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INSIDE

Review of The International Liver Congress™ 2013: the 48th Annual Meeting of **EASL**

Amsterdam, The Netherlands

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ISSN 2053-4221 — May 2013 • emjreviews.com

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European Medical Journal - Hepatology is published annually.
For subscription details please visit www.emjreviews.com

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ISSN

Welcome

Kelly-Ann Lazarus, Editor

Hello, and welcome to the first edition of *European Medical Journal – Hepatology*, in which we aim to provide all specialists with a high quality independent review of presentations from the 48th Annual Meeting of the European Association for the Study of the Liver, the International Liver Congress™, alongside the latest news and revelations in the field.

Through EASL's embracing of innovative technologies and online education possibilities, combined with a multitude of presentations from the forefront of hepatology research, the Amsterdam-hosted event was undoubtedly one to remember, with topics spanning from potential hepatitis cures to alcoholic liver damage, the highlights of which can be seen in our Congress Review section.

As previously mentioned, we also look to provide both the most prevalent breakthroughs in scientific research and stories of human interest, including a remarkable report on a 19-year-old girl, suffering from acute liver failure while in her 39th week of pregnancy, successfully surviving a 12-hour surgery following the delivery of her child.

Accompanying these sections are articles written by revered hepatologists from across the whole of Europe, covering such topical issues as applying the PIRO concept in cases of chronic liver failure, treating hepatic encephalopathy, and the hepatitis B virus.

I would also like to warmly thank our editorial board listed opposite for their continued support in ensuring the quality of this journal, with all of us here at *European Medical Journal* hoping this edition is the start of a great source of topical information in the world of hepatology.

Kelly-Ann Lazarus, **Editor**

Robust protection against recurrent episodes of hepatic encephalopathy[1]

Significant reductions in episodes† of hepatic encephalopathy and hospitalisation rates‡ have been demonstrated with XIFAXAN® 550 b.d. and concomitant lactulose*[1]. XIFAXAN® 550 b.d. provides a cost-effective treatment option[2] that enhances quality of life for patients.[3]

† p<0.001 ‡ p=0.01
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XIFAXAN® has varying availability and licensing internationally. Before prescribing, consult your country approved prescribing information, available from your local distributor or Norgine Ltd.

Adverse events should be reported to your regulatory agency. Adverse events should also be reported to your local distributor or Norgine Limited, Norgine House, Moorhall Road, Harefield, Uxbridge, Middlesex UB9 6NS, United Kingdom. Email: globalmedinfo@norgine.com

References: 1. Bass, N.M., *et al.* N Engl J Med, 2010; 362(12): 1071-81. 2. Norgine data on file. 3. Sanyal, A., *et al.* Aliment Pharmacol Ther, 2011; 34(8): 853-61. 4. XIFAXAN® 550 Summary of Product Characteristics, Dec 2012.

XIFAXAN® 550 is indicated for the reduction in recurrence of episodes of overt hepatic encephalopathy in patients ≥ 18 years of age.[4]

Rifaximin-α is licensed under the Trade Names of XIFAXAN®, TARGAXAN®, and others. Please note that Trade Names and licensed indications may vary throughout Europe and between countries.

INT/XIF/0313/0182
Date of preparation: March 2013.

NORGINE

Foreword

Prof. Dr. Markus Peck-Radosavljevic

Associate Professor of Medicine,

Medical University of Vienna, Austria

Dear Colleagues,

It is a pleasure for me to introduce the inaugural issue of the *European Medical Journal – Hepatology*. As you might know, the *European Medical Journal* is dedicated to reporting on major European conferences and this issue reports on the most important international liver conference in 2013, the International Liver Congress™ (ILC), organised by the European Association for the Study of the Liver (EASL) in Amsterdam, April 24-28th. The ILC attracted over 9,600 participants and was a major success both for its educational content as well as the breakthrough scientific advances presented.

> *"The field of hepatology is definitely one of the most fast-moving fields in all of medicine at the moment."*

This year the most outstanding clinical presentations came from the field of drug development for chronic hepatitis C, with several large phase-III studies reporting their final and most promising data at the ILC. In addition, important advances in the management of liver cancer, for example the ART-score for selecting patients for intervention treatment of liver cancer, were presented, and outstanding basic and translational results like novel approaches to eliminate cccDNA in hepatitis B-infected hepatocytes were discussed. The field of hepatology is definitely one of the most fast-moving fields in all of medicine at the moment.

I hope you will enjoy this years' coverage in the *European Medical Journal – Hepatology* and already now look forward to the undoubtly exciting news to be covered at the most important liver meeting in 2014, The International Liver Congress™ 2014 in London.

I wish you enjoyable reading,

Markus Peck-Radosavljevic

Associate Professor of Medicine, Vice-Chairman, Department of Gastroenterology and Hepatology, Medical University of Vienna, Austria, Fellow of the Austrian College of Physicians, Member of American Association for the Study of Liver Disease, the European Association for the Study of the Liver (EASL), the American Gastroenterologic Association, the Austrian Transplant Association, the Austrian Society for Infectious Diseases, Austrian Association for Gastroenterology and Hepatology; Secretary General of the Austrian Association for Internal Medicine; Vice-Secretary of EASL.

EASL ANNUAL MEETING 2013

THE INTERNATIONAL LIVER CONGRESS 2013

RAI EXHIBITION CENTRE, AMSTERDAM, THE NETHERLANDS

24TH - 28TH APRIL 2013

EMJ EUROPEAN MEDICAL JOURNAL

Welcome to the *European Medical Journal* review of the 48th Annual Meeting of the European Association for the Study of the Liver

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WELCOME TO THE *EUROPEAN MEDICAL J*
INTERNATIONAL L

THE European Association for the Study of the Liver (EASL) held its 48th Annual International Liver Congress™ (ILC) in Amsterdam, The Netherlands, this year, furthering its aim to present studies in clinical hepatology and liver disease for the benefit of patients worldwide since 1966.

Not only famous for its safe and easy-going atmosphere, this green city holds urban areas dedicated wholly to environmental innovation, the European Commission having named Amsterdam one of the eight finalists for the first-ever European Green Capital Awards in 2009.

Home of 180 inhabitant nationalities, and with 58% of said population cycling on a daily basis, it seems only fitting that a city with such a diverse, healthy-living culture has become a popular hub for a variety of medical congresses.

Held at the Amsterdam RAI Exhibition and Convention Centre within the Zuidas business district, the Congress aimed to provide the opportunity for its 9,500+ clinicians and scientists, from across the globe, to hear the latest hepatology news, research, treatments, and perspectives on liver disease from outstanding experts in the field.

Technology was high on EASL's agenda, with attendees given the chance to explore and sign up to their new revolutionary eLearning website, Livertree™, the society's official portal to online education. In a bid to attract audiences to more trusted sources of information, its "revolutionary" search tool enables hepatologists to find educational material of the past eight years, and was officially launched after the International Liver Congress™.

Coinciding with branching out into eLearning education with Livertree™, EASL announced their latest publication, *The Burden of Liver Disease in Europe: A Review of Available Epidemiological Data.*

A comprehensive publication reviewing 260 epidemiological studies published in the last five years, the literature review is a continuation of the association's focus on raising awareness amongst decision makers in Brussels concerning the constant need to tackle liver disease.

Brand new revelations into the hepatitis virus, particularly hepatitis B and C, dominated the

OURNAL REVIEW OF EASL'S 48TH ANNUAL LIVER CONGRESS™

Congress, with EASL Secretary General Professor Mark Thursz declaring that hepatologists are currently living in "a brave new world" for infected patients, as a myriad of Phase III experimental drug reports show significant potential.

This was accompanied with findings resulting from further exploration into established territory. Greater scrutiny into the effects of cardiovascular exercise, alcohol and body weight on the liver's health embodies EASL's goal of in-depth topic examination.

With such exciting, groundbreaking research coming through, on top of the innovations carried out by EASL, hepatologists will no doubt wonder what news will emerge from this "brave new world" by the time of next year's International Liver Congress™ in London from 9th-13th April 2014.

NEW FORUM "VIROCHANNEL" IS BORN

AS the attendance of such conferences as The International Liver Congress™ continues to grow on an annual basis, a new bilingual (English & French) website www.virochannel.com launched this month, looking to combine in-depth congress coverage with a private social media forum. Its key concept: To combine the ideas of Youtube and Facebook for an online community focused on a specific medical area.

ViroChannel is owned and operated by l'Actuel Communications, a specialised division of the Clinique médicale l'Actuel: Centre of Excellence HIV – STI – Hepatitis, based in Montreal, Quebec, Canada. Led by Editor-in-Chief Doctor Réjean Thomas, a recognised figurehead of patient support in the global medical community, ViroChannel looks to tailor its suitability towards the worldwide audience of virologists, physicians, pharmacists, nurses, clinicians, and other healthcare professionals, encouraging them to share and compare ideas in order to better the care of patients, "for a common global concern".

"ViroChannel is a living, breathing community that depends upon the participation of our members for its continued growth and evolution," Doctor Thomas said.

"I believe that through the sharing of our ideas, we can envision the future of healthcare and inspire our colleagues to take action that will turn the ideas into reality."

With the site filming and broadcasting short videos featuring experts covering the most contemporary and relevant scientific research presented at the foremost virology conferences across the world, ViroChannel would be of interest to those professionals who are unable to attend significant conferences and want to watch highlights of congresses online.

In particular, such features as the "Ask the expert" educational series – in which invited authorities are interviewed on specific issues, new treatment options,

ONLINE: A completely free service, Virochannel enables members to see Congress highlights, connect with other specialists and through a private social media forum.

"ViroChannel is a living, breathing community."

- Doctor Réjean Thomas

and recent trial results – offers great insight. With over 1,000 worldwide virology specialists already registered, private and public forums moderated by ViroChannel's Scientific Committee members, featuring such names as Doctor Jean-Michel Pawlotsky, will offer a vast spectrum of perspectives and ideas of medical practice. Membership to the independent, private social media network is free, whereupon participants will receive breaking medical news, watch both live and on-demand conference broadcasts and educational videos, share in ViroChannel's slide library, download practice aids, and join discussions within the forum.

In attending conferences, the team continues to strengthen the number of users, next stopping at IAS Kuala Lumpur 2013, with Doctor Thomas adding: "Time you spend on ViroChannel will be filled with the most important and relevant content and programs in the virology community today."

THE EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER (EASL) LAUNCHES LITERATURE REVIEW AT THE INTERNATIONAL LIVER CONGRESS™ (ILC)

AT its flagship event, The International Liver Congress™ (ILC), a meeting which brings together over 9,500 hepatologists from all over the world, EASL launched its latest publication, a literature review entitled *The Burden of Liver Disease in Europe: A Review of Available Epidemiological Data*.

The literature review is a comprehensive publication which reviews 260 epidemiological studies published in the last five years, with a view to outline the current state of evidence on the burden of liver disease in Europe and its causes.

Alcohol consumption, viral hepatitis B and C, and metabolic syndromes related to obesity are the leading causes of cirrhosis and primary liver cancer in Europe.

Each of the major causes of liver disease is amenable to prevention and treatment but epidemiological data is scarce. Additional data is urgently needed to provide reliable information, without which it will not be possible to implement cost-effective prevention programmes and novel treatments to tackle liver disease and avoidable deaths in Europe.

The literature review is one of the cornerstones of EASL's work in Brussels where the Association works, often in collaboration with other stakeholders, to raise awareness of the importance of a comprehensive strategy to tackle liver disease. It is estimated that liver disease affects 6% of the EU's population (approximately 29 million people).

Liver disease is also reported to be the EU's fifth biggest killer, accounting for at least one in six deaths. Despite these alarming figures, little is done in terms of policy although there are opportunities presented by the many areas relevant to liver disease, including: public health, research, communicable diseases, nutrition and obesity, alcohol, organ donation and transplantation, cancer, and rare diseases.

At the ILC, the EASL EU Booth provided EASL members and other conference participants with the opportunity to find out more about EASL's work in policy and advocacy including information about the recently formed Friends of the Liver MEP Group which is chaired by Stephen Hughes MEP.

The Interest Group acts as a forum for important issues surrounding liver disease to be discussed at EU level. It brings together experts from the liver community (physicians, patients, researchers, health economists, nurses and allied professionals) and European policymakers to discuss key topics and activities related to liver disease. In this way, MEPs receive valuable information relevant to their current work and are better able to hold the European Commission to account as it drafts new proposals that impinge on the liver community.

The EU booth at ILC was also an opportunity to showcase EASL's support for consortia wishing to apply for grants under the European Commission's Framework 7 programme. EASL allocates approximately 60,000 Euros per year to this initiative.

To give congress participants exposure to the types of projects the Commission supports, a poster display highlighted the different liver disease-related projects which have, over the years, been successful in obtaining commission funding. The successful projects included, PROLIFICA, HEPTROMIC, and FLIP.

The EASL Literature Review can be downloaded at http://www.easl.eu/_eu-policy/eu-literature-review. A limited number of hard copies is also available on request.

PROBIOTICS HELP H.E. NEURO ISSUES

PROBIOTICS could become the go-to treatment for hepatic encephalopathy (HE), after it was announced on the Thursday of EASL that they significantly reduce the development of the disease.

Studying 160 cirrhotic patients over a period of nine months, research found probiotics significantly improved patients' arterial ammonia levels after three months treatment, with twice as many patients taking a placebo developing overt HE compared to patients taking a capsule-form probiotic.

EASL's Treasurer, Professor Mauro Bernardi, calling hepatic encephalopathy an "insidious disease", said: "Treatment normally involves the use of antibiotics or laxatives to suppress the production of toxic substances in the intestine but there is still a great deal of room for improvement, so it will be exciting to see the results of further studies to determine if clinicians have a new form of treatment on the cards."

Ammonia is thought to be one of the main mediators of cerebral dysfunction in HE. By enriching the gut flora with non-urease producing microorganisms, probiotics decrease ammonia production. Hepatic encephalopathy, caused by an accumulation of toxins in the blood which would normally be removed by the liver, causes range of neuropsychiatric issues such as personality changes, intellectual impairment, confusion, and coma, at times leading to death.

"It will be exciting to see the results of further studies to determine if clinicians have a new form of treatment on the cards."

- Professor Mauro Bernardi

COCKTAIL OF ALCOHO LETHAL LINK FOR EUR

ALCOHOL and body weight's deadly impact o liver disease came under greater scrutiny a study of over 107,000 women were revealed on EASL Congress.

Although the effects of a high alcohol intake and bo the liver are well-known, combinations of the two f with low BMI and a high alcohol intake hold a gre liver disease than women with high BMI and lower a

"There has been a need for a large population st factors' influences on each other," EASL's Scientif Dr. Daniele Prati said. "These findings will have a how we can help millions of people across the worl liver disease."

Within the study, women across the United Kingdo low or high BMI (<25 or ≥25) and a low or high alc units per week), with Dr. Prati adding that further re order to determine the exact thresholds for each fa risk of chronic liver disease.

NEW NASH DIAGNOSIS

A BRAND new non-invasive test for non-alcoholic steatohepatitis (NASH) was demonstrated through Professor J. Abrigo's "Phosphorus Magnetic Resonance Spectroscopy in Non-Alcoholic Fatty Liver Disease" study on Wednesday at EASL 2013.

The imaging technique, phosphorus magnetic resonance spectroscopy (P-MRS), allows non-invasive *in vivo* assessment of hepatocellular metabolism, and offers an alternative to the risks and unpleasantness from the current 'gold standard' procedure of a full liver biopsy.

Though the results now require prospective confirmation in independent cohorts, P-MRS shows a fair diagnostic accuracy for non-alcoholic fatty liver disease (NAFLD), through highlighting distinct alterations in adenosine triphosphate (ATP) and

OL, WEIGHT AND LIVER DISEASE SHOWS ROPE, THE HARDEST-DRINKING CONTINENT

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Other studies revealed that patients with alcoholic cirrhosis, who also have fatty liver disease and are overweight, obese, or are type 2 diabetic, are also more likely to develop hepatocellular carcinoma.

With Europe deemed the heaviest-drinking region in the world according to the World Health Organization (WHO) and over 20% of its population aged 15 and over admitting to heavy episodic drinking at least weekly, calls for improved methods of diagnosis are increasing. Physicians often rely on indirect evidence of alcohol abuse, such as information from family members, questionnaires and laboratory testing.

"Women are at particular risk as they are twice as sensitive as men to alcohol-related liver damage and developing a more severe form of the disease at lower doses with shorter durations of alcohol consumption," Doctor Prati added.

Harmful and hazardous alcohol consumption is a net cause of 7.4% of all ill-health and early death in the EU

- European Commission

S TECHNIQUE FOUND

phosphodiester (PDE) levels. The research was achieved by using 31P MRS and studying liver metabolite changes from 132 patients with biopsy-proven NAFLD.

