pooled data for >1,000 patients with cirrhosis from 12 Phase II/III trials on the 3D regimen±RBV showed that 1.2% of patients decompensated lower than the expected 7% per year during the trial. Lower baseline albumin, prior non-selective beta blocker use for varices, and lower baseline HCV RNA were independently associated with hepatic decompensation events.[29] Excellent safety and efficacy are also seen in patients (N=509) with GT1a/b compensated cirrhosis on LDV/SOF+RBV, with SAEs observed in only 3% of patients.[30]

As mentioned, high SVR rates are achieved for cirrhotic patients on DAAs in trials, however in the real world, clinicians may be more lenient with certain baseline factors such that some patients who might be on the borderline of decompensation will be treated. Efficacy and safety data are still excellent in most of the real-world experiences.

Many real-world cohorts currently include patients with advanced fibrosis or cirrhosis. The AMBER cohort (N=186) in Poland was a heterogeneous group of patients with HCV GT1, 76% of whom had F3/F4 fibrosis. Similar to trial results, these patients, followed for 12 or 24 weeks on OBV/PTV/r±DSV±RBV, once again exhibited low rates of decompensating events (1.6%) but not out of proportion to either what was expected or what was seen in the trial.[31]

In Israel, 12 sites recruited 661 GT1-infected patients with F3/F4 fibrosis treated with OBV/PTV/r+DSV±RBV. Similar to Phase III trial results, SVR rates of 99% were achieved, with only 1% of patients having hepatic decompensation (half of these continued therapy and achieved SVR).[32] The ABACUS study in Italy included a large population of cirrhotic patients and, once again, an SVR rate >95% was observed with very few SAEs and a low rate of decompensation.[13]

Multiple real-world cohorts in Spain with patients on OBV/PTV/r±DSV±RBV showed excellent SVR rates. In Madrid, only 2% of GT1b-infected or cirrhotic patients (N=823) had SAEs.[33] Another retrospective, multicentre analysis in Spain, of GT1 and GT4-infected patients (N=139) with or without fibrosis, showed low rates of SAEs and no hepatic decompensation events.[34] A cohort (N=177) in Barcelona also exhibited excellent efficacy and safety results, although with more SAEs (23%), but fewer discontinuations and no decompensation cases during interim analysis.[35]

In the USA, the observational analysis of >4,000 treatment-naïve veterans with HCV treated in routine medical practice with LDV/SOF±RBV for 8 or 12 weeks revealed slightly lower SVR rates (87–91%) amongst cirrhotic patients. RBV was prescribed at the discretion of the investigator, so it is not known what impact the addition of RBV to a 12-week over a 24-week regimen would have had.[36] The US TRIO cohort contained pharmacy data from centres that agreed to participate. Data confirmed that while efficacy of treatment with LDV/SOF >12 weeks was good for cirrhotic or treatment-naïve GT1a/b patients, the addition of RBV and/or the extension of treatment to 24 weeks for treatment-experienced cirrhoticswould be needed to achieve the desired SVR. Prescribing DAAs outside of the FDA-approved labelling had a negative impact on SVR rates.[37]

An extensive early access programme in Europe provided access to DCV before market authorisation to >7,000 patients in urgent need of HCV treatment and who had no other treatment options. Patients with mixed GTs that had severe liver disease were treated with DCV/SOF±RBV. SVR rates achieved at 24 weeks were high in patients with CP-A cirrhosis.[38]

While DAAs are well tolerated, with high rates of SVR in the real world and as reported in clinical trials in patients with compensated cirrhosis, none of the mentioned regimens are approved in decompensated cirrhosis. The contraindications are based on either the label or recommendations (Table 1).

Table 1: Label recommendations for hepatitis C virus genotype-1 infected patients with compensated and decompensated cirrhosis.[4,6-8]

Regimen	Child–Pugh A Mild hepatic impairment	Child–Pugh B Moderate hepatic impairment	Child–Pugh C Severe hepatic impairment
OBV/PTV/r+DSV±RBV	Recommended	Not recommended	Contraindicated
LDV/SOF±RBV	Recommended	Recommended	Recommended
SMV+SOF±RBV	Recommended	Not recommended	Not recommended
DCV+SOF±RBV	Recommended	Recommended	Recommended

OBV: ombitasvir; PTV: paritaprevir; r: ritonavir; DSV: dasabuvir; RBV: ribavirin; SOF: sofosbuvir; SMV: simprevir; DCV: daclatasvir; LDV: ledipasvir.

Table 2: Pharmacokinetics of direct-acting antivirals with severe renal impairment (estimated glomerular filtration rate <30 mL/min/1.73 m²).[6-8,45,47]

Direct-acting antiviral	Increase in AUC compared with healthy subjects (%)
Ombitasvir	No change
Paritaprevir	≤50
Ritonavir	114
Dasabuvir	≤50
Ledipasvir	Not relevant
Sofosbuvir GS-331007	171 451
Simeprevir	62
Daclatasvir*	51
Grazoprevir	65
Elbasvir	86

*Estimated for creatinine clearance level=15 mL/min
AUC: Area under the curve.

ALLY-1 (DCV+SOF+RBV for 12 weeks)[39] and SOLAR-2 (LDV/SOF+RBV for 12/24 weeks)[40] trials in patients with decompensated cirrhosis produced good SVR rates in CP-A[39] and CP-B, while for CP-C the results are small in number and are variable to suboptimal.[39,40]

Treatment of HCV GT1 or GT3-infected patients (N=467) with decompensated cirrhosis (however, only 10% CP-C) also highlighted that more data are still required for GT3, particularly for patients with more advanced disease.[41] Similar observations have been made from the European early access programme.[38] In summary, real-world experience shows good efficacy results for CP-B, while those for CP-C remain questionable.

Real-World Experience: What Have We Learned About Chronic Kidney Disease?

Professor Antonio Craxí

HCV-infected patients have a 23% higher risk of presenting with CKD compared with patients without the virus.[42] In a hospital population of US veterans (N=100,518) with HCV, 11.2% had renal impairment with a very high incidence of 16.7/1,000 patient-years.[43] Furthermore, in a population where the risk of HCV infection is intrinsically high, such as dialysis patients, the prevalence of HCV is very high (3–68%)[44] against the background rate of HCV in that specific population and perhaps for specific countries.

CKD is defined as kidney damage with or without impaired kidney function for ≥3 months with implications for health. Renal function is evaluated using estimated glomerular filtration rate (eGFR), a measure of the flow rate of the kidneys, calculated from the creatinine clearance level (CrCl), and the age, sex, and race of the individual. Renal damage may have many causes; along with HCV, cryoglobulinaemia and nephritis are also quite prevalent causes. Many of the current drugs cannot be used or must be used with caution for patients with severe renal impairment, and renal function may affect the pharmacokinetics and pharmacodynamics of the medication (Table 2).[7,8,45-47] HCV seropositivity by itself is also a significant risk factor for proteinuria in the general population.[48] A meta-analysis of >800,000 patients from nine different observational studies showed a rather high prevalence of HCV infection, making these patients intrinsically predisposed to have renal damage. In many cases patients with HCV are almost 1.5-times more at risk of having proteinuria than those who are not infected.[48] It is therefore very important that physicians recognise how HCV predisposes patients to renal deficiency.

The current American Association for the Study of Liver Diseases (AASLD)[49] and EASL guidelines[50] state that treatment, particularly in patients with cryoglobulinaemia, is imperative and should be

prioritised to avoid organ damage outside the liver. At this stage however, no universal treatment is suggested. Patients on long-term haemodialysis should also be prioritised or considered for HCV therapy due to an increased risk of nosocomial transmission and also because being HCV-negative increases the chances of being considered for a renal transplant.

Currently, regimens approved for use in patients with mild or moderate renal impairment include: OBV/PTV/r+DSV±RBV; LDV/SOF; EBR/GZR; SOF; SMV+SOF; and DCV+SOF. However, patients with severe renal impairment show a significant difference in terms of eGFR and CrCl when treated with drugs such as OBV, LDV, or SOF (rather low SVR rates when combined with RBV). At present, OBV/PTV/r+DSV±RBV and EBR/GZR are the approved treatment options for patients with severe renal impairment and end-stage renal disease (ESRD). These patients, including those on haemodialysis, exhibit up to 95% SVR rates without the use of RBV (RUBY-I study).[51] Patients on EBR/GZR showed similar rates (94–99%); however, the frequency of SAEs was rather significant at 14% (C-SURFER study).[9,52] These SVR rates are comparable to those observed in non-renal patients.

Following successful clinical trials, there are now numerous national and international cohorts assessing HCV treatments in patients with renal insufficiency (potentially a consequence of liver disease) in the real-world setting. In a Spanish cohort of 100 patients with advanced CKD (37% on dialysis), 47 have shown a promising SVR rate of 89% following treatment with various regimens, with no significant safety problems.[53] Another cohort from Spain (N=33) on the 3D regimen with or without a 200 mg dose of RBV produced 100% real-world SVR rates, comparable to those seen in Phase III clinical trials.[54] In the ABACUS study in Italy, involving GT1-infected patients with cirrhosis and treated with 3D+RBV, all patients with CrCl <30 mL/min and very low eGFR (i.e. with severe renal deficiency) achieved a high SVR,[13] comparable to those reported in clinical trials. In the HCV TARGET, real-world SVR rates of 80–100% were achieved in patients treated with SMV+SOF±RBV across baseline eGFR and independent of renal failure. However, safety data show significant numbers of cases of anaemia when CrCl was low, as well as progressive worsening of renal function with CrCl ≤30 mL/min; similar patterns were observed for adverse events.[53]

SOF-based regimens have been used widely for patients with HCV as well as those with renal failure. Although not recommended in the summary of product characteristics for SOF, real-world studies in North America and Europe have evaluated SOF-containing regimens in patients with severe renal impairment or ESRD on haemodialysis.[55] Two studies from France reported 86–100% SVR rates, although some treatment-related issues have occurred; however, pharmacokinetics of the drug in a patient on dialysis are entirely different from those with renal failure and no dialysis.[56,57] In the US and Canada, renal impairment with DAA therapy was also evaluated and compared with that of first-generation protease inhibitors. The first-generation inhibitors cause problems in 25% of patients, regardless of the response rate, while SOF-based regimens caused problems with renal function resulting in renal impairment in 14% of patients.[58]

While current treatment is effective in a clinical setting, on-treatment renal impairment can occur with some DAA regimens. Safety profiles need to be explored further and in larger cohorts, and need to be carefully considered when treating HCV-infected patients with severe renal impairment.

Question and Answer Session

Professor Ira Jacobson

Q: Does a patient with ESRD, who previously achieved an SVR with treatment, wait for a HCV-negative kidney transplant or be treated right away with a HCV-positive kidney?

A: Using HCV-positive organs in HCV-infected patients regardless of the status will significantly improve the chances of patients with transplants because you can treat these patients. They will get re-infected, possibly with a different strain of HCV, and they can be retreated.

Summary and Close

Professor Ira Jacobson

After almost three decades of clinical trials, a virological cure is now seen in >95% of HCV-infected patients. The medication is effective in a broad range of patients, including those historically considered difficult-to-treat. It is easy to adhere to, has fewer contraindications, and very few serious side effects.

All the real-world studies and registries combine over 43,000 patients, and show that DAAs are well tolerated with very high rates of SVR in clinical practice. However, some populations still have unmet medical needs. These include patients with: RAV; severe renal impairment; GTs 3, 5, and 6; decompensated cirrhosis; DAA failures; and hepatocellular carcinoma. Hepatocellular carcinoma poses a number of issues such as screening, prevention, pathogenesis, and timing of antiviral therapy. The question also remains with regard to compensated cirrhotics: will their improved Model for End-Stage Liver Disease (MELD) scores derail them from their trajectory towards transplantation, yet leave them at the risk of life-threatening complications, and poor quality of life? Another set of patients that could see improved SVR rates are the CP-C group.

Overall, it appears that next-generation regimens will address many of these unmet needs. For example, ASTRAL data show a 95% SVR rate to SOF and velpatasvir for GT3. SURVEYOR-I and II trials present effective pan-genotypic NS5A inhibitors ABT-493 and ABT-530 for patients with or without cirrhosis. Finally, triplet regimens consisting of advanced protease and NS5A inhibitors combined with nucleotide polymerase inhibitors show promise both for initial therapy, with the potential to shorten duration of therapy for many patients, and for re-treatment of patients who have failed to have SVR with first-generation regimens. As this field evolves, the next-generation regimens in HCV will have pan-genotypic efficacy (GT1–6), a high barrier to resistance, efficacy in patients with unmet medical needs, and simple dosing.

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EFFICACY AND SAFETY OF ELBASVIR/GRAZOPREVIR VERSUS SOFOSBUVIR/ PEGYLATED INTERFERON/ RIBAVIRIN IN TREATMENT-NAÏVE AND PEGYLATED INTERFERON RIBAVIRIN PRIOR TREATMENT FAILURE SUBJECTS WITH CHRONIC HEPATITIS C VIRUS GENOTYPE 1 OR 4 INFECTION

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BACKGROUND AND AIMS

Direct-acting antiviral agents (DAAs) are the standard of care for the treatment of chronic hepatitis C virus (HCV) infection and there are currently no head-to-head trials directly comparing DAA regimens. Treatment with sofosbuvir (SOF), pegylated interferon alpha (PEG), and ribavirin (RIBA) was the standard of care at the time our protocol was designed. The addition of SOF to PEG/RIBA increased efficacy compared with PEG/RIBA alone. The adverse effects due to PEG/RIBA remained. In the NEUTRINO trial, evaluating SOF/PEG/RIBA for 12 weeks, the overall sustained virological response rate at 12 weeks (SVR12) was 90%. There was a difference in efficacy between cirrhotic and non-cirrhotic patients, and between patients infected with genotype 1a and 1b. The most common adverse events and laboratory abnormalities were flu-like syndrome, anaemia, and neutropenia.

STUDY DESIGN

We investigated elbasvir/grazoprevir (Zepatier™), recently approved in the USA and Canada for genotypes 1 and 4, and in Canada for genotype 3 in combination with SOF. This is an all-oral, once-daily, fixed-dose combination of elbasvir (NS5A inhibitor) and grazoprevir (NS3/4A protease inhibitor). There is broad activity against most genotypes *in vitro*. There is high efficacy in treatment-experienced patients, patients with cirrhosis, patients with HIV/HCV co-infection, and patients with end-stage kidney disease, based on results of previous trials. This was a randomised, open-label study conducted in the European Union and Turkey that enrolled treatment-naïve and PEG/RIBA treatment-experienced patients. All patients were to be treated for only 12 weeks.

Patient Characteristics

Patients are well distributed with respect to gender, age, race, baseline viral load, genotype, presence of cirrhosis, and prior treatment status.

The majority of patients are genotype 1b, reflecting the regions involved in the trial. Approximately 17% of enrolled patients had cirrhosis according to their Metavir score.

EFFICACY

Statistical Analysis

Efficacy was assessed in the full analysis set population, which includes all randomised patients who received at least one dose of study medication. The primary efficacy endpoint was achievement of a SVR12 (negative virus in the blood). The analysis followed a stepwise approach. First non-inferiority of efficacy was assessed, then superiority of safety, then superiority of efficacy. Non-inferiority was assessed by examining the difference in efficacy between the two groups. If the lower bound of the 2-sided 95% confidence interval (CI) was >10%, then non-inferiority was achieved. If non-inferiority was met, then superiority of safety was assessed. If the superiority of safety was achieved, then superiority of efficacy was assessed. Finally, if the lower bound of the 2-sided 95% CI for the difference between SVR12 rates in the treatment groups was >0, then superiority of efficacy was achieved.

Results

In the elbasvir/grazoprevir group, the overall SVR12 rate was 99.2%. In this group, all patients achieved SVR12 and one patient was classified as a failure due to discontinuation/loss to follow-up. The patient discontinued from the trial at treatment Week 12 and HCV RNA was negative. In the SOF/PEG/RIBA group, the overall SVR12 rate was 90.5%. Eleven patients relapsed after treatment. One patient did not achieve SVR12 due to study discontinuation at treatment Week 1. The reason for discontinuation was the presence of a flu-like syndrome. The difference in efficacy rates between the two groups was 8.7%, and the lower bound of the 95% CI was 3.6%. This finding confirmed both non-inferiority and superiority of efficacy of the elbasvir/grazoprevir regimen. In genotype 1a, the SVR12 rates were 100% in both arms. In genotype 1b, SVR12 in the elbasvir/grazoprevir group was 99.0%, and in the SOF/PEG/RIBA group it was 90.4%. Nine genotype 1b patients in the SOF/PEG/RIBA group relapsed. In genotype 4, SVR12 was 100% in the elbasvir/grazoprevir group, and 60.0% in the SOF/PEG/RIBA group, where two of the six patients relapsed.

Subgroups Analysis

There was a significant difference between the two groups favouring elbasvir/grazoprevir in male patients, patients with non-CC IL28B genotype, in patients with cirrhosis, in patients with high viral load, and in prior PEG/RIBA null-responders.

SAFETY

Statistical Analysis

Superiority was assessed in the 'all subjects as treated' population, which includes all randomised patients who received at least one dose of study medication. Superiority of safety was achieved if the proportion of subjects in the elbasvir/grazoprevir group who experienced a Tier 1 adverse event (drug-related adverse events, anaemia, neutropenia, depression, hepatic event of clinical interest) was lower than the proportion of subjects in the SOF/PEG/RIBA group. Tier 1 safety events were chosen because they provided objective assessment concerning broad tolerability, haematological side effects (which are of particular concern for PEG/RIBA containing regimens), and liver-related laboratory abnormalities (which have been reported previously in patients receiving high doses of grazoprevir). Tier 2 adverse events included any adverse event, any drug-related adverse event, and any serious adverse event.

Results

A significantly lower number of patients experienced a Tier 1 adverse event in the elbasvir/grazoprevir group compared with SOF/PEG/RIBA. This difference is influenced by the haematological side effects, including anaemia and neutropenia. Importantly, no subject in either group experienced a hepatic event of clinical interest. Of the Tier 2 adverse events, a greater proportion of patients

in the SOF/PEG/RIBA group experienced adverse events, drug-related adverse events, and serious adverse events.

CONCLUSIONS

a) Superior efficacy of the 12 weeks fixed-dose combination of elbasvir/grazoprevir in genotype 1 and genotype 4 infected patients compared to 12 weeks of SOF/PEG/RIBA has been demonstrated, including high efficacy in important subpopulations such as prior PEG/RIBA null-responders, patients with cirrhosis, and patients with a high baseline viral load.

b) Superior safety profile of elbasvir/grazoprevir compared with 12 weeks of SOF/PEG/RIBA is based on the following facts:

- No serious drug-related adverse events
- No discontinuations due to drug-related adverse events
- Superior haematological safety profile
- No hepatic safety events

THE REAL-WORLD ISRAELI EXPERIENCE OF TREATING CHRONIC HEPATITIS C, GENOTYPE 1 PATIENTS WITH ADVANCED FIBROSIS USING PARITAPREVIR/RITONAVIR/OMBITASVIR AND DASABUVIR, WITH OR WITHOUT RIBAVIRIN: A LARGE MULTICENTRE COHORT

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Background and Aims

The paritaprevir/ritonavir/ombitasvir and dasabuvir with or without ribavirin (3D±R) regimen is approved in the USA and Europe for chronic hepatitis C (CHC) patients with genotype (GT)-1 and 4. This regimen has been demonstrated to be safe, well tolerated, and highly efficacious, achieving a sustained virological response rate of >95% at follow-up Week 12 (SVR12) in non-cirrhotic as well as cirrhotic patients in Phase III registration trials. 'Real-world' (RW) treatment may differ from registration trials in many aspects such as efficacy and tolerability as included patients may have heterogeneous compliance patterns and significant comorbidities. Data on RW treatment experience with 3D±R regimen is limited.

This regimen has recently been approved in Israel for GT-1, CHC patients with advanced fibrosis only (stages F3 and F4). We have collected data from a large cohort in order to evaluate safety and efficacy in a RW setting.

Methods

Data on demographics, clinical features, safety, and virological response were collected from 12 centres in Israel. To date, 661 patients initiated the 3D±R regimen for 12 or 24 weeks, of whom 410 were cirrhotic. The primary efficacy end-point was a SVR12; special attention was paid to serious adverse events (SAEs) and early treatment discontinuation.

Results

Of the 661 patients, 44% were males, mean age was 60 years old (19–84) with 28% aged ≥65 years, 410 (62%) were treatment-experienced with pegylated interferon and ribavirin, GT-1b was dominant (86%), and 22 patients received a liver transplant. There were 410 (62%) patients who were cirrhotic, 28% of whom had oesophageal varices and/or platelet count <90,000/mL, and 10% had a Model for End-Stage Liver Disease (MELD) score >10. To date, 633 patients have completed therapy; 528 reached the point of SVR12 and in 432 patients the polymerase chain reaction (PCR) results of SVR12 were available.

In regards to safety, SAEs were observed in 25 (3.8%) patients: severe anaemia (11 patients), severe infection (5 patients), and others. Twenty-three (3.4%) patients discontinued therapy prematurely (including 4 of 22 post-transplant patients), eight of them due to hepatic decompensation manifested by ascites, and elevated bilirubin in most cases. Decompensation occurred from Day 2 and up to 8 weeks after initiation of therapy. Three of the eight patients were older than 75 years old (77, 78, 80), three had a baseline MELD score >10, five had portal hypertension, and three had experienced previous decompensation. One patient died due to multi-organ failure. In all others, decompensation had been resolved.

Efficacy of the treatment was determined by interim intention-to-treat (ITT) analysis, which showed negative PCR at the end-of-treatment in 579 of 582 patients (99.5%) and a SVR12 in 412 of 432 patients (95%). After excluding patients who did not achieve a SVR12 for reasons other than virological failure (virological relapse was noted in four patients), the modified ITT SVR12 was 99% (412 out of 416). The SVR12 rate was similar among non-cirrhotic and cirrhotic patients. Among 22 post-transplant patients, 19 achieved a SVR12 (86%).

Conclusion and Discussion

Treatment with the 3D±R regimen in a RW setting is highly effective in GT-1 CHC patients with advanced fibrosis including those with cirrhosis achieving a high rate of SVR12. Treatment with this regimen appears to be safe and the vast majority of adverse events are manageable. However, caution should be used and closer monitoring may be required in older patients with portal hypertension and a borderline MELD score (>10) or Child–Pugh Class A, and especially in those who have experienced decompensation in the past, in order to avoid hepatic decompensation.

THE ROLE OF INFLAMMASOME IN PATHOGENESIS OF ALCOHOLIC LIVER DISEASE

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Inflammation defines the progression of alcoholic liver disease (ALD) from reversible to advanced stages. Inflammation in ALD is dependent on Kupffer cells (KC), the resident liver macrophages. Traditionally, activation of KC is explained by the gut–liver axis theory, whereby alcohol increases translocation of bacterial components, such

as lipopolysaccharides (LPS), from the gut to the liver and increases production of tumour necrosis factor (TNF)-α by KC, thereby triggering liver inflammation.[1]

However, translocation of LPS from the gut to the liver is non-specific and therefore it is not known how KC discriminate truly noxious pro-inflammatory signals from background noise.[2] This is unlikely to be explained by high amplitude signals, because in ALD there is a relatively low increase of LPS.[3] The alternative hypothesis is that in addition to LPS derived from the gut, KC require a co-stimulatory signal from a different source. This researchproposes that hepatocytes damaged by alcohol release co-stimulatory signals for inflammation in ALD.

It has been shown that culture supernatants of hepatocytes damaged by ethanol double the production of TNF-α in response to LPS by KC, suggesting that damaged hepatocytes release pro-inflammatory signals.[4] This is consistent with the danger model of immune activation, according to which not only immune cells but also damaged tissues control inflammation.[5] This occurs via signalling triggered by danger signals that are released from host cells undergoing necrosis. Some danger signals, such as uric acid or adenosine triphosphate (ATP), feed into two signal pathways that are mediated by inflammasomes, which are intracellular multimeric complexes that sense danger signals and activate interleukin (IL)-1β and IL-18.[2]

Historical data showed that patients with alcoholic hepatitis have high levels of IL-1 in the blood or that alcohol induces hyperuricaemia,[6,7] however the role of the inflammasome in ALD has never been formally evaluated. Firstly, we have shown that media from damaged hepatocytes, but not from healthy hepatocytes, activate the inflammasome in KC primed by LPS.[4] Secondly, activation of the inflammasome in livers of alcohol-fed mice occurred in KC, but not in hepatocytes.[8] Thirdly, mice deficient in caspase-1, the effector caspase of the inflammasome, are protected from alcohol-induced liver inflammation. Finally, using mice with cell-specific deficiency in caspase-1 either in KC or in hepatocytes, we demonstrated that the effect of the inflammasome is KC specific in alcohol-induced liver inflammation.[8]

A wide variety of danger signals are released from damaged hepatocytes exposed to alcohol and many will activate the inflammasome. As a proof-of-principle, we identified two sterile danger signals: uric acid and ATP. Both are increased in the blood of healthy individuals after drinking ethanol and they are also released by hepatocytes exposed to ethanol.[9] Both are recognised by the inflammasome sensor protein, NLRP3, which is required for inflammasome activation in ALD.[9] In KC, activation of the inflammasome was ameliorated by depletion of ATP or uric acid with apyrase or uricase, respectively, and completely prevented by depletion of both. Mice overexpressing uricase, or mice treated with allopurinol or probenecid, thereby depleted of uric acid, do not activate caspase-1, suggesting that uric acid is required for activation of the inflammasome *in vivo*. In addition, mice deficient in ATP receptors showed similar prevention of inflammasome activation in the liver upon depletion of uric acid.[4,9]

Finally, we focussed on IL-1β, one of the two effectors of cytokines activated by the inflammasome. To date, there are no published data on IL-18 in the context of ALD. IL-1β is one of the few cytokines competing for its receptor with an endogenous antagonist, IL-1 receptor antagonist (IL-1Ra). Anakinra is a recombinant form of the natural IL-1Ra. Administration of IL-1Ra improved liver histology and inflammation in a dose-dependent fashion in a mouse model of ALD.[8]

With the discovery of the role of the inflammasome in ALD, IL-1 has become a potential therapeutic target that may be safer than TNF-α because it is not required for antimicrobial defence. Consequently, anakinra and rilonacept (an IL-1 blocking antibody) are in two currently ongoing clinical trials.[10,11] As ALD is a complex disease mostly caused by factors beyond IL-1β, other treatments are combined with IL-1 inhibition in these trials.

In summary, these new data substantially broaden the complexity of inflammation in ALD. Thus, inflammation in ALD is not only dependent on

gut-derived LPS, but also requires a second signal in the form of sterile danger molecules released from hepatocytes which have been damaged by alcohol. Both signals are integrated by the inflammasome, thereby releasing IL-1β. It is hypothesised that this mechanism may enable KC to distinguish true inflammatory signals from background noise. Future studies will show whether these principles can be successfully implemented into clinical applications and treatments in alcoholic hepatitis.

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ALCOHOLIC HEPATITIS: CURRENT AND FUTURE TREATMENTS

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Alcoholic hepatitis (AH) is a clinical syndrome characterised by the recent onset of jaundice and liver failure, which occurs in a minority of patients with ongoing alcohol abuse. The diagnosis is usually based on a history of heavy alcohol use, findings from blood tests, exclusion of other liver diseases, and is confirmed through a liver biopsy, usually performed by a transjugular route. Histological findings included steatosis, hepatocyte ballooning, and an inflammatory infiltrate with polymorphonuclear neutrophils. Severe AH is commonly defined by a Maddrey's discriminant function ≥32. This severe form, which occurs predominantly in patients with a background of cirrhosis, is associated with a poor short-term prognosis and a 3-month mortality of 30–50%. European[1] and USA guidelines for alcoholic liver disease recommend the use of corticosteroids or pentoxifylline in patients with severe AH. A meta-analysis using individual patient data from five recently published randomised controlled trials showed that 28-day survival rates were higher for corticosteroid-treated patients than for non-corticosteroid-treated patients. A survival benefit was also observed at 1 month for corticosteroid treatment versus placebo after adjustments for baseline determinants of prognosis in the largest randomised controlled trial in severe AH to date.[2] The applicability of corticosteroid therapy is limited by concerns about the risks of infection. N-Acetylcysteine in combination with corticosteroids has been shown to improve short-term survival. Only short-term increases in survival can be expected with available therapies. No treatment has been found to increase patient survival >3 months. Malnutrition is present in almost every patient with AH, and is associated with impaired survival. Recently however, our

group demonstrated in a randomised controlled trial that the systematic administration of intensive enteral nutrition using a feeding tube in addition to corticosteroid treatment did not improve survival rates when compared with conventional nutrition and corticosteroids.[3] Interestingly, we observed that regardless of the allocated therapy, daily calorie intake was associated with 1 and 6-month mortality. Patients with a daily calorie intake <21.5 kcal/kg of body weight had a significantly higher risk of death and infections. Absence of serum bilirubin decrease or a Lille score >0.45 at Day 7 of steroid therapy identified non-responders and is associated with a very poor prognosis (survival rate 25–30% at 6 months).[4] No medical therapeutic option is available for those patients. Liver transplantation in highly selected non-responders has been demonstrated as effective and associated with a low risk of alcohol relapse, although this strategy challenges the classical 6-month rule of abstinence and thus results from a multicentre clinical trial are eagerly awaited.

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HEPATITIS C DIAGNOSTICS AND POINT OF CARE TESTING: TOOLS TO ENHANCE SCREENING AND DIAGNOSIS

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Globally there are an estimated 115 million people with antibodies to the hepatitis C virus (HCV) and 2 million of them are HIV co-infected.[1] Critically, the most recent World Health Organization (WHO) guidelines launched at ILC 2016 recommend prioritisation criteria rather than eligibility criteria for HCV treatment to facilitate a 'test and treat' approach. This recognises the benefits of early treatment, such as the reduction of liver and non-liver related morbidity[2] and decreased risk of transmission, while acknowledging the lack of resources in countries and the need for a progressive scale-up over time.

There are no comprehensive screening programmes for HCV in low and middle-income countries, meaning that the majority of people are unaware of their status. One barrier is the lack of fit-for-purpose and quality assured tests that can be used at the point-of-care. Prior to the introduction of new, all oral, pan-genotypic, direct-acting antivirals (DAAs), toxic and often ineffective interferon-based treatment was used. This required a much more comprehensive set of tools to diagnose, genotype, stage, and monitor the treatment's toxicity and a person's treatment response. A major advantage of DAAs is the facilitation of an extremely simplified diagnostic and monitoring strategy. Now, testing can be reduced to a qualitative virological test at diagnosis, optional infrequent monitoring, including of alanine transaminase, creatinine, and haemoglobin (all readily available tests), and a qualitative test of cure (sustained virological response at 12 weeks).

The majority of those with chronic HCV have viral loads >10,000 IU/mL, and preliminary evidence shows that the few people failing DAA therapy rebound with viral loads >1,000 IU/mL,[3] yet current guidelines still recommend an analytical sensitivity down to <25 IU/mL with no evidence of whether this is necessary for an acceptable clinical sensitivity. Clarification of this is urgently needed so that a feasible limit of detection can be reached that is supported by guidelines for uptake at country

level, particularly for manufacturers of point-of-care tests, and for the validation of dried blood spots as a sample type, especially when measuring core antigen.

For a simplified diagnostic strategy to be realised, a number of aspects need to be addressed: 1) political will and funding from countries and donors; 2) simple capillary blood-based point-of-care tests for serological screening and qualitative core antigen or RNA testing (supported by a clear market for quality-approved tests); 3) evidence to support a simplified diagnostic strategy together with guidelines that recommend (i) test and treat approaches to obviate the need for staging, (ii) pan-genotypic regimens to obviate the need for genotyping, and (iii) no viral load monitoring; and 4) access to affordable DAA regimens for people living in all low and middle-income countries. Failing to implement such a strategy may prevent advances in DAA therapy from reaching people in developing countries, and they will continue to unnecessarily bear the burden of HCV disease.

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FUNCTIONAL IMPROVEMENT OF HEPATOCYTE-LIKE CELLS WITH TRANSGENE ELIMINATING FROM HUMAN INDUCED PLURIPOTENT STEM CELLS

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Induced pluripotent stem cells (iPSCs) have become an important tool in human disease therapy and have previously been generated by introducing four Yamanaka factors via a retrovirus. However, these genes are integrated into the host genome and so cannot be used in a clinical application. Foreign genes result in insertion mutations, residual expressions, and reactivation of transgenes during differentiation, which prevent their differentiation potential. In this report, we introduce a transgene-free iPSC generation method and its differentiation into hepatocytes.

MATERIALS AND METHODS

We generated transgene-free iPSCs with the Cre–loxP system, which was used to remove integrated transgenes from the genome. Human newborn foreskin fibroblasts were infected with a transgene expressing polycistronic lentivirus for 3 days, after which they were switched to a mTeSR1 media. The media was switched every day. In order to confirm the pluripotency of the colony, live staining was conducted with a TRA-1-60 antibody. TRA-1-60-expressing colonies were selected and cultured on a matrigel coated dish. To remove transgenes from the iPSCs colony, they were treated with Cre recombinase for 2 hours and then cultured until colonies grew out. We confirmed that transgenes were not expressed in the genome by polymerase chain reaction (PCR). We found efficient methods for the differentiation of iPSCs into a homogeneous population of functional hepatocytes. For endoderm differentiation, the iPSCs were treated with activin A for 6 days, and then with hepatocyte growth factor and

fibroblast growth factor 4 for 5 days. Human iPSC (hiPSC)-derived hepatocyte marker expression was confirmed by PCR, immunocytochemistry, and periodic acid–Schiff staining.

RESULTS

Two weeks after transgene lentivirus infection, pluripotent colonies appeared in the feeder-free conditions of the mTeSR1. The colonies expressed TRA-1-81, Oct4, Nanog, Sox2, and Myc as well as TRA-1-60. Transgenes were removed by Cre recombinase. Without transgenes, pluripotent marker expression was not altered. hiPSC-derived hepatocytes expressed not only hepatocyte specific markers, but also demonstrated markers of liver function, such as albumin, α-fetoprotein, the asialoglycoprotein receptor, glycogen storage, and cytochrome P450 activity. In conclusion, we have generated transgene-free hiPSCs and functional hepatocytes *in vitro*, and these results represent a step forward in the development of clinically applicable patient-specific hepatocytes for cell-based therapeutics.

LESS OESOPHAGEAL VARICEAL BLEEDING IN CHRONIC HEPATITIS B PATIENTS WITH CIRRHOSIS WHO USE STATINS: AN ALTERNATIVE FOR PROPHYLAXIS?

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Hepatitis B virus infection is a major health issue and the main cause of cirrhosis worldwide. Oesophageal variceal bleeding (OVB) is an important complication of portal hypertension (PHT) due to cirrhosis and a major cause of death. Non-selective beta blockers (propranolol and nadolol), as well as endoscopic variceal ligation (EVL), are recommended for primary prophylaxis of variceal bleeding. However, some patients are intolerant to propranolol and nadolol, whereas others have limited access to technically competent EVL centres.

Statins reduce the risk of cirrhosis and its decompensation in chronic viral hepatitis. Statins induce vasodilation, and reduce fibrosis progression and portal pressure, possibly by inhibiting endothelin-1. PHT correlates well with OVB, although direct demonstration of the ability of statins to prevent a first OVB occurrence is lacking.

The Taiwanese National Health Insurance (TNHI) programme was launched in March 1995. To date, >99% of the residents of Taiwan are enrolled. We conducted a nationwide cohort study using the TNHI research database to evaluate the risk of first OVB in statin users. A total of 139,738 chronic hepatitis B (CHB) patients with cirrhosis were identified from 1997–2009. Patients in the statin cohort (n=2,222) were matched 1:1 with those not taking statins based on age, sex, hepatic impairment, and inception point. For comparable severity of hepatic impairment, we use the presence of ascites and hepatic coma in matching the case and control. The cohorts were followed for up to 10 years, starting from the date of initial statin use (or match date) until the first episode of variceal bleeding, withdrawal from insurance, or December 2009.

After adjustment for competing mortality, the cumulative incidence of first OVB was lower in the statin cohort than in the non-statin cohort (relative risk=0.167; 95% confidence interval [CI], 0.124–0.225; P<0.001). The adjusted hazard ratio (AHR) of statin users for OVB was 0.057 (95% CI: 0.041–0.080; P<0.001) after controlling for age, sex, use of a beta blocker, CHB treatment, hepatocellular carcinoma, comorbidity index, EVL, non-statin lipid-lowering drug use, and triglyceride-lowering drug use. The AHRs of statins were 0.063, 0.060, and 0.056 in the subgroups of 28–45, 46–120, and >120 yearly time-weighted mean cumulative defined daily doses (TWM cDDDs) of statins, respectively, compared with non-users (<28 yearly TWM cDDDs). Furthermore, statin users have lower overall mortality.

This population-based nationwide cohort study demonstrated the protective effect of statins for first episode of OVB in CHB patients with cirrhosis. Statins reduce the occurrence of OVB independent of non-selective beta blockers or EVL, which is the current recommendation for primary prophylaxis. This association was stronger with higher doses and longer durations of statin use. This study provided evidence to further support the pleiotropic effect of statins in advanced liver diseases. A randomised controlled trial is warranted to confirm the results of this study before statins can be recommended as an alternative for primary prophylaxis of OVB.

THE EFFECT OF MTORC1 INHIBITION ON NON-ALCOHOLIC FATTY LIVER DISEASE AND SUBSEQUENT HEPATOCELLULAR CARCINOMA DEVELOPMENT

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Since mTORC1 is activated in up to 50% of hepatocellular carcinoma (HCC) cases, there has been much interest in the use of mTORC1 inhibitors for HCC treatment. mTORC1 is also activated in response to obesity, which greatly enhances HCC risk in both humans and rodents. We hypothesised that mTORC1 inhibition attenuates obesity-induced fatty liver (non-alcoholic fatty liver disease) and its subsequent HCC development.

Rapamycin treatment improved fatty liver in high-fat diet fed obese mice; surprisingly however, such mTORC1 inhibition also resulted in increased interleukin (IL)-6 production, and activation of signal transducer and activator of transcription 3 (STAT3), which has previously been shown to enhance HCC development. To determine the direct effect of long-term mTORC1 suppression in hepatocytes, we generated liver-specific *Raptor* knockout (*Rpt* KO) mice. Unexpectedly, complete mTORC1 inhibition by *Rpt* ablation resulted in not only increased IL-6 production and STAT3 activation, but also strongly potentiated diethylnitrosamine-induced HCC development in high-fat diet-induced obese mice as well as lean mice. Next, we established *Tsc1* knockout (*TSC1* KO) mice, in which mTORC1 is constitutively hyperactivated. It has been reported that mTORC1 inhibition attenuated liver damage, fibrosis, and spontaneous HCC development in *TSC1* KO mice. We then generated *TSC1/p62* double knockout mice to investigate the role of *p62*, a multifunctional protein, accumulated in *TSC1* KO mice livers. In the absence of *p62*, liver damage and fibrosis observed in *TSC1* KO mice were reduced, and most importantly, the tumourigenesis was completely abolished.

mTORC1 inhibition suppresses liver damage, fibrosis, and tumourigenesis in the mTORC1-hyperactive state (Figure 1). However, in both normal and obese

mice, the adverse effects of mTORC1 inhibition outweigh its benefits. Recently, increased liver damage was also observed during clinical trials of an mTOR inhibitor, everolimus. Remarkably, a trial for HCC (EVOLVE-1) did not meet the primary endpoint. Hyperactivation of *Akt* and aberrant liver regeneration, which is also seen after rapamycin treatment and in *Rpt* KO mice livers, may be reasons to be concerned about long-term mTORC1 inhibition as an HCC treatment. Patients with HCC usually underlie a chronic liver disease, typically liver cirrhosis, and require regeneration in which mTORC1 should be active to maintain their liver functions. In conclusion, mTORC1 inhibition may not be a broadly effective treatment for HCC patients.

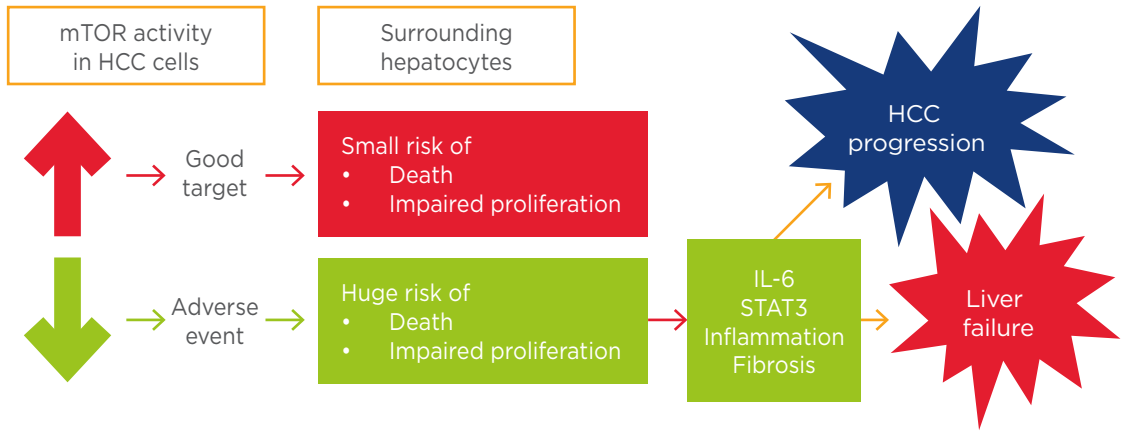

Figure 1: Therapeutic potential of mTOR inhibition.
HCC: hepatocellular carcinoma; IL-6: interleukin-6; STAT3: signal transducer and activator of transcription 3.

STRUCTURE AND FUNCTION OF THE BILIARY EPITHELIUM: SECRETIN'S REGULATION OF LIVER FIBROSIS

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Cholangiocytes represent 3–5% of cells within the liver; however, they play a crucial role in bile modification and the regulation of hepatic homeostasis. Understanding the physiology of cholangiocytes has contributed to recent advances; one example is the mechanisms of liver fibrosis that originate from cholangiopathies, specifically primary sclerosing cholangitis (PSC). Previous work from our laboratory has shown that cholangiocytes proliferate in response to cholestatic injury and

secrete a number of hormones, peptides, growth factors, and cytokines. These mediators act through autocrine/paracrine mechanisms to modify bile secretion, regulate biliary proliferation, and activate other cell types, such as hepatocytes, stellate cells, and mast cells to initiate the inflammatory and fibrotic response to liver injury. Heterogeneity exists within the biliary tree between large and small cholangiocytes, which proliferate in response to cAMP-dependent and Ca^{2+}-dependent mechanisms, respectively. Large cholangiocytes express the secretin receptor (SR) and secrete secretin (Sct) that stimulate biliary HCO_{3-} secretion and promote cholangiocyte proliferation via a cAMP/PKA/ERK dependent pathway. We have shown that the Sct/SR axis is vital for cholangiocyte proliferation during cholestasis, but its role in hepatic fibrosis, specifically in PSC, has not been investigated.

Our team has demonstrated that the Sct/SR axis is upregulated in models of cholestasis, such as bile duct ligated (BDL) rats; ablation of the Sct/SR axis significantly decreases cholangiocyte proliferation, bile duct mass, and liver fibrosis in this model. Furthermore, chronic Sct treatment significantly upregulates the fibrotic reaction following BDL. It was hypothesised that this process occurs via downregulation of *let-7a* and *miR-125b*, resulting in an increased expression of nerve growth factor, vascular endothelial growth factor, and transforming growth factor beta-1 (TGF-β1), which promote activation of hepatic stellate cells (HSC).

The Sct/SR axis and TGF-β1 expression are also upregulated in a mouse model of PSC, the $Mdr2^{-/-}$ mouse. Treatment of $Mdr2^{-/-}$ and BDL mice with a SR antagonist significantly decreases TGF-β1 gene transcription, cholangiocyte TGF-β1 secretion, and TGF-β1 serum levels, whereas treatment with Sct upregulates TGF-β1 secretion from cholangiocytes. Treatment of HSC with cholangiocyte supernatant from BDL mice promotes HSC proliferation and expression of fibrotic markers (FN1, α-SMA). In human PSC samples we also found a significant increase in the Sct/SR axis, as well as increased levels of TGF-β1 and decreased expression of *let-7a* and *miR-125b*, which correlates with the $Mdr2^{-/-}$ and BDL models. These findings suggest the Sct/SR axis not only promotes cholangiocyte proliferation, but also regulates hepatic fibrosis via TGF-β1 activation of HSC in $Mdr2^{-/-}$ and BDL mice models, which may be an important target for developing pharmacological therapies for PSC.

INCIDENCES OF ALL MALIGNANCIES IN PATIENTS WITH CHRONIC HEPATITIS B RECEIVING LONG-TERM ORAL NUCLEOS(T)IDE ANALOGUE TREATMENT: A STUDY OF 44,494 SUBJECTS

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Around the world, there are millions of patients with chronic hepatitis B (CHB) receiving long-term oral nucleos(t)ide analogues (NUCS) as the antiviral treatment. The treatment efficacy of these agents has been established, however some doctors and patients may still have some concerns about their safety in long-term use. In particular, entecavir, one of the most commonly prescribed NUCS, showed potential carcinogenic effects in animal studies. However, supraphysiological doses of entecavir were used in animal studies. The risks in humans are still to be defined.

Our group aimed to determine the cancer risks in CHB patients who did or did not receive NUCS treatment. In this retrospective cohort study,

we retrieved data from the Hospital Authority, the sole public healthcare provider in Hong Kong. We minimised the biases from confounding factors using sophisticated statistical methods, including a 3-year landmark analysis and propensity score weighting.

We first identified >100,000 subjects with viral hepatitis; 44,494 CHB patients (39,712 untreated and 4,782 treated) were included in the final analysis. At a median follow-up of 4.7 years, malignancies had occurred in 835 (2.1%) versus 274 (5.7%) of NUCS-treated versus untreated subjects, respectively. When compared with the general population in Hong Kong, the incidence of hepatocellular carcinoma was increased in CHB patients. The incidence of other cancers was either similar or lower, with the exception of lymphoma and cervical cancer.

NUCS-treated patients had similar risks to develop all cancers (weighted hazard ratio 1.01, 95% confidence interval [CI]: 0.82–1.25, p=0.899), when compared to untreated patients. However, the trend showed that NUCS-treated patients had a higher risk of developing colorectal cancer (weighted hazard ratio: 2.17, 95% CI: 1.08–4.36, p=0.029) and cervical cancer (weighted hazard ratio: 7.33, 95% CI: 1.72–31.17, p=0.007), which fell short of statistical insignificance after Bonferroni correction multiple comparisons for 13 different outcomes (p>0.0038).

Similar observations were found in all subgroups of different genders, age groups, and choice and duration of treatment. The only exception was a higher risk of colorectal cancer in treated female patients, with a weighted hazard ratio 5.78 (95% CI: 1.81–18.42, p=0.003). Nonetheless, the absolute number of events was small (5 and 15 in NUCS-treated and untreated patients, respectively), leading to a very wide 95% CI in the hazard ratio.

In conclusion, this large-scaled population-based study does not suggest an increased risk of all cancers in NUCS-treated CHB patients. The risks of colorectal and cervical cancers in NUCS-treated female patients require independent confirmation. Before further data are available, screening for colorectal and cervical cancers should be encouraged in such patients according to international recommendations.

PHASE III STUDY OF TENOFOVIR ALAFENAMIDE COMPARED WITH TENOFOVIR DISOPROXIL FUMARATE IN PATIENTS WITH HBeAG-NEGATIVE, CHRONIC HEPATITIS B: WEEK 48 EFFICACY AND SAFETY RESULTS

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Tenofovir alafenamide (TAF), a novel prodrug of tenofovir (TFV), is more stable in plasma and more efficiently delivers TFV into lymphoid cells and hepatocytes at lower systemic TFV exposures than tenofovir disoproxil fumarate (TDF) when administered at approximately one-tenth the TDF dose. TDF can cause renal and bone toxic effects related to high plasma TFV concentrations. TAF-containing regimens can provide improved renal and bone safety compared with TDF-containing regimens in the therapy of HIV-1 infected patients maintaining a similar efficacy.[1] TFV has been widely used in the treatment of patients with chronic hepatitis B (HBV). It is a potent nucleotide analogue, with a very high efficacy in suppressing viral replication for both treatment-naïve and treatment-experienced patients. However, the effect on hepatitis B surface antigen clearance is minimal and the majority of patients require life-long therapy with the potential risk of renal and bone effects.

In this Phase III study, patients with hepatitis B envelope antigen (HBeAg)-negative were randomised 2:1 to receive TAF 25 mg once daily or TDF 300 mg once daily, each with matching placebo, and treated for 48 weeks. The primary efficacy analysis was the percentage of patients with HBV DNA <29 IU/mL at Week 48; the study was powered to demonstrate non-inferiority in efficacy of TAF with a 10% margin. Safety was assessed by the emergence of treatment adverse events. Two key safety endpoints were defined by changes in bone mineral density (changes in hip and spine bone mineral density [BMD]) and markers of bone formation and resorption. The second key endpoint was renal safety, evaluated by changes in serum creatinine, proteinuria, estimated glomerular filtration rate, and renal tubular function. Viral resistance was performed by population sequencing in patients with virologic breakthrough, or viraemia at discontinuation.

Four hundred and twenty-five patients were randomised and treated at 105 sites in 17 countries. The majority were males, mean age was 46 years, and 72% were Asian; all HBV genotypes were included and 21% were previously treated with nucleos(t)ides.

The primary endpoint: virological response, defined by HBV DNA <29 IU/mL at Week 48, was observed in 93.0% with TAF and 93% with TDF, meeting the primary hypothesis of non-inferiority. A greater percentage of TAF-treated patients achieved normalisation of serum alanine aminotransferase values. Patients on TAF experienced smaller declines in hip and spine BMD than TDF. Significantly smaller decreases in Cockcroft–Gault estimated glomerular filtration rate compared to TDF, with improved markers of renal tubular function were observed. The rates of discontinuations and serious adverse events were low and similar in the two arms. No resistance development was observed in the four patients (two per group) who qualified for testing. In summary, in treatment naïve and experienced HBeAg-negative patients with chronic HBV, TAF 25 mg was non-inferior in efficacy when compared with TDF 300 mg with improved bone and renal parameters.

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NON-ALCOHOLIC FATTY LIVER DISEASE NATURAL HISTORY

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Non-alcoholic fatty liver disease (NAFLD) is a spectrum of histopathological changes ranging from bland steatosis to steatohepatitis with or without increasing fibrosis, leading to hepatocellular carcinoma in a small but significant number of patients. The disease is multifactorial and there is a large inter-individual variability in disease progression. The prevalence of NAFLD in developed countries ranges between 20% and 30% of the population, and is strongly associated with the frequency of obesity in each country. Very few studies have investigated the prevalence of non-alcoholic steatohepatitis (NASH) in the general population because the diagnosis of NASH requires a liver biopsy. In an American study performed in the general population the prevalence of NASH was 12.2%, and in bariatric surgery series the prevalence ranged between 13% and 56%.