NAFLD sufferers can be separated into two categories; those with relatively benign simple steatosis, and those with NASH, an advanced form of the disease which can progress into cirrhosis and hepatocellular carcinoma. NASH is becoming increasingly widespread, with its presence estimated in around 3% of the population of the western world. Although techniques such as insulin resistance blood tests or ultrasound imaging can detect NAFLD and NASH, differentiating between the two can only be achieved through studying a small sample of liver acquired via a fine needle.

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PHASE III TRIALS IN HCV TREATMENTS SHOW POTENTIAL

DIRECT-ACTING antiviral agents (DAAs) demonstrate encouraging potential in hepatitis C (HCV) treatment, according to new data from phase III trials presented on the first day of EASL.

The anticipated trials conducted among HCV patients with a range of genotypes (GT 1 to 6), with two trials in particular, POSITRON and NEUTRINO, have been garnering attention due to both their high success rate and low adverse effects.

Professor Mark Thursz, EASL Secretary General, said: "Hepatitis C treatment in the last four years has undergone an amazing revolution. Prior to that we had just pegylated interferon and ribavirin and the average treatment success rate was 50%. We are now looking at regimes going into market early next year where we can expect a 90% cure rate from this infection."

"Furthermore, the new drugs that we are using will be suitable for patients who couldn't previously access therapy. So really, it is a brave new world for patients with hepatitis C."

The POSITRON study consisted of interferon (IFN)-ineligible, IFN-intolerant, or IFN-unwilling cirrhotic and non-cirrhotic GT 2 and 3 HCV-infected patients treated with a combination of sofosbuvir and ribavirin over 12 weeks. With the SVR12 rate of 78%, all 278 patients became HCV RNA negative, while only 2% of patients discontinued treatment in the sofosbuvir and ribavirin group due to adverse effects.

> "It is a brave new world for patients with hepatitis C."
>
> *- Professor Mark Thursz*

Meanwhile, the NEUTRINO trial is deemed a short, simple and effective option for HCV-infected patients, with 90% SVR12 in treatment naive genotype 1, 4, 5 or 6 HCV-infected patients following a treatment combination of sofobuvir, peginterferon alfa-2a and ribavirin for 12 weeks. A total of 327 patients were enrolled, 292 classed as genotype 1, 28 as genotype 4, and seven as genotype 5 and 6.

Other studies focused on protease inhibtor data, with STARTVerso™1 using Faldaprevir, an oral once-daily protease inhibitor combined with peginterferon alfa-2a and ribavirin to treat 652 patients, finding 88% were eligable to halt treatment after 24 weeks.

VITAL THERAPIES DEVELOPING BIO-ARTIFICIAL LIVER

A BIO-ARTIFICIAL liver incorporating live human hepatoblastoma cells, designed to treat patients suffering life-threatening acute liver failure, has been developed by Vital Therapies, Inc, following a $150 million fundraising effort devoted towards the groundbreaking technology.

Comprising of four cartridges of immortalised human liver cells and mounted on a bedside unit, ELAD® is manufactured to provide liver support continuously for up to 17 days without the cells losing their ability to perform, potentially allowing time for the patient's native liver to regenerate to a healthy state, or to stabilise the patient until a suitable donor organ can be found for transplantation.

Acquired from a human liver tumour, the company suggests cells can be grown in unlimited quantities to be stored and shipped. ELAD® has been tested by over 150 patients so far in seven clinical trials, as well as a compassionate-use program, with data from the Phase 2b, to be announced later on at the American Transplant Congress in Seattle.

LIVERTREE™ TO BE ONE-STOP WEBSITE

FOLLOWING the ILC Congress in Amsterdam, EASL have officially launched Livertree™, a revolutionary eLearning Portal for liver research and education.

Attendees at the Congress had the chance to explore the portal pre-launch of the one-stop educational website, boasting of featuring the most advanced search tool ever built for the field of liver research.

Joel-Zvi Chetzroni, CEO of the MULTILEARNING Group and architect of Livertree™, said: "This is the most advanced eLearning portal ever built for a medical society across all therapeutic areas."

"With this level of technology and the new approach in online education, medical associations are step by step attracting back their audience who is currently flirting with privately owned/sponsored educational websites. The audiences are going back to medical associations, the trusted source of education in their field."

> "This is the most advanced eLearning portal ever built for a medical society across all therapeutic areas."
>
> *- Joel-Zvi Chetzroni*

Centralising and organising thousands of EASL educational materials published throughout the past eight years, EASL has combined this accumulated knowledge with eLearning components such as accredited courses (UEMS) and breakthrough concepts in online education options concerning EASL lectures.

"The launch of Livertree™ during our International Liver Congress™ has been a phenomenal success on site, and since this is an educational benefit only given to our members, we had the pleasure to welcome more than 350 new members on site during the Congress," said Gregoire Pavillon, Executive Director of EASL.

"We strongly feel that Livertree™ will have a much greater impact than expected on our society."

On visiting the site, EASL members gain access to a patent-pending concept that provides them with the ability to retrieve clear and concise sections of lectures without the need to enter a single keyword.

> The Hepatology Tree, specifically built for Livertree™, has over 1,500 branches already
>
> *- EASL*

cccDNA MAY HOLD KEY TO CURING HBV

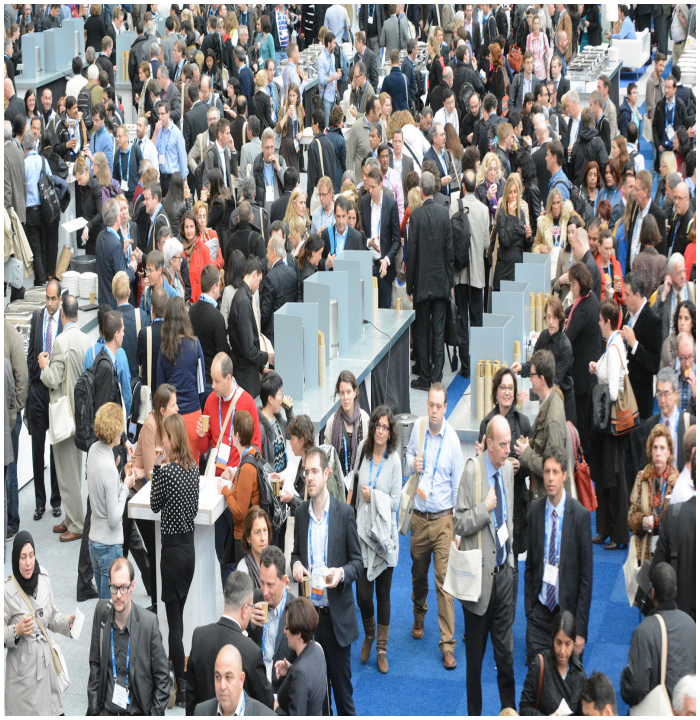
THE key to curing chronic hepatitis B virus (HBV) infection may be in covalently closed circular DNA (cccDNA), according to *in vitro* and *in vivo* study results presented on Thursday 25th April at EASL.

Three experimental studies demonstrated effective HBV cccDNA targeting or depletion using novel therapeutic approaches, which potentially could result in a cure. This would be an advancement from life-long treatments currently used to suppress the long-term persistence of HBV cccDNA, which is organised into mini-chromosomes within the nucleus of infected cells by histone and non-histone proteins.

EASL Educational Councillor Professor Fabien Zoulim said: "In chronic hepatitis B infection, the viral genome forms a stable mini-chromosome – the covalently closed circular DNA (cccDNA) – which can persist throughout the lifespan of the hepatocyte."

"Current treatments focus on suppression of HBV and discovery of compounds directly targeting cccDNA has been one of the major challenges to curing HBV infection; but these preliminary data show novel therapeutic approaches can be applied to successfully target cccDNA with the long-term aspiration of finding a cure."

COUNCILLOR: Professor Fabien Zoulim



The first experimental study found that liver regeneration induces a strong reduction of viral replication and cccDNA level, while not achieving full cccDNA eradication due to *de novo* infection returning without antiviral treatment. Conclusions suggested the induction of hepatocyte turn-over combined with viral-suppressing antiviral drugs may accelerate clearance of the viral mini-chromosome.

The second study suggested targeting epigenetic control of nuclear cccDNA mini-chromosomes to suppress HBV transcription, its replication potentially highlighting other therapeutic approaches, while the third said lymphotoxin beta receptor (LTbR) agonisation represented a basis for a novel alternative approach to curing chronic HBV infection as a whole.

HBV carries a 20-30% risk of death through cirrhosis, and a 25% likelihood of the patient developing liver cancer. Although primarily endemic in China and other parts of Asia due to childhood infection, the World Health Organization estimates two billion people are infected worldwide.

CARDIO AIDS POTENTIAL CANCER CURE

REGULAR exercise reduces the chance of developing liver cancer, maintaining hope for those at risk from hepatocellular carcinoma (HCC), as revealed by a study on mice, presented at EASL.

Two groups of mice, one fed a controlled diet, the other high fat, were divided into separate exercise and inactive groups and put to run on a motorised treadmill for 60 minutes a day, five days a week. Following 32 weeks, 71% of the controlled diet mice developed tumours larger than 10mm, compared to 100% of the inactive group.

EASL Educational Councillor, Professor Jean-Francois Dufour, said: "The results could eventually lead to some very tangible benefits for people staring down the barrel of liver cancer, and I look forward to seeing human studies in this important area in the future."

"The prognosis for liver cancer patients is often bleak as only a proportion of patients are suitable for potentially curative treatments, so any kind of positive news in this arena is warmly welcomed."

With over half a million new cases diagnosed worldwide annually, HCC causes 47,000 yearly deaths in Europe alone.

Professor Dufour added: "It's been previously unknown whether regular exercise reduces the risk of developing HCC. This research is significant because it opens the door for further studies to prove that regular exercise can reduce the chance of people developing HCC."

The conclusion adds additional weight to the benefits of exercising not only to avoid cancer, but to help recuperate and prevent recursion. Professor Robert Thomas, a consultant oncologist for Bedford Hospital, said while speaking to Macmillan Cancer Support: "Traditionally doctors and nurses have often advised patients to rest during chemotherapy and radiotherapy, or after cancer treatment. In this case, however, rest is not best."

He added: "Regular exercise during these treatments has significantly been shown to improve psychological well-being, improve mood and prevent what is sometimes called 'chemo brain'."

BRAND NEW TACE RANKING METHOD

A SCORING system measuring which hepatocellular carcinoma (HCC) patients would benefit the most from transarterial chemoembolisation (TACE) was revealed at the International Liver Congress 2013™.

The Assessment for Retreatment with TACE score (ART-score) is observed following the first TACE session, based on liver function and tumour response, and their impact on a patient's overall survival. The ART-score differentiated two groups (0-1.5 points; ≥2.5 points) with differences in prognosis (median overall survival: 23.7 months vs. 6.6 months; $p<0.001$) which was confirmed in an independent external validation cohort. In addition, a higher ART-score was associated with major adverse events after the second TACE session ($p=0.011$).

Professor Markus Peck-Radosavljevic, EASL's Vice-Secretary, said: "These findings represent an important discovery, as they will enable physicians to identify and treat those HCC patients who will benefit from repeat TACE sessions."

HEPATIC ENCEPHALOPATHY: A PARADIGM SHIFT

Summary of Presentations given at the Norgine Symposium, 48th Annual Meeting of EASL, Amsterdam, April 25th 2013

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Disclosure: The speakers received honorarium from Norgine for participating in this symposium.
Acknowledgements: Writing assistance provided by Trilogy Writing and Consulting Ltd.
Support: The publication of this article was funded by Norgine. The views and opinions expressed are those of the authors and not necessarily of Norgine.
Citation: EMJ Hepatol. 2013;1:20-30.

Hepatic Encephalopathy: A Paradigm Shift

Dieter Häussinger

The most recent understanding of the pathogenesis of hepatic encephalopathy (HE) is that heterogeneous precipitating factors cause a low-grade cerebral oedema and an oxidative stress response with formation of reactive oxygen and reactive nitrogen species (ROS/RNS). This triggers multiple changes in signalling pathways and causes protein and RNA modifications, which result in alterations in gene expression and neurotransmission. These events in turn alter synaptic plasticity and lead to disturbances of oscillatory networks in the brain that are responsible for the cognitive and motoric symptoms of HE **(Figure 1)**.[1,2,3] This pathophysiological series of responses has been demonstrated in both, the human brain and in animal experiments.

Gene expression in the human cerebral cortex controls individuals with liver cirrhosis with and without HE. As shown by whole genome gene expression analysis, 434 genes are specifically upregulated in HE in the brain **(Figure 2)**. Upregulation of these genes are specific for HE and cannot be detected in cirrhotic patients without HE.[4]

This new pathophysiological concept is not the only paradigm shift; there are new diagnostic methods, new aspects of sociomedical relevance and new treatment options available to treat HE.

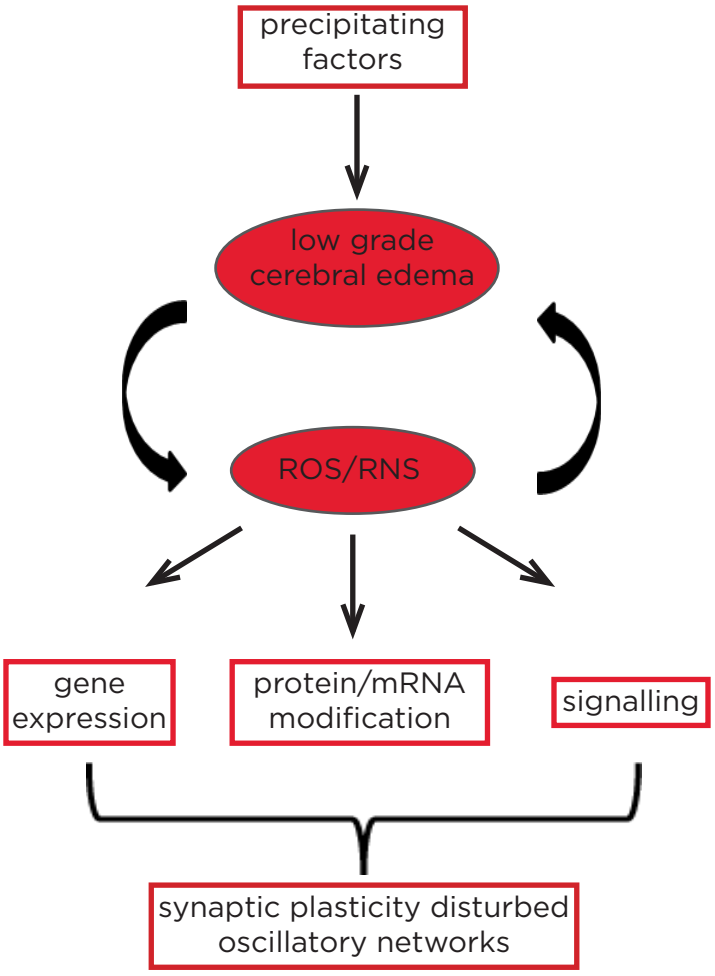


Figure 1. The pathogenesis of hepatic encephalopathy.

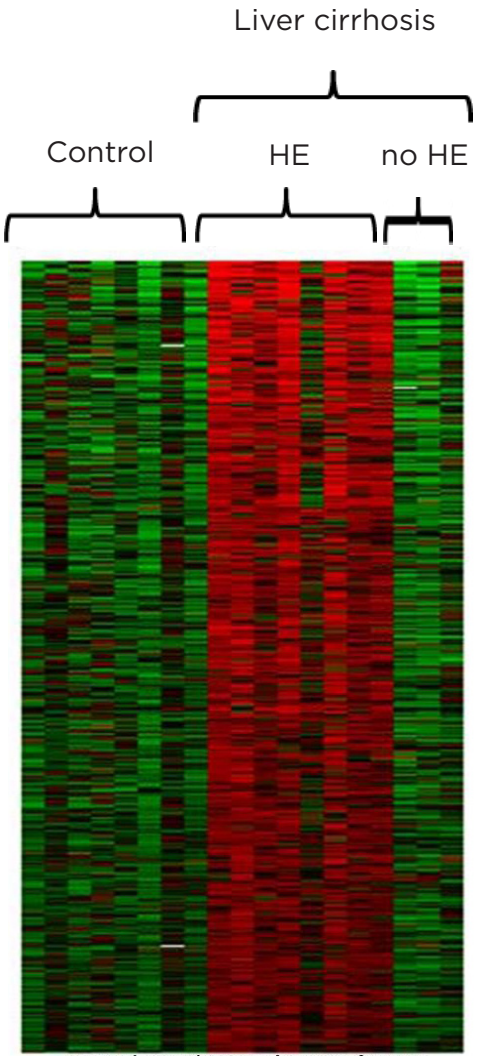


Figure 2. Gene expression in the human cerebral cortex.