Several studies have described the natural history of NAFLD. Patients with NAFLD have a decreased survival compared with the general population. Cardiovascular disease is the leading cause of death and liver-related death is the third most common cause, after non-liver related malignancy. Age and diabetes are the strongest predictors of mortality in NAFLD patients. Decompensated liver

disease develops in 5%, and in some studies up to 10% of patients. In the USA, NAFLD is the second leading cause of liver transplantation today. NAFLD is also associated with future development of diabetes, which develops in a large proportion of patients.

Even though the natural history of NAFLD has been described to a large extent, several issues remain to be resolved. Three important issues are the 'bland steatosis issue', 'the NAFLD Activity Score (NAS) issue', and 'the alcohol issue'.

Bland steatosis (steatosis without significant necroinflammation and/or fibrosis) is considered a benign state. This view should be altered, since a significant number of patients do develop significant fibrosis over time. In studies of patients with two liver biopsies, between 44% and 64% of patients with bland steatosis had developed fibrosis at the second liver biopsy.

The NAS has been used in most clinical trials to evaluate efficacy during the past few years. In two recent large follow-up studies the NAS was unable to predict mortality in NAFLD patients. Fibrosis was the strongest predictor of survival irrespective of NAS score. This is probably explained in part by the relatively large impact of steatosis grade on the final score, as NAS is the composite score of steatosis grade, ballooning, and lobular inflammation. For future research, especially clinical drug trials, we need surrogate endpoints that are strongly associated with future clinical events.

Even though the disease is named non-alcoholic, patients are allowed to drink alcohol on a regular basis. The amount of alcohol consumption that should be considered the threshold for classification of 'non-alcoholic', and thus NAFLD, varies. The National Institute for Health Research (NIHR) Clinical Research Network on NAFLD/NASH states 140 g/week in men and 70 g/week in women; the European Association for Study of the Liver (EASL) position statement suggests 210 g/week in men and 140 g/week in women; and the American Association for Study of Liver Diseases/American Gastroenterological Association (AASLD/AGA) guidelines propose 294 g/week in men and 196 g/week in women. There are very few studies with robust methodology that have investigated the interplay between NAFLD and alcohol. There are increasing data demonstrating that moderate alcohol consumption protects from the development of NAFLD. Unpublished data from Sweden show that phosphatidylethanol, a newly developed marker of alcohol consumption, was associated with significant fibrosis and necro-inflammatory changes in NAFLD patients. This suggests that there could be a hidden drinking problem in some of our NAFLD studies. Moreover, very few NAFLD studies take into account the variability of alcohol consumption many individuals display during a lifespan.

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EDITOR'S PICK

In this next article, Luca Quagliata of the Institute of Pathology, University Hospital of Basel, Basel, Switzerland, discusses the integration of the histological, clinical, and molecular classifications of hepatocellular carcinoma. This is a field of ongoing research without any final conclusions, and thus the data is difficult to interpret. This article not only provides a useful overview of these differing systems but also addresses the difficulty of applying them in a dynamic combination, shedding light on an important avenue for future research.

Prof Markus Peck-Radosavljevic

CLINICAL, HISTOLOGICAL, AND MOLECULAR CLASSIFICATION OF HEPATOCELLULAR CARCINOMA: HOW DO THEY GET ALONG?

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ABSTRACT

Hepatocellular carcinoma (HCC) will soon become a prominent part of the medical and economic burden on many Western countries' healthcare systems. This review will discuss some emerging scenarios concerning the different classifications of HCC from the clinical, histological, and molecular perspective and to what extent they are integrated with each other. Beginning with an overview of the current numbers and facts regarding HCC, it touches upon the latest development of the epidemiological scenario. It is noteworthy that besides viral hepatitis infection, the fast growing rate of individuals affected by metabolic syndromes represents an additional influential factor on the rising incidence of HCC. However, despite recognised epidemiological evidence, too little is known about the molecular mechanisms that favour HCC development and progression. For instance, long non-coding RNAs playing a major role in the HCC carcinogenesis process have only recently been recognised. Although high cure rates are achieved for clinically asymptomatic patients when small tumours are detected, HCC is typically silent with few severe symptoms until its advanced stages. Patients with severe clinical signs are seldom good candidates for any type of curative therapy. Microscopically, HCC cells resemble normal liver cells to a variable degree, depending on the tumour differentiation status. Pathologists often use a panel of markers to assist HCC differential diagnosis. From a molecular perspective, HCC presents as a highly heterogeneous tumour entity. Despite considerable research efforts, to date no molecular classification has been introduced in clinical practice. A number of classifications have been suggested to stratify HCC patients by the likelihood of survival, with the aim of identifying those with the best chance of being successfully treated. These different systems do not seem to work well in conjunction and the various involved disciplines have so far failed to achieve their common goal. Co-ordinated initiatives involving clinicians, pathologists, biologists, and bioinformaticians are needed to achieve a comprehensive classification of HCC.

Keywords: Hepatocellular carcinoma (HCC), molecular classification, sorafenib, long non-coding RNA (lncRNA).

HEPATOCELLULAR CARCINOMA: AN OVERVIEW OF THE NUMBERS AND FACTS

Unlike most malignancies, mortality from liver cancer has increased significantly over the past 20 years.[1,2] Hepatocellular carcinoma (HCC) accounts for up to 85% of liver tumours.[3] HCC has one of the widest variations in incidence in different parts of the world amongst all tumour types.[4-6] In fact, while HCC is the fifth most common cancer in men and the seventh in women worldwide, it represents the most common cause of death from cancer in East Asia and Sub-Saharan Africa, which have the greatest number of cases.[6,7] HCC incidence in the USA and Europe has doubled over the past two decades.[8] Furthermore, epidemiological evidence anticipates that in Western populations the HCC burden will continuously increase over the next 20 years.[3] This is mostly due to the mounting number of patients with advanced hepatitis C virus (HCV) and/or non-alcoholic steatohepatitis.[9] Although it is clear that HCC will soon become a prominent part of the medical and economic burden on the healthcare systems of Western countries, too little is currently being done to efficiently stem this alarming phenomenon.[1,10,11] Conversely, the incidence of HCC has substantially decreased in other areas, such as China and Hong Kong.[12] This phenomenon can be explained by looking at the epidemiological fluctuation in risk factor exposure, such as the decline of the hepatitis B virus (HBV) infection rate due to vaccination, and a marked reduction of exposure to aflatoxins from grains as a result of the introduction of improved hygiene standards. Aflatoxins are a family of mycotoxins produced by fungi of the *Aspergillus* genus known to be powerful experimental carcinogens. Aflatoxin B contamination of food, predominantly grains and peanuts, is most common in China and Southern Africa.

Nowadays, it is estimated that globally up to 80% of HCC is associated with HBV or HCV infection.[6,8] The risk of developing HCC is increased 5 to 15-fold in chronic HBV carriers and up to 17-fold in HCV infected patients.[3] Though the viruses display a similar tropism, they are different, being implicated in the alteration of distinctly different molecular pathways, and the precise mechanisms by which they can cause HCC onset are not yet fully defined. However, many new lines of evidence now suggest that the pathogenesis of HCC is immune-mediated, as an indirect result of the cycle of inflammation-necrosis-regeneration that is typical of chronic hepatitis.[13]

Metabolic syndromes represent a growing issue influencing HCC incidence. Nearly 30% of new cases of HCC in the USA have no identified aetiological agent and recent data seems to point to non-alcoholic fatty liver disease related cirrhosis as the main predisposing factor.[9] In Western populations, heavy alcohol consumption and alcohol related liver disease is the second most common HCC risk factor after HCV infection.[3] Finally, hereditary diseases also contribute to the HCC burden, with hereditary haemochromatosis, tyrosinaemia, and α1-antitrypsin deficiency playing a major role.[14]

Irrespective of geographical location, HCC occurs more frequently in men than women; it is also worth noting that age at diagnosis can be significantly different depending on the geographical area.[6]

In conclusion, despite the well-known risk factors briefly described above, such as gender, age, viral infection, alcohol intake, diabetes, obesity, ethnicity, and portal hypertension,[2,4,7,15] there is little known about the mechanisms that favour HCC development and progression.[4]

CLINICAL FACTS AND CLASSIFICATION: COMPREHENSIVE OR LIMITED?

HCC is typically a silent disease with few clinically severe symptoms until its advanced stages.[16] Upper abdominal pain along with weight loss and hepatomegaly, with signs of decompensated liver disease like jaundice or ascites, are very common at presentation.[3] Given that most patients live with underlying liver cirrhosis, complications such as hepatic decompensation in the form of accumulating ascites, hepatic encephalopathy, or obstructive jaundice occur at a significant rate.[3]

Radiographic imaging is still the most frequently used approach to evaluate patients with a suspected severe liver tumour.[17] Once symptomatic, HCC is easily detectable by ultrasound, computed tomography (CT) scan, magnetic resonance imaging (MRI), or angiography. Ultrasonography-based investigation for surveillance/detection of early stage tumours has become the method of choice, easily detecting nodules with diameters of 2–3 cm in size.[17] HCC nodules are exclusively supported by an arterial blood supply, typically

present as a hypervascular lesion with washout in the venous phase. A number of widely used contrast agents can be employed in combination with ultrasound, CT, or MRI to depict blood flow in lesions <1 cm.[17] According to the guidelines of the European Association for the Study of the Liver (EASL),[2] and those of the American Association for the Study of Liver Disease (AASLD), the detection of a typical vascular profile with at least one dynamic imaging technique is already sufficient for a diagnosis of HCC for lesions >2 cm in size.[2] Thus, no pathological examination of tissue for these cases is required. Conversely, for lesions that range between 1 and 2 cm, a minimum of two dynamic imaging techniques are necessary, and there is a possibility of having to perform a biopsy for radiologically atypical lesions.[2]

In the early stages of HCC, the majority of tumour nodules appear to grow within an encapsulated mass. Once enlarged, they tend to infiltrate, damage, and destroy the adjacent tissue by substantially replacing the normal parenchyma of the liver and generating a number of characteristic satellite nodules.[18] Microvascular invasion and/or intrahepatic metastases are very common events observed in up to 60% of tumours <5 cm in diameter and >95% for those >5 cm.[16] Thrombosis of the portal vein along with its branches appears in 65–75% of advanced tumours, and in the hepatic veins in about 20–25% of cases. Invasion of the large bile ducts with obstructive jaundice can also occur, but it is a comparatively rare event (~5% of cases).[3] Although metastases are common in advanced HCC, with almost half of patients having at least regional lymph node and lung involvement, most patients eventually die as a result of liver failure. In advanced stages of the disease, HCC patients have a median survival time of 1–3 months. Although longer, survival expectations in patients with earlier stages who are not eligible for resection are still extremely low: ~20% at 1 year and ~8% at 2 years.[19] These numbers demonstrate the harsh reality of HCC.

The tumour, nodes, metastases (TNM) system, laid out by the International Union against Cancer (UICC), is available for HCC. Often however, different staging systems are combined to more accurately define HCC status, for example by integrating features of the underlying liver disease, the functional state of the liver, and the size of the tumour mass. Studies comparing different staging systems suggest that the Barcelona Clinic Liver Cancer Classification (BCLC),[20] which incorporates tumour extent, liver function, and overall patient performance status, represents the best option to identify treatable patients as well as to predict survival.[20]

HCC management has advanced considerably in the past 20 years.[16] High cure rates are achieved for patients with small tumours that are detected whilst clinically asymptomatic;[21,22] however, most patients only present in the advanced stages of the disease. HCC patients with severe clinical signs are rarely good candidates for any type of curative therapy.[16] Some improvements in the direction of palliative therapy have recently been achieved, though most efforts have been correctly pointed towards surveillance and early diagnosis in high-risk populations. Current BCLC recommendations for HCC therapy suggest that Stage 0 and A (very early and early HCC, respectively) patients should receive curative treatment; surgical resection is optimal for Stage 0 and A1, and transplantation or ablation for Stage A2, A3, or A4. Surgical resection is mostly effective for small tumours in patients with no underlying liver disease. For patients with multiple small tumours and compensated cirrhosis, liver transplantation is the best option for curing the underlying liver disease as well as the tumour.[23] Patients selected according to the Milan criteria (i.e. solitary HCC ≤5 cm or up to three tumours each ≤3 cm) have a 1-year survival of 81% and 5-year survival of 51%.[24] Transplantation is clearly not always available, thus percutaneous tumour ablation (ethanol or radiofrequency) has become the most widely used treatment for early but unresectable tumours.[25] At the same time, since HCC receives its blood supply from the hepatic artery rather than the portal vein, angiographic embolisation of the artery has been used to produce tumour necrosis and prolong survival. Conversely, palliative therapy is recommended for Stage B and C (intermediate or advanced HCC, respectively), with chemoembolisation for Stage B and sorafenib for Stage C. Sorafenib is a multiple tyrosine kinase inhibitor that effectively blocks several receptors' activity, such as vascular endothelial growth factor receptor, platelet-derived growth factor receptor, and the RAF serine/threonine kinases along the RAF/MEK/ERK pathway.[26] Nevertheless, sorafenib has shown a consistent but limited survival benefit in HCC (10–12 weeks increased survival) accompanied by a number of moderate-to-severe

side effects.[26,27] Finally, patients with Stage D (end-stage HCC) receive symptomatic treatment. This makes HCC somewhat unique among cancers, having no standard cytotoxic therapy.[16] Overall, it is imperative to identify new therapeutic targets as well as biomarkers to predict response to therapy.

HISTOLOGICAL CLASSIFICATION: THE OLD, THE NEW, AND THE UNKNOWN

Microscopically, HCC cells resemble normal liver cells to a variable degree, mostly depending on the tumour differentiation status.[18] Nuclei are often clear and prominent, with a concomitant high nuclear–cytoplasmic ratio. Commonly, light hyperchromatism and nuclear irregularity are observed. HCC cells typically have distinct cell membranes and a modest amount of eosinophilic, finely granular cytoplasm.[18] In the earliest stages, HCC-transformed cells simply grow within pre-existing liver cell plates.[18,28] In this case, they retain the reticulin framework, and not infrequently preserve the portal tracts.[18] Nonetheless, the cells of such well-differentiated tumours have high nuclear–cytoplasmic ratios, generating the typical nuclear crowding appearance. By further proliferating, these tumours produce abnormal structural patterns with thin trabeculae and/or pseudoglands.[29]

In 1954, Edmondson and Steiner suggested grading HCC on a scale from I–IV, having increasing nuclear irregularity, hyperchromatism, and nuclear–cytoplasmic ratio, accompanied by diminished differentiation status.[30] This system holds substantial importance to both the amount and appearance of the cytoplasm, and the nuclear–cytoplasmic ratio. Thus, Grade 4 tumours have very scant cytoplasm even if the nuclei might be minimally anaplastic. The correlation between the Edmondson–Steiner grade and HCC prognosis is still disputed, but generally tumour grade is a weak independent predictor of the clinical course, providing very little prognostic information.[18] Finally, the Edmondson–Steiner grading system is highly subjective and relies heavily on the pathologist's expertise. A univocal system to comprehensively grade HCC has not yet been established.

Several markers are currently used to assist the differential diagnosis of HCC. Glycoprotein I is the most useful and is frequently employed in distinguishing HCC from other malignancies.[31] Hepatocyte paraffin 1 stains for urea cycle enzyme carbamoyl phosphate synthetase 1 in liver mitochondria, which is positive in about 90% of all HCC cases, showing a typical granular pattern in most liver specimens (however it is not specific to the hepatocytes). Additional markers that are useful in the diagnosis of HCC include heat shock protein 70, the glutamine synthetase, annexin A2, and arginase-1.[18,31] None of these markers alone are sufficient for a definitive diagnosis and a combination of multiple markers is normally the best approach.

MOLECULAR CLASSIFICATION: MUCH EFFORT, LITTLE HELP, BUT HIGH HOPES

From a molecular point of view, HCC presents as a highly heterogeneous tumour entity.[32] This is not entirely surprising due to the wide range of its aetiologically associated factors.[7,33] Several molecular approaches such as coding-gene expression profiling (either by microarray or massive RNA sequencing), along with deep DNA sequencing analysis and array comparative genomic hybridisation (CGH) have identified the key alterations favouring the onset of HCC.[32]

A number of genomic rearrangements are consistently observed in HCC samples independent of their aetiology. For example, the amplification of the chromosome 6p21 (also containing the *VEGFA* gene) is observed in approximately 6–8% of all HCC cases.[34] Interestingly, patients with *VEGFA*-amplified HCCs show an improved survival compared with non-amplified cases under sorafenib treatment.[35] A meta-analysis of several sets of independently performed CGH experiments, including a total of 169 HCC samples, highlighted that overall, chromosomal gains are most abundant in numerous specific large (i.e. 1q, 6p, 8q, 17q, and 10q) and two narrow (5p15.33 and 9q34.2–34.3) genomic regions, while as many as 88 significant losses are repeatedly present in the 4q, 6q, 8p, 9p, 13q, 14q, 16q, and 17p18 regions.[36] Performing whole exome and/or genome sequencing, several studies have attempted to combine copy number variation with single nucleotide variation data and/or gene expressing profile, with the aim of establishing a molecular classification of HCCs.[15,33,37-39] Such classifications effectively enable scientists to group HCCs on the basis of specific dysregulation of a limited set of molecular pathways. A recent HCC classification has established a molecular signature based on the combined evaluation of as few as

five genes including *HN1, RAN, RAMP3, KRT19*, and *TAF9*. This 5-gene signature can independently define any other clinical and pathological tumour features to predict HCC patients' outcome when treated by surgical resection.[40] Additionally, a recent meta-analysis of HCC data, comprising several hundred HCC tumours, identified two main subclasses: S1-S2 and S3.[41] The S1-S2 subgroup is characterised by: more aggressive HCCs presenting with severe genetic instability; the impairment of tumour suppressor *TP53*; the activation of pro-survival signals controlled by *E2F1* and *MET* pathways; *KRT19* positivity; a high rate of cellular proliferation; a larger tumour mass; low differentiation status; higher incidence of tumour recurrence, and a poorer prognosis overall.[41] Further subclassification highlights that the S1 group shows the activation of the transforming growth factor β pathway while the S2 group shows positivity for stemness markers, such as *EpCAM, AFP*, and *GPC3*, in addition to the insulin-like growth factor 2 pathway activation. Those patients in the S3 class are characterised by less aggressive features, such as recurrent somatic mutations in exon 3 of *CTNNB1* along with the expression of specific genes, such as *GLUL, LGR5*, and *SLC1A2*, but little alteration of canonical WNT pathway genes. They also have smaller and more differentiated tumours. All of these features partially preserve the normal hepatocyte function in S3 patients; this results in an overall better prognosis.[41]

It should be noted that all of the previously cited HCC molecular classifications were established using resected tumours, introducing a selection bias towards patients who have no liver cirrhosis and are at an early stage of the disease. A recent study set out to develop a molecular classification system using liver biopsy instead of resection specimens, thus removing the biases associated with a given stage of HCC (Makowska et al., accepted). This study has challenged all previously reported data, suggesting that clear-cut differences in HCC might be missed by merely looking at the gene expression profile or mutation spectrum.

In conclusion, despite considerable research efforts, to date no molecular classification has been introduced in clinical practice, and currently all HCC cases are treated according to their stage rather than their molecular subtype.

LONG NON-CODING RNAS: THE DARK SIDE OF THE LIVER

The unprecedented fast progress of deep sequencing technology, along with the improvement of bioinformatics tools to conduct complex whole genome data analysis,[42] has revealed that while >70% of the human genome is transcribed into RNA,[43] only as little as 2–5% of the RNA produced is eventually translated into proteins.[44] The next challenge is to unravel the biological functions of the vast amount of non-coding RNA (ncRNA) transcripts and to define their impact on cell physiology.[44]

ncRNAs are broadly grouped into two major classes: 1) transcripts shorter than 200 nucleotides, namely small ncRNAs, mainly including Piwi-interacting RNAs, small interfering RNAs, and microRNAs; and 2) long non-coding RNAs (lncRNAs) ranging in length from 200 nt to ~100,000 kb, with an mRNA-like transcript structure and a very low conservation rate across species. Nevertheless, the definition of ncRNAs, far from being complete, remains a topic of debate as our understanding of their functions grows.[45-47] For most of the predicted lncRNAs the potential functions and mechanisms of action are still undetermined; one significant discovery is that most lncRNAs show a tissue-specific pattern of expression.[47]

One major contribution, aiming to ameliorate the current molecular classification of HCC, may occur with the integration of lncRNA expression profiles with existing data sets. Lately, a growing critical mass of researchers have started to focus their activities on the implications of lncRNA alterations in pathophysiology.[48,49] To date, a number of lncRNAs have been proven to be associated with HCC disease development and progression.[42,43,50] Plasma HULC (Highly Upregulated in Liver Cancer) was one of the first lncRNAs to be examined in HCC, isincreased in a consistent proportion of HCC plasma[51] and tissue samples, and is associated with histological grade and HBV infection.[52] Such findings envision the use of lncRNAs as non-invasive novel diagnostic and/or prognostic biomarkers, which may also allow monitoring of disease progression. Other compelling examples include metastasis-associated lung adenocarcinoma transcript 1, reported to be associated with metastasis formation and HCC recurrence.[53] The HOXA transcript at the distal tip was found to be

highly upregulated in HCC and was able to predict both disease progression and patient outcome.[54]

Defining ncRNA functions, expression patterns, and regulatory mechanisms will be critical to fully appreciate their biological relevance in controlling liver functionality and pathological conditions, such as HCC.

COMPREHENSIVE CLASSIFICATION OF HEPATOCELLULAR CARCINOMA: A MAJOR UNMET NEED

A number of staging systems have been recommended to stratify HCC patients by the likelihood of survival and to identify those with the best chance of being successfully treated. This review has attempted to assess different perspectives on defining some of the important characteristics of HCC. Far from being exhaustive, the aim of this paper was to underline that despite the efforts of numerous researchers in recent years with relation to the definition of HCC pathophysiological features, the different disciplines involved have so far failed to achieve their common goal. This is due, on one hand, to the high complexity of the studied subject, but also to the lack of co-ordinated initiatives involving clinicians, pathologists, biologists, and bioinformaticians simultaneously. Such co-operative attitudes have only very recently emerged[16] and will represent a fundamental step in achieving a comprehensive classification of HCC. One might think of a common data-sharing platform for the HCC research community where all relevant information could be readily available for researchers to use. Often such platforms are only curated for one specific aspect (e.g. molecular features, such as DNA sequencing data) and are poorly annotated for the others (clinical and histological).

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THE INTERNATIONAL LIVER CONGRESS (ILC) 2016: RIFAXIMIN USE IN CIRRHOSIS-RELATED HEPATOLOGICAL DISORDERS AND NEW PERSPECTIVES

Summary of the presentations on rifaximin from the Annual European Association for the Study of the Liver (EASL) meeting held in Barcelona, Spain, from 13th–17th April 2016

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ABSTRACT

Rifaximin (RFX)-α is a broad-spectrum antibiotic that targets commensal gut bacteria and reduces the excess ammonia produced by the gut bacteria of patients with cirrhosis. This innovative agent has been approved in most European countries for several therapeutic indications, including the prevention of episodes of overt hepatic encephalopathy (HE) in adult patients. New data on RFX use in HE were presented at the International Liver Congress (ILC), namely the annual meeting of the European Association for the Study of the Liver (EASL) held in Barcelona, Spain, from 13th–17th April 2016.

The beneficial effects of RFX have been attributed to the antibiotic action against a broad spectrum of gut bacteria, accompanied by the advantage of its very poor systemic absorption generating a gastrointestinal tropism. More recently, data are accumulating to suggest that other non-antibacterial effects contribute to RFX efficacy, making it a very interesting option for enteric diseases. RFX is thus explored outside of HE, in both cirrhotic and non-cirrhotic patients.

This review aims to highlight the presentations from ILC 2016 focussing on RFX developments in clinical research.

Keywords: Rifaximin (RFX), bacterial flora, hepatic encephalopathy (HE), cirrhosis, ascites, steatosis, bacterial peritonitis, non-alcoholic fatty liver disease (NAFLD), gut microenvironment, gut microbiota, inflammation.

INTRODUCTION

Rifaximin (RFX)-α (registered names, Normix®, Alfa Normix®, Colidimin®, Flonorm®, Lormyx®, Refero®, Spiraxin®, Targaxan®, Tixteller®, Tixtar®, Xifaxan®, Xifaxanta®, and Zaxine®) is a broad-spectrum antibiotic that targets commensal gut bacteria, including Gram-negative and Gram-positive aerobes and anaerobes, and that reduces the excess ammonia produced by the gut bacteria of patients with cirrhosis.[1] This innovative agent has been approved in most European countries for several therapeutic indications, including the prevention of episodes of overt hepatic encephalopathy (HE) in adult patients. HE is a debilitating complication that occurs as the main presentation of liver failure in cirrhotic patients; about 30–45% of these patients develop severe HE, which can be life-threatening.[2-4] Indeed, HE manifests as neuropsychiatric symptoms including disorientation, confusion, inappropriate behaviour, and personality changes.[5]

The indication approval for RFX use in HE followed pivotal clinical trial data demonstrating its efficacy

and safety.[6,7] New data on RFX use in HE was presented at the International Liver Congress (ILC), namely the annual meeting of the European Association for the Study of the Liver (EASL), held in Barcelona, Spain, from 13th–17th April, 2016. The beneficial effects of RFX have been attributed to the antibiotic action against a broad spectrum of gut bacteria, accompanied by the advantage of its very poor systemic absorption generating a gastrointestinal tropism. More recently, data are accumulating to suggest that other non-antibacterial effects contribute to RFX efficacy, making it a very interesting option for enteric diseases.[8] RFX is thus explored outside of HE, in both cirrhotic (cirrhosis has been linked to a pro-inflammatory milieu and hyperammonaemia) and non-cirrhotic patients.

This review aims to highlight the presentations from ILC 2016 focussing on RFX developments in clinical research.

NEW CLINICAL DATA ON HEPATIC ENCEPHALOPATHY

Efficacy and Safety of Rifaximin in Acute Hepatic Encephalopathy

Crisafulli et al.[9] initiated a randomised controlled trial to evaluate the impact of RFX dosage and combination with lactulose on HE stabilisation and resolution, in addition to the length of the patient's stay in the emergency department (ED). Seventy-seven patients were randomly assigned to either RFX 400 mg 4-times per day plus lactulose (Group A, n=39) or RFX 400 mg 3-times per day plus lactulose (Group B, n=38). Unsurprisingly, Group A patients experienced a faster HE reversion (3.35±1.16 versus 5.41±1.41 days, p<0.05), full disappearance of all symptoms (4.12±0.86 versus 6.33±0.59 days, p<0.05), and a shorter ED stay (5.7±3.8 versus 8.2±4.1 days, p<0.05).

In addition, this treatment arm showed a more significant decrease of ammonium between admission and 24 hours, versus Group B (35.23±8.56% versus 18.53±8.05%, p<0.005). When the baseline ammonium was >100 mg/dL, the difference between both treatment arms was even more significant (40.57±6.12% versus 20.74±6.56%, p<0.001). These results suggest that RFX plus lactulose, at both normal and high doses of RFX, is safe and effective as a first-line attack therapy in the context of the medical emergency that is acute HE.

Cost-Effectiveness of Rifaximin in the Prevention of Hepatic Encephalopathy Recurrence

HE generates a huge impact on patients' lives and represents a significant burden on healthcare systems.[2,10] At ILC 2016, new real-world clinical resource use data from the IMPRESS retrospective observational study were presented.[11] This study was conducted in 11 specialist National Health Service (NHS) centres in the UK, encompassing the medical records of 145 HE patients who had received RFX. Of note, 61% of patients were male, the mean age was 60.9±11.5 years, and 119 patients (82%) were on concomitant lactulose therapy. Child–Pugh score was recorded for 46% of patients, of which 10% were Class A, 54% Class B, and 36% Class C.

Resource use analyses were conducted in the 6 and 12 months pre and post-RFX initiation periods, only on patients who were alive at the end of each respective investigation period (6 and 12 months). At 6 months, RFX therapy was associated with significant reductions in hospital resources, namely mean hospital bed days per patient (28.6±3.1 versus 11.9±2.3, p<0.001), and hospitalisation frequency (2.2±0.2 versus 1.0±0.1, p<0.001) as compared with pre-RFX initiation. Total hospital bed days (n=101) were also reduced (mean 2,890 versus 1,206 days).

Similar findings were observed at 12 months, including significant reductions in mean hospital bed days per patient (31.7±3.6 versus 16.4±2.9, p<0.001) and hospitalisation frequency (2.7±0.3 versus 1.7±0.2, p=0.002), as compared with pre-RFX initiation. Total hospital bed days (n=99) were also reduced (mean 3,138 versus 1,621 days).

Of note, this study was the first to evaluate and demonstrate reductions in critical care bed days with RFX between the pre and post-initiation periods of 6 months (7.9 versus 2.0 days, p=0.046) and 12 months (11.3 versus 2.4 days, p=0.017). Significant reductions in hospital re-admissions and ED visits were observed, but only for the pre and post-initiation periods of 6 months.

Overall, RFX was well tolerated with only three patients (2%) reporting adverse events and four (3%) developing *Clostridium difficile* infection (none of these groups discontinued therapy). Interestingly, these findings are strongly aligned with those from another study conducted in seven liver centres across the UK that highlighted marked

reductions in the number of hospital admissions and hospital length of stay, demonstrating the cost-effectiveness of RFX for HE prophylaxis.[12]

RIFAXIMIN FOR SPONTANEOUS BACTERIAL PERITONITIS

Spontaneous bacterial peritonitis (SBP) is a serious and life-threatening liver cirrhosis complication with a high recurrence rate of 70% at 1 year.[13] Norfloxacin, a fluoroquinolone, is widely used for secondary prophylaxis to prevent recurrences of SBP in patients with liver cirrhosis and ascites. Due to the emergence of quinolone-resistant and Gram-positive SBP however, some specialists have suggested the use of RFX, which does not appear to promote the emergence of bacterial resistances.[13]

A randomised controlled trial of RFX versus norfloxacin in 262 cirrhotic patients with ascites and a previous episode of SBP[14] was conducted.[15] All patients were randomly assigned to receive either 1,200 mg RFX (n=103) or 400 mg norfloxacin (n=92), daily, for 6 months.

RFX was more effective than norfloxacin, since the recurrence rate of SBP was significantly lower in the RFX group (3.88% versus 14.13%, p=0.041) when compared with the norfloxacin group. Likewise, the mortality rate was significantly decreased in the RFX group (13.74% versus 24.43%, respectively; p=0.044). Regarding the safety profile of both regimens, RFX was associated with a lower rate of side effects versus norfloxacin (p=0.033), which makes intestinal decontamination with RFX a more attractive treatment option than norfloxacin based on the findings of this study.

RIFAXIMIN FOR NON-ALCOHOLIC FATTY LIVER DISEASE

Non-alcoholic fatty liver disease (NAFLD) may involve pro-inflammatory cytokines and increased insulin resistance, thus contributing to hepatic steatosis and BMI elevation. In an open-label, prospective, multicentre cohort study, the effect on NAFLD of a daily administration of 1,100 mg RFX for 6 months was evaluated in 126 NAFLD patients (42 steatosis and 84 non-alcoholic steatohepatitis [NASH]).[16]

The NASH group showed significant reductions in BMI, gamma glutamyl transferase (γ-GGT), alanine aminotransferase (ALT), endotoxin, pro-inflammatory cytokines (interleukin [IL]-6), tumour necrosis factor-α, IL-10, and cytokeratin-18 (CK-18). Similarly, patients with steatosis showed reductions in ALT, γ-GGT, and homeostasis model assessment score (a measure of insulin resistance). However, RFX therapy did not show a significant effect onserum levels of aspartate aminotransferase and lipid profile. Overall, RFX appeared to modify the pathogenesis of NASH through the reduction of serum endotoxin and improvement of insulin resistance, BMI, pro-inflammatory cytokines, and CK-18.

RIFAXIMIN FOR ASCITES

Refractory ascites (diuretic-resistant ascites and diuretic-intractable ascites) occurs in nearly 17% of cirrhotic patients.[17] An open-label, prospective, single-centre study aiming to evaluate standard diuretic therapy plus midodrine and RFX (800 mg RFX/day) against standard diuretic therapy in 400 cirrhotic patients (randomised at a ratio of 1:1 to either arm) with refractory or rapidly recurrent ascites was conducted.[18]

Adding RFX and midodrine led to a complete response in 78% of patients, partial response in 18%, and no response in 4% versus 15%, 55%, and 30% in the control group, respectively. By improving systemic and renal haemodynamics, as well as providing significant improvements on diuresis and weight loss, the combination therapy helped reduce paracentesis needs and control ascites. Midodrine and RFX also significantly improved short-term survival (12.6±3.2 months versus 6.6±2.2 months, p=0.000).

The authors concluded that adding RFX and midodrine to standard medical therapy is mandatory and advised to improve the systemic haemodynamics, the control of ascites, and short-term survival.

PRECLINICAL STUDY ON RIFAXIMIN AND SYSTEMIC/INTESTINAL INFLAMMATION

To assess the action of RFX on intestinal barrier, inflammatory milieu, and ammonia generation independent of the direct effect on microbiota, a preclinical study was conducted on germ-free 10-week-old GF C57/BL6 male mice; some of them were colonised with cirrhotic human stools, and RFX was administered to a subgroup of each group. RFX promoted intestinal homeostasis by

changing intestinal permeability and inflammatory markers, which could suggest a positive influence of RFX beyond antibiotic activity.[19] A beneficial impact was observed on serum ammonia through elevated small bowel tissue glutaminase, as well as a 3-fold increase in caecal glutamine content (p=0.02), even in the absence of microbiota. RFX positively altered the microbial functionality of the intestinal barrier without changing its composition, and beneficially impacted systemic and intestinal inflammation.

CONCLUSION

The evidence on the 'eubiotic' effects of RFX beyond its antibiotic properties, through changes in the metabolic function of the gut microbiota and microenvironment, continues to accumulate. New data presented at ILC 2016 further ascertained the clinical efficacy and safety profile of RFX in a range of hepatological and enteric disorders, including real-life data, which mirror the findings from pivotal clinical trials.

Although definitive studies on the effect of RFX on gut microbiota in larger cohorts of both healthy volunteers and patients are needed, the evidence for the use of RFX in several enteric diseases is becoming more robust, and supports the potential of this innovative compound to have a significant and positive impact on treatment outcomes and quality of life for patients.

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NONSURGICAL TREATMENT FOR LOCALISED HEPATOCELLULAR CARCINOMA

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ABSTRACT

The majority of patients (worldwide) diagnosed with hepatocellular carcinoma (HCC) in 2016 will not be candidates for a potentially curative therapy; however, patients with disease localised to the liver will have options for treatment that are proven to be safe, effective, and worthy of consideration. Transarterial radioembolisation and transarterial chemoembolisation continue to evolve, as does stereotactic external beam radiation therapy with photons or protons. Nonsurgical therapies can provide substantial improvements in quality of life and survival rates compared with best supportive care. This review considers the current use of, and medical evidence for, intra-arterial therapies and external beam radiation options in the nonsurgical management of HCC.

Keywords: Yttrium, selective internal radiation therapy, transarterial chemoembolisation (TACE), transarterial embolisation, external beam radiation therapy (EBRT), proton, stereotactic body radiation therapy (SBRT), radioembolisation, microspheres, gastrointestinal cancer.

INTRODUCTION

Hepatocellular carcinoma (HCC) accounts for 85% of liver cancers worldwide and is the most common malignancy of the hepatobiliary tract. In 2012, 783,000 cases of HCC included 338,000 cases of pancreas adenocarcinoma.[1] HCC is the third leading cause of cancer mortality worldwide. Fewer than 30% of patients with HCC can be approached with curative intent therapies, such as surgical resection, transplantation, or ablation for tumours <3 cm.[1]

This review discusses current treatment options for liver HCC in >70% of patients who are not candidates for curative-intended treatment. Intervention categories include hepatic-arterial therapies, such as radiation implantation and external beam radiation therapy (EBRT). Promising new systemic therapies and immunotherapy agents that may prove helpful in the future are beyond the scope of this discussion.

INTRA-ARTERIAL THERAPIES

The portal venous system supplies ≥75% of the blood flow to normal liver lobules, and the hepatic arteries supply 75–100% of the blood flow to primary or metastatic solid tumours of the liver. Treatment strategies exploiting this vascular anatomy rely on the hepatic arterial flow to deliver chemotherapy, radiation particles (microspheres), or occlusion to cause hypoxic cell death.

Transarterial radioembolisation (TARE) uses inert microspheres (100–800 µm in diameter) released proximally to the HCC tumour; these cause complete obstruction of blood flow to the downstream tissue resulting in cell death in the tumour and normal liver cells where collateral vessels are not close enough for diffusion of oxygen and nutrients. The most common and successful non-radiation approach is transarterial chemoembolisation (TACE) delivery of a cytotoxic agent combined with ischaemia-inducing obstructive particles directly to the tumour. Historically, conventional transarterial chemoembolisation (cTACE) used heterogeneous

particles given in conjunction with multiple chemotherapy agents, with and without lipiodol as the vehicle. TACE using drug-eluting beads (DEB-TACE) purports to use same size microparticles for reproducible saturation and delivery of chemotherapy.

CONVENTIONAL TRANSARTERIAL CHEMOEMBOLISATION

Chemotherapy agents are typically emulsified in lipiodol, an oily contrast agent believed to increase intratumoural retention of a cytotoxic agent. Embolisation of the target vessels is performed via delivery into the hepatic artery of gelfoam, calibrated poly(vinyl alcohol), or acrylic copolymer gelatin particles that cause irreversible occlusion of the feeding vessel. The use of calibrated particles is increasing globally due to the ability to size particles according to target vessel. Vessel occlusion after injection of calibrated particles results in lower peak plasma concentration and increased drug retention within tumours. Therapeutic benefit obtained from adding a cytotoxic agent to bland embolisation was challenged by two clinical trials in the 1990s[2,3] and two meta-analyses,[4,5] both of which suggested that the antitumour effect is mainly driven by ischaemia.

Randomised controlled trials[6,7] in selected patients with preserved liver function have provided data supporting the use of TACE for palliative treatment of unresectable HCC. In a Spanish trial,[6] patients with preserved liver function and no main portal vein thrombosis (PVT) were treated with fixed interval chemoembolisation, embolisation, or best supportive care. The 2-year survival rate after TACE was 63% compared with 27% in untreated patients (p=0.009). A trial in Hong-Kong[7] comprised patients with lobar or branch PVT with preserved liver function and a 2-year survival rate of 31%, again superior to the 11% observed in the control group (p=0.002). Three meta-analyses[4,5,8] confirmed that TACE improves survival of patients and it is now the standard treatment for patients in the intermediate stage of the Barcelona Clinic Liver Cancer (BCLC) staging system (multinodular HCC, relatively preserved liver function, absence of cancer-related symptoms, and no evidence of vascular invasion or extrahepatic spread).[9]

The range of patients treated by TACE in clinical practice greatly exceeds the margins of the BCLC intermediate stage. As a result, reported survivals are heterogeneous ranging from 53–90% at 1 year, 11–67% at 2 years, and 8–26% at 5 years.[10-18] The median survival average is 16 months, even in the most recent series with unrestricted patient selection.[19-21] Median survival ranges reported by stages are 16–45 months in the early BCLC Stage A, 15.6–18.2 months in intermediate BCLC Stage B, and 6.8–13.6 months in the advanced BCLC Stage C. Prognosis after TACE largely depends on liver function, tumour burden,[10,12,16-18] presence of portal vein invasion, and response to treatment. TACE is contraindicated in patients with PVT as occlusion of arterial blood flow may induce liver failure; however, super-selective TACE may not be harmful in specific patients with segmental branch invasion.

TACE is a safe procedure, although it is frequently followed by side effects such as post-embolisation syndrome, which occurs in >40% of patients and includes nausea, abdominal pain, and fever symptoms that tend to be mild and short-lived. A transient decline in liver function after TACE appears in 20–45% of patients, and acute liver decompensation is reported in 0.1–3% of cases.[22,23] Mortality rates of 0.003–10% in the different series[4,17,18] reflect differences in the target population and TACE regimen. Liver functional reserve is key to an optimal selection and patients should be Child–Pugh Class A or B7 without ascites. A recent consensus from a panel of experts recommends a series of absolute and relative contraindications for the treatment of patients in the intermediate and advanced stages.[24]

TRANSARTERIAL CHEMOEMBOLISATION USING DRUG-ELUTING BEADS

DEB-TACE slowly releases embolising particles, previously loaded with cytotoxic agents, into the tumour. Embolising particles contain a sulfonate-modified poly(vinyl alcohol) hydrogel (DC Beads®, Biocompatibles, Surrey, UK) or a sodium acrylate and vinyl alcohol copolymer (HepaSphere™, BioSphere Medical, Inc., Rockland, MA, USA). Trials investigating embolising particles loaded with doxorubicin show that systemic exposure to this drug is significantly reduced when compared with conventional TACE.[25] In an international randomised trial comparing cTACE with DEB-TACE using DC Beads, the primary endpoints of superiority of DEB-TACE in achieving objective tumour response at 6 months and producing fewer treatment-related serious adverse events in the first 30 days were not met.[26] Tumour response rates were 52% and 44% and time-to-progression was 7.1 months

and 6.4 months for DEB-TACE and cTACE, respectively. A similar 6-month response rate of 51% was reported for HepaSphere Microspheres in a multicentre study.[27] A prospective randomised comparison of DEB-TACE and bland embolisation using the same unloaded particles showed that despite producing a significantly better response rate at 9 months (55% versus 31%), 12-month survival was similar (85.3% versus 86%).[28] Although DEB-TACE does not improve survival over cTACE, DEB-TACE provides a way to perform TACE in a standardised way, and when optimal patients are selected, the beneficial effect of TACE can challenge that of percutaneous ablation. Recent reports from two centres, comprising 300 patients in the early and intermediate stages, show 3-year and 5-year survival rates of 62–66% and 22–38%, respectively.[29] Major complications, including liver abscess, cholecystitis, and pleural effusion, occurred in 4.1% of patients in the Greek series;[28] in the Spanish series,[25] 1.6% of patients had liver failure; the death rate was 10%, of which 0.96% of cases were attributed to treatment.

TRANSARTERIAL RADIOEMBOLISATION

During TARE treatment, radioactive microspheres are injected intra-arterially for internal radiation treatment.[30] Two types of microspheres are available: radioactive glass microspheres (TheraSphere®; MDS Nordion, Ontario, Canada) and resin (SIR-Spheres®; Sirtex Medical Limited, Sydney, Australia). Both types use ^{90}Yttrium as the radiation-emitting isotope. Due to the small diameters of 25–45 μm, radioactive microspheres produce no significant ischaemic effect unlike the >100 μm particles used in TACE. Patients are candidates for TARE if their liver function is preserved (serum total bilirubin <2 mg/dL) and there is no ascites or hepatic encephalopathy present.[30,31]

Clinical trials comparing TARE with other therapies with a sufficient number of patients to answer the question of superiority have not been performed; however, Level II evidence is available from cohort series published in the last 5 years.[32-37] TARE has been used to treat unresectable patients who are not candidates for TACE (advanced stage due to symptoms, PVT, or intermediate stage with very large tumours or extensive bilobar involvement).[38] A case-controlled study with poor TACE candidates indicated that TARE might improve survival compared with experimental therapies or best supportive care (16 months versus 8 months, $p<0.05$).[32] Intermediate stage patients analysed by tumour stage and treated by TARE reached a median survival of 16–18 months[35,37,39] compared with median survival achieved by TACE. Broadly equivalent survivals are also reported in retrospective analyses of single institutions. The remaining treatment options for patients in the intermediate stage who fail to respond to TACE include the antiangiogenic and antiproliferative targeted agent sorafenib or TARE.

Sorafenib is the mainstay for treating advanced HCC; for example, cases exhibiting vascular invasion, extrahepatic disease, or deteriorated performance status patients with at least partially preserved liver function. TARE has no macroembolic effect[40] and can be applied safely to patients with PVT, which offers a median survival of 6–13 months, similar to 6.5–10.7 months reported in the Phase III clinical trials of sorafenib in the same group of patients. In patients with only branch or segmental PVT, survival extends from 10 to 14 months.[34,35,38,41] Due to this growing body of Level II evidence, TARE is now included in the guidelines adopted by the European Society for Medical Oncology (ESMO), the European Society of Digestive Oncology (ESDO), and the National Comprehensive Cancer Network (NCCN).

TARE is used to reduce tumour size to within acceptable limits for liver transplantation, to render non-operable patients operable, or to simplify surgery. Downsizing from UNOS (United Network for Organ Sharing) T3 to T2 was achieved more often with TARE than with TACE (58% versus 31%, $p=0.023$).[42] Atrophy of the radiated lobe after TARE and contralateral lobe hypertrophy resulting from injection of high activity of ^{90}Yttrium in a lobar hepatic artery may be valuable and contribute to resectability.[43] In a group of 21 UNOS T3 stage patients, 29% were downstaged and underwent surgical resection or liver transplantation, with a 3-year survival rate of 75%,[44] comparable with the survival rates in patients with early-stage disease who are treated radically at the time of diagnosis.

Rare complications after TARE resulting from the irradiation of non-tumoural tissues include pneumonitis, cholecystitis, gastrointestinal ulcerations, and liver damage. Liver toxicity is the most challenging adverse event in HCC patients as the majority of these tumours arise in cirrhotic livers, with some degree of reduced functional reserve. A variable incidence of liver

decompensation including ascites (0–18%) or encephalopathy (0–4%) has been reported.[36,37] The incidence of radioembolisation-induced liver disease, characterised by jaundice and ascites appearing 4–8 weeks after TARE in cirrhotic patients, was 9.3% in the largest series reported.[45]

Combinations with Systemic Agents

Clinical trials of sorafenib with intra-arterial therapies are disappointing. Time-to-progression among patients with >25% tumour necrosis or shrinkage at 1–3 months following one or two TACE sessions where participants received sorafenib, was not better than time-to-progression of patients receiving placebo (5.4 months versus 3.7 months, respectively, p=0.25).[46] When continuous sorafenib or placebo was given concurrently with DEB-TACE, safety was not an issue[47] and the hazard ratio for time-to-progression was 0.797 in favour of sorafenib (95% confidence interval: 0.588–1.080, p=0.072). Overall survival was comparable.[48,49] The addition of TARE to sorafenib for intermediate and advanced stage patients is currently being studied in the randomised controlled SORAMIC trial (NTC01126645). An interim analysis of the first 40 patients randomised to radioembolisation with ^{90}yttrium resin microspheres followed by sorafenib (n=20) or sorafenib only (n=20) in this study showed that there were no significant differences in adverse events or Grade 3/4 toxicities between the combination and control arms. A Phase II study comparing DEB-TACE plus sorafenib with DEB-TACE plus placebo has not reached the median overall survival, but the time-to-progression is not statistically different between the two arms.[51] Two retrospective studies and one prospective study suggest that sorafenib with TACE is safe, with varying evidence of an advantage in time-to-progression when used in combination.[52-54]

EXTERNAL BEAM RADIATION THERAPY

Radiation therapy (RT) to liver tumours is limited by the relative radiosensitivity of the sinusoid endothelium, compared with the significantly higher doses of radiation required to confidently destroy HCC cells.[55] Normal tissue complication probability (NTCP) models are based on observed complications after radiotherapy in a specific organ, with known daily and total dose data and specific clinical outcomes measured.[56,57] The accepted endpoint in hepatic NTCP models is radiation induced liver disease (RILD), classically reported in terms of TD5/5 and TD50/5; the total dose of photon radiation, typically to the whole liver, creates a 5% rate of RILD by 5 years post-radiation, and a 50% rate respectively.[56-58]

External Beam Radiation Therapy for Hepatocellular Carcinoma

Three dimensional conformal radiotherapy (3DCRT) and intensity modulated radiotherapy (IMRT) have been mainstays of advanced treatment delivery using computed tomography based datasets to target tumours while sparing normal surrounding tissues. Stereotactic body radiotherapy (SBRT) is a specialised form of 3DCRT that delivers very high single fractions of daily radiation; up to five in total. There are many challenges with EBRT for HCC; however, there has been success using image-guided radiation therapy to assist in delivery of 3DCRT, IMRT, and SBRT, along with respiratory motion compensation and tumour visualisation. Proton beam radiation therapy (PBRT) utilises a different type of energy to photon-based radiation, and represents a treatment that has the physical characteristics to provide superior dose deposition compared with 3DCRT.

Indications for External Beam Radiation Therapy in Hepatocellular Carcinoma

RT has a well-proven ability to sterilise tumours similarly to other local ablative approaches such as radiofrequency ablation.[59] In the BCLC classification, Stage 0 and early Stage A patients who cannot undergo surgical resection, transplant, or radiofrequency ablation, are candidates for RT. In Stages B and C, RT has efficacy in situations where TACE has been ineffective or is unsuitable, such as in patients with portal vein invasion where TACE is contraindicated and TARE may be impossible or ineffective.[59]

STEREOTACTIC BODY RADIATION THERAPY FOR HEPATOCELLULAR CARCINOMA

SBRT for HCC offers an increased ability to spare normal liver tissue from receiving tolerance doses of radiation. Four prospective studies and four retrospective single institution reports have been reported in the literature (2006–2011) with cohort sizes ranging from 8–60 patients. Despite the lack of larger, randomised controlled data, the positive outcomes in all stages of HCC are proven with a wide array of fraction sizes and total doses. Excluding the eight-patient

study, the remaining three studies used at least five different fractionation schedules adjusted for Child–Pugh A or B. One-year survival ranged from 48–79% in the heterogeneous groups.[60-62]

PROTON BEAM RADIATION THERAPY FOR HEPATOCELLULAR CARCINOMA

PBRT offers increased control of radiation dose deposition at any depth in the body.[63] Prospective studies are positive regarding toxicity and tumour control with encouraging overall survival in selected HCC patient groups in Eastern and Western populations.[63] There are 10 prospective studies which have analysed PBRT. Each study reports on greater numbers of patients than those which have looked at SBRT (76-318).[64-67] The outcomes of superiority of SBRT or PBRT in HCC patients are unknown; however, it is likely that SBRT and PBRT will be complementary to each other based on factors such as tumour size, distribution, and location in the liver. Dawson[63] suggested that photon beams (3DCRT, IMRT, SBRT) are best employed in Child–Pugh Class A patients with tumours of <6 cm in size, in the right lobe, near the dome. Protons may be utilised best in Child–Pugh Class B, tumours that are >8 cm, and those that are central and/or medial in the liver.[59] Only Level IIa evidence supports any form of radiation in HCC; however, combined with the retrospective reports of hundreds of patients, there is significant evidence supporting radiotherapy in all stages of HCC.[59]

CONCLUSIONS

HCC patients unable to receive curative approaches can derive significant benefit in quality of life and survival if eligible for the intra-arterial or external therapies presented. New technologies exploiting both approaches are currently in clinical testing, and include external radiation using carbon ion beams, combined chemotherapy, TARE, and variations on TACE both mechanically and via the chemotherapy agent deployed.