HE Epidemiology

Peter Jepsen

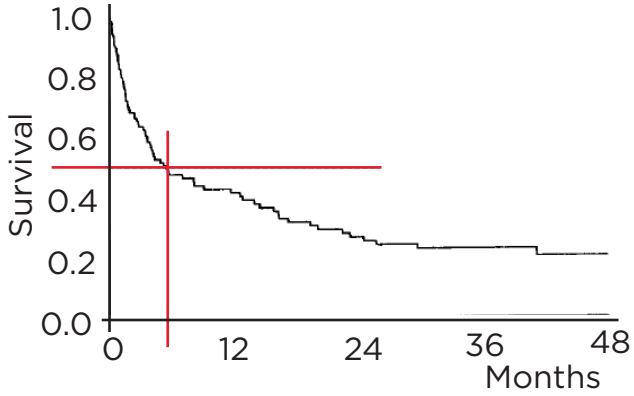
The International Society for Hepatic Encephalopathy and Nitrogen Metabolism (ISHEN) issued a consensus statement (2011)[5] emphasising that hepatic encephalopathy (HE) is a continuum of worsening cognitive function. However, in clinical practice HE is defined by clinically relevant categories; patients who are unimpaired, patients with covert HE and patients with overt HE. The transition from unimpaired to covert HE is defined by the results of specialised tests and the transition from covert to overt HE is defined by flapping (asterixis). The specialised tests used to diagnose covert HE is a huge research topic in its own right and is not discussed here.

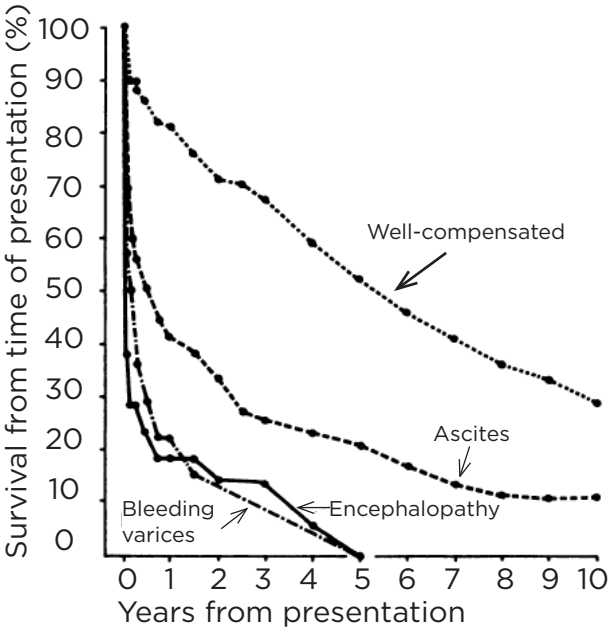
Although surprisingly few studies have examined how HE affects life expectancy, it is evident that overt HE is associated with a short survival time. Bustamente et al. (1999)[6] reviewed 111 patients with overt HE and found an expected residual survival time of approximately 6 months. In a landmark study of patients with alcoholic cirrhosis, Saunders et al. (1981)[7] compared the impact of various cirrhosis complications and also found that HE was associated with a survival time of approximately 6 months. More recently a Danish study[8] of alcoholic cirrhosis patients found that with the improvements in the management of variceal bleeding, HE was clearly the most lethal cirrhosis complication and has the greatest impact on patient mortality **(Figure 3)**.

Covert HE is not associated with the steep short-term mortality seen with overt HE. This was shown in a study[9] of 271 cirrhosis patients with mixed aetiology, a significant difference in survival between patients with overt HE and patients with covert HE was observed. These results confirm what would be expected because covert HE is an earlier manifestation of the spectrum of neurocognitive impairment in cirrhosis.

HE affects not only mortality but quality of life (QoL). QoL was compared in 544 cirrhosis patients with or without HE in the largest QoL study to date.[10] The patients included in the study were not suffering from the most severe forms of HE because they had to have the ability to fill out a questionnaire. The Nottingham Health Profile and the SF-36, both of which are generic measures of health-related QoL, were used to assess the effects of HE on the physical and mental domains of the patients. The 2 groups of patients with cirrhosis, those with HE and those without HE, were compared with a baseline population sample. The results showed that cirrhosis with or without HE does not cause pain. However, cirrhosis, and to an even greater extent HE, affects the physical domains of the patient. The Nottingham Health Profile showed that energy, mobility and sleep were significantly affected in patients with HE and the SF-36 indicated that physical function was affected. Overall, the results showed that the physical domains were more affected by HE than the mental domains. However, this is possibly due to HE patients' poor insight into their own mental capacity.

It is less clear whether covert HE affects QoL. Studies have shown conflicting evidence; with the milder forms of HE it is difficult to disentangle the effects of cirrhosis on QoL from covert HE.[10] In addition, the differences in cirrhosis severity, aetiology and the diagnostic criteria make it difficult to compare studies. However, it is clear that covert HE does cause problems with attention, visuospatial abilities and psychomotor speed, for example patients with





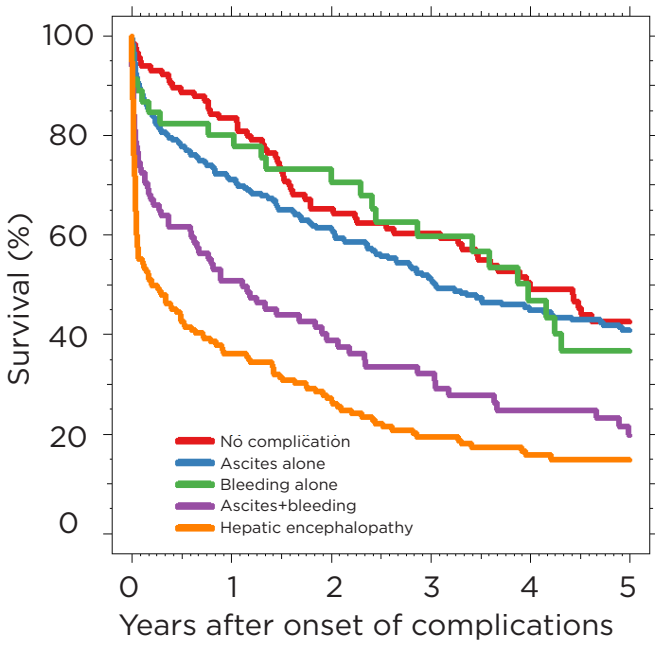


Figure 3. Survival with overt HE.

covert HE have difficulties in completing complicated work tasks and driving a car.

HE imposes a burden on the caregivers of cirrhosis patients. Montagnese et al.[11] measured the caregiver burden in 31 caregivers (94% were relatives). The caregivers completed a questionnaire (The Caregiver Burden Inventory) that focused on the time devoted to care for the patient and the psychological, physical, social and emotional burden they experienced. The results showed that the burden on the caregiver was markedly greater for caregivers to patients with overt HE than for caregivers to cirrhosis patients without HE. The study concluded that in HE patients there is a burden on both the caregivers and the patients themselves.

Overt HE is a relatively uncommon presentation at the time of cirrhosis diagnosis. In a study[12] of 1,115 cirrhosis patients only 10% of the study population were reported to have overt HE when cirrhosis was diagnosed. Similarly, this prevalence was shown in a study of 250 patients with alcoholic cirrhosis,[7] 10% had overt HE at the time of cirrhosis diagnosis. A further study[8] of 466 Danish patients showed that 11% had overt HE at the time of cirrhosis diagnosis. The results of these studies indicate that in the majority of patients, there is time to intervene and prevent the development of HE.

The presence of covert HE is much less clearly defined. Several studies have found that the prevalence of covert HE in cirrhosis patients is 25% to 50%, although some have reported a prevalence as high as 75%; the difference seen in the results are due to the variance of the study population and diagnostic criteria.[13-15] Covert HE is a warning sign that overt HE may ensue. This was shown in a Dutch study[16] of 116 cirrhosis patients with mixed aetiology. Twenty-five patients had covert HE, and in 2.5 years of follow-up 56% of these patients developed overt HE.

Patients who have already developed cirrhosis complications are much more likely to develop HE. The risk of a first episode of overt HE was compared between patients who had not developed cirrhosis complications and patients who had already had variceal bleeding or ascites. Those who had not developed complications had a 5 year risk of a first episode of overt HE of 7% compared with 26% in those with other complications **(Figure 4)**. These findings are consistent with the observations that overt HE is a rare first complication of cirrhosis.[8]

In studies of patients with viral cirrhosis, a recent

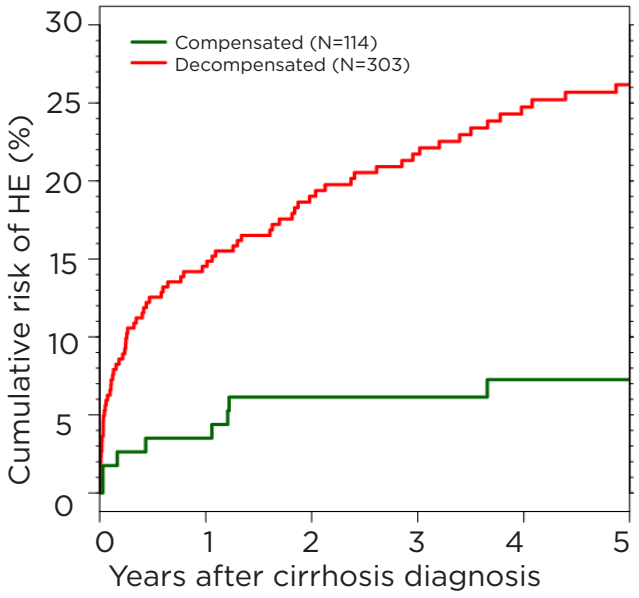


Figure 4. Risk of overt HE in alcoholic cirrhosis.

Cuban study[17] examined the risk of overt HE in 402 patients with compensated hepatitis C virus (HCV) cirrhosis. Those without varices had a 5-year risk of overt HE of approximately 5% compared with 15% in those who had non-bleeding varices. These findings are consistent with an Italian study that reported a 5-year risk of overt HE of 9% and a 10 year risk of 25%[18] in compensated cirrhosis. Conversely a further study[19] found a much lower risk of overt HE, approximately 5% after 10 years. This lower risk may be explained by the differences in diagnostic criteria for overt HE.

The vaptan trials were conducted to examine whether patients with ascites might benefit from satavaptan treatment. The 3 trials included a total of 1,198 cirrhosis patients with ascites, 25% of whom had previously had an HE episode. Data from the 1-year follow-up period showed that 27% of the patients had at least one episode of overt HE.[20]

The different risk estimates shown in the various studies is probably due to the differences in the prevalence of other risk factors for developing HE. The risk factors for developing HE are usually divided into precipitants and more remote risk factors. Precipitants are those risk factors that manifest immediately before overt HE occurs, including constipation, dehydration, infections, variceal bleeding and medications. These produce inflammation or an increase in nitrogen load. The more remote risk factors for developing overt HE include previous episodes of overt HE, covert HE, ascites, and hyponatraemia. In addition, Jepsen et al. (2012)[21] found that poor galactose elimination capacity (GEC, a measure of hepatic metabolic function) is a strong risk factor for overt HE but is not a risk factor for ascites or variceal bleeding. The role of other risk factors in the development of HE such as cirrhosis aetiology and comorbidity remain largely unknown.

In summary, HE is a continuum; in its overt form it is associated with a very high mortality with an expected survival time of approximately 6 months. The covert form of HE is an early warning sign of overt HE, however covert HE causes its own specific problems. HE affects QoL and is a burden on caregivers as well as on the patients themselves. The prevalence of HE at the time of cirrhosis diagnosis is 10% however, the risk of development is highly variable ranging from approximately 5% to more than 25% after 5 years.

Strategies to Improve the Diagnosis and Management of Hepatic Encephalopathy

Rajiv Jalan

The changing paradigm of hepatic encephalopathy (HE) illustrates the current problems in the management of the syndrome. This includes the interaction between ammonia and inflammation and how new concepts of acute and chronic liver failure will impact on how HE is understood and treated in the future.

Two factors lead to the development of the syndrome, liver disease and progression (which is linked with liver injury and maybe limited or on-going), and coincidently increased bacterial translocation as a result of a number of interacting factors. It is thought that these two factors lead to the development of the complex progression of hepatic fibrosis. This leads to compensated cirrhosis or decompensated cirrhosis. Decompensated cirrhosis is typically associated with the syndrome of hepatic encephalopathy.

Whether there is fibrotic liver disease, compensated cirrhosis or decompensated cirrhosis the issue is complicated by the effect of the 'second hit'. A 'second hit' implies the effect of a superimposed hepatic event such as exacerbation of liver disease with drugs, viruses or toxins, or an extrahepatic event such as infection, trauma, variceal bleeding, insertion of a transjugular intrahepatic shunt or surgery. This can lead to the development of HE which is a precipitated syndrome. Cirrhotic patients depict altered host response to injury, which is associated with multiple organ dysfunction, and a resulting

syndrome that is referred to as acute or chronic liver failure. HE is one of the characteristic complications.

Peter Ferenci (1998)[22] defined the types of HE: Type A associated with acute liver failure; Type B associated with portal systemic bypass, no intrinsic hepatocellular disease; Type C associated with liver cirrhosis and portal hypertension/or portal-systemic shunts. This definition remains a useful tool for discerning the types of HE and classifying patients.

Overt HE is just the tip of the iceberg. A larger proportion of patients lie in the domain of unrecognised syndrome referred to as minimal hepatic encephalopathy. Pre-minimal HE is being increasingly recognised as a sub-group of patients who have normal neuropsychological test results but have an increased number of associated symptoms such as fatigue, undue anxiety, autonomic dysfunction and depression. This area of HE is where understanding needs to be increased and therefore, where the changing paradigm of the perception and treatment of HE is predicted.

Patients with minimal HE (MHE) have a poor quality of life (QoL), they are tired, lack concentration and some cannot drive. It is in this patient group that differences can be made and new treatments developed. If patients with MHE develop infection or bleeding complicates the syndrome and can lead to the overt form of HE.

An analysis of 1,400[23] patients identified the features of patients with HE in acute on chronic liver failure. The analysis defined one group of patients that had no other attendant organ dysfunction i.e. no acute-on-chronic liver failure (ACLF), and showed that in this group mortality rates were very low (4%). In contrast, the analysis found that if a patient had associated organ dysfunction identified by high bilirubin, high creatinine, low sodium and a marked inflammatory response, a precipitating event and a higher Model End Stage Liver Disease (MELD) there is an increased risk of mortality (30%). These results show that patients with associated organ dysfunction are likely to have a higher mortality; however this mortality is not necessarily dependent on the severity of HE but on the ancillary features. This is a paradigm shift in the understanding of overt HE identifying possibly two or more sub-classes of patients. Therefore reclassification of the features of HE will be necessary in the future.

At present, HE is graded using the West Haven Criteria for semi-quantative grading of mental state; from Grade 1 to Grade 4. The diagnosis and categorisation of patients with Grades 2, 3 and 4 is straightforward; the problem is diagnosing Grade 1 HE. This is because there is a variety of symptomatology that is difficult to define. Grade 1 HE is defined as trivial lack of awareness, euphoria, anxiety, shortness of attention span and impaired performance but these symptoms could be applied to most people in particular sets of circumstances who do not have HE! It is essential that sub-classifications of this early form of HE are developed to enable clear definition and clear diagnosis of the syndrome.

In the pathophysiology of HE, low grade cerebral oedema is very important. The cell that is swollen is the astrocyte. Astrocytes are located very close to the blood vessels in the brain and form part of the blood-brain barrier. In severe acute liver failure, the astrocytes become very swollen. It is thought that this swelling is due to ammonia, although cerebral inflammation can also cause the astrocytes to swell. High levels of ammonia can be seen in different categories of HE patients[24] but whether the levels correlate with outcomes is unknown. Ammonia causes cells in the brain to swell; it is thought that this is caused by the accumulation of the metabolite glutamine allowing attraction of water into the cell which in turn leads to cell swelling. In the last 15 years it has become apparent that the role of ammonia in this process is complex. Ammonia is produced in the gut, not necessarily by the action of bacteria, but by the uptake of glutamine into the gut, the metabolic generation of ammonia is one of the future targets of therapy. The role of the kidney is very important both in ammoniagenesis and ammonia excretion,[25] millimolar quantities of ammonia are excreted in the urine every day providing a huge opportunity to impact on ammonia concentrations.

The classical understanding of the pathogenesis of HE is that liver failure results in increased ammonia which causes brain oedema. However, the alteration of bioenergetics should be considered, whether the alteration of bioenergetics is pathophysiologically important or is a consequence of increased ammonia is unknown. The alteration of bioenergetics interacts significantly with the whole systemic inflammatory response which may act both inside and outside the brain through the blood brain barrier. This leads to the increased activity and/or expression of transcription factors which may lead to brain oedema and consequently neurological dysfunction.

Clinical data confirms that ammonia is synergistic with inflammation in the pathogenesis of HE.[26-31] Gut permeability and its modulation is an important

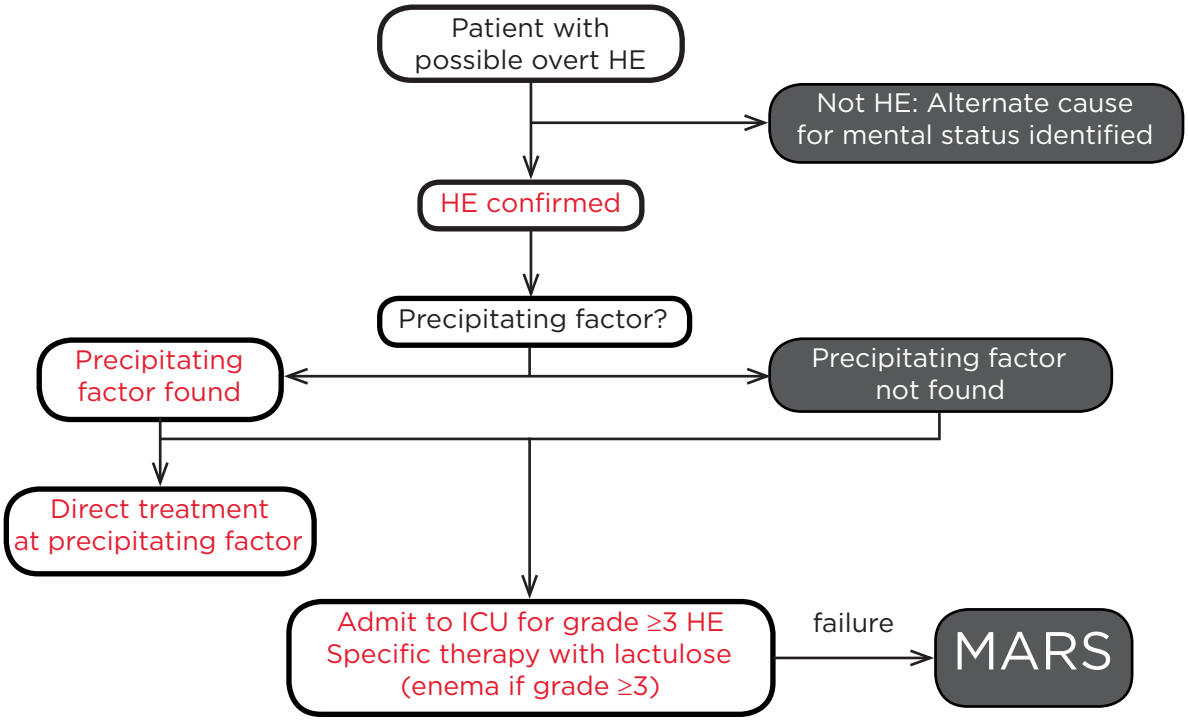


Figure 5. The treatment algorithm for overt HE.

factor in the inflammatory response, endotoxemia is thought to prime the circulation in the brain by upregulation of the Toll-like receptor 4. This increases permeability, alters liver function, primes the circulation, organs, kidneys and brain resulting in the predisposition to the effect of the 'second hit'. The outcome of this process is HE.

The importance of targeting the gut is highlighted by the significant difference seen in the development of HE following an acute variceal bleed between patients who are treated early with lactulose compared with those who are untreated.[32] This suggests that the occurrence of the overt form of HE can be prevented. Therefore, the prevention of overt HE is a major treatment goal.

Treatment of patients with large portosystemic shunts is very difficult to manage. Riggio et al (2005)[33] compared patients who did not have HE and were having transjugular-intrahepatic-portosystemic-shunts (TIPS) inserted. Seventy-five patients were randomised to 3 arms; lactitol, rifaximin or no treatment. The results showed that there was no difference in patient outcome with any of the treatments used as prophylaxis to prevent HE following TIPS. Therefore, the role of shunting in the development of these syndromes is important. However, rifaximin treatment has illustrated the principle that modulating the gut can lead to a reduction in the recurrence of HE.[34]

In patients who don't respond to treatment, albumin dialysis may be useful. Albumin is being used as an adsorbent; it was thought that this molecule is a volume expander but it is actually a very important antioxidant and has a lot of other functions and is critically involved in the process of binding toxins and removing endotoxins. Seventy patients who had failed all forms of treatment in intensive care were studied, and extracorporeal albumin dialysis led to a significantly greater wake up rate in patients treated with this device and the coma time was reduced. Although no difference in survival with the treatment was seen, the study showed that if the patient responded to therapy and the HE improved, the patients were more likely to survive.[35]

The treatment algorithm for overt HE **(Figure 5)**, includes the following steps; confirmation of the cause and other possible causes ruled out. If HE is confirmed the precipitating factor should be found and treated.[36] If the precipitating factor is not found the patient should be treated with lactulose (lactulose appears to be the best treatment at the present time) and albumin dialysis considered.

In conclusion, ammonia and inflammation are synergistic, but may be independent of each other, in the development of the syndrome of HE, providing 2 important targets for therapy. Current approaches for HE are improving. Lactulose is the main treatment for MHE and for the primary prophylaxis of bleeding.

Rifaximin is used for secondary prophylaxis, and in severe acute HE, albumin dialysis is usefully. In the future, there will be several more drugs available to treat HE initiating a paradigm shift that will enable improved classification of patients. There is no strategy that has been shown to reduce ammonia consistently in cirrhosis; however two new agents show promise (HPN-100 and ornithine phenylacetate). There will be more development in this area in the future.

Prevention of Recurrence of Overt Hepatic Encephalopathy

Fred Poordad

The algorithm for the management of a patient with possible overt hepatic encephalopathy (HE) involves confirming the HE and then searching for precipitating factors. However, in patients with progressive recurrent HE approximately 80% of the time a clear precipitating factor is not found. If precipitating factors are identified, treatment should be directed at these (this does not always involve long term therapy). When precipitating factors are not found the patient should be treated by admission to intensive care (HE grade 3 or above) and specific therapy for the underlying cause as well as HE therapy with lactulose or rifaximin commenced.

There are various management options for patients with recurrent overt HE these include non-absorbable disaccharides (lactulose and lactitol) and non-absorbable antibiotics (rifaximin and neomycin). Rifaximin and neomycin are both FDA approved, though only neomycin is approved for the treatment of acute HE. Other therapies such as sodium benzoate are not currently licensed in the EU to treat HE.[37]

The rationale for the use of non-absorbable disaccharides is to lower ammonia by metabolic trapping. Non-absorbable disaccharides are thought to work by protonating ammonia to ammonium and enhancing the excretion of the compound, however there are other mechanisms involved such the inhibition of bacterial ammonia production and the purgatory effect of non-absorbable disaccharides which remove bacteria from the colon.[38] Yet studies using lactulose or lactitol do not show this to be an effective treatment over placebo in HE.[39] Conversely in clinical practice, even though no effect on mortality has ever been shown, these treatments do appear to be effective, but it is difficult to show this in clinical trial settings. The most challenging aspect of treating patients with non-absorbed disaccharides is that they can cause a tremendous number of adverse events (AEs), which include abdominal bloating, gas/flatulence, unpredictable diarrhoea and, if used to extremes, can lead to volume contraction and electrolyte abnormalities. These AEs are distressing for the patient, often to the point of the patient becoming non-adherent to therapy. Bajaj et al (2010)[40] showed that patients with HE treated with lactulose typically experienced a recurrence within 9 months. 3 out of 4 of these patients required hospital admission, and 39% of those admitted to being non-compliant to lactulose treatment. In addition, 8% of the patients experienced lactulose-associated dehydration. The multivariate analysis predictors of recurrence showed the 2 variables that predicted re-admission and recurrence of HE were non-adherence to lactulose treatment (OR, 3.26) and a high Model for End-stage Liver Disease (MELD) score (OR, 1.14).

Sodium benzoate increases ammonia metabolism and renal elimination. It is not FDA or EU approved for the treatment of HE. Limited clinical studies exist regarding the use of sodium benzoate in the treatment of HE, and one study examining basal/post glutamine challenge ammonia levels in cirrhotic patients suggested a note of caution in its use.[41] Unsurprisingly sodium benzoate has not been widely adopted as a treatment for HE.

Non-absorbable antibiotics are thought to reduce the production of gut-derived ammonia by decreasing the bacteria that produce it. However, it has become apparent that there are other mechanisms involved suggesting that the complete mechanism is not fully understood. There are few clinical studies that assess this mechanism in relation to neomycin treatment for HE. Neomycin is absorbed at a rate of up to 5%, therefore it is not truly a non-absorbed antibiotic. In addition, the use of neomycin is limited due to its ability to cause hearing loss[42] and it has the potential to cause nephrotoxicity, particularly in patients with high MELD scores. Consequently, neomycin is not a recommended choice for the treatment of HE.

The efficacy and safety of rifaximin (Xifaxan®) in HE has been studied extensively both in the EU and the US. The trials include a double-blind, randomised, dose-finding multi-centre study,[47] open-label studies,[44-46] several comparative randomised controlled trials against neomycin,[46-49] paromomycin,[50-52] lactulose[46,53-55] and lactitol,[56] and one multi-national placebo-controlled trial.[34]

A review[39] of randomised trials that compared non-absorbable disaccharides with antibiotics and placebo

showed that overall antibiotics produced a positive effect in the management of HE and were superior to non-absorbable disaccharides in improving HE. This indicates that although disaccharide treatment is a well-established treatment, and often the first-line treatment for HE, (possibly due to its relatively low cost), the efficacy of these compounds need to be assessed.

The treatment options for HE have distinct advantages and disadvantages. Treatment efficacy estimates in patients with overt HE[57] based on pooled data (generated primarily for pharmacoeconomic evaluation) showed varying clinically significant improvements in patients treated with lactulose, lactitol, neomycin and rifaximin of 68%, 69%, 64% and 90%, respectively. Though these were not head-to-head comparisons in the same trial, the estimates provide relative differences in an overview of the efficacy of the different treatments.

Following recovery from overt HE the goal is to maintain the patient in remission. It must be accepted that it is not a curable disease and that a further episode is likely, therefore the aim is to delay the next episode of HE for as long as possible. Historically, lactulose has been the standard treatment (possibly due to a lower medication cost per patient). However, rifaximin appears to have a superior tolerability and efficacy profile.[39] In addition, early data suggests that preventing hospital admission could decrease morbidity associated with in-patients and decrease overall healthcare costs.[58]

A Phase III randomised, double-blind, placebo-controlled trial evaluated rifaximin for the maintenance of HE remission.[34] The inclusion criteria were that patients had experienced at least 2 episodes of West Haven Grade 2 or higher HE in the 6 months prior to enrolment, and at the time of enrolment were in remission (Grade 1 or Grade 0 disease). The patients were randomised to receive rifaximin or placebo over a 6-month period; due to their previous HE episodes, 90% of the patients were taking concomitant lactulose. It was deemed unethical to remove lactulose and randomise the patients to placebo alone; therefore the background treatment for the majority of the patients was lactulose. The primary endpoint of the study was the first HE breakthrough. The results showed no HE breakthrough in 77.9% of patients receiving rifaximin versus 54.1% of patients receiving placebo (HR: 0.42; P<0.0001), 86.4% of patients receiving rifaximin did not require hospitalisation for an episode of HE compared with 77.4% receiving placebo (HR: 0.50; P=0.01) during the 6 month study period. No significant differences in drug-related adverse events (AEs) were seen between the 2 groups and no novel emergent AEs were seen **(Table 1)**. Of particular importance, there was no emergent clinically meaningful resistance, no bacterial over-growth and no propensity fungal infections. In addition, following this 6-month study, a 3-year open-label maintenance trial[34] was performed. This was a commitment to the regulatory authorities to collect safety data over an extended period of time in patients who received long-term antibiotics; the study showed that the effectiveness of the rifaximin did not change over time.

Adverse event	Xifaxan® 550mg b.i.d. (n=140) n (%)	Placebo (n=159) n (%)
Any event	112 (80.0)	127 (79.9)
Nausea	20 (14.3)	21 (13.2)
Peripheral edema	21 (15.0)	13 (8.2)
Ascites	16 (11.4)	15 (9.4)
Fatigue	17 (12.1)	18 (11.3)
Diarrhea	15 (10.7)	21 (13.2)
Dizziness	18 (12.9)	13 (8.2)
Headache	14 (10.0)	17 (10.7)

Table 1. Rifaximin (Xifaxan®) in HE: rate of adverse events.

Quality of life (QoL) was assessed in HE patients treated with rifaximin compared with placebo in a pivotal Phase III trial.[59] The patients were administered the Chronic Liver Disease Questionnaire (CLDQ) at baseline and every 4 weeks until the end of treatment. The CLDQ is a disease specific instrument to assess health-related QoL; it incorporates 29 items across 6 domains and a 7-point scale with higher scores indicating improved QoL. The area under the curve for CLDQ was normalised by exposure time to calculate the time-weighted average. The mean time-weighted average for overall QoL (P=0.0093) and all 6 subdomains in the rifaximin arm were significantly greater compared with the placebo arm. The results indicated an improvement in QoL in patients that were treated with rifaximin **(Figure 6)**.

Breakthrough HE can be prevented with 6 months treatment of rifaximin in 1 out of 4 cases, and 1 out of 9 cases of hospitalisation-related HE can be avoided.[34] This was demonstrated in a single

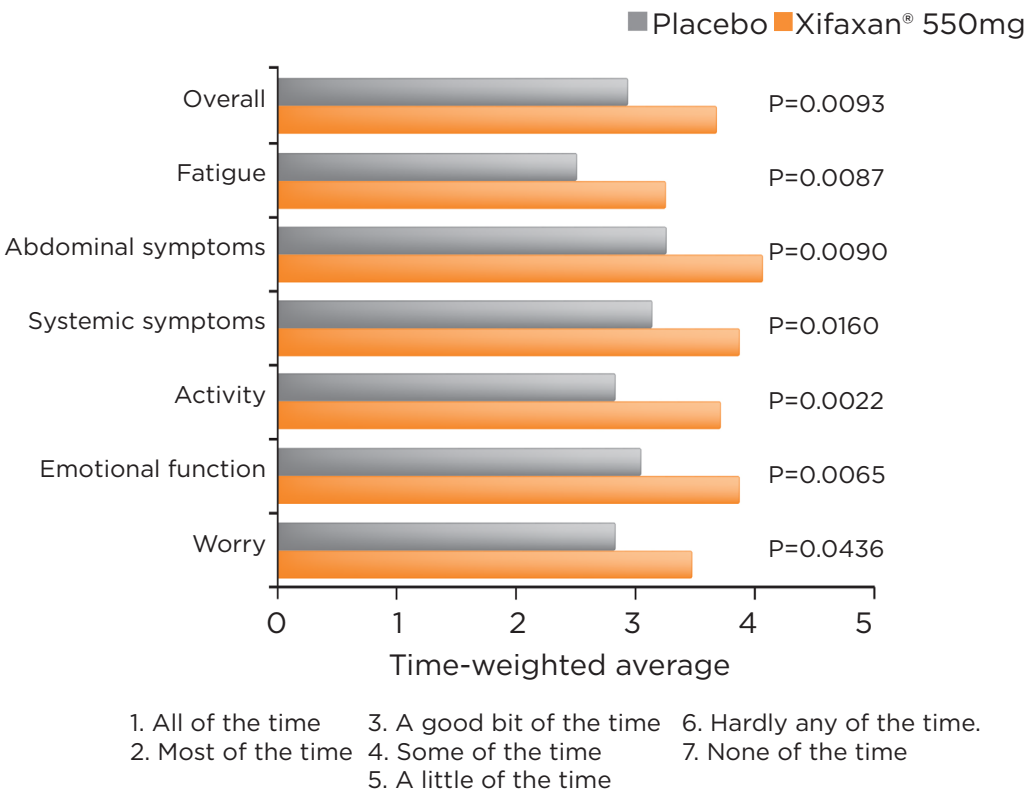


Figure 6. Chronic Liver Disease Questionnaire results with rifaximin treatment in HE.

centre study comparing rifaximin and lactulose in the management of HE.[58] The average length of hospital stay for the lactulose group was 5.0 days compared with 3.5 days in the rifaximin group (P<0.001). The total annual cost of hospitalisation for the lactulose group was $13,285 compared with $7,958 in the rifaximin group, showing a significant cost differential of $5,327.