TACE is a heterogeneous group of procedures in terms of materials, extent and selectivity of vessel occlusion, and timing of repeated sessions. Good tumour responses are generally observed when a reduced number of smaller tumours are embolised in a selective fashion through a distinct feeding vessel. Two positive clinical trials and three meta-analyses report that TACE is the standard of care for HCC patients in the intermediate stage. DEB-TACE is a more recent standardised way of performing TACE with similar outcomes. Compared with TACE, evidence supporting the use of TARE in the treatment of HCC patients comes from consistent, large cohort series involving patients with more advanced HCC, those unsuitablefor other locoregional therapies, or patients who have failed TACE.

TACE and TARE should be considered complementary tools. TARE can be an alternative to repeated TACE for patients who fail to respond to initial TACE, and as a first option in those patients who are poor candidates for TACE. Results of ongoing clinical trials will soon establish if sorafenib or other targeted therapies improve the outcome of HCC patients treated by TACE and TARE.

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FATTY LIVER DISEASE: A CROSSTALK BETWEEN LIPID SPECIES

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ABSTRACT

The role of different lipid species such as free fatty acids and sphingolipids in non-alcoholic fatty liver disease (NAFLD) has been extensively studied during the last decade. In addition, free cholesterol accumulation in hepatocytes plays a crucial role in the transition from steatosis to steatohepatitis. However, the contribution of these lipids to NAFLD pathology is often evaluated individually. This review attempts to enclose the main metabolic and signalling connections between lipotoxic lipid species, and how their homeostasis is disrupted in NAFLD.

Keywords: Fatty liver, non-alcoholic fatty liver disease (NAFLD), lipotoxicity, sphingolipid, cholesterol, ceramide, fatty acids (FA), palmitic acid (PA).

INTRODUCTION

In 1953, Hokin and Hokin discovered that some lipid species located in the cell membrane were able to participate in cell signalling.[1] Since then, the role of lipids in different pathologies has become an intense area of research; despite this, the pathogenesis of non-alcoholic fatty liver disease (NAFLD) is still poorly understood. The most prevalent hypothesis, proposed in 1998,[2] postulates the convergence of two hits, the first consisting of the accumulation of lipid droplets or steatosis, followed by the onset of the second hit, which induces inflammation and tissue damage, defined as steatohepatitis. This hypothesis implies that steatosis is a sensitisation factor for the reactions of the second hit. Due to this preconception, many *in vivo* dietary studies have been performed with unspecific high fat diets, sometimes without a lipid composition disclosure and often named as 'Western diets'.[3,4] While this is a useful approach to achieving some clinical features found in patients, such as steatosis, it hampers elucidation of the key detrimental lipid species that are participating in stress, inflammation, and apoptosis signalling pathways. There is compelling evidence that hepatic triglycerides (TG) are not the effectors of cellular toxicity in NAFLD, but rather a defence mechanism to avoid free fatty acid (FFA) accumulation, which can trigger cell death pathways.[5] Likewise, many reports suggest an important lipotoxic role of cholesterol and sphingolipid species.[6,7]

Fatty liver disease therefore has a complex pathology, which comprises many concomitant cell alterations.[8] In patients, plasma serum and liver biopsies show different lipid signatures for each stage of the pathology.[9-12] This connection between lipid composition and disease severity underscores how important it is to further characterise the contribution of these molecules to NAFLD pathology and how they interact with each other in order to advance treatment and prevention strategies.

The purpose of this review is to describe the lipid metabolic changes induced by the main lipotoxic lipids in NAFLD.

FATTY ACIDS

Fatty acids (FA) are carboxylic acids with a non-ramified aliphatic chain of different length. In mammals, FA usually contain up to 28 carbons in even numbers, but the most abundant length in biological tissues ranges from 14–20. In addition, the presence of one or more double bonds between carbons further increases the diversity of these molecules. Saturated FA (SFA), with no double bonds, account for 30–40% of the FA present in animal tissues, in order of abundance: palmitic acid (PA) (15–25%), stearic acid (10–20%), myristic acid (MA) (0.5–1%), and lauric acid (<0.5%).[13]

The role of medium-long-chain FA in NAFLD has been more extensively studied than shorter chain FA (<16 carbons), probably because of the faster catabolism of the latter and therefore lower evidence of toxic effects in the cell.[14] Nowadays, it has been demonstrated that FFA species can have opposite roles in NAFLD, particularly when it comes to comparing SFA with unsaturated ones. Buettner et al.[15] observed that high fat diets with different fatty acid compositions triggered different outcomes: a high intake in polyunsaturated FA reduced expression of lipogenic genes regulated by sterol regulatory element-binding protein 1 (*SREBP-1c*) and increased peroxisome proliferator activated receptor (PPAR)-α-regulated lipolytic genes, whereas diets rich in SFA and monounsaturated FA induced hepatic steatosis. PA is the source of FA and ceramide synthesis in the endoplasmic reticulum (ER) of the cell and is also the most abundant FA in Western diets. However, an intracellular increase in non-esterified PA can be fatal, since this molecule induces mitochondrial dysfunction,[16] lysosomal permeabilisation,[17] ER stress, and autophagy alteration.[18] Similar lipotoxic effects have been observed with other SFA such as stearic acid.[19,20] On the other hand, many *in vitro* studies report that unsaturated fats like oleic acid could protect cells against PA lipotoxicity.[21-23]

The line between 'good' and 'bad' blurs when considering insulin resistance in the literature. Buettner et al.[15] found that an olive oil enriched diet led to insulin resistance in rats. Conversely, it has been shown that the polyunsaturated FA oleic acid, the main olive oil component, has the ability to induce PPAR-α thereby promoting FFA oxidation, safe storage into TG, and conferring protection against insulin resistance.[24] Of note, studies that report the participation of unsaturated fats in insulin resistance are often performed with lipid mixtures,[25] making it difficult to exclude a possible intervention of other lipid species in the pathogenic mechanism.

FATTY ACIDS AND SPHINGOLIPIDS

PA is the precursor of ceramide, the simplest sphingolipid, which belongs to a family of lipids present in biological membranes and segregated in particular domains, where they participate in different signalling events. Sphingolipid synthesis occurs in the ER and begins with the condensation of the amino acid L-serine with PA to form 3-ketosphinganine. This first reaction of the pathway is achieved by the enzyme serine palmitoyltransferase and is the limiting step. Afterwards, 3-ketosphinganine reductase reduces 3-ketosphinganine to sphinganine followed by action of dihydroceramide synthase, which adds a fatty acid to sphinganine to form dihydroceramide. The six dihydroceramide synthase isoforms described so far exhibit specificity towards different FA, giving rise to heterogeneous ceramide species.[26] Finally, dihydroceramide desaturase (DES) creates a double trans 4,5 bond thereby converting dihydroceramide into ceramide. Interestingly, Beauchamp et al.[27] found that DES can be modified by an irreversible lipidation in its N-terminal residue with MA through a process called myristoylation, which is carried out by the enzyme N-myristoyltransferase. Myristoylation increases the activity of DES implying that MA, a 14 carbon saturated FA present in mammalian milk, can potentiate the synthesis of ceramide. Once synthesised, ceramide traffics towards the Golgi apparatus, where it is modified to generate more complex sphingolipids: ceramide trafficking, by the ceramide transfer protein to the Golgi, is used for sphingomyelin synthesis by the enzyme sphingomyelin synthase through the addition of a phosphocholine or phosphoethanolamine group in the first carbon of ceramide with concomitant generation of diacylglycerol.[28] Alternatively, ceramide can also travel to the Golgi apparatus by vesicular transport to be converted into glucosylceramide upon the addition of glucose by the enzyme glucosylceramide synthase. The addition of sialic acids to glucosylceramide generates complex sphingolipids and gangliosides. Besides the aforementioned *de novo* synthesis, ceramide can also be generated from acid (ASM) or neutral (NSM)

sphingomyelinases and from the internalisation of membrane sphingolipids through endocytic pathways to the lysosomes, where they are hydrolysed by sphingomyelinases or glucosidases to produce ceramide, a process known as the salvage pathway. Many routes therefore converge into the generation of ceramide. Importantly, ceramide and other sphingolipid species are bioactive lipids that contribute to a myriad of cellular processes; the alteration of their metabolic homeostasis is a potential therapeutic target, based on modulating their levels towards a desired functional outcome.[29]

In a fatty liver scenario, the increase in PA due to its higher dietary intake and liver import fuels *de novo* ceramide synthesis. Moreover, PA and other SFA can act as toll-like receptor 4 agonists,[30] a signalling pathway shown to induce ceramide synthetic enzymes in macrophages,[31] but whether this occurs in hepatocytes is still under debate.[32,33] PA could also indirectly trigger ceramide synthesis through its lipotoxic effects. For example, reactive oxygen species or cytokines like tumour necrosis factor alpha (TNF-α) lead to a rapid accumulation of ceramide through ASM.[34] This event is of particular relevance in lipid rafts, membrane microdomains with a high concentration of sphingolipids, cholesterol, and signalling molecules. The produced ceramide is capable of spontaneously self-aggregating and reorganising lipid rafts, thereby fusing them and creating large signalling platforms. Consequently, ceramide plays a crucial role in cellular signalling by clustering receptors together.[35]

Similarly to PA, ceramide accumulation triggers lipotoxic pathways like inflammation, ER stress, mitochondrial dysfunction and permeabilisation, autophagy alteration, and lysosomal cathepsin D activation, as reviewed in detail elsewhere.[25,36-38] Some of these pathways can in turn induce ceramide and toxic derivatives like gangliosides, creating a vicious cycle. On the other hand, sphingosine-1-phosphate or ceramide-1-phosphate have been reported to be anti-apoptotic and mitogenic.[29] Recently, an effort has been made to decipher the role of different ceramide species in cellular signalling. Ceramide C16:0 is toxic for macrophages[39] but the contrary has been observed in human head and neck squamous cell carcinomas, where C16:0 was anti-apoptotic and C18:0 pro-apoptotic.[40] Therefore, the role of ceramide species seems to be specific to each cell type and further investigation is required to characterise their cellular effects.

It is important to note that there is an overlap of the targeted organelles and lipotoxic outcomes reported with PA and its product ceramide in the literature. Since PA can be converted into ceramide or trigger ceramide synthesis by other means, particular care is suggested before attributing lipotoxic roles to PA. In this regard, we have recently determined that ER stress is aggravated by *de novo* synthesised ceramide after PA treatment in primary hepatocytes.[41] Importantly, we have also validated *in vivo* the potential toxic role of MA when ingested in combination with PA through a sustained ceramide production, causing serious disruption of lipid metabolism homeostasis, ER stress, inflammation, and steatohepatitis.

FATTY ACIDS AND CHOLESTEROL

Cholesterol is a crucial sterol for animal cell membranes. The molecule intercalates between phospholipids, positioning the hydroxyl group near the polar and the steroid rings in the apolar zones. This interaction immobilises and packs the membrane, diminishing its fluidity and permeability, but at the same time protects the membrane against phase transition due to a higher distance between aliphatic chains. Besides its structural function, cholesterol is highly abundant in lipid rafts, where it participates in the formation of caveolae, and is the precursor of steroid hormones, biliary acids, and vitamin D.

Synthesis of cholesterol is regulated by its availability. The main negative feedback mechanism is the inhibition of the sterol regulatory element-binding protein 2 (SREBP-2), a transcription factor that activates cholesterol biosynthetic and uptake enzymes like 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase and low-density lipoproteins (LDL), respectively. Similarly, cholesterol can also directly bind to HMG-CoA reductase to induce its degradation by the proteasome.[42] A high dietary intake of cholesterol therefore reduces its endogenous synthesis. This regulation might be compromised in case of a concomitant increase of other lipid types, mainly TG, which provide a source of FA. FA β-oxidation would produce acetyl-CoA, required for the first step of *de novo* cholesterol synthesis through the mevalonate pathway. However, acetyl-CoA availability is controlled by the different subcellular localisation of these metabolic pathways, meaning that

previous export from the mitochondria to the cytosol, through the citrate transporter, is required.[43]

Cholesterol homeostasis can also be regulated by the nuclear receptors liver X receptor (LXR) and farnesoid X receptor (FXR). Oxysterols activate LXR, inducing the transcription of genes involved in cholesterol transport and clearance, thereby making it a potential therapeutic target for atherosclerosis.[44] On the other hand, LXR activation also induces lipogenic transcription factors like SREBP-1c, meaning that cholesterol could indirectly influence steatosis. In the context of fatty liver disease, LXR inhibition instead of activation may therefore be more beneficial.[45]

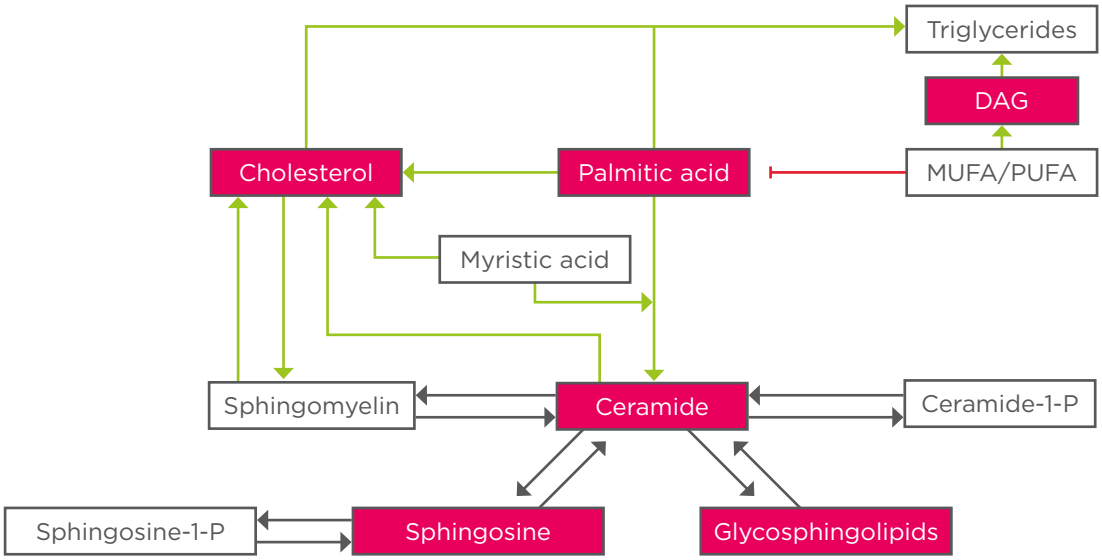

Figure 1: Crosstalk between lipid species in fatty liver disease.
Lipotoxic lipid species are highlighted in red, dark arrows represent enzymatic reactions, green and red arrows refer to changes through stress or metabolic pathways.
MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; DAG: diacylgylcerol.

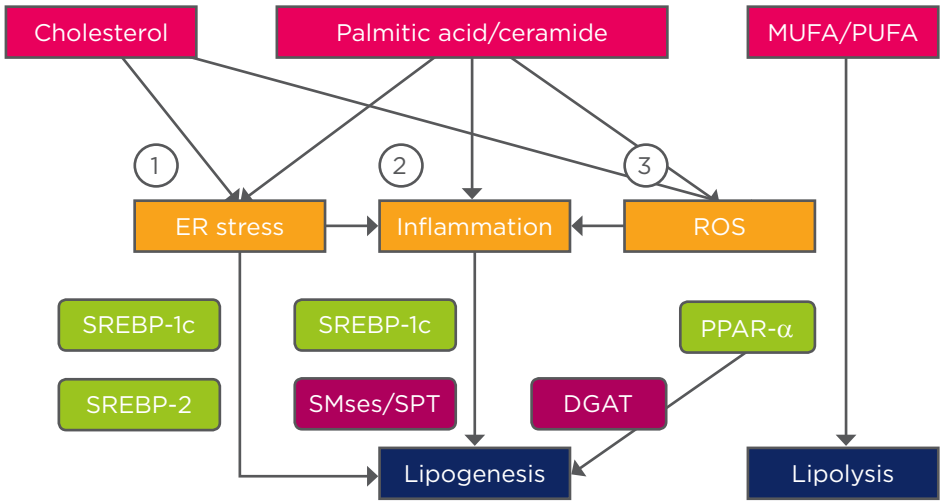

Figure 2: Pathways affecting lipid homeostasis in non-alcoholic fatty liver disease.
1) Ca^{2+} perturbation, ER membrane alteration; 2) TLR4; 3) Mitochondrial dysfunction.
Transcription factors are highlighted in green and enzymes in purple.
TLR4: Toll-like receptor 4; DGAT: Diglyceride acyltransferase; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; ER: endoplasmic reticulum; ROS: reactive oxygen species; PPAR-α: peroxisome proliferator activated receptor alpha; SPT: serine palmitoyltransferase; SREBP: sterol regulatory element-binding protein; SMse: sphingomyelinase.

Cholesterol catabolism into bile acids begins with its conversion to 7-α-hydroxycholesterol by cholesterol 7-α-monooxygenase (CYP7A1), the rate-limiting step of the pathway.[46] Bile acids bind FXR, which represses CYP7A1, acting as a negative feedback loop. Besides its role in maintaining the balance between cholesterol and bile acids, FXR also induces lipolysis by activating PPAR-α and repressing SREBP-1c.[47] Accordingly, FXR deficiency in mice results in a hepatic and serum increase of cholesterol and TG,[48] which are NAFLD hallmarks. FXR activation by natural and synthetic agonists is thus currently regarded as one of the main therapeutic strategies for NAFLD.[49] However, clinical studies show that treatment with FXR agonists has some undesired side effects such as pruritus or increased LDL cholesterol,[50] so further research is required to develop FXR modulators with a higher specificity for lipolytic activity.

The increase in lipotoxic FFA, such as PA, in the fatty liver promotes ER stress, which has been shown to activate SREBP-2.[51] In relation to this, we have recently observed that mice that are fed diets with PA or PA plus MA have ER stress, higher HMG-CoA reductase gene expression, and hepatic cholesterol.[41] Curiously, a high intake in MA, which is not regarded as lipotoxic per se, has been found to affect serum lipoproteins and hepatic cholesterol levels.[52] The authors attributed this change to a negative correlation of MA with the scavenger receptor Class B Type I, a high density lipoprotein receptor, and cholesterol catabolic enzymes, but they did not analyse ER stress markers. Moreover, since the authors used a mixture of different fats, the participation of other lipid types in this outcome cannot be discarded.

Conversely, an increase in ER membrane cholesterol-loading can perturb the organelle calcium stores, thereby triggering ER stress and engaging in a vicious cycle.[53] The unfolded protein response induces lipogenic genes expression, enhancing the accumulation of FA, lipotoxicity, and synthesis of other lipotoxic lipids like ceramides. Our group has also reported that free cholesterol accumulation can affect other organelles like mitochondria, by depleting mitochondrial glutathione and sensitising hepatocytes to inflammatory cytokines[54] and lysosomes, by impairing mitophagy and the subsequent removal of damaged mitochondria.[55]

CHOLESTEROL AND SPHINGOLIPIDS

The relationship between cholesterol and sphingolipids has been less directly addressed, but these two lipids seem to be tightly regulated. Cholesterol preferentially binds to sphingomyelin in lipid membranes and concomitant changes in their levels have been reported in several studies, as reviewed in detail by Ridgway[56] and briefly synthesised in the following lines. An obvious link between cholesterol and sphingomyelin is their presence and crucial role in lipid rafts, suggesting that a proper ratio is necessary for the functioning of these particular membrane regions. In fact, a sphingomyelin decrease in the plasma membrane by exogenous sphingomyelinase treatment downregulates SREBP-2, and induces cholesterol esterification and its retrograde transport towards the ER. However, it is unclear if the local ceramide formation by this sphingomyelin hydrolysis participates in the process. In contrast, a decrease in cholesterol may not mean a decrease in sphingomyelin. This might be due to the need for sphingomyelin to continue other important functions for the cell and to maintain the membrane structure.

An imbalance of these lipids is seen in a number of pathologies. Niemann–Pick diseases are inherited lysosomal storage disorders with severe metabolic and in some cases neurological consequences. Acid sphingomyelinase deficiency and resultant sphingomyelin accumulation in lysosomes is the cause of Niemann–Pick Type A and B, whereas Type C (NPC) is caused by NPC 1 or 2 protein deficiency, involved in cholesterol efflux from lysosomes. A hallmark of all Niemann–Pick variants is fatty liver disease, with accumulation of sphingomyelin, cholesterol, and other lipid types in lysosomal compartments and other cell locations.[57,58] Due to the close relationship between cholesterol and sphingomyelin and because these pathologies share common traits, treatment of patients with sphingomyelinases could be an effective therapy regardless of their genetic defect. In fact, NPC patients also have a low ASM activity due to secondary sphingomyelin accumulation, and treatment with sphingomyelinases has proven effective against cholesterol loading in the lysosomes of NPC cells.[59]

Finally, and similarly to SFA, other sphingolipids like ceramide and gangliosides could induce ER stress,[60] thereby activating SREBP-2 and cholesterol synthesis, as we have recently observed

in vivo.[41] Cholesterol levels can therefore be influenced by sphingolipids or vice versa and further research will be required to discern their respective lipotoxic roles in NAFLD.

LIPID HOMEOSTASIS AND GENETIC POLYMORPHISMS

Comparative studies of candidate genes in NAFLD patients have found some polymorphisms associated with an altered lipid metabolism. For example, a single nucleotide polymorphism (SNP) in the microsomal triglyceride transfer protein (MTTP) was identified in its promoter region.[61] MTTP is involved in very low density lipoprotein (VLDL) synthesis and export, but patients with the SNP variant are predisposed to liver and serum TG accumulation due to reduced MTTP protein expression. However, experimental design with larger cohorts might be required to confirm findings in comparative studies, since other works often do not report the same correlations.[62]

Genome-wide association studies have also identified different SNPs associated with NAFLD, as reviewed in detail by Anstee and Day.[63] Among them, the SNP rs738409 (Ile148Met) in the patatin-like phospholipase domain-containing 3 (*PNPLA3*) gene is currently the most strongly associated with TG accumulation and NAFLD biochemical markers. *PNPLA3* is a triacylglycerol lipase, but curiously the SNP presence is not only related to steatosis but also to steatohepatitis and fibrosis.[64] Further research is therefore needed to fully elucidate the mechanisms by which this allelic variant promotes such pathogenic outcomes.

CONCLUSION

Lipids play a crucial role in cell signalling pathways and metabolism. The study of individual lipid species is a powerful tool to look for possible therapeutic strategies, but it is also important to understand the way these molecules' metabolic and signalling pathways are interconnected. Figure 1 summarises how the main lipotoxic species can affect each other in the context of fatty liver and Figure 2 shows the main pathways that disrupt lipid homeostasis. NAFLD research has evolved towards a hunt for detrimental lipids. Considering the interconnectivity summarised in this review, *in vitro* and *in vivo* studies should systematically perform a detailed quantification of lipid species and metabolic gene expression, which should be regarded as necessary as assessing transaminases in serum. For that purpose, lipidomics have become crucial in identifying changes in a myriad of these molecules with high sensitivity,[65] thereby providing the basis to look for molecular pathogenic mechanisms.

Further work needs to be done to fully elucidate NAFLD complexity,[66] but it is clear that its inherent lipid accumulation translates into a lipid homeostasis dysregulation, with many lipid types being affected simultaneously and more importantly, many of them having specific biological functions.

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IMMUNOPATHOGENESIS OF HEPATITIS B VIRUS INFECTION AND RELATED COMPLICATIONS

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ABSTRACT

Chronic hepatitis B (CHB) is a serious consequence of hepatitis B virus (HBV), which infects and replicates in the liver. It is characterised by prolonged hepatitis B surface antigen seropositivity; this can lead to both cirrhosis and hepatocellular carcinoma (HCC). The infection begins when HBV binds its only known functional receptor, sodium taurocholate cotransporting polypeptide (NTCP), which was identified recently. The discovery of NTCP was a significant breakthrough in the field of HBV research, and has facilitated the establishment of a susceptible hepatoma cell line model for studying the mechanisms underlying HBV pathogenesis. Following productive HBV infection, both cellular and humoral immune cells and molecules, such as T cells and chemokines, are activated to resolve infection by destroying HBV-infected hepatocytes. However, host immunity to HBV is not always protective, most likely due to immune evasion mechanisms employed by HBV. These mechanisms may result in viral persistence, accumulation of mutations, and aberrant epigenetic alterations that lead to HCC. Here we highlight our current understanding of the HBV replication cycle, immunopathogenesis, and related mechanisms underlying the progression of CHB to advanced liver disease, along with the attendant complications.

Keywords: Chronic hepatitis B (CHB), epigenetic alterations, immunopathogenesis, hepatocarcinogenesis.