Rifaximin is a semisynthetic antibiotic that is a derivative of rifamycin. It has broad coverage against gram-positive and gram-negative bacteria, aerobes and anaerobes. Rifaximin is a non-systemic antimicrobial with absorption of less than 0.4%, it is concentrated in the gastrointestinal tract and excreted in the faeces[60] and has a very low propensity to produce clinically meaningful resistance. Dose finding studies have shown that 1100mg is the appropriate dose per day for the treatment of HE and multiple doses of rifaximin do not result in accumulation.[61] In addition, no clinically relevant drug interactions have been observed.[62,63]

In conclusion, there are limited therapeutic options for HE, most of the historical therapies have multiple side effects. There is now a broad spectrum, non-absorbed antibiotic therapy, rifaximin (Xifaxan®) that is well tolerated, effective and is suitable for long term use.

Future research for new therapeutic options will focus on enhancing survival in advanced liver disease.

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ADRENAL INSUFFICIENCY (AI) IN CIRRHOSIS

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Disclosure: No potential conflict of interest.
Citation: EMJ Hepatol. 2013;1:32-37.

ABSTRACT

Adrenal dysfunction or insufficiency (AI) in cirrhosis, also described as hepato-adrenal syndrome, is an only recently recognised entity. It is estimated that at least 10% of patients with compensated cirrhosis and over 30% with decompensated cirrhosis have adrenal insufficiency, defined by an abnormal result in the adrenocorticotropic hormone (ACTH) stimulation test. This could increase the risk of cardiocirculatory compromise, infections, and decompensation in these patients but as yet has to be confirmed. An important problem is that diagnosis of adrenal insufficiency in liver disease is difficult, as symptoms can be subtle and overlap with those due to cirrhosis. Furthermore, laboratory testing and reference standards have not been clearly defined. There is evidence that critically ill patients with cirrhosis and AI have a worsened outcome compared with similar patients that do not have AI. However, there is no clear consensus about diagnosis or treatment, in particular regarding steroid replacement therapy, for AI in patients with cirrhosis.

This review will give a brief overview of AI in patients with liver disease, first describing diagnostic tests for AI without liver disease and subsequently the available tests and their pitfalls in the setting of liver disease. As this clinical entity is increasingly recognised, the focus of research will likely change from prevalence and diagnostic studies to mechanisms and therapy, both of which are not defined at present.

Keywords: Adrenal insufficiency, cirrhosis, hepato-adrenal syndrome, CIRCI, diagnosis

ADRENAL INSUFFICIENCY IN PATIENTS WITHOUT LIVER DISEASE

AI results from a deficient production of hormones secreted by the cortical layer of the adrenal gland. This deficiency can be either primary, related to the adrenal gland itself, secondary, through pituitary disease with decreased production of ACTH or tertiary, due to hypothalamic involvement leading to a disruption in the hypothalamus-pituitary-adrenal (HPA) axis caused by a deficiency in corticotropin releasing hormone (CRH). AI is a rare disease in the Western world with primary AI having an estimated prevalence of 35-60/10^6.

Symptoms depend on the acuity of onset, but chronic AI may be oligosymptomatic with fatigue, lassitude and weight loss. Overt AI may only become apparent through intercurrent illness when activation of the HPA axis fails. This may then lead to an impaired cardiovascular response and increased risk of infection.

A particular entity is the relative adrenal insufficiency described in critically ill patients (Critical Illness-related Corticosteroid Insufficiency – CIRCI), where there is inadequate adrenal cortisol secretion relative to the severity of the illness in patients with evidence of systemic inflammation. This may be through an inability to increase cortisol secretion or possibly through end-organ (tissue) resistance.[1]

Assessment of the HPA Axis

Several investigations are available to test the HPA axis. Basal cortisol levels are measured at 8-9am, a time coinciding with the peak in the diurnal variation in cortisol secretion. Generally, total

plasma cortisol is measured, which is a surrogate marker for free plasma cortisol (the biologically active form) in patients with normal plasma protein synthesis. Values <138nmol/l are highly suggestive of AI, whereas values above 415nmol/l essentially exclude AI.

If basal cortisol levels are low or there is a clinical suspicion of AI, ACTH levels should be measured, again, between 8-9am coinciding with the diurnal peak of secretion. In primary AI these exceed 100pg/ml (22pmol/l), however, normal plasma ACTH values do not rule out mild secondary AI.[2] Therefore, dynamic testing is required to establish the diagnosis of AI.[3]

Stimulation tests involve synthetic ACTH in a high or low dose (HDSST or LDSST) or corticotropin. The insulin-induced hypoglycaemia test (IIT) is now rarely used due to obvious concerns about patient safety when inducing hypoglycaemia.

The high dose SST can be performed any time of the day. Blood samples are taken at baseline and 30-60 minutes after intravenous or intramuscular administration of 250mcg of ACTH 1-24 (Synacthen). Post-stimulation levels of over 550nmol/l exclude primary AI. This test has been criticised for its supraphysiologic stimulation of the HPA axis and the possibility that this dose may stimulate adrenal secretion, resulting in levels above 550nmol/l despite a degree of adrenal insufficiency. The HDSST may be most appropriate in critically ill patients.

The low dose SST (LDSST) has therefore been proposed.[4] Here, a 1mcg dose is given intravenously after obtaining a baseline plasma cortisol sample. Further samples are taken at 20 and 30 minutes. The normal response is a cortisol plasma concentration above 500nmol/l. However, the disadvantage of this test is that it has yet to be validated in acute hypothalamic-pituitary disorders and in the critically ill,[5] and therefore is only most appropriate for non-critically ill patients.

The administration of corticotropin-releasing hormone differentiates primary from secondary AI. In primary AI, the elevated levels of ACTH will rise even further in response, whereas in a pituitary disorder causing secondary AI the low ACTH levels will not respond to CRH. CIRCI is defined as a difference of basal to post-stimulation cortisol levels (delta cortisol) of >250nmol/l (9mcg/dl) after HDSST, or a random total plasma cortisol of <276nmol/l (10 mcg/dl).[1]

Management

Overt adrenal insufficiency requires hormone replacement. This is generally achieved with a short-acting corticosteroid, such as hydrocortisone, taken orally in two to three divided daily doses. Hydrocortisone has some mineralocorticoid activity. Different replacement protocols are available, the most commonly chosen is the twice daily oral hydrocortisone replacement with 2/3 of the dose in the morning (20mg) and 1/3 in the evening (10mg) in an effort to mimic the diurnal variation.

The management of patients with CIRCI is controversial. Current recommendations state that the benefit of treatment with glucocorticoids appears to be limited to patients with vasopressor-dependent septic shock and patients with early severe acute respiratory distress syndrome.[1]

ADRENAL INSUFFICIENCY IN PATIENTS WITH LIVER DISEASE

Diagnosis of AI in patients with liver disease is not a straightforward task. Under normal circumstances cortisol is bound to over 90% by cortisol-binding globulin (CBG) and albumin, while only 10% is unbound (free) and metabolically active.[6] Measurement of cortisol levels in patients with cirrhosis is therefore complicated by decreased protein synthesis (CBG and albumin), and binding (mainly to albumin) thus the measured total cortisol underestimates the free cortisol so that this results in overdiagnosis.[7] In critically ill patients with decreased serum albumin levels, overdiagnosis of AI has been reported[8-9] when using total serum cortisol measurements. This is likely to be due to a decrease in CBG. Not surprisingly, CBG levels were also found to be decreased in patients with cirrhosis, this being worse in patients with decompensated (Child C) disease.[10-11] As serum-free cortisol concentration is expensive and difficult to measure, salivary cortisol has been proposed as a surrogate marker. In non-cirrhotic patients salivary cortisol concentrations were in concordance with free cortisol concentrations, even in patients with hypoalbuminaemia and CBG abnormalities.[12] In patients with cirrhosis, Galbois et al.[13] found a better correlation between salivary cortisol and free cortisol than between total and free cortisol levels. Using total serum cortisol for diagnosis led to overestimation of AI in this study. Similarly, in a study of 125 patients with cirrhosis salivary cortisol and serum free cortisol were also closely correlated (in the whole population, Spearman coefficient at T0: r=0.69; P<0.0001).[14] However, the fact that liver

diseases such as alcoholic liver disease, primary biliary cirrhosis and primary sclerosing cholangitis can lead to salivary gland pathology may complicate this test.

Test interpretation, however, remains controversial. In addition, the spectrum of liver diseases, including their acuity, severity and aetiology is very diverse. Thus, different degrees of neurohormonal activation and levels of circulating endotoxins and inflammatory cytokines exist, which may possibly potentiate the effects of concurrent AI.[15] Overall the available evidence suggests a.) AI is common in patients with acute or chronic liver disease and b.) its prevalence increases with more severe hepatic disease. Clinical symptoms are rarely a guide to diagnosis as they overlap with those of cirrhosis in stable patients and many patients with acute or decompensated liver disease are critically ill. The suggested,[1] most appropriate form of stimulation test for critically ill patients with and without liver disease is the HDSST, measuring the peak but more importantly the change in cortisol concentration (delta cortisol). It is likely that patients with decompensated liver disease also fall into the above category, and their adrenal function should be assessed according to CIRCI criteria. The majority of these patients have evidence of systemic inflammatory response syndrome. To date, there are no clear guidelines for diagnosing AI in patients with stable cirrhosis outside the intensive care setting. Apart from the question concerning which stimulation test to choose, the most accurate test for the measurement of cortisol concentration has yet to be established. Similar to intensive care patients, patients with cirrhosis often have low serum protein levels. Measuring total (protein-bound) cortisol concentration may lead to the over-diagnosis of AI in this setting.[13, 16] Free cortisol concentrations have only rarely been assessed in cirrhosis due to the expense and difficulty in measurement. Several studies have assessed salivary cortisol concentration in comparison with total and/or free cortisol.[13-14] Salivary cortisol may therefore be an appropriate surrogate marker for plasma free cortisol concentration,[12] however, collection may be difficult in the ICU environment and contamination may affect the measurement.

Patients that have adrenal insufficiency or CIRCI overall have a worse prognosis in particularly in the setting of sepsis and septic shock, as discussed below. So far corticosteroid replacement has only been recommended in two critically ill patient groups: those with septic shock, particularly when persistently hypotensive and poorly responsive to fluids and vasopressors, and those with early, severe acute respiratory distress syndrome (ARDS).[1]

AI in Stable or Decompensated Liver Disease

Over the last 20 years, 11 studies have been published looking at the prevalence of AI in these groups as well as the correlation with severity of disease.

McDonald et al.[10] performed both SST and IIT on 38 patients with non-alcoholic liver disease and 40 healthy controls. There was a significant 64% reduction in maximal increments of plasma cortisol after IIT, and a 39% reduction after SST. A negative correlation between peak cortisol response and severity of hepatic dysfunction was also observed.

Ten years later Ziets at al.[17] used the CRH test to assess the HPA axis in 52 patients with cirrhosis. This was abnormal in 58% of patients using a peak cortisol level of <550nmol/l or <250nmol/l increase. Again, HPA dysfunction was more common in more severe liver disease. There was no difference between patients with cirrhosis due to alcohol or viral hepatitis.

A study of 88 patients with cirrhosis found a relatively low prevalence of AI (9%) using salivary cortisol measurements after SST.[13] However, this may have been due to active alcohol consumption in >50% of subjects, as alcohol induces adrenal cortisol production.[18] Peak total, delta total and peak-free plasma cortisol were measured during SST in 43 compensated cirrhotics.[16] The prevalence was higher using CIRCI criteria as opposed to standard criteria (47% vs. 39%), and lowest when peak-free cortisol concentrations were used (12%). Neither CIRCI criteria nor peak-free cortisol concentrations have been validated in this patient group.

We performed LDSST on 101 stable cirrhotics without evidence of infection or haemodynamic compromise.[19] The overall prevalence of AI was 38% with a higher prevalence in more advanced liver disease.

In a study from France, 125 consecutive patients were assessed for AI with SST. Basal and peak total, salivary and free cortisol were measured. AI was found in 7.2% as defined by peak cortisol concentration below 510nmol/l. As about a quarter of these patients were septic, use of CIRCI criteria may have been more appropriate for evaluation of AI. Patients who did fulfill their criteria for AI, however, had more severe liver disease. Both groups had similar basal salivary

and free cortisol concentrations.

Over the last years the increasing interest in AI associated with different stages of cirrhosis has led to several interesting abstracts at the EASL conferences[20-23]. All of these studies assessed CIRCI criteria after a high dose of SST. Acevedo et al.[20] compared 10 patients with compensated with 188 patients with decompensated cirrhosis. RAI was found in 27% using CIRCI criteria, however this was similar between the two groups. There was no significant difference in mortality in patients with and without AI.

The same group measured delta cortisol in 166 patients with advanced cirrhosis after SST.[21] RAI was defined by CIRCI criteria and was associated with more severe circulatory dysfunction, septic shock, more severe infections, lower serum sodium and increased hospital mortality. Delta cortisol levels <250nmol/l and/or peak plasma cortisol was used after SST to establish diagnosis of RAI in 85 patients with cirrhosis and non-infected ascites.[23] AI was present in 39% and associated with a higher mortality (33% vs. 10%).

In a study of 37 patients with cirrhosis and acute variceal haemorrhage, RAI was present in 38% and associated with a higher risk of not controlling bleeding at day 5.[22] Our group[24] compared 20 patients with cirrhosis and variceal haemorrhage to 60 stable cirrhotic patients and 14 healthy volunteers using LDSST and SST for diagnosis of AI. We could not demonstrate a difference of the prevalence of AI between the two groups of cirrhotics using SST. However, when LDSST was used, the prevalence of AI was higher in patients with acute variceal haemorrhage. These patients also had higher basal and peak cortisol concentrations than stable cirrhotics associated with similar delta cortisol levels, suggesting an inadequate adrenal response with respect to the severity of the patients' condition (CIRCI).

AI in Critically Ill Patients with Liver Disease With or Without Sepsis

Two other studies have looked at acutely decompensated, septic patients with cirrhosis and assessed the prevalence of AI in this setting. Tsai et al.[25] performed SST on 101 patients with cirrhosis and severe sepsis. AI was diagnosed on the basis of peak or delta serum cortisol levels (<414nmol/l or <250nmol/l). AI was diagnosed in 52% and was related to severity of liver disease, a lower mean arterial pressure, increased inotropic requirements and increased hospital mortality (81% vs. 38%).

Another study from the same year[26] evaluated a similar patient group and diagnosed AI in 68% with a higher prevalence in more advanced disease (CTP-C 76% vs. CTP-B 25%). Stress doses of hydrocortisone were given to patients with AI (50mg/QID). This group was compared to a selection of patients in whom investigation of adrenal function was not undertaken. Shock resolution and mortality rate was improved in the group of patients receiving hydrocortisone. However, Arabi et al.[27] performed a randomised, double-blind, placebo-controlled trial of low dose hydrocortisone replacement (50mg/QID) in septic patients with cirrhosis. This led to a significant reduction in vasopressor doses and higher rates of shock reversal but no reduction in 28-day mortality. It was also associated with an increase in shock relapse and gastrointestinal bleeding.

In 45 patients with acute liver failure AI was diagnosed in 62% using reference ranges from a healthy population.[28] Delta and peak cortisol values were lower in patients with haemodynamic instability, ventilator dependence and in those who died or required transplantation.

Marik et al.[29] performed LDSST in 340 patients with acute or acutely decompensated liver disease as well as recently or previously transplanted patients admitted to the intensive care unit. AI was found in 33% of patients with fulminant hepatic failure, 66% of patients with acutely decompensated cirrhosis, 61% of patients previously transplanted and in 92% of patients recently transplanted and maintained on steroid-sparing immunosuppressive regimen.

AI in Liver Transplant Recipients

Assessment of adequate adrenal function after liver transplantation is even more complicated by the recent surgery and steroid-containing immunosuppressive regimens. We assessed the intraoperative administration of 1000mg of methylprednisolone in 90 consecutively enrolled patients undergoing first elective liver transplantation, irrespective of adrenal function.[30] Requirements for vasopressors, invasive ventilation, fluid administration, haemofiltration, and length of intensive care stay were all significantly reduced in patients who received the methylprednisolone bolus intraoperatively.

AI has been reported in post-transplant patients

with steroid sparing regimen[31] and after steroid withdrawal.[32] Subclinical/latent adrenal insufficiency may adversely affect outcome in the operative and postoperative liver transplant setting, which maybe complicated by infection, hypotension and severe blood loss. An inadequate adrenal reserve is likely to significantly impair an appropriate stress response in these patients.

SUMMARY AND FUTURE STUDIES

AI is present in at least 10% of patients with cirrhosis, and the prevalence increases with more severe liver disease. The pathophysiology of AI in cirrhosis has yet to be elucidated. In the general population the most common cause for AI is primary adrenal dysfunction (pred. autoimmune adrenalitis). In liver disease however, hypothalamic-pituitary impairment[10] has been suggested as the predominant cause for AI. Possible explanations include the increased concentration of circulating cytokines, which may interfere with appropriate activation of the HPA axis.[33] Other potential causes include the use of CNS depressants, pituitary/adrenal ischaemia or haemorrhage and infections. All of these conditions are known to have an effect on the HPA axis.[34] The potential effect of increased brain water seen in acute and chronic hepatic encephalopathy is also unknown. As far as we are aware, there are no post mortem studies of adrenal or pituitary anatomy and pathology in patients with cirrhosis.

The end-organ effects of latent AI in liver disease are unclear. However, in times of physiologic stress, such as variceal haemorrhage, ascites formation or infection, patients with AI seem to have a worse prognosis. In particular with regards to circulatory dysfunction AI may play an important role in patients with cirrhosis. In an already vasodilated cirrhotic patient, cortisol deficiency may lead to more profound hypotension with detrimental effects on end-organ perfusion, particularly on the kidneys, and response to vasoconstrictors. Currently it is unknown whether AI in cirrhosis has direct effects on cardiac function. However there are similarities between cirrhotic cardiomyopathy (CM) and AI-associated CM, both of which may only become apparent in stressful conditions.[15]

An interesting question is whether treatment for decompensation in these patients should include cortisol replacement and whether decompensation could be delayed or prevented.

In summary, studies are needed to facilitate the diagnosis of AI and a consensus is required to define diagnostic criteria. The effect of AI in compensated and decompensated liver disease, as well as the optimal treatment/replacement strategy for these patients, has to be further assessed in randomised controlled trials.

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ACUTE-ON-CHRONIC LIVER FAILURE: APPLYING THE PIRO CONCEPT

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Disclosure: No potential conflict of interest
Citation: EMJ Hepatol. 2013;1:38-43.

ABSTRACT

Acute-on-chronic liver failure (ACLF), a clinical syndrome associated with a dismal prognosis, occurs acutely in previously stable cirrhotic patients. An important feature of this syndrome is the potential for reversibility if it is recognised early and supportive measures are instituted before multi-organ failure ensues. In response, there have been recent efforts to better define and understand the pathophysiological basis of the condition so as to aid early diagnosis and management. The PIRO concept is conceptually useful as it indicates a distinction between the insult and the response. Interventions that target inflammation may adversely impact on the ability to control the infection, and interventions that target infection may not be useful if pathophysiological process is being driven through inflammation. A classification based on the PIRO concept may allow the categorisation of patients into distinct pathophysiologic and prognostic groups and allow a multidimensional definition of ACLF.

Keywords: Acute-on-chronic liver failure, cirrhosis, liver.

INTRODUCTION

An early and proper diagnosis of acute-on-chronic liver failure (ACLF), together with the identification of indicators associated with disease severity, is critical for outcome prediction and therapy. Although this clinical entity is well recognised, it remains poorly defined due to the extreme heterogeneity in the mode of presentation. In order to clinically describe the group of patients referred to as ACLF, we adopted the definition that these patients would have an acute deterioration in liver function over a short period (up to four weeks), associated with a precipitating event in patients with well-compensated liver disease, characterised by organ failure.[1,2] The high prevalence of ACLF and mortality rates associated with it, remains an important healthcare issue as according to reported literature, short-term mortality rates vary from 46% to 89%.[3] In recent years, knowledge regarding the pathophysiology of ACLF has largely increased with the aim of improving the survival rates of these patients.

The pathophysiology of ACLF can be explained using the PIRO concept, which was initially developed for use in the sepsis setting.[4] Using this concept in ACLF, '**P**' stands for *predisposition*: predisposing factors that make a cirrhotic individual more likely to develop ACLF and organ failure. '**I**' represents the acute *insult or precipitating event*. '**R**' stands for the *inflammatory/immune response*, which occurs as a consequence of the acute insult. '**O**' signifies *organ dysfunction*, which is the final sequelae of the inflammatory response generated following the acute insult.[5] These could represent the four most important factors determining outcome.

PIRO SYSTEM

Predisposition (P)

In a large prospective study of ACLF patients conducted over six years,[2] it was clearly highlighted that the following factors predispose to the development of organ failure (OF) in cirrhotic patients with an acute deterioration:

- Male gender
- Higher bilirubin level
- Lower albumin level
- Higher Child-Pugh (CP) score
- Higher Model of End Stage Liver Disease (MELD) score
- Hospital admission in the preceding six months with hepatic decompensation.

Regarding factors that predict survival following the development of organ failure, the researchers found that patients who survived had the following characteristics: lower serum bilirubin, serum creatinine levels, Child-Pugh score and MELD scores, as well as a shorter prothrombin time, activated partial thromboplastin time, and higher albumin levels.

Although patients that survived following the development of organ failure had lower Child-Pugh score and MELD scores compared to the non-survivors, these scores may not accurately predict survival in ACLF. The literature suggests that once extrahepatic organ failure has developed, then organ failure scores such as the Sequential Organ Failure Assessment (SOFA)[6] or the Acute Physiology, Age, and Chronic Health Evaluation (APACHE)[7] may be more useful in predicting outcome and survival.[4,8]

Precipitating Events/Acute Insult (I)

In the majority of patients, ACLF usually develops following an identifiable precipitating event. Since patients with ACLF have previously stable disease on the background of either well-compensated or decompensated disease, and thus have a variable functional mass at the time of insult, the precipitants in ACLF are widely variable.[9] These precipitants may directly affect the liver or may be a consequence of an extrahepatic insult **(Table 1)**.

The most common precipitating event in ACLF is infection.[2,9,10] According to the literature, it accounts for up to 47% of all precipitating events.[2] In addition to bacterial infections, such as spontaneous bacterial peritonitis and urinary tract infections, viral infections are also recognised precipitants of ACLF. For example, acute hepatitis A infection superimposed in a patient with chronic hepatitis B cirrhosis is associated with a higher risk of ACLF, and thus a high mortality rate.[11] Cirrhotic patients who develop acute hepatitis B and E infection also have a high risk of developing ACLF, and of death.[12-13]

Hepatic precipitants of ACLF	Extra-hepatic precipitants of ACLF
Alcoholic Hepatitis	Infection
Acute Viral Hepatitis (A,B,E)	Variceal Haemorrhage
Drug Induced Liver Injury	Surgery
Portal Vein Trombosis	Trauma
Ischemic Hepatitis	

Table 1. Common precipitating events in acute-on-chronic liver failure (ACLF)

Variceal haemorrhage accounts for approximately 20% to 30% of all precipitating events. Alcoholic binge drinking is a contributory factor in between 50-70% of cases studied in the UK, but significantly lower in the patients in the CANONIC study where alcohol binge, as a precipitating event, was observed in about 20% of patients.[14] It is worth mentioning that in any given patient, there may be more than one insult leading to ACLF, and in a proportion of patients, there are no identifiable precipitants.

Inflammatory Response (R)

Following the initial insult, there is an altered host response to injury, resulting in an excessive systemic inflammatory response. This induces tissue damage and subsequent organ failure. Deregulated inflammation is considered a hallmark of ACLF and the mechanism by which this arises is multifactorial.[2,10] A hyperdynamic circulation is a common aspect in patients with systemic inflammatory response syndrome. In fact, in these patients with enhanced cytokine production, there are severe disturbances of the cardiovascular system: the circulation becomes hyperdynamic, cardiac output increases, and both blood pressure and systemic vascular resistance decrease.[15]

In addition to the precipitating insult (which may be infectious or non-infectious), there is abundant evidence in the literature to suggest that cirrhotic patients have increased bacterial translocation, secondary to increased intestinal permeability and changes in intestinal microflora.

Despite the insults, ACLF patients are unable to

generate an adequate immune response due to a dysfunctional innate immune system.[3,5,8] Firstly, in patients with ACLF, the Kupffer cells (resident macrophages of the liver and main effectors of innate immune system) are bypassed due to the presence of multiple intra and extrahepatic shunts, leading to failure to effectively clear endotoxins. In addition, the reduced protein synthesis that occurs in cirrhosis results in defective complement production. This means that the opsonisation capacity of the Kupffer cells is reduced and thus, bacterial phagocytosis is impaired.[3] Another contributing factor to an altered host response in ACLF is a phenomenon known as "immune paralysis", akin to that seen in sepsis, and associated with a high mortality. It has been demonstrated that in ACLF patients, there is reduced TNF-α production following stimulation with lipopolysaccharide. There is also a reduced expression of HLA-DR (an antigen-presenting receptor complex) on peripheral monocytes.[16]

The consequence of the immune dysfunction is that the initiating insult overwhelms the host immune system, leading to an exaggerated release of proinflammatory cytokines into the systemic circulation. This results in systematic inflammatory response syndrome (SIRS).[3,8,17] SIRS is defined as the clinical expression of an abnormal generalised inflammatory reaction in organs distant from the initiating insult.[17] It is reported that up to 42% of ACLF patients who develop organ dysfunction have evidence of SIRS despite the absence of infection in these patients.[2] In addition, the presence of SIRS is associated with more severe encephalopathy, an increased incidence of bacterial infection, and renal failure.[18] SIRS also occurs more frequently in non-survivors of ACLF compared with survivors.[2]

Innate immune dysfunction also results in macro-circulatory dysfunction. It is known that patients with ACLF have a hyperdynamic circulation and a high cardiac output. There is also peripheral vasodilatation and a low systemic vascular resistance, resulting in a reduced effective arterial volume and a decrease in mean arterial blood pressure. The consequence of these circulatory changes is end-organ hypoperfusion. This, in combination with a SIRS response, may result in organ failure, multi-organ failure and ultimately death.[8]

Organ Dysfunction (O)

Organ failure plays a central role in the clinical course of ACLF, occurring in one third of patients. Following the development of organ failure, there is an estimated mortality rate of 55% and 65% in the Intensive Care Unit (ICU)[19,20] and in hospital, respectively.[21] In addition to the liver, the organs commonly affected include the kidneys, brain, circulatory system and adrenal glands.

Liver: The hallmark of the liver manifestation of ACLF is hyperbilirubinaemia and coagulopathy. Hyperbilirubinaemia arises as a result of reduced detoxification function of the liver. In addition, the reduction in the liver's synthetic function leads to a decrease in the production of coagulation factors, and as a consequence, coagulopathy results. Hyperbilirubinaemia and coagulation failure have been identified as independent predictors of mortality in ACLF.[2,5,22,23] Histopathologically, it appears that the presence of cholestasis is associated with increased risk of infection, and balloon degeneration may be associated with a poorer outcome. The mechanism of hepatocyte cell-death in this syndrome remains unclear and requires further studies.

Kidney: Renal function is almost universally altered in patients with ACLF.[24] The most common causes of kidney failure are prerenal azotemia, hepatorenal syndrome, and acute tubular necrosis.[25] SIRS is present in approximately 40% of patients with functional renal failure. In addition, the presence of SIRS in patients with functional renal failure was associated with an in-hospital mortality rate of 68%.[26] Further evidence that SIRS plays an important role in kidney failure associated with ACLF, is derived from studies which have shown that the use of anti-inflammatory agents, such as pentoxifylline, improves renal function or significantly decreases the risk of developing renal failure in patients with alcoholic hepatitis.[27,28]

Hepatorenal syndrome (HRS) is the development of functional renal failure in patients with advanced liver disease. Type 1 HRS, which occurs in an acute setting, is a rapidly progressive decline in renal function associated with a mortality rate of 80% at two weeks.[29] Our current understanding is that HRS develops as a result of the haemodynamic dysregulation associated with portal hypertension. In this setting, splanchnic vasodilatation leads to decreased effective arterial volume, resulting in severe renal vasoconstriction and hypoperfusion. Activation of the sympathetic nervous system through a hepatorenal reflex arc is also thought to play a role. In addition, there is altered renal blood flow autoregulation in patients with HRS. However, attempts to restore this circulatory dysfunction with the use of splanchnic vasoconstrictors (terlipressin)

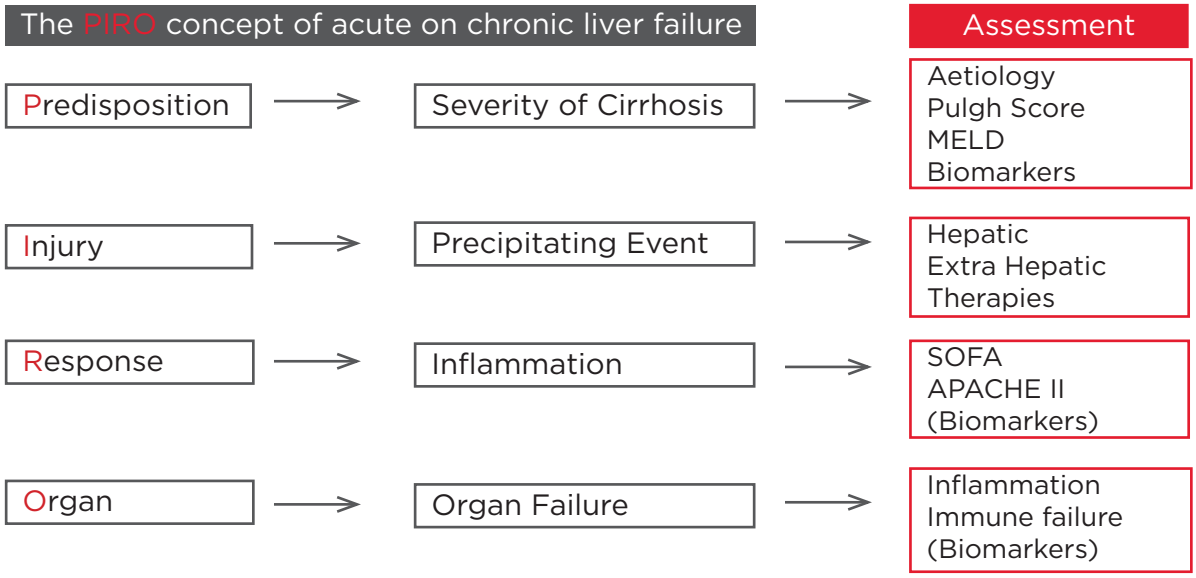

Figure 1. The PIRO concept of acute-on-chronic liver failure. In patients with ACLF, it is useful to think about the PIRO concept in determining pathogenesis and prognosis. Predisposition is indicated by the severity of the underlying illness. Injury by the nature and severity of the precipitating event. Response by host response to injury, which determines the severity of inflammation and risk of infection. Organs by the extent of organ failure.[2]

and volume expanders (albumin) only improves renal function in about 40%-50% of patients.[30] These observations indicate that other pathophysiological mechanisms may be responsible for the development of HRS. Indeed, recent studies have demonstrated histopathological evidence of renal tubular cell-death associated with apoptosis, increased renal tubular expression of toll-like receptor-4, increased urinary markers of acute kidney injury such as neutrophil gelatinase-associated lipocalin (NGAL), and more recently, urinary toll-like receptor-4 providing possible novel biomarkers and approaches to therapy.[31]

Brain: Hepatic encephalopathy (HE) may be due either to a precipitating factor or as a consequence of ACLF. It is a feature in up to 75% of patients with ACLF.[10] Brain oedema is central to the development of HE in ACLF. Hyperammonemia occurs in ACLF because of a reduction in the liver's detoxifying abilities. This high level of ammonia then diffuses into the brain. Once in the brain, it combines with glutamate to form glutamine. An increase in brain glutamine levels results in astrocyte swelling. Hyponatremia, another common finding in ACLF patients, exacerbates astrocyte swelling due to differences in osmolality between the intracellular and the extracellular compartment,[32,33] and systemic inflammation also plays a role in astrocyte swelling and cerebral oedema. Patients with evidence of infection or SIRS are more likely to develop severe encephalopathy.[34] It is likely that a synergy exists between hyperammonemia and inflammation. The high level of ammonia may prime the brain to the deleterious effect of superimposed inflammation by induction of microglial cells, which have a huge repertoire for cytokine production. This explains why measures which reduce the ammonia levels or levels of endotoxemia reduce the occurrence of HE.[5, 35-37]

Adrenals: Adrenal insufficiency occurs in 68% of patients with cirrhosis and septic shock. Although the mechanism responsible for this is not clear, it may be related to reduction in adrenal blood flow, which occurs as a consequence of circulatory changes in ACLF. In addition, high levels of pro-inflammatory cytokines, seen in ACLF, inhibit cortisol synthesis. Adrenal insufficiency may contribute to the development of multi-organ failure in ACLF.[38]

CONCLUSIONS

ACLF is a devastating condition associated with a high mortality rate. Despite this, treatment options are limited and mainly supportive. The shortage of cadaveric livers means that liver transplant, the definitive treatment option, is not available to most patients with ACLF. There is an urgent need to better define the condition as well as further understand the patho-physiological basis of the condition. The PIRO system is conceptually useful as it indicates a distinction between the insult and the response, thus providing a framework for a better pathophysiological understanding of this syndrome.