INTRODUCTION

Chronic hepatitis B (CHB) results from hepatitis B virus (HBV), which belongs to the *Hepadnaviridae* family and the genus *Orthohepadnavirus*. CHB remains a significant contributor to morbidity and mortality related to sequelae such as cirrhosis and hepatocellular carcinoma (HCC).[1] Recent statistics show that 240–400 million people have CHB worldwide, with the Pacific Islands, Africa, and Asia representing hyperendemic areas.[2,3] More than 750,000 HBV-infected people die annually due to liver failure from cirrhosis and HCC. This still occurs despite the availability of HBV vaccines; recently, the FDA approved therapeutic agents such as nucleos(t)ide analogues and pegylated interferon, for use in the management of HBV.[4]

HBV is a prototype virus of 42 nm in diameter, with infectious double-shelled Dane particles that are indicated by the presence of hepatitis B surface antigen (HBsAg) in the blood. It is significantly pathogenic, leading to both acute and chronic infection.[5] HBV is non-cytopathic, and therefore hepatic damage due to HBV infection results as a consequence of prolonged and aggressive activation of host immunity, aimed at eradicating the virus.[5,6] Antiviral and antitumour immune responses are complex, with both the innate and adaptive systems affected and their dysfunction implicated in the progression of disease. More research efforts in understanding the early stages of HBV infection, host immunity, pathogenesis, and mechanisms underlying HBV progression to cirrhosis and HCC are urgently needed. This may lead to the development of therapeutic interventions that will elicit robust HBV-specific immune responses that clear the virus without damaging the hepatocytes. To this end, this review discusses research that elucidates mechanisms underlying HBV entry into the cell, immunopathogenesis, and hepatocarcinogenesis.

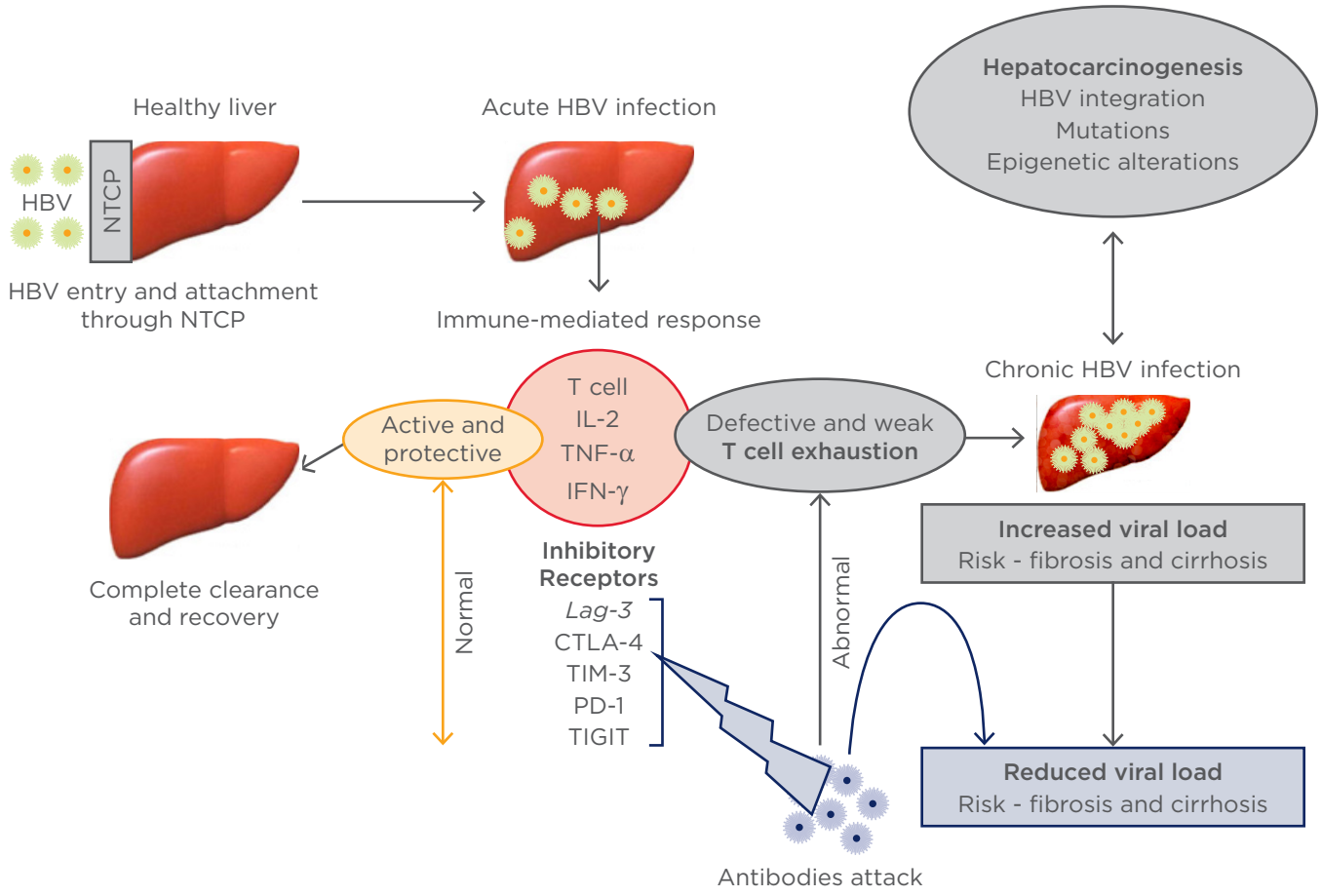

Figure 1: Impairment of T cell responses and cytokine disruption by the inhibitory cell receptors.
The effect on inhibitory receptors is associated with increased viral replication and aggressive liver disease. Blockade of the inhibitory receptors by specific antibodies restores T cell differentiation and cytokine production leading to resolved HBV infection with minimal liver damage.
HBV: hepatitis B virus; NTCP: sodium taurocholate cotransporting polypeptide; IL-2: interleukin 2; TNF-α: tumour necrosis factor alpha; IFN-γ: interferon gamma; Lag-3: lymphocyte activation gene 3; CTLA-4: cytotoxic T lymphocyte antigen 4; TIM-3: T cell immunoglobulin domain and mucin domain 3; PD-1: programmed cell death 1; TIGIT: T cell immunoreceptor with Ig and ITIM domains.

DISCOVERY OF THE HEPATITIS B VIRUS ENTRY RECEPTOR AND HEPATITIS B VIRUS REPLICATION

Horizontal, perinatal, and sexual transmission through contact with infected individuals and various bodily fluids are the predominant modes of HBV infection.[7] HBV replication has been extensively studied over the last two decades; this led to the seminal discovery of the sodium taurocholate cotransporting polypeptide (NTCP) receptor, which facilitates HBV attachments and entry (Figure 1). NTCP, also known as SLC10A1, is abundantly expressed in the liver. It is involved in the transportation and clearance of bile acids from portal blood into hepatocytes. Yan et al.[8] demonstrated that the preS1 region of the large protein L selectively interacts with the NTCP receptor following the binding of heparan sulfate proteoglycans, and this enables HBV attachment and entry into hepatocytes. HBV requires DNA polymerase and reverse transcriptase to replicate through RNA intermediates known as pregenomic RNA.[5] Following entry into the hepatocytes, a HBV nucleocapsid is released into the cytoplasm and transported into the host cell's nucleus to deliver relaxed circular (rc)DNA. In the nucleus, the rcDNA is repaired and converted to covalently closed circular super-coiled DNA, which serves as a template for the transcription of four viral RNA transcripts. The 3.5 kb pregenomic RNA transcript is then reverse-transcribed within the core particles. The resulting viral DNA within the core particles may either be enveloped and exported

or recycled back to the nucleus, thus promoting HBV persistence in liver.[6-11]

Knockdown of NTCP expression in duck primary hepatocytes infected with duck hepatitis B virus significantly decreased HBV infection, confirming that NTCP is indeed required for viral entry.[12] Since the discovery of HBV, researchers have struggled to find an easily accessible model for studying HBV infection. It is now possible to achieve >50% infection efficiency in human-derived HepG2 and Huh7 cell lines expressing exogenous NTCP systems.[12,13] Yan et al.[14] showed that the spinoculation involving centrifugal inoculation of HepG2 cells expressing NTCP significantly enhanced HBV infection, which was dramatically reduced after treatment with HBV entry inhibitors such as irbesartan, which target NTCP.[15] Even though further studies are urgently required to establish a model that will achieve 100% HBV-infection efficiency in *in vitro* or *in vivo* systems, the illustrated data support the evidence that NTCP is required for HBV attachment and entry. These findings provide new opportunities in HBV research for comprehending the early steps of HBV infection.

CHRONIC HEPATITIS B IMMUNE PATHOGENESIS

HBV is non-cytopathic, highlighting the complex but important interaction between the virus and host in causing HBV-related liver disease. Several studies on HBV transgenic mouse models have led to insightful knowledge on viral immunopathogenesis and the various strategies HBV utilises to escape immune responses.[6,10] The liver is an immune-privileged organ as it is the site for both humoral and cell-mediated immunity involving resident macrophages (including Kupffer and natural killer cells) and CD4/CD8 T cells, which play significant roles in fighting against invading pathogens and malignant transformation.[6]

Acute HBV infection usually resolves spontaneously with robust production and activation of protective immune responses from neutralising antibodies, Kupffer cells, natural killer cells, CD4/CD8 T cells, interleukin (IL)-2, interferon gamma (IFN-γ), and tumour necrosis factor alpha (TNF-α) cells.[16] Some individuals fail to clear the virus and progress to CHB infection; this is likely due to various factors including a long incubation phase before an immune response develops, an absence of 'danger signals' and viral evasion, resistance, deletion, alteration, and expansion of the immune system.[17] Immune system anomalies are associated with immature dendritic cells and neutrophils, impaired production of cytokines (IFN-γ, IL-2, and TNF-α), and T cell exhaustion (the progressive loss of CD4/CD8 T cell function; Figure 1).[17,18] These anomalies are attributable to HBV-mediated abnormal regulation of *lymphocyte activation gene 3* (*Lag-3*), cytotoxic T lymphocyte antigen 4 (CTLA-4), T cell immunoglobulin domain and mucin domain 3 (TIM-3), programmed cell death 1 (PD-1) receptor, CD244/2B4, CD160, and T cell immunoreceptor with Ig and ITIM domains (TIGIT),[19-21] which are outlined in Figure 1.

Immunoregulatory and inhibitory cell surface receptors are expressed on B cells, T cells, and cancer cells.[19-21] PD-1 and CTLA-4 are the well described immunoregulatory receptors in CHB infection and cancer. The primary role of PD-1 is to minimise T cell activity in peripheral tissues at the time of an inflammatory response to infection. Upregulation of PD-1 was observed in CHB patients with elevated HBV DNA and alanine aminotransferase (ALT) levels, and this correlated with increased viral replication and active liver disease. Genome-wide microarray analysis in both acute and chronically HBV-infected mice also sheds some light by showing direct correlation of T cell impairment and upregulation of PD-1.[22] Mechanisms underlying PD-1 activation in CHB infection have not yet been defined completely. However, it is known that PD-1 depends on its interaction with PD-L1 and PD-L2 ligands to negatively regulate T cell responses, cytokine induction, and proliferation.[23,24] Most recently, it was shown that induction of PD-1 is partially regulated by endogenous transforming growth factor, which regulates T cell proliferation and differentiation.[25]

In cancer cells, upregulation of PD-1 promotes immune resistance by blocking activation of T cell receptor signalling pathways responsible for presenting invading pathogens to the major histocompatibility complex.[18,25] One of the milestones that has recently emerged in the field of immunotherapy is the possible blockade of immunoregulatory receptors with specific antibodies to restore and enhance CD4/CD8 T cell immunity. Blockade of the inhibitory effect of PD-1 elicited robust production of HBV-specific CD8 T cell responses and resulted in intermediate T cell differentiation that significantly lowered viral replication, HBV DNA, and ALT levels in CHB patients.[26,27] In cancer patients, PD-1 blockade

resulted in tumour regression following inhibition of adaptive immune resistance.[23] In a mouse model with productive HBV infection, the PD-1/PDL1 complex led to upregulation of intrahepatic PD-1 expressing CD8/CD4 T cell immunity, suggesting a strong correlation between upregulation of PD-1 and increased HBV.[28] The use of anti-PD-1 antibodies in this mouse model blocked the interaction of PD-1 with PD-L1, leading to resolved HBV infection and reversal of the immune dysfunction. Li et al.[29] and other studies corroborated these findings by showing that a combination of therapeutic vaccination and PD-1 blockade in chronic HBV infection enhanced T cell immunity, leading to resolution of CHB.[29,30] Overall, these data suggest a new approach in the immunotherapeutic field to resolve CHB using PD-1 blockade and monoclonal antibodies.[26,30]

CTLA-4 is another significant immunoregulatory agent that becomes dysregulated during CHB infection. Several studies show that CTLA-4 negatively regulates T cell responses. In the early activation of HBV-specific CD8 T cells, induced by IFN-γ, CTLA-4 becomes upregulated, leading to increased levels of *Bcl-2*-interacting mediator of cell death, a novel member of the *Bcl-2* family that promotes apoptosis via transcription of E2F-dependent mechanisms.[19] A synergistic relationship between CTLA-4 with T cell receptor and CD28 impairs T cell responses by suppressing the production of IL-2 and T cell proliferation through activation of the apoptosis pathway.[24,27] Blocking the CTLA-4 pathway results in the suppression of the apoptosis, leading to an increased expansion of IFN-γ-producing HBV-specific CD8 T cells.[20] These may provide novel immunotherapeutic targets to eradicate CHB infection while minimising the risk of HCC progression.

CHB infection is characterised by persistent HBsAg seropositivity in infected individuals for >6 months.[5] Laboratory and histological assessments through screening for the presence of HBsAg, hepatitis B envelope antigen (HBeAg), hepatitis B core antibody (anti-HBc), hepatitis B surface antibody (anti-HBs), HBV DNA, and ALT levels aid in differentiating the five clinical phases of CHB infection as illustrated in Figure 2. The division of CHB phases by the HBeAg status is clinically relevant, but is also indicative of the role of the HBeAg in inducing or regulating an immunological response.[6] The first phase is immune tolerance, which is characterised by HBeAg seropositivity and normal ALT and liver histopathology, thought to be due to the tolerogenic effect of HBeAg. This was shown in the transgenic mice with deletion of specific T cell subsets and predominance of a Th2 response with the production of anti-inflammatory cytokines IL-4, IL-5, and IL-10.[31]

The second immune active phase is characterised by high HBV DNA and ALT levels, corresponding to the necroinflammatory activity seen in liver tissue associated with progressive disease. Immunologically, this phase corresponds to and is mediated by the secretion of proinflammatory cytokines, such as TNF-α, IFN-γ, and IL-20, and the increase in hepatitis C antigen-specific CD8 T cells seen in the liver. Contrastingly, seroconversion to HBeAg negative status and the appearance of hepatitis B envelope antibody (anti-HBe) in the serum is associated with decreased inflammation, suggesting the loss of the tolerogenic effect of HBeAg.[31] Patients in the third phase of CHB, immune clearance, have anti-HBe antibodies and undetectable HBV DNA, normal ALT, and histology, associated with a lower but not eliminated risk of complications. Immune tolerance may be due to hyporesponsiveness of endogenous IL-12, and subsequent failure to induce HBV-specific memory CD8 T cell responses. The fourth phase involves immune evasion and is associated with increased risk of fibrosis, resulting from various factors such as viral integration (which disguise the virus from the immune system) and loss of antigenicity.[6,31] The immune reactivation phase may occur with or without HBeAg expressing genomes, and is characterised by elevated HBV DNA and fluctuating ALT levels; these are associated with an increased risk of severe liver injury, which is likely explained by the prevailing proinflammatory cytokine milieu and reduction of CD8 T cells.[31,32]

Most recently, the FDA reported an increased fatal risk of HBV immune reactivation in patients receiving immunosuppressive therapy such as anti-CD20 agents ofatumumab and rituximab, which block the humoral immune responses by disrupting the function of B cells. Other immunoregulatory agents that may lead to HBV reactivation include etanercept, leflunomide, cyclosporine, and TNF-α inhibitors. It is therefore highly recommended that patients be tested for the presence of HBsAg, anti-HBc antibodies, and HBV DNA levels before they are treated with these immunoregulatory agents. Appropriate treatment of HBV must be immediately initiated in patients who develop HBV reactivation following treatment with ofatumumab

and rituximab; ofatumumab and rituximab must be stopped immediately to prevent acute flares that may lead to liver transplant and death.[33]

UNDERLYING MECHANISMS OF HEPATOCARCINOGENESIS

Liver cirrhosis and HCC remain the major consequences of CHB. Globally, HCC is the third leading cause of global cancer-related deaths after colorectal and lung cancers.[34] The available HBV therapeutic agents significantly reduce viral replication, but they still do not correct aberrant genetic and epigenetic alterations that promote progression of HBV to liver cirrhosis and malignant transformation of hepatocytes.[35] The progression of HBV infection to HCC occurs in a series of steps generally following a sequence of CHB infection, fibrosis or cirrhosis, dysplastic nodule formation, and HCC development as illustrated in Figure 3. The average period of HBV infection progression to cancer is about 20–30 years, and 8–10 years after the development of cirrhosis.[36-38]

It is believed that over repetitive cycles of CHB infection, HBV DNA integrates into the host genome at preferential sites known as chromosomal fragile sites (e.g. *FRA1A, FRA2C, FRA4E,* etc.), which are frequent sites for translocation of chromosomes, oncogene amplification, and deletion of tumour suppressor genes in cancer.[39] This increases the propensity for the accumulation of mutations and epigenetic alterations that may lead to fibrosis and ultimately cirrhosis and HCC development.[39]

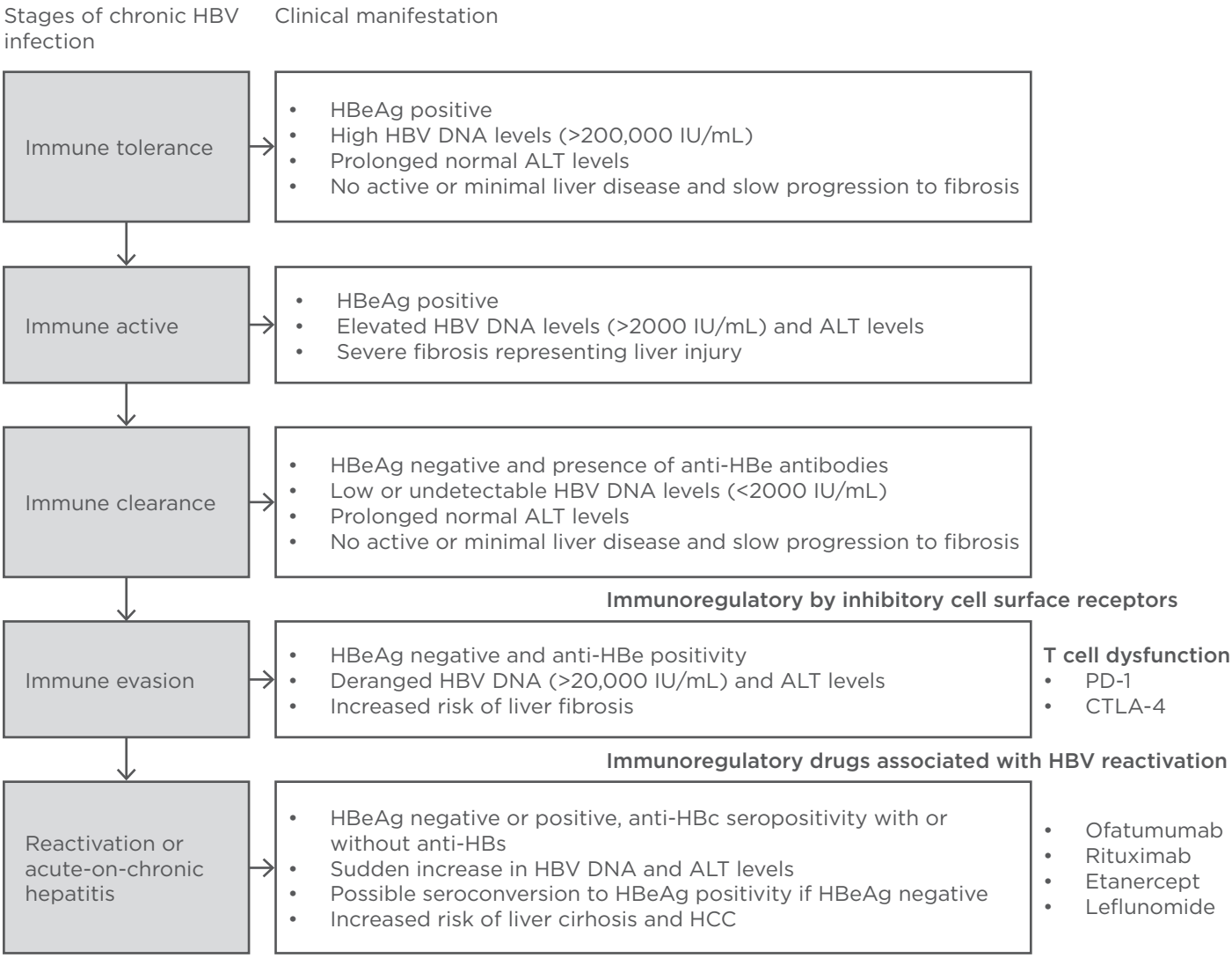

Figure 2: Chronic HBV replication phases and clinical manifestation.
HBV: hepatitis B virus; HBeAg: hepatitis B envelope antigen; ALT: alanine aminotransferase; anti-HBe: anti-hepatitis B e-antigen; HCC: hepatocellular carcinoma; anti-HBc: hepatitis B core antibody; PD-1: programmed cell death 1; CTLA-4: cytotoxic T lymphocyte antigen-4.

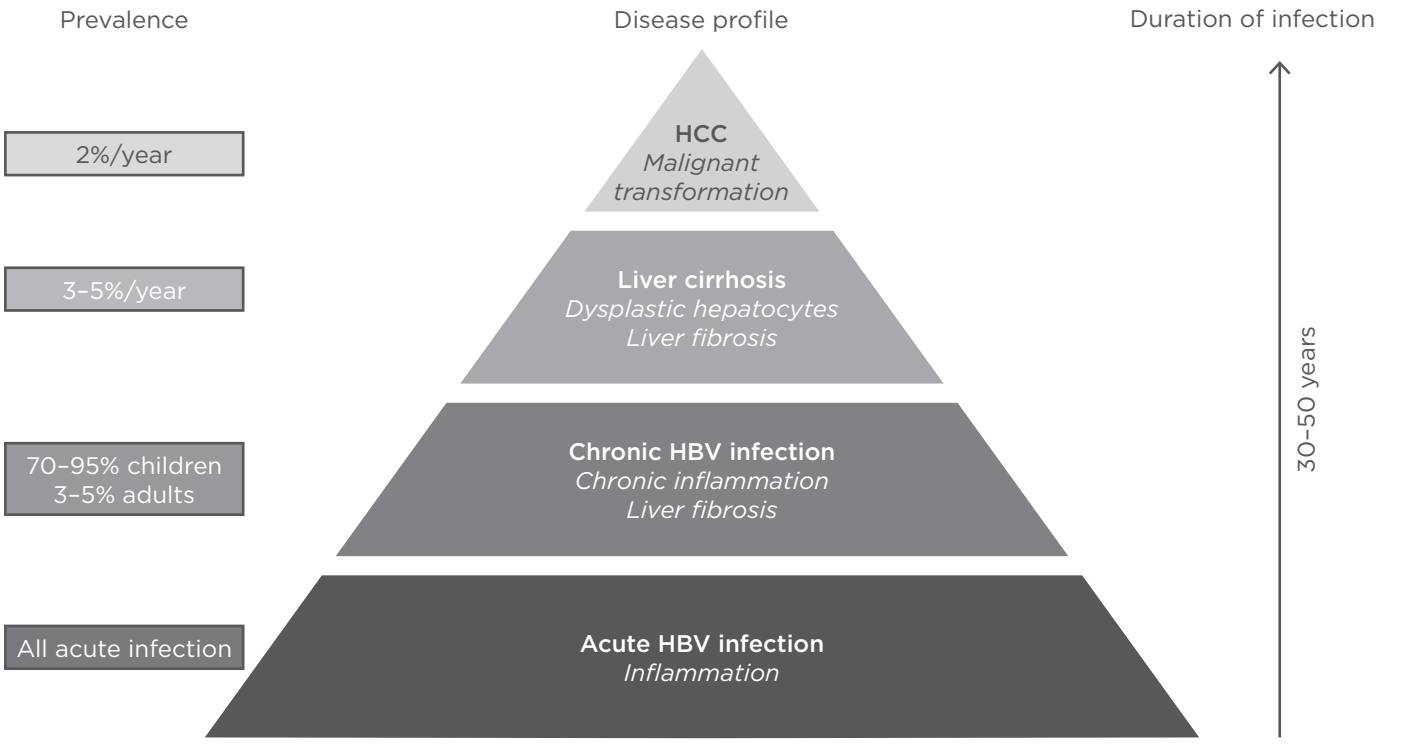

Figure 3: Chronic HBV infection, cirrhosis, and HCC development.
About 70–95% of children and 3–5% of adults infected with hepatitis B virus (HBV) may progress to chronic HBV infection associated with chronic inflammation and fibrosis.
HCC: hepatocellular carcinoma.