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CELLULAR CONSEQUENCES OF Z ALPHA-1 ANTITRYPSIN ACCUMULATION AND PROSPECTS FOR THERAPY

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Disclosure: No potential conflict of interest
Citation: EMJ Hepatol. 2013;1:44-51.

ABSTRACT

The Z variant (Glu342Lys) of α_1-antitrypsin polymerises and accumulates in the hepatocyte endoplasmic reticulum predisposing to neonatal hepatitis and liver cirrhosis. The resultant secretory defect leaves the lungs vulnerable to elastolysis and early-onset emphysema. There is currently no cure for the liver or lung disease other than organ transplantation. This review discusses the evolving understanding of the molecular pathogenesis of the condition and how this has led to the emergence of novel treatment strategies for α_1-antitrypsin-related liver disease.

Key words: Alpha-1 antitrypsin, polymerisation, liver disease, emphysema.

INTRODUCTION

Alpha-1 antitrypsin (AT) is a member of the serine proteinase inhibitor superfamily. It is primarily synthesised in hepatocytes and secreted into plasma from where it enters into the lungs and protects the alveoli from unregulated neutrophil elastase activity.[1,2] The normal variant is termed M-AT, according to its isoelectric point.[1,2] Severe α_1-antitrypsin deficiency most commonly occurs due to a point mutation in the α_1-antitrypsin gene, resulting in a substitution of a glutamic acid for a lysine at position 342 (^{342}GluLys); Z-AT.[1-4] Despite being known about for 50 years, it remains under-recognised. It affects 1 in 2000 in northern Europe, 1 in 4500 in the US and 1 in 5000 in the UK.[3,4] It is now well established that self-aggregation (polymerisation) of Z-AT leads to retention of Z-AT as periodic acid Schiff–positive (PAS-positive), diastase resistant inclusions within the hepatocyte endoplasmic reticulum (ER), which predisposes to neonatal hepatitis and liver cirrhosis.[5-7] The resultant secretory defect (10–15% of the levels of those of the normal M allele) predisposes to early-onset emphysema.[1,2] Retrospective studies have identified that up to 25% of those with Z-AT may suffer from liver cirrhosis or liver cancer in late adulthood.[8] Other than organ transplantation, there is no effective treatment for Z-AT-related disease.[1,2,7-16] Thus, reducing polymerisation and/or aggregation of Z-AT remains the major goal in the treatment of Z-AT-related liver disease.

α_1-ANTITRYPSIN DEFICIENCY

Mechanisms of Z α_1-Antitrypsin Deficiency

The Z mutation disturbs the normal reactive centre loop-β-sheet A tertiary relationship to promote a highly specific intermolecular linkage, whereby the reactive loop of one molecule, inserts into the β-sheet A of a second and so on to form Z-AT polymers **(Figure 1A)**. This results in ER accumulation of Z-AT polymers and retention within hepatocytes.[5] Formation of Z-AT polymers is accelerated *in vitro* by acidity (pH<6), and increased temperature.[7] Oxidation of the Z-AT monomer promotes accelerated formation of oxidised Z-AT polymers **(Figure 1B)**.[17] To date, over 120 variants of α_1-antitrypsin have been identified, some of which are prone to polymerisation.[1,2] The rates of polymer formation of these mutants correlate with the degree of retention of protein within

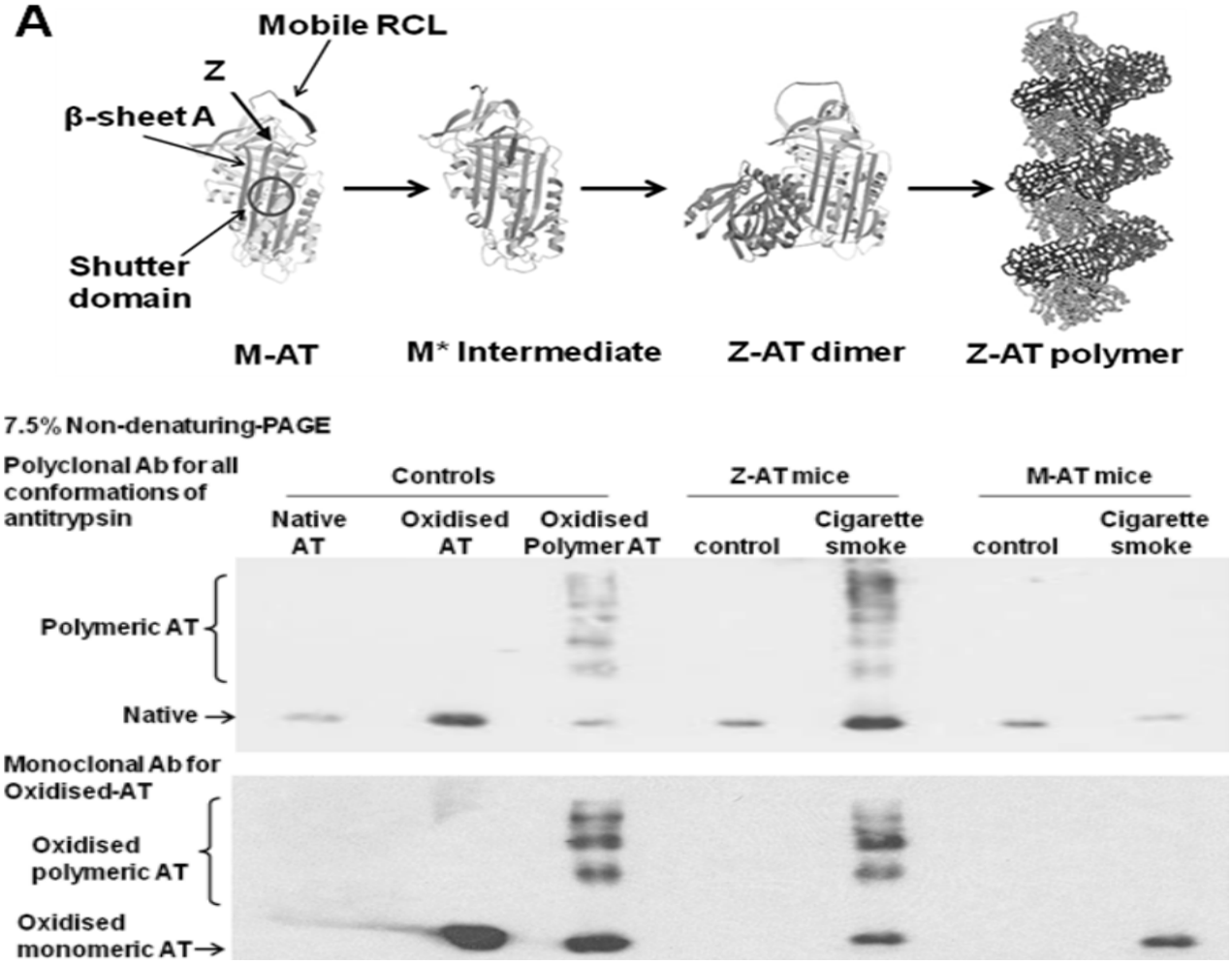

Figure 1. Polymerisation of Z-alpha-1 antitrypsin

Figure 1A. Mechanism of Z α_1-antitrypsin polymerisation

The structure of α_1-antitrypsin is centred on β-sheet A and the exposed mobile reactive centre loop. Polymer formation results from the Z-AT (E342K at P17; Z) or other mutations in the shutter domain, which destabilise β-sheet A to favour partial loop insertion and the formation of an unstable intermediate (M*). β-sheet A can accept the loop of another Z-AT molecule, to form a Z-AT dimer, which then extends into Z-AT polymers. The individual molecules of AT within the polymer are shown in different shades of grey.[7]

Figure 1B. Western blot analysis of bronchoalveolar lavage fluid for Z-AT polymers in cigarette smoke-exposed mice models

Proteins were analysed on 7.5% non-denaturing PAGE

Top gel: a polyclonal anti-human antitrypsin antibody, detected α_1-antitrypsin polymers in transgenic mice for Z-AT and monomeric species in control (non-cigarette smoke exposed) Z-AT, and both cigarette-exposed and control transgenic mice for M-AT.

Bottom gel: a monoclonal anti-human oxidised α_1-antitrypsin antibody, detected oxidised polymeric Z-AT in cigarette smoke-exposed Z-AT mice, and monomeric oxidised α_1-antitrypsin in cigarette smoke-exposed M-AT mice. Oxidised conformations of α_1-antitrypsin were not detected in control Z-AT or M-AT mice. Results suggest that cigarette smoke induces the formation of Z-AT oxidized polymers.[17]

hepatocytes, and the degree of plasma deficiency.[18] Other polymerising mutants of α_1-antitrypsin resulting in hepatic inclusions and plasma deficiency include: Siiyama (Ser53Phe) and Mmalton (^{52}Phe deleted), S (^{264}GluVal) and I (^{39}Arg→Cys) variants of α_1-antitrypsin.[7] These polymerising variants can also interact with the Z variant to form heteropolymers, inclusion bodies and liver cirrhosis.[7,18] The process of polymerisation is a generalised mechanism by which serpin mutants are associated with disease: polymerisation of antithrombin, α_1-antichymotrypsin, C1-inhibitor and neuroserpin mutants is associated with thrombosis, emphysema, angioedema, and familial encephalopathy with neuronal inclusion bodies, respectively.[7]

Lung Disease in Z α_1-Antitrypsin Deficiency

Interestingly, despite the severe deficiency of such a major proteinase inhibitor, there is variability in the expression of pulmonary disease even in members of the same family.[19-21] Several aspects of the pulmonary disease in PiZZ individuals are variable, for example age at the onset of lung disease, pulmonary function (spirometric measures of airflow obstruction and reduction in gas transfer factor), asthma-related phenotypes, extent of chronic bronchitis and bronchiectasis, distribution of emphysema (basilar predominance vs. diffuse emphysema), disease-related morbidity and mortality.[22] These findings suggest that modifier genes, environmental exposure, and gene-environment interactions also determine disease expression.[21,23] Cigarette smoking is clearly associated with rapid progression of the lung disease in PiZZ homozygotes. However, this in itself does not fully explain the variability seen in the severity of emphysema in the PiZZ group. Occupational exposure to mineral dust and the use of kerosene heaters have been associated with reduced FEV1 in non-smokers, independent of smoking status.[22,23]

PiZZ individuals with COPD have increased lung neutrophils and increased leukotriene B4 and interleukin-8.[24-27] Z-AT polymers have been identified in bronchoalveolar lavage fluid and explanted lung tissue from Z-AT homozygotes.[24,25] Interestingly, these extracellular Z-AT polymers are co-localised with and chemotactic to neutrophils.[24,28]

Liver Disease in Z Homozygotes

Following screening of 200,000 PiZZ neonates, Sveger identified variability in the clinical presentation, suggesting involvement of genetic and environmental gene modifiers in the expression of liver disease.[6] A more detailed description of the clinical aspects of the liver disease related to Z-AT is described elsewhere.[7,9-13]

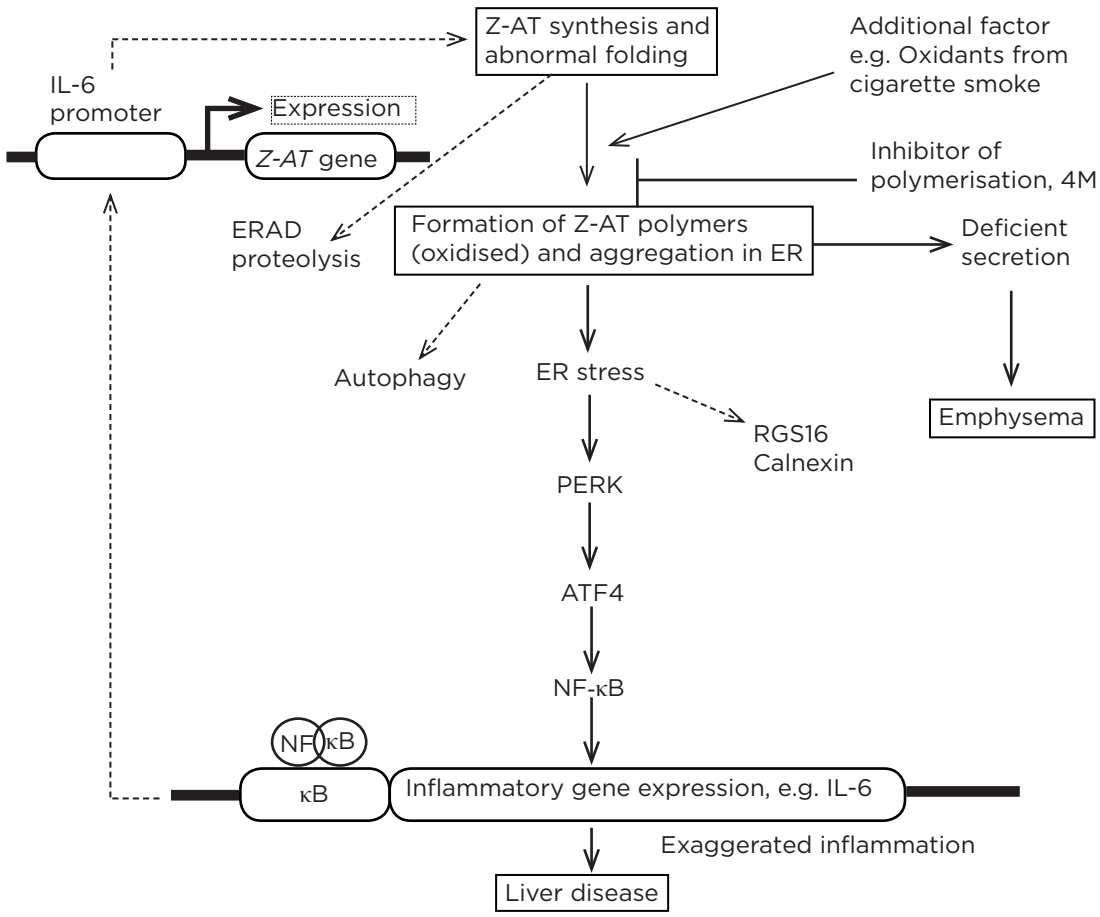

Figure 2. Proposed mechanism of liver disease in PiZZ individuals

Misfolded Z-AT protein is recognised by the quality control apparatus of the hepatocyte and degraded by the endoplasmic reticulum (ER) associated degradation (ERAD) proteasomal pathway. However, Z-AT proteins can escape this process and form Z-AT polymers which aggregate. These intracellular-inclusion bodies initiate the ER overload response. See main text for more details.