Aberrant DNA methylation of promoter CpG islands is the primary epigenetic change seen during the course of HBV infection as it progresses to cirrhosis and HCC. Such methylation is detected at higher rates in HCC cells and less so in hepatocytes at the stage of CHB infection, fibrosis, and cirrhosis.[40] In a study by Tseng et al.,[38] high HBsAg levels were found to be associated with a risk of developing HCC even in the presence of low HBV DNA levels. This finding may be due to a higher degree of viral HBsAg integration into the host genome that would result in mutations and epigenetic alterations, particularly DNA methylation that affects both HBV and host genome.[40]

Evidence linking the DNA methylation process and CHB infection is extremely compelling. DNA methylation involves the attachment of a methyl group to the DNA coding sequence. This process is driven by the action of DNA methyltransferases, and may be classified as hypomethylation and hypermethylation.[41] In CHB infection, integrated HBV DNA becomes methylated as part of the innate immune defence mechanism to alter viral gene expression, leading to repressed viral replication.[40] For instance, toll-like receptors and IL-4, which form an important part of the innate immune system, were reported to be epigenetically regulated, leading to the activation of cellular pathways, such as the nuclear factor kappa B pathway, that result in reduced viral replication.[42-44] Over time, the same methylation system that initially benefitted the host may start methylating the surrounding host promoter regions, causing aberrant activation or silencing in the transcription of genes including immunoregulators, tumour suppressors, and oncogenes, which are critical for the development of liver cancer.[40]

A large number of genes with distinct physiological functions have been found to be hypermethylated in the genome of patients with CHB or HBV-related HCC. These include classical genes such as *RAR-β2, DLEC, IGJBP-3, LINE-1, RB1, ASPP1, E-cadherin, GSTP1, hTERT, caveolin-1,* and *p16*^{INK4a} genes. Aberrant methylation of these genes leads to silencing of gene transcription and perturbed cellular signalling pathways such as ubiquitination, DNA repair, proliferation, and apoptosis, which may lead to the development of HBV-related HCC.[45-49]

Although hypermethylation has been labelled as the key epigenetic regulator of gene transcription, other processes such as histone modification and microRNA (miR) expression have emerged as equally important in driving carcinogenesis. Genome-wide studies identified DNA methylation, histone modifications, and miR expression profiling across all the samples containing CHB and HBV-related HCC.[50-52] However, amongst genome-wide studies there are discrepancies and data variations due to lack of proper, normal controls, heterogeneity of disease, variations in sample sources, and use of different technologies for analysis, suggesting the need for further validation.

Zhao et al.[50] demonstrated that epigenetic alterations are multi-step events with unique profiling in different stages of progression from CHB to HCC development. MiR are small single-stranded RNA molecules that interact with messenger RNA targets to negatively regulate gene transcription.[53] In HBV-related HCC, aberrant regulation of miR expression was observed and emerged as a potential indicator for diagnosis, prognosis, and therapeutic targets. For instance, in cirrhotic liver tissues containing underlying HBV-related HCC, *miR-17-92, miR-17-5p, miR-21, miR-29, miR-34a, miR-93, miR-96, miR-100, miR-101, miR-122, miR-124, miR-199a/b, miR-222, miR-224, miR-425,* and *miR-529* have been aberrantly regulated.[54-63] Distinct expression profiling of miRs is often associated with tumour development, aggressiveness, recurrence, metastases, and poor prognosis.[61-63]

Accumulated evidence indicates the significant role of epigenetic alterations in driving HBV pathogenesis and related carcinogenesis. Many studies have shown that epigenetic alterations are reversible[42] and thus serve as potential targets for the development of markers for early diagnosis, prognosis, and therapeutic interventions.

FUTURE DIRECTIONS

Ample evidence highlights that CHB persists as a result of an impaired innate and adaptive immune system including immunoregulatory inhibitory cell receptors such as PD-1 and CTLA-4, which play a significant role in driving HBV progression to fibrosis, cirrhosis, and HCC. Several studies showed that the blockade of immunoregulatory inhibitory effects of cell receptors using antibodies restores T cell proliferation and cytokine production, leading to reduced viral replication and minimal inflammatory activity in the liver. Notably, epigenetic alterations could be considered part of the innate immune system aimed at suppressing viral replication. Unfortunately, similar epigenetic processes may also be hijacked and implicated in HBV hostile activities, resulting in the alterations of important genes, including immunoregulatory and tumour suppressors, which play critical roles in hepatocarcinogenesis. Future studies are required to enhance our understanding of the epigenetic consequences of HBV-host interactions in immunopathogenesis. This will aid in identifying novel potential immunotherapeutic targets that will help in the eradication of HBV infection and ultimately, progression to cirrhosis and hepatocarcinogenesis.

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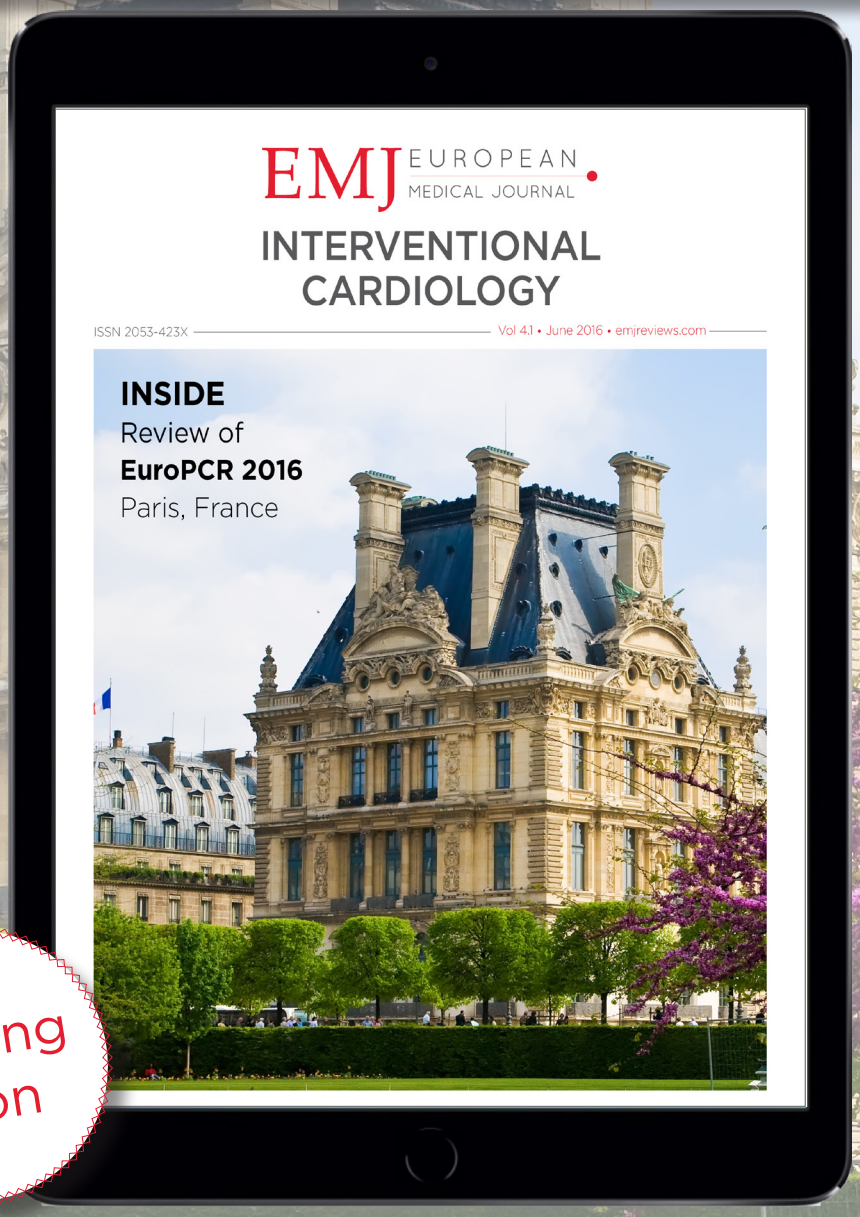

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MANIFESTATION OF NON-ALCOHOLIC FATTY LIVER DISEASE/NON-ALCOHOLIC STEATOHEPATITIS IN DIFFERENT DIETARY MOUSE MODELS

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH), which are usually associated with obesity and metabolic syndrome, are considerable health and economic issues due to the rapid increase of their prevalence in Western society. Histologically, the diseases are characterised by steatosis, hepatic inflammation, and if further progressed, fibrosis. Dietary-induced mouse models are widely used in investigations of the development and progression of NAFLD and NASH; these models attempt to mimic the histological and metabolic features of the human diseases. However, the majority of dietary mouse models fail to reflect the whole pathophysiological spectrum of NAFLD and NASH. Some models exhibit histological features similar to those seen in humans while lacking the metabolic context, while others resemble the metabolic conditions leading to NAFLD in humans but fail to mimic the whole histological spectrum, including progression from steatosis to liver fibrosis, and thus fail to mimic NASH. This review summarises the advantages and disadvantages of the different dietary-induced mouse models of NAFLD and NASH, with a focus on the genetic background of several commonly used wild-type mouse strains as well as gender and age, which influence the development and progression of these liver diseases.

Keywords: Non-alcoholic fatty liver disease (NAFLD), mouse model, high-fat (HF), methionine and choline-deficient (MCD), high-carbohydrate, genetic background.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) have many medical and economic implications worldwide and in all age groups, confirmed by clinical studies which indicate an almost 2-fold increase in mortality for NAFLD patients.[1-11] NAFLD and NASH are closely associated with obesity, insulin resistance, and glucose intolerance, and further represent the hepatic manifestation of metabolic syndrome (MS).[1,2,12-14] The diseases include different stages and display a broad spectrum of hepatic pathological features, ranging from mild steatosis to significant inflammation, fibrosis, and finally, cirrhosis.[13,15-17] Additionally, in humans NAFLD and NASH are histologically characterised by hepatocellular degenerations like ballooning and the formation of Mallory–Denk bodies.[18]

Experimental studies regarding NAFLD/NASH often use mouse models, due to their similarity to human anatomy, genetics, and physiology. Many aspects of the diseases can be studied in a cost and time-effective manner (e.g. body composition can be monitored, metabolic parameters can be assessed, and pathohistological alterations can be induced within a relatively short time).[19-30] Nevertheless, mouse models are restricted by the available sample amount and the difficulties of invasive or surgical procedures (such as in haemodynamic studies) in mice due to their size.[31,32] For modelling NAFLD/NASH in mice, several different wild-type (WT) strains (e.g. C57BL/6, Balb/c, and 129Sv) fed with special diets (e.g. high-fat [HF], methionine and choline-deficient [MCD], and high-fructose diets) have been previously used.[22-30] These dietary mouse models attempt to mimic hepatic and metabolic conditions

occurring during human NAFLD/NASH. Depending on the experimental setup, the models comprise distinctive features of human NAFLD/NASH, including hepatic injury (e.g. steatosis, lobular inflammation, ballooning, and perivenular fibrosis) and metabolic abnormalities (e.g. Type 2 diabetes and increased triglyceride and cholesterol levels). Furthermore, genetically modified mice (e.g. leptin$^{-/-}$ and acetyl-CoA oxidase$^{-/-}$ mice) are used to model human NAFLD and NASH.[24,33-35] Thus, various mouse models are already established for investigating the complex pathophysiological mechanisms and influential factors that contribute to human NAFLD/NASH development, though the approaches, along with the results, vary greatly depending on the research question.[5,36] The use of different genetic backgrounds for dietary and genetic mouse models is another variable, profoundly influencing outcomes of the respective studies. This review will summarise the phenotypes of dietary-induced mouse models of NAFLD and NASH and assess the influence of genetic background, gender, and age on the manifestation of these diseases in the respective mouse models.

DIETARY-INDUCED NON-ALCOHOLIC FATTY LIVER DISEASE AND NON-ALCOHOLIC STEATOHEPATITIS IN MICE

Methionine and Choline-Deficient Model

The induction of NAFLD/NASH in mice by a MCD diet is based on impaired phosphatidylcholine synthesis and the subsequent reduction in very low density lipoprotein (VLDL) production, leading to hepatic triglyceride accumulation and thus steatosis (Table 1).[37] Furthermore, methionine and choline restriction promotes oxidative stress through the induction of hepatocyte microsomal cytochrome P450 2E1 (CYP2E1) expression and thereby increases reactive oxygen species formation.[38,39]

Mice fed with a MCD diet develop steatosis within 3 weeks of feeding, expanding to extensive macrovesicular steatosis, including hepatic lymphocyte and neutrophil infiltration, after 8–10 weeks. Pericellular and perisinusoidal fibrosis is also observed after the respective feeding duration.[39,40] The inflammatory response is increased in mice fed with a MCD diet (Table 1); liver macrophages are activated by the transcription factor nuclear factor kappa B, leading to increases in tumour necrosis factor alpha, interleukin 6, and transforming growth factor levels.[41,42] Moreover, expression of intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and macrophage chemotactic protein-1 is increased, promoting hepatic infiltration and activity of neutrophils and macrophages.[43]

Although the hepatic histological features and inflammatory response of MCD models reflect the conditions in human NAFLD/NASH, this model type does not resemble human metabolic physiology; serum triglyceride, cholesterol, insulin, glucose, and leptin levels are not increased and mice do not exhibit peripheral insulin resistance. Furthermore, mice lose up to 25% body weight due to methionine and choline restriction (Table 1).[41,44-46] This problem can be overcome by combining methionine-defined (0.1%) and choline-deficient feeding with HF content, which results in immediate development of hepatic steatosis, inflammation, and fibrosis along with moderate weight loss.[47] Additionally, it has been shown that development of NAFLD/NASH in MCD animal models is species and strain-dependent. When three different rat strains and C57BL/6 mice were subjected to a MCD diet for 4 weeks, all rat strains developed steatosis, but inflammation was rare and fibrosis absent. In contrast, C57BL/6 mice showed necroinflammation and some exhibited focal pericellular fibrosis, while steatosis was minor compared with rats.[48] The MCD model is a widely used and well-established model for investigations concerning inflammatory and fibrotic events in NAFLD and NASH in short-term courses of treatment compared with HF feeding. However, it is questionable if this model resembles the pathophysiological mechanisms of human NAFLD/NASH, which are closely associated with those of MS.

Models of High-Fat Feeding

HF diets attempt to induce NAFLD/NASH-associated liver injury in the context of obesity and MS (Table 1). These diets are composed of 45–75% fat with variations in saturated/unsaturated fat and cholesterol content. However, a study in which mice were fed with HF diets, varying in saturation of the fatty acids (FAs) and in cholesterol content, showed that steatosis and hepatic inflammation are almost independent of saturation of dietary FAs, and that different dietary FAs are mainly stored as oleic acid in mouse livers.[30] Furthermore, dietary cholesterol seems to play a more pivotal role in the development of hepatic inflammation

and thus may promote progression from NAFLD to NASH in mice.[30,49]

Lieber et al.[25] reported a liquid HF diet composed of 71% fat, 11% carbohydrates, and 18% protein. This diet was shown to induce steatosis and inflammation in combination with metabolic abnormalities in rats and mice. Though the liver histology closely resembled features of human NAFLD, no signs of fibrosis were observed within this model, making it ineligible for studies regarding the progression of NAFLD to NASH.[25,50,51] Another study investigated HF diet-induced NAFLD and NASH in a longitudinal approach by feeding mice a diet containing 60% fat (enriched in saturated FAs), 20% carbohydrates, and 20% protein for up to 50 weeks. After 10 weeks of HF diet, feeding-induced obesity and hyperinsulinaemia were observed, with glucose intolerance occurring after 12 weeks. Steatohepatitis was observed after 19 weeks of the HF diet, demonstrating that metabolic abnormalities are induced prior to development of steatosis and hepatic inflammation in HF diet-fed mice. Although features of human NAFLD were seen in this model, fibrosis was also not observed, even after 50 weeks of treatment.[27]

The use of HF diet mouse models is considered advantageous as the models are associated with relatively low experimental requirements and do not include non-physiological procedures to induce increased levels of triglycerides, hepatic inflammatory cell infiltration, obesity, and insulin resistance. However, most HF diet mouse models do not include fibrosis and induced liver injury is relatively minor compared with that seen in the MCD model (Table 1). Furthermore, periods of dietary treatment to induce features of NAFLD are lengthy, and results may vary because of treatment duration and experimental setup.

Models of High-Carbohydrate Feeding

Increased carbohydrate content can also be used to induce steatosis in mice (Table 1). Diets with varying carbohydrate content, composed of 30–65% glucose or fructose are given for 8 weeks, with fructose acting as the most powerful sugar to induce NAFLD. These mice exhibit obesity, steatosis, and inflammation in the context of metabolic changes, e.g. insulin resistance, increased levels of total cholesterol and triglycerides, and elevated liver enzymes.[52-57] An excessive fructose intake is thought to increase *de novo* lipogenesis and visceral adipose tissue formation, resulting in increased portal delivery of free FAs to the liver and thus in hepatic triglyceride accumulation and inflammation (Table 1).[57-60]

Table 1: Comparison of metabolic status and liver histology of commonly used dietary and genetic mouse models.

Model	Metabolic status	Liver histology
Dietary models		
MCD[38-45]	No increased weight and obesity, no dyslipidaemia, no insulin resistance	Macrovesicular steatosis, lymphocyte and neutrophil infiltration, pericellular and perisinusoidal fibrosis
High-fat[24,26,29,48-50]	Weight increase and obesity, dyslipidaemia, glucose intolerance, insulin resistance	Steatosis, inflammatory cell infiltrates, no fibrosis
High-carbohydrate[51-56]	Obesity, dyslipidaemia, insulin resistance	Steatosis, inflammatory cell infiltrates, no fibrosis
Western-type[56,60-64]	Obesity, dyslipidaemia, insulin resistance	Steatosis, inflammation, fibrosis
Genetic models[24,33-35,66-68]		
ob/ob and db/db mouse	Obesity, insulin resistance	Steatosis, no inflammation, fibrosis only after stimulation e.g. MCD diet
Acyl-CoA oxidase deficient mouse	No increased weight and obesity	Steatosis, inflammation, became resistant to steatosis at the age of 6–8 months
SREBP-1c transgenic mouse	Insulin resistance, diabetes	Steatosis, inflammation, perivenular and pericellular fibrosis

MCD: methionine and choline-deficient; SREBP-1C: sterol regulatory element-binding protein-1c.

Most often, increased carbohydrate uptake is combined with HF and high-cholesterol feeding; these diets are termed Western-type diets. Western-type diets can lead to hepatic triglyceride accumulation and steatosis along with hepatic inflammation and fibrogenesis, representing conditions similar to those seen in human NASH (Table 1).[29,57,61-64] Thus, a combination of HF and high-carbohydrate content in the respective diets may cause a synergistic effect, which induces hepatic and metabolic conditions that better resemble the features of human NAFLD and NASH.[65] Nevertheless, these models produce conflicting results that are more dependent on species and formulation of the particular diet, implicating the need for a strict experimental setup to generate reproducible results.

Genetic Mouse Models

Many genetic models have been established to investigate the role of the respective genes in NAFLD/NASH development and all are associated with hepatic lipid accumulation, though they concern various different pathways. Frequently used genetic models include mice that exhibit a mutation in the leptin (ob/ob) or the leptin receptor (db/db) gene, resulting in leptin deficiency or resistance to the actions of leptin. Subsequently, hyperphagia, obesity, metabolic abnormalities, and the spontaneous development of NAFLD, but no liver fibrosis, emerge (Table 1).[24,34,35,66,67] Another category of genetic models are those which affect β-oxidation of long-chain FAs and hepatic triglyceride export; the mice, lacking acyl-CoA oxidase as an example, exhibit severe steatosis and inflammation, but no features of MS.[33] Overexpression of the transcription factor sterol regulatory element-binding protein-1c (SREBP-1c) in mice leads to dysregulation of adipocyte differentiation and thus to insulin resistance, diabetes, and hepatic triglyceride accumulation, but this model is limited by a lack of obesity and metabolic abnormalities.[68]

INFLUENCE OF GENETIC BACKGROUND ON NON-ALCOHOLIC FATTY LIVER DISEASE/NON-ALCOHOLIC STEATOHEPATITIS DEVELOPMENT IN WILD-TYPE MICE

For humans it is evident that genetic variations influence the development and progression of NAFLD and NASH. For mouse models of diet-induced NAFLD and NASH, it is known that genetic background has a substantial influence on the observed NAFLD/NASH-associated pathological features.[69]

It has been demonstrated that when fed a MCD diet, the widely used inbred mouse strain, C57BL/6, is more prone to developing NAFLD/NASH-associated liver injury (e.g. lipid accumulation, oxidative stress, and fibrosis) compared with the C3H/HeN strain.[70] Moreover, it was shown that when fed an MCD diet, C57BL/6 mice are more susceptible than Balb/c mice to NAFLD/NASH development through genetic or epigenetic mechanisms influencing macrophage activation.[71] Nevertheless, a comparison between seven WT mouse strains, which included a quantitative trait locus (QTL) analysis, identified the A/J strain as most susceptible to NAFLD development when fed with a MCD diet by exhibiting the highest serum alanine aminotransferase (ALT) levels and relatively high hepatic triglyceride content. In addition, for ALT and liver weight, QTLs on numerous chromosomes were identified, once more demonstrating the polygenic nature of NAFLD and NASH.[72] QTL analyses are beneficial to identify chromosomal loci that control even small physiological effects in polygenic diseases such as NAFLD and NASH. When effects are measured in F2 intercrosses and compared with their parental inbred strains, QTL analyses are even more powerful and generate an overall picture of the number of QTLs that are segregating.[73] Such QTL analyses on hepatic fibrosis in inbred mice and respective F2 intercrosses identified several loci that are involved in hepatic fibrosis, e.g. *hepatic fibrogenic gene 1* and *2* and *complement factor 5*.[74-76] An additional interesting approach combined a choline-deficient and L-amino acid defined diet with HF diet feeding and compared C57BL/6J (most prone to NAFLD development upon HF diet feeding, see the following) to A/J mice. Steatosis and elevated serum ALT levels were observed in both strains, while inflammation was minor in A/J mice and fibrosis could only be induced in C57BL/6J mice.[47]

When two mouse strains, C57BL/6 and 129Sv, were exposed to a HF diet, the mice exhibited similarities as well as substantial differences in metabolic and hepatic response. Both strains showed increased weight, serum cholesterol levels, and steatosis, while serum triglyceride levels were reduced. Differences between the two strains were obvious in the development of various metabolic features, for example C57BL/6 mice

exhibited a more severe phenotype of obesity, glucose intolerance, and insulin response. Higher hepatic triglyceride accumulation and lower serum triglyceride levels were also observed in C57BL/6 mice compared with 129Sv mice.[50,64,77] Moreover, 129Sv mice developed features of MS and steatosis only when fed the HF diet, while the C57BL/6 strain also showed a response to the low-fat (LF) control diet. The observed differences between the two strains may be due to an induction of SREBP-1c and stearoyl-coenzyme A desaturase-1 (SCD-1) expression and activity in C57BL/6 mice. These results implicate a role for dietary fat content and genetic predisposition in the development of NAFLD by SREBP-1c and SCD-1 action.[77] A comparison of C3HeB/FeJ, C57BL/6NTac, C57BL/6J, and 129P2/OlaHsd fed with a HF diet gained dissimilar results; 129P2/OlaHsd and C3HeB/FeJ exhibited macrovesicular steatosis associated with a high hepatic triglyceride accumulation, and only microvesicular steatosis was exhibited in the two C57BL/6 strains. However, the conflicting results achieved by this study may be explained by differences in the composition of the used HF diet and treatment duration as well as genetic variations among the used mouse strains.[78]

A comparison of C57BL/6 and DBA/2 mice fed with a Western-type diet for 16 weeks also resulted in significant differences in susceptibility to NAFLD development. C57BL/6 mice exhibited a more severe degree of steatosis, including increased hepatic triglyceride levels and a greater peripheral insulin resistance compared with DBA/2J mice, though both strains developed obesity and severe hepatic insulin resistance. These observations suggest that peripheral rather than hepatic insulin resistance determines the degree of hepatic steatosis, and that development of peripheral insulin resistance may be determined by genetic factors that influence the susceptibility of different WT mouse strains to develop steatosis. Furthermore, development of obesity seems to be independent from genetic factors.[79] Another study compared over 100 mouse strains for their susceptibility to HF and high-sucrose diet-induced NAFLD and found great differences in hepatic triglyceride accumulation. These differences may be caused by mitochondrial function, as well as the gut microbiome.[80]

Lastly, a study investigating 10 inbred mouse strains observed that even on a LF diet, hepatic triglyceride content varied due to the genetic background of the mice. Balb/c mice exhibited the highest hepatic triglyceride levels, most probably due to a short-chain acyl-CoA dehydrogenase deficiency, resulting in impaired β-oxidation of short-chain FAs. C57BL/6 mice showed an intermediate hepatic triglyceride accumulation and SWR mice displayed the lowest hepatic triglyceride content. Hepatic lipogenesis and triglyceride secretion were reduced in these mice while FA oxidation was higher than in both Balb/c and C57BL mice, suggesting that an increased hepatic triglyceride export protects SWR mice from hepatic triglyceride accumulation. Moreover, SWR mice appear to have low rates of lipolysis in adipose tissue, indicated by decreased plasma free FA levels when compared with Balb/c and C57BL mice, leading to decreased hepatic triglyceride production. This study also showed that C57BL mice expressed high levels of SCD-1, but nevertheless increased export and FA oxidation seem to restrain hepatic triglyceride contents, compared with Balb/c mice with lower SCD-1 expression. Although this study proves that genetic factors profoundly influence hepatic triglyceride accumulation, the factors and their contributing effects remain undetermined.[81] Susceptibility of different commonly used WT mouse strains to the development of NAFLD/NASH features is summarised in Table 2.

In NAFLD/NASH research, the C57BL/6 strain represents a widely used WT model. For this strain, a heterogeneous metabolic response to HF diet feeding was reported.[28,82,83] When C57BL/6 mice were fed a HF diet, the mice split in different metabolic subgroups, including lean non-diabetic, lean diabetic, and obese diabetic individuals.[83] Also, in respect to hepatic injury, different phenotypes were observed.[28,29,84] Thereby, the fat content of the used diets determined the development of liver disease in C57BL/6 mice. When fed a LF diet some C57BL/6 mice exhibited normal liver histology, while others developed benign hepatic lipid accumulation. HF diet feeding also resulted in two different hepatic phenotypes, including mice with macrovesicular steatosis and mice with more severe liver injury, exhibiting ballooning, Mallory–Denk body formation, and inflammatory cell infiltration in addition to steatosis.[28] Furthermore, heterogeneous occurrence of fibrosis due to a HF, high-fructose, and high-cholesterol diet was reported.[29]

Table 2: Susceptibility of different common mouse wild-type strains to develop features of NAFLD/NASH.

Model	Metabolic status	Liver histology
MCD[69,70]	-	C57BL/6>Balb/c=C3H/HeN
High-fat[71,77]	C57BL/6=129Sv: weight gain, elevated serum cholesterol, reduced serum triglycerides C57BL/6>129Sv: obesity, glucose intolerance, insulin response	C57BL/6>129Sv: steatosis 129P2/OlaHsd=C3HeB/FeJ>C57BL/6: steatosis
Western-type[78]	C57BL/6=DBA/2J: obesity, hepatic insulin resistance C57BL/6>DBA/2J: peripheral insulin resistance	C57BL/6>DBA/2J: steatosis
Low-fat[79]	-	Balb/c>C57BL/6>SWR: hepatic triglyceride accumulation

MCD: methionine and choline-deficient.

GENDER AND AGE EFFECTS ON NON-ALCOHOLIC FATTY LIVER DISEASE/NON-ALCOHOLIC STEATOHEPATITIS DEVELOPMENT AND PROGRESSION IN WILD-TYPE MICE

Another important aspect of NAFLD/NASH development is gender. For humans, it was shown that women have a higher prevalence of progression from NAFLD to NASH due to higher fibrotic activity.[85-87] Such gender-specific differences in the development of NAFLD/NASH have also been reported in mice. A study in rodents fed a MCD diet compared not only different species and strains but also gender, and found an increased susceptibility to NAFLD/NASH among male rodents.[48] Mice fed with diets rich in carbohydrates exhibited differences in the degree of inflammation; males showed steatosis in combination with inflammation, whereas females developed steatosis without signs of inflammation.[88] Additionally, it was shown that dietary cholesterol content influences hepatic triglyceride accumulation, especially in female mice.[30]

Similarly, ageing may affect the development and progression of NAFLD/NASH in humans and mice.[89-93] In humans, age only seems to be a risk factor for NAFLD in females.[94,95] Nevertheless, age increases the risk of progression to steatohepatitis, fibrosis, and mortality.[10,96-98] Similar observations were made for rodents. Studies comparing young, middle-aged, and old C57BL/6 mice fed a HF diet have found more liver damage and inflammation in the older mice, although the development of steatosis and the metabolic status were similar in all age groups.[91,92] Another study on different-aged C57BL/6 mice showed that hepatic triglycerides significantly accumulate in older mice and that lipogenic genes are upregulated, even on a LF diet.[93] However, the gender and age aspect remains controversial in both humans and mice, and therefore is a point of discussion in the published literature.

CONCLUSION

Dietary treatment in mice, with either methionine and choline restriction or over-nutrition, is a powerful model for human NAFLD and NASH, and will possibly help to refine diagnosis, prevention, and treatment of the diseases. Nevertheless, all dietary mouse models possess limitations, either in lacking the metabolic context of the human disease or in progression of NAFLD to NASH, as well as inconsistency between different approaches. The latter limitations may exist not only because of different formulation of the diets, but also because of different genetic backgrounds, varying age, and the respective gender of the mice used. Thus, more focus should be directed towards the genetic, age, and gender differences of the WT mice and their implication in the susceptibility to develop NAFLD/NASH, which may generate new insights in genetic determinants of human NAFLD and NASH.

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NON-MALIGNANT PORTAL VEIN THROMBOSIS IN LIVER CIRRHOSIS: DIAGNOSIS AND TREATMENT

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ABSTRACT

Portal vein thrombosis (PVT) is considered a common complication of liver cirrhosis. Its prevalence increases with liver disease severity, reaching 25% in patients awaiting liver transplantation (LT). The majority of patients with cirrhosis are diagnosed incidentally with PVT during routine ultrasound in their cirrhosis follow-up. Doppler ultrasound is the recommended first-line investigation. Computed tomography or magnetic resonance angiography are the best methods to assess the extent of the PVT. The natural history of PVT in liver cirrhosis is not very well defined, but in the context of LT the deleterious effects of PVT are better known. There are no consensus guidelines about the treatment of PVT in cirrhotic patients and although anticoagulation is considered as the first-line therapy, the evidence regarding this treatment is based on a small series of patients. Nonetheless, it seems that anticoagulation therapy is useful in cirrhotic patients with PVT, particularly in patients who are candidates for a LT, in order to maximise the recanalisation rate and prevent thrombus progression. This treatment must be administered as soon as possible following a prophylactic treatment to avoid variceal bleeding, otherwise it seems to have a broad safety profile. A transjugular intrahepatic portosystemic shunt would be the alternative procedure for patients with no response to anticoagulation therapy or where portal hypertension complications occur.

Keywords: Portal vein thrombosis (PVT), liver cirrhosis, anticoagulation.

INTRODUCTION

The portal vein is an 8 cm conduit that originates from the confluence of the superior mesenteric and splenic veins posterior to the neck of the pancreas. It accounts for 75% of the blood supply to the liver.

Portal vein thrombosis (PVT) is an obstruction of the portal vein trunk and/or its branches by a blood clot, which includes the splenic, superior mesenteric, and inferior mesenteric veins. It can present in a variety of conditions, including cancer, infections, myeloproliferative diseases, inflammatory conditions, following ablative therapy for hepatocellular carcinoma (HCC), and cirrhosis.[1] From a clinical point of view, there are two types of PVT:

- Acute: sudden formation of a thrombus within the portal vein, which was not detected during the previous biannual ultrasound. Occlusion may be complete or partial
- Chronic (portal cavernoma): replacement of the normal portal vein by a network of hepatopetal collateral veins. It functions as a portoportal shunt[2]

Portal Vein Thrombosis and Cirrhosis

To date, there has been no published consensus on management of non-malignant PVT in liver cirrhosis. Moreover, there have only been a few studies about the incidence and prevalence of PVT in cirrhosis. In any case, PVT is the most common thrombotic event in cirrhotic patients. The prevalence is between 0.6% and 26%, depending on the method used for its evaluation.[3-5] A large, observational, prospective study in Italy, estimating

the prevalence of PVT evaluated by ultrasound with power-Doppler in a cohort of patients with liver cirrhosis of any aetiology and severity, is ongoing and will provide useful clinical data.[6] A recent prospective study found a cumulative incidence of PVT of 4.6%, 8.2%, and 10.7% at 1, 3, and 5 years, respectively.[7] Other studies that included patients with more severe cirrhosis at baseline found a higher incidence (7.4%, 12.8%, and 16.4% per year).[8-10] In spite of previous data, there are no specific recommendations regarding early detection of PVT in cirrhosis aside from the screening of HCC.

In cirrhotic patients the pathogenesis of PVT appears multifactorial. Slow blood flow in the portal venous system increases the probability of developing thrombi,[9] but the reproducibility of portal vein flow velocity measurements between different equipment and operators make it difficult to find absolute values.

It is now recognised that cirrhosis is associated with the hypercoagulability of plasma. In these patients, plasma is partially resistant to anticoagulation mediated by thrombomodulin. This situation is probably the result of two common alterations in cirrhotic patients: elevated levels of factor VIII (a procoagulant driver) in combination with decreased levels of protein C (an anticoagulant driver).[11]

Systemic thrombotic risk factors such as *factor V Leiden* mutation and the *G20210A prothrombin* mutation may play a role in the formation in PVT, however there are some contradictory results in the area.[7,12] Other risk factors for PVT include endoscopic sclerotherapy of oesophageal varices,[13] gastrointestinal infections,[14] and bacterial translocation/endotoxinaemia, which might be mitigated by with enoxaparin.[15]

DIAGNOSIS

Most patients with cirrhosis are diagnosed with asymptomatic PVT during routine ultrasound. The sensitivity and specificity of Doppler ultrasound are 89% and 92%, respectively,[16] so it is the primary method of choice in this context. If Doppler ultrasound shows portal vein patency, no further studies are indicated. However, ultrasound limitations can cause a false positive result, for example due to their accuracy being clearly influenced by the operator skill,[17] or very low flows.[18] The main ultrasound findings of PVT include hyperechoic material within the vessel lumen, absence of flow, and an inability to identify or dilate the portal vein. When portal cavernoma has developed, tortuous vessels in the porta hepatis are present.[19]

It is important (from a prognosis and treatment point of view) to distinguish between partial and complete PVT. In occlusive PVT, a thrombus leaves no channel for blood flow. Contrast enhanced ultrasound is more sensitive than Doppler ultrasound, since it can visualise hypoechoic or small thrombi (≤3 mm).[20] Furthermore, during the arterial phase, contrast enhanced ultrasound allows identification of the malignant thrombus origin when there is vascularisation (the thrombus appears hyperechoic).[21,22]

Enhanced computed tomography and magnetic resonance imaging are the best methods to assess the extent of the PVT. In addition, they provide information about the development of collateral circulation, the status of adjacent organs, and are indicated if intestinal ischaemia or HCC are suspected.[2]

CLINICAL PRESENTATION AND NATURAL HISTORY OF PORTAL VEIN THROMBOSIS

In patients with cirrhosis, diagnosis of PVT is growing in relation to the increased frequency of liver imaging. The most common scenario is therefore the detection of asymptomatic PVT during routine ultrasound examination. Complications of PVT include variceal bleeding, failure of endoscopic control of bleeding, intestinal ischaemia (in patients with extension of the thrombus into the superior mesenteric vein), and portal biliopathy (causing partial or complete bile duct obstruction).[5,14,23]

The risk of developing PVT increases with the severity of the liver disease, although the development of PVT is a marker rather than a direct cause of cirrhosis progression.[7] On the other hand, some studies show that PVT has little influence on prognosis and is not associated with an increased risk of death or reduced chance of undergoing transplantation.[23,24] A recent meta-analysis concluded that PVT appears to increase mortality and liver decompensation from ascites, but the small number of included studies limited widespread conclusions.[25] It is likely that the prognosis of cirrhosis with PVT will be influenced by the degree of occlusion of the venous lumen

and the clinical situation of the patient at the time of diagnosis.

Natural history studies have identified relatively high rates of recanalisation in cirrhotic patients with non-malignant PVT. In two different reports, PVT improved in 47.6% and 45% of patients, respectively.[23,26] In another recent study, the spontaneous repermeation rate was even higher (70%).[7] In all three studies most patients only had a partial thrombosis, which could have influenced these results, as stated before. Nonetheless, these data show that in a percentage of patients thromboses will progress. If a simultaneous worsening in the liver function exists, the patient could be a candidate for liver transplantation (LT); in this context, the deleterious effects of PVT are better understood.

Portal Vein Thrombosis and Liver Transplantation

PVT may adversely affect the outcome of LT but currently it is considered only a relative contraindication for LT.[27,28] The prevalence of PVT in patients awaiting LT has a very broad range (between 2.1% and 23.3%). PVT presents important challenges in patients undergoing LT due to the technical requirements of clot removal or alternate vascular reconstructions.[29] The Yerdel classification[29] is the most widely accepted in defining not only the morphology of PVT, but also the presence of suitable collateral vessels that could be useful for an extra-anatomical reconstruction of portal flow; allowing appropriate graft selection and planning of the transplant surgical procedure.

The impact of PVT on LT has not been clearly defined, probably because most studies are retrospective and only some of them evaluate patients with occlusive and non-occlusive PVT separately. Again, the separation between occlusive and non-occlusive thrombosis is very important; in patients with partial PVT, post-transplant mortality outcomes are no different from non-PVT patients but it is significantly increased in patients with complete PVT.[13,30] Data from a high volume centre suggests that PVT is an independent predictor of mortality post LT, with occlusive PVT conferring an additional increase in mortality at 30 days.[31]

PVT following LT carries a poor prognosis. The rate of *de novo* PVT occurrence post-LT is 0–2%.[29] On the other hand, patients with PVT at the time of LT also have a higher risk of recurrent PVT after transplantation (rates of 2–3%).[32] In the case of transjugular intrahepatic portosystemic shunt (TIPS) insertion for the treatment of PVT, the incorrect positioning of its distal tip must be avoided due to its possible interference with LT.

For all the reasons noted above, the treatment of cirrhotic patients with PVT seems especially appropriate in candidates for LT, so they can achieve a complete/partial recanalisation or at least prevent extension of thrombus, particularly to the superior mesenteric vein.

TREATMENT

There is no consensus about the standardised treatment of PVT in cirrhotic patients. How to choose the best candidate, the best moment, and the best type of treatment are questions without a clear answer, because the natural history of PVT in cirrhosis is still a matter of debate.[33] A reasonable decision in this context could be made on a case-by-case basis,[34] but it would be desirable to establish a model for the general management of these patients. There are several therapeutic strategies in patients with cirrhosis and PVT: anticoagulation, TIPS, and thrombolytic therapy.

ANTICOAGULATION

In spite of the reservations that will be noted below, anticoagulation is the first-line therapy in cirrhotic patients with PVT. There are seven studies published to date that have evaluated this treatment in these patients.

Primary Prevention

Villa et al.[15] performed a randomised controlled trial to evaluate the results of enoxaparin in preventing PVT in patients with advanced cirrhosis. There was no thrombosis in the active arm (n=34 patients) compared with 27.7% PVT in the control arm (n=36 patients), with a follow-up of 2 years. No relevant side effects or haemorrhagic events were observed. Though several limitations of this trial have been identified,[35] a recent study with cirrhotic rats showed that prolonged administration of enoxaparin improves portal hypertension and liver cirrhosis, probably by potentiating fibrosis regression. These results could help to explain the results of the study of Villa et al.[15] In any case, before the prophylactic use of anticoagulant therapy is recommended in cirrhotic patients, further confirmative studies are required.

Secondary Prevention

It is likely that anticoagulation treatment is frequently used in clinical practice, but there are only scarce data about its results. In Table 1 the six studies published are shown (including a total of 193 patients). Only a few patients had a portal cavernoma. Treatment (warfarin and/or low-molecular-weight heparin [LMWH]) was associated with complete recanalisation rates between 36% and 75%. Thrombus progression was reported between 0% and 15%.

In two of these studies, the early administration of anticoagulation was associated with a greater probability of recanalisation.[38,39] In the three studies compared treatment with control subjects, the recanalisation and thrombus progression rates were favourable to the treated group.[36,39,41] Amitrano et al.[37] and Delgado et al.[38] reported re-thrombosis rates after stopping anticoagulation between 27.2% and 38%. These results outline the possibility of maintaining anticoagulation therapy in the long-term at least for those patients awaiting LT.

Only two patients developed severe bleeding complications: one cerebral haemorrhage and one significant vaginal bleeding.[39,40] Delgado et al.[38] reported five bleeding complications, probably in relation to therapy, and identified a platelet count <50x10^9/L as the only parameter significantly related to a higher risk of bleeding. Chen et al.[41] reported four other severe bleeding events. Of these 11 patients, 10 were treated with vitamin K antagonists (VKA) alone. As previously suggested, this fact could support a greater safety profile of LMWH.[42] On the other hand, a recent study in a group of cirrhotic patients with upper-gastrointestinal bleeding reported that anticoagulation therapy is not necessarily associated with an increased morbidity/mortality.[43] No other significant side effects were observed during the treatment. No deaths were found to be associated with this therapy.

All the patients must be screened to grade varices prior to receiving anticoagulation therapy. There is also no consensus on the prophylactic treatment of variceal bleeding in these patients, but it seems logical that it must follow recognised international guidelines.[44]

Our group recently published results related to 27 cirrhotic patients with non-malignant PVT. Twenty-six patients received anticoagulation: 23 LMWH and 3 VKA. The median time from diagnosis to the initiation of treatment was 2 weeks. The complete recanalisation rate was 57.6%. The median time to achieving this complete response was 10 months (95% confidence interval: 3–17). Re-thrombosis occurred in 35.7% of patients. Patients with no response to treatment did not show progression of thrombosis. Only two patients, one of them with 30,000 platelets, presented a bleeding complication (mild in both cases). No significant differences regarding the appearance of portal hypertension related complications were observed. Patients with a MELD score <8 achieved recanalisation within a significantly shorter timeframe compared with the other patients (p=0.04).[45]

The VKA should be used with regular laboratory monitoring. Though cirrhotic patients are usually treated with doses aimed at 2.0–3.0 international normalised ratio, this value might not be representative of the real anticoagulation.[46]

Table 1: Summary of studies about the use of anticoagulation therapy with secondary prevention in patients with portal vein thrombosis and liver cirrhosis.

Study	Type of study	No. patients	Type of anticoagulation	Recanalisation complete/partial/none (%)	Progression (%)
Francoz et al.[36]	Prospective	19	LMWH and warfarin	42/0/53	5
Amitrano et al.[37]	Prospective	28	LMWH	75/7/11	7
Delgado et al.[38]	Retrospective	55	LMWH and/or warfarin	45/15/40	0
Senzolo et al.[39]	Prospective	33	LMWH	36/27/21	15
Werner et al.[40]	Retrospective	28	Warfarin	39/43/18	0
Chen et al.[41]	Retrospective	30	Warfarin	Improved: 68 None: 18	13

LMWH: low molecular weight heparin.

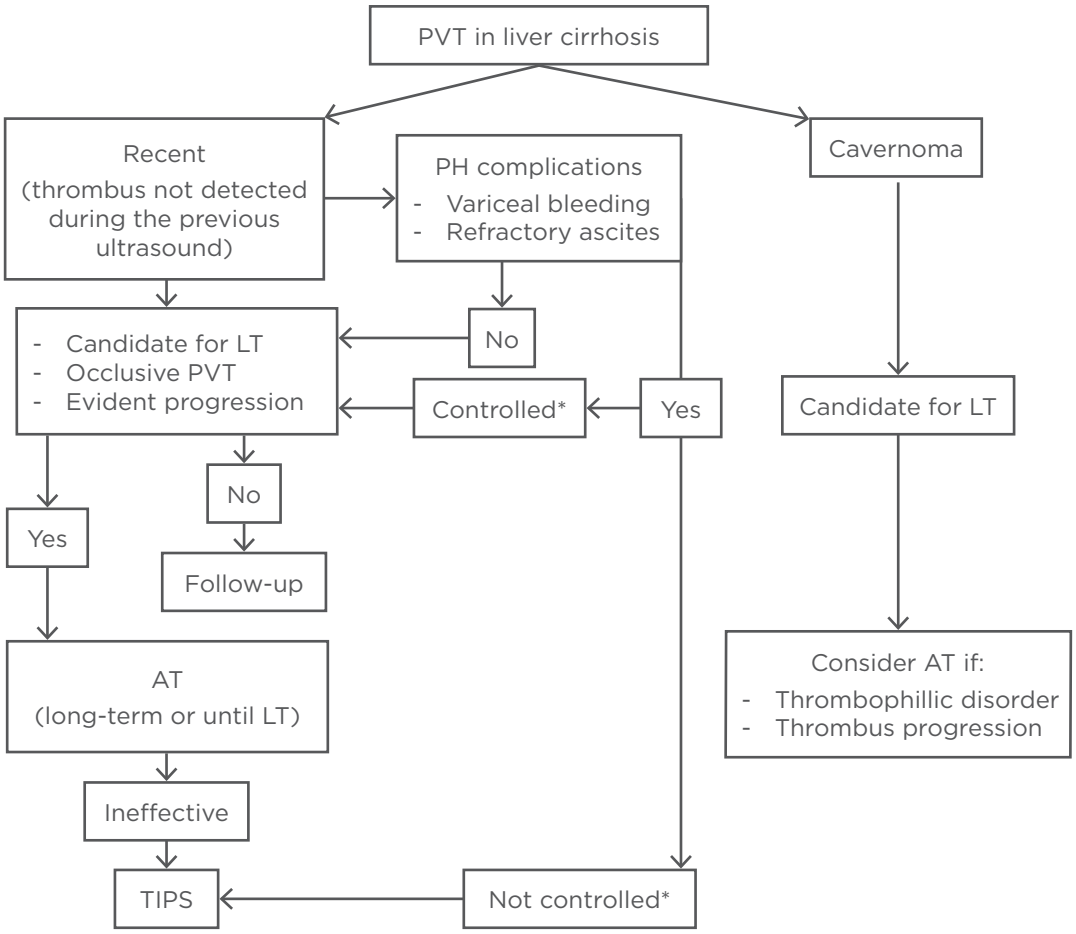

Figure 1: Algorithm for treatment of non-malignant portal vein thrombosis in liver cirrhosis.
PVT: portal vein thrombosis; LT: liver transplantation; AT: anticoagulation therapy; TIPS: transjugular intrahepatic portosystemic shunt; PH: portal hypertension.
*Controlled or not by endoscopic therapy, paracentesis, drugs.

LMWH does not seem to require laboratory monitoring to adjust dosage. In any case, the anti-Xa assay in cirrhosis is not representative of the real anticoagulation.[46] A thrombin generation test might be considered for monitoring the anticoagulation effect in this group of patients, but this needs to be evaluated.[39]

Newer oral anticoagulants represent an attractive option for cirrhosis patients due to ease of administration, and although hardly studied in this population, a recent publication comparing their safety to traditional anticoagulation displayed similar rates of bleeding in a cohort of selected cirrhosis patients.[47] However, due to the predominant hepatic metabolism of these agents, their use is not advisable in decompensated cirrhosis.

Taking into account all of the above, although further controlled studies with more patients are clearly needed, it seems that anticoagulation therapy is useful in cirrhotic patients with PVT, particularly in patients who are candidates for a LT, in order to maximise the recanalisation rate and prevent thrombus progression. This treatment is most efficacious when administered as early as possible and it seems to have a broad safety profile. In this sense, LMWH appears to be safer, but at present it is not possible to recommend a specific type of LMWH or its optimal dosage in order to set up liver-specific guidelines. There is not a defined regularity of ultrasound surveillance to monitor PVT across the duration of therapy, but a reasonable interval would be every 3 months.

TRANSJUGULAR INTRAHEPATIC PORTOSYSTEMIC SHUNT

The reported technical success rate for TIPS in cirrhotic patients with PVT is 75–100%.[48-52] However, many of these studies are retrospective and the indication for TIPS was the treatment of portal hypertension complications but not PVT itself.

Treatment with TIPS may be feasible if portal cavernoma is present but is not an option if a patent intra hepatic portal vein branch is lacking.[48]

The role of anticoagulation therapy in post-TIPs patients remains undefined. A recent trial shows that anticoagulation may not be necessary in certain patients with PVT and the presence of a superior mesenteric vein thrombus may be used to predict recanalisation failure.[53]

The procedure-related complication rate is 0–17%[35] and the two main postoperative complications are the risk of developing hepatic encephalopathy and shunt dysfunction, with incidences of 7–32% and 0–50%, respectively.[54] These incidences have, however, been reduced with the use of covered stents, and the long-term outcome of TIPS in cirrhotic patients with PVT is excellent.[49]

THROMBOLYSIS AND PERCUTANEOUS PORTAL VEIN RECANALISATION

Thrombolytics (tissue plasminogen activators) can be infused into the portal vein indirectly (injection into the superior mesenteric artery through the femoral or radical artery) or directly (via a percutaneous transhepatic or transjugular intrahepatic approach).[14,55] PVT can be recanalised percutaneously with balloon angioplasty or by the placement of stents.[56] Even though these procedures may be effective in patients with cirrhosis and PVT, experience is scarce and complications may be serious.

CONCLUSION

Diagnosis of PVT is occurring more frequently in patients with cirrhosis (above all, in those with more severe liver disease) because of the increasing use of ultrasound in their follow-up. Doppler ultrasound is the recommended first-line investigation in this context. When PVT is identified the use of computed tomography and magnetic resonance imaging should be considered to rule out associated HCC.

There are no clinical guidelines regarding treatment of PVT in cirrhotic patients. However, patients with cirrhosis and occlusive PVT, LT candidates, or those with an evident thrombus progression, should receive anticoagulation therapy, either long-term or until LT. This therapy should be administered as soon as possible but only following prophylactic treatment of oesophageal varices. TIPS would be the best alternative procedure if anticoagulation therapy is ineffective or if hypertension portal complications occur.

If PVT is diagnosed when cavernoma is already present, anticoagulation therapy would only be indicated for patients with a thrombophilic disorder or in cases of thrombus progression, mostly in candidates for LT. Though PVT is a risk factor for LT, it is not considered an absolute contraindication even when PVT is complete. At this point, on the basis of all considerations and taking into account the published studies, we propose an algorithm for management of PVT (Figure 1).

In our opinion, specific guidelines about management of PVT in cirrhotic patients should be established in the near future. It seems clear that there is a lack of randomised control trials regarding management of PVT, mainly pertaining to the role of anticoagulation in these patients, not only about its efficacy, but also its safety and laboratory monitoring. With respect to the possible benefits of primary prophylaxis, it will be necessary to confirm initial findings and perhaps define the target population (all the cirrhotic patients, independent of liver function, prioritise those patients with slow portal flow, etc.).

Moreover, recent studies have shown that the activation of the coagulation factors may stimulate fibrogenesis.[57] This same mechanism could, simultaneously, lead to the occurrence of PVT, as mentioned earlier. In the area of research done by Villa et al.[15] it would be desirable to confirm the role of anticoagulation therapy in the prevention not only of the development of PVT but also its effect on worsening of liver disease.

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