Activation of the ER-Overload Response in Severe α_1-Antitrypsin Deficiency

The pathophysiology of liver injury in PiZZ homozygotes is a consequence of the gradual accumulation of aggregated Z-AT in the ER of Z-AT cells. The relationship between the intracellular aggregation of misfolded Z-AT proteins and ER stress has been intensively studied.[9,29-43] The aggregated Z-AT activates the ER-overload response **(Figure 2)**.[30,33] A key step in the regulation of the ER overload response involves transmembrane transducers for sensing ER stress such as transmembrane Protein Kinase RNA (PKR)-like ER Kinase PERK, regulator of G-protein signalling 16 (RGS16) and calnexin.[33-39] Calnexin is a transmembrane ER chaperone in liver cells that binds to misfolded proteins suggesting the importance of proteasomal activity in disposal of misfolded proteins.[34-39]

In particular, activation of PERK leads to phosphorylation of eukaryotic translation initiation factor 2α (eIF2α), which causes a general inhibition of protein synthesis, and induction of the transcription factor ATF4 that binds to the amino acid response element to encode factors involved in the inflammatory response.[32] We and others have also shown that ER accumulation of Z-AT polymers is associated with activation of PERK, which when activated induces NF-κB activity, in keeping with activation of the ER overload response.[28,33] Because IκB has a shorter half-life than NF-κB, PERK-mediated translational attenuation shifts the ratio of IκB to NF-κB, thereby freeing NF-κB to translocate to the nucleus, where it induces the transcription of genes involved in the inflammatory response, as demonstrated by significant secretion of inflammatory cytokines such as IL-6.[32,33] Increased IL-6 activity could perpetuate the liver injury by increasing translation of Z-AT via its well characterised IL-6 promoter[33,44,46] that would subsequently lead to further aggregation and accumulation of Z-AT, and so on. The level of RGS16 upregulation in the liver of PiZZ individuals is associated with the hepatic levels of Z-AT polymer inclusion bodies.[9,36]

Autophagy in Severe α_1-Antitrypsin Deficiency

The importance of autophagy in PiZZ individuals was demonstrated by the identification of three gene products; ATG5, ATG6 and ATG16, which are necessary for digestion or degradation of aggregated Z-AT.[34,36] Autophagy also determines how much aggregated Z-AT accumulates in the ER. The presence of aggregated Z-AT, rather than the soluble Z-AT, specifically activates autophagy. This was supported by the finding that accumulation of a mutant α_1-antitrypsin Saar (AT Saar) that does not aggregate, induced activation of unfolded protein response (UPR) and ER-associated degradation (ERAD) or the proteasomal degradation pathway that degrades soluble Z-AT.[34,36]

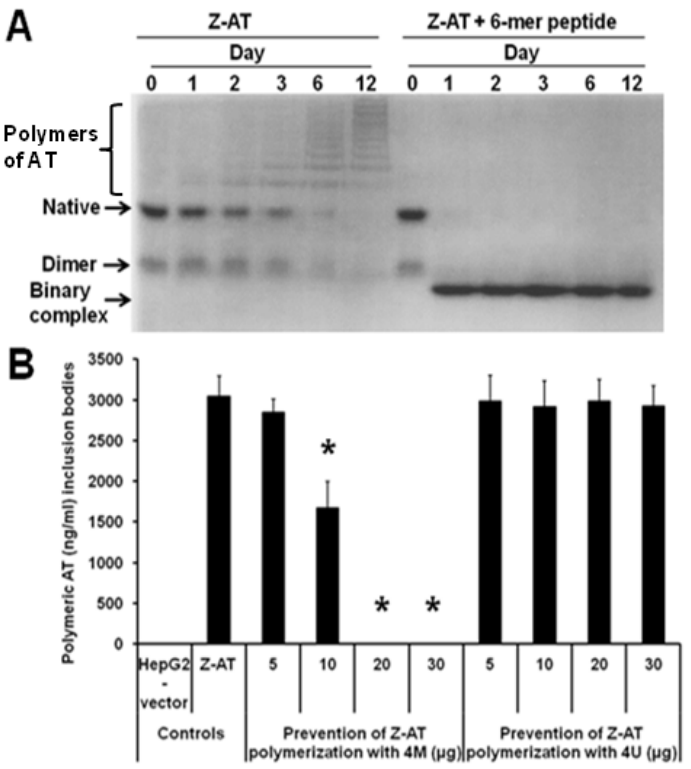

Figure 3. The effect of synthetic peptides on Z-AT polymerisation

Figure 3A. The 6M peptide inhibits Z-AT polymerisation in vitro
7.5% (w/v) non-denaturing PAGE demonstrating the formation of Z-AT polymers over 12 days (left). The six-mer peptide completely inhibited Z-AT polymerisation. Each lane contains 10μg of α_1-antitrypsin.[16]

Figure 3B. 4M prevents formation of Z-AT polymers *in vivo*
A cell model of Z-AT was developed using HepG2 cells transfected with the human Z-AT gene. Enzyme linked immunosorbent assay (ELISA) showed a dose dependant prevention of accumulation of Z-AT polymers in inclusion bodies. 20μg of 4M completely prevented accumulation of intracellular Z-AT polymers.[33] 4U refers to a control structurally unrelated peptide which had no effect on polymerisation.

Potential Treatment Options for Severe α_1-Antitrypsin Deficiency

To date, there is no effective specific treatment for Z-AT-related liver disease. A major distinction

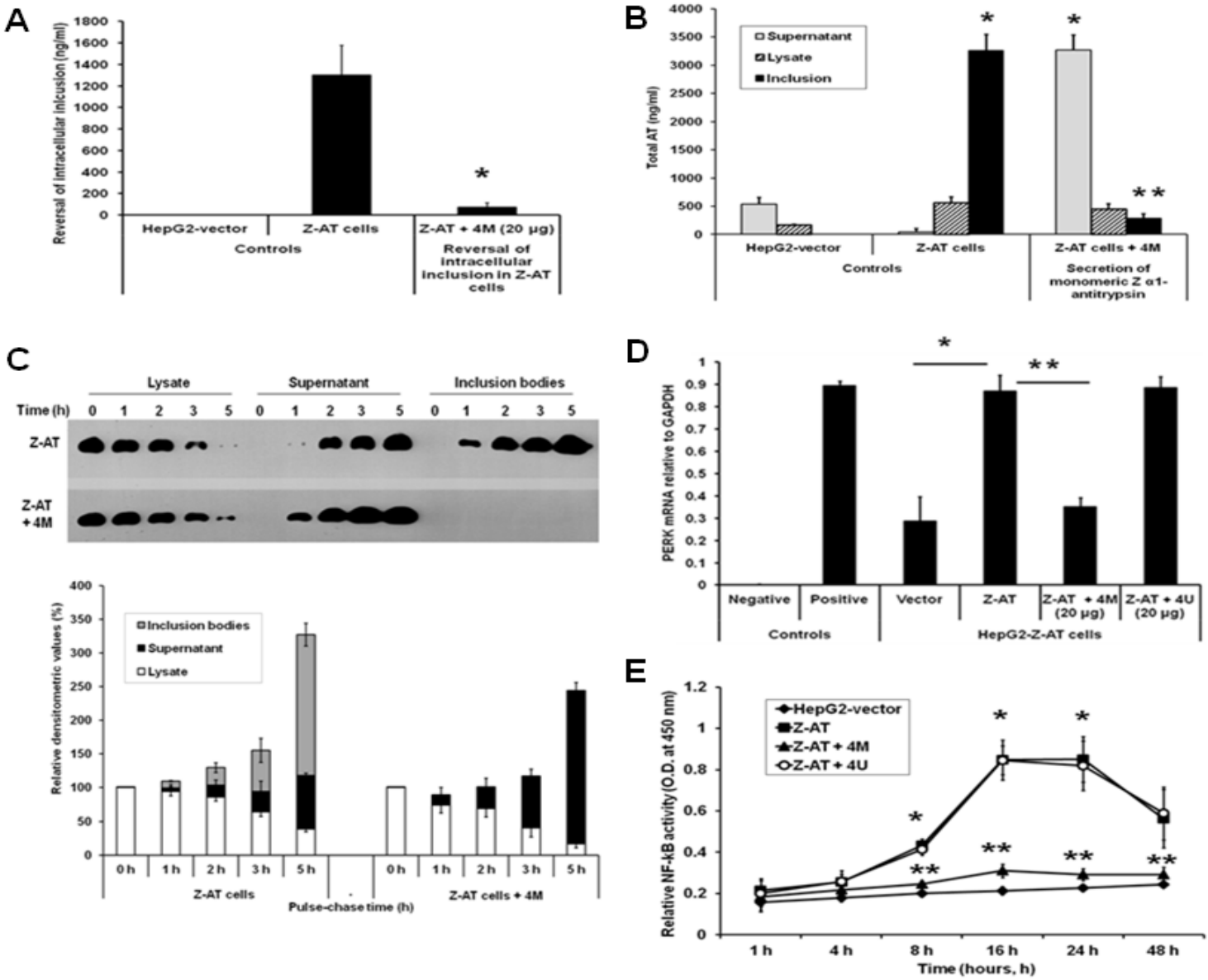

Figure 4A. 4M dissociates intracellular Z-AT polymers
Z-AT cells were incubated for 24 hours, which allowed retention of Z-AT polymers as inclusion bodies before adding in 4M peptide (P<0.001). 4M dissociated over 90% of Z-AT polymers in inclusion bodies thereby resulting in the monomeric conformation of the Z-AT in the inclusion (P<0.001).[33]

Figure 4B. 4M facilitates secretion of Z-AT monomers
The dissociation of inclusion Z-AT polymers with 4M resulted in the secretion of Z-AT monomers (P<0.001) in the supernatant (**).[33]

Figure 4C. 4M prevents intracellular polymers and facilitates secretion of Z-AT monomer
Top gel: Analysis of metabolic labelling by Western blot demonstrates the distribution of Z-AT proteins in Z-AT cell lysate, supernatant and accumulation in inclusion bodies. Treatment with 4M completely prevented aggregation of intracellular Z-AT and subsequently increased secretion of α_1-antitrypsin into the supernatant. **Bottom graph:** 4M increased the concentration of monomeric Z-AT in the supernatant and reduced inclusion body α_1-antitrypsin. Levels of α_1-antitrypsin from pulse-chase for lysates (white bars), supernatants (black bars) and inclusion bodies (grey bars) are presented on histograms. Results are expressed as relative to percentage control, t=0 (100%), showing a time-dependent aggregation into inclusion bodies of Z-AT cells and secretion of AT from Z-AT+4M cells into supernatant.[33]

Figure 4D. 4M reduces activation of PERK in Z-AT cells
RT-PCR analysis demonstrated that ER retention of Z-AT resulted in 3.04-fold upregulation of PERK mRNA in hepatic Z-AT cells when compared to vector control (P<0.001). Treatment with the inhibitor of polymerisation, 4M was able to significantly inhibit Z-AT-induced upregulation of PERK mRNA in Z-AT cells (**) (P<0.001). In contrast, the unrelated four-mer peptide (4U) had no effect on PERK mRNA.

Figure. 4E 4M inhibits NF-κB activation in Z-AT cells
Activation of ER-overload response in Z-AT cells induced by ER retention of Z-AT is further supported by upregulation of NF-κB, which could be inhibited by 4M; (**) (P<0.001).

between pathogenesis of liver and lung disease in severe α_1-antitrypsin deficiency is gain of function and loss of function, respectively. The liver disease relates to the intracellular accumulation of Z-AT polymers in hepatocytes, rather than unopposed elastolysis in the lung due to lack of α_1-antitrypsin. Therefore, antitrypsin augmentation therapy is used for the lung disease, but is not indicated for liver disease relating to severe α_1-antitrypsin deficiency.

Several strategies are currently being studied for treatment of severe α_1-antitrypsin deficiency-related liver disease including: gene therapy, the potential of combining human induced pluripotent stem cells (iPSCs) with genetic correction, enhancing autophagic clearance of aggregated Z-AT by drugs or transfer of a hepatocyte-directed master gene, and inhibitors of Z-AT polymerisation.[1,2,7-17,33-40] Other strategies under evaluation also include gene therapy using small-interfering RNA, ribozymes and peptide nucleic acid.[30]

Studies have suggested that enhancing the clearance of Z-AT polymers/aggregates by drugs; carbamezepine (CBZ), rapamycin or liver-directed transfer of transcription factor EB (TFEB) is of benefit in cell and animal models.[41-43] CBZ promotes autophagic and proteasomal degradation of both soluble Z-AT and insoluble Z-AT polymers, and was found to decrease the hepatic load of Z-AT and hepatic fibrosis in a mouse model of Z-AT deficiency-associated liver disease. Although CBZ changes the rate of intracellular degradation of Z-AT, it does not enhance Z-AT secretion. Rapamycin activates autophagy by inhibiting rapamycin kinase.[42] TFEB gene transfer enhances autophagy and reduces activation of NF-κB and IL-6.[43]

Other studies have targeted secretion of α_1-antitrypsin by interfering with Z-AT polymerisation in PiZZ individuals using 4-phenylbutyric acid and glycerol and imino sugar compounds.[30] However, neither of these resulted in any change in plasma concentrations of α_1-antitrypsin in Z-AT.

Another approach to treat Z-AT-related liver disease would be to inhibit the formation of Z-AT polymers. An allosteric cavity that is distinct from the interface involved in polymerisation was identified as a target for rational structure-based drug design to block polymer formation. From a library of 1.2 million commercial drug-like compounds/chaperones, only four compounds reduced the rate of Z-AT polymerisation *in vitro*.[15] One compound; CG blocked Z-AT polymerisation, had no effect on α_1-antichymotrypsin, antithrombin, wildtype and mutant Syracus neuroserpin. Although CG blocks Z-AT polymerisation/reduces Z-AT aggregates in a cell model of the disease, it does not increase the secretion of Z α_1-antitrypsin from the cells.

Inhibition of Polymerisation by Targeting Strand 4a

It was recognised that polymerisation could be prevented by synthetic peptides with homology to strand 4a.[5,44] However, these peptides are large and also anneal to M-AT and other members of the serpin superfamily such as α_1-antithrombin.[5,16] and therefore were unsuitable as therapeutic agents. Understanding the distinct conformation adopted by the Z-AT protein facilitated the design of a shorter six-mer synthetic peptide (Ac-FLEAIG-NH_2 (6M)) **(Figure 3A)**. This six-mer peptide preferentially bound to Z-AT rather than M-AT and other common circulating serpins.[16] However, the specific binding of the six-mer to pathologic Z-AT renders the Z-AT as inactive, and therefore would not address the predisposition of PiZZ homozygotes to develop emphysema.

Recently, a shorter four-mer synthetic peptide, Ac-TTAI-NH_2 (4M) has been identified that specifically anneals to the pathologic Z-AT.[33,45] Our cell studies showed that it entered the ER of the Z-AT cell without any cytotoxicity, and was able to abrogate Z-AT polymerisation in vivo **(Figure 3B)**. It was also able to dissociate existing intracellular Z-AT polymers/aggregates **(Figure 4A)**.[33] In addition, metabolic labelling demonstrated that in a cell model it was able to significantly restore normal secretion of Z-AT **(Figure 4B,C)**. Furthermore it reduced the ER overload response as demonstrated by inhibition of PERK-dependant NF-κB activity **(Figure 4D,E)**. Our findings uniquely demonstrate the potential of this strategy for prevention of Z-AT polymer formation in the liver, with preservation of inhibitory function in the tissues.

SUMMARY

The scientific community has achieved great understanding of the molecular mechanisms for of Z-AT related liver and lung disease. The recognition of the underlying mechanism of Z-AT aggregation and its cellular consequences, and the understanding of degradation pathways and gene corrective approaches, signify an exciting time in the field of Z antitrypsin related liver disease. Ongoing work will define whether these strategies alone or in combination will come to fruition and provide amelioration from this devastating disease.

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IL28B POLYMORPHISMS IN INTERFERON-TREATED PATIENTS WITH CHRONIC HEPATITIS B

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Disclosure: No potential conflict of interest
Citation: EMJ Hepatol. 2013;1:52-56.

ABSTRACT

Pegylated interferon (Peg-IFN) may achieve a sustained off-treatment response in 20-30% of the patients with chronic hepatitis B (CHB). However, given the high cost of treatment, the frequent side effects, and the lack of effectiveness in a large proportion of patients, there have been attempts to identify the subjects who are most likely to benefit with such therapy. Response rates may be significantly increased by careful selection of patients based upon baseline serum alanine aminotransferase, HBV DNA levels, and viral genotype. Recently, genome-wide association studies identified polymorphisms of the interleukin 28B (IL28B) gene as a potent predictor of sustained viral response in chronic hepatitis C patients treated with Peg-IFN plus Ribavirin, encouraging similar studies in HBV. Overall, these studies failed to provide convincing evidences that IL28B genotype significantly impacts on response to Peg-IFN in chronic hepatitis B (CHB) patients, though these studies are very heterogeneous in terms of patient populations, methodology, baseline features, treatment regimens, duration of follow-up, and ethnicity, making new studies in larger cohorts very much needed.

Keywords: Chronic hepatitis B, pegylated interferon alpha, sustained response, HBsAg clearance, IL28B polymorphism, rs12979860, rs8099917.

INTRODUCTION

Antiviral therapy of chronic hepatitis B (CHB) patients is aimed to improve quality of life and survival by preventing progression of liver damage to cirrhosis, hepatic decompensation, hepatocellular carcinoma (HCC), and death.[1-3] This goal can be achieved if hepatitis B virus (HBV) replication can be suppressed in a sustained manner, either by short-term treatment with pegylated interferon (Peg-IFN), or long-term therapy with entecavir and tenofovir.[4] The main advantages of Peg-IFN over nucleoside/nucleotide analogues (NUC) are the absence of resistance, the immunomodulatory properties that induce a direct inhibition of viral replication, and the enhancement of the host's antiviral immune response.[5-9] The major hindrances to the wide usage of Peg-IFN are the need for parenteral therapy and the clinical and laboratory monitoring, its side-effects profile, and the lack of effectiveness in a large proportion of patients.

To increase its cost-effectiveness, several strategies have been suggested; such as a pre-treatment selection of ideal candidates based upon low viral load, high alanine transaminase (ALT) levels, and viral genotype. However, this approach may be cumbersome as biochemical and virological markers tend to fluctuate over time in HBV-infected patients, viral genotype is not routinely performed in all centres, and more importantly, these variables systematically failed to identify good responders at individual level. Assessment of early on-treatment hepatitis B surface antigen (HBsAg) kinetics is currently the best predictor of non-response to Peg-IFN, as patients with minimal or zero decline of HBsAg or HBsAg levels >20,000 IU/ml have no chance of achieving a sustained response, making Peg-IFN withdrawal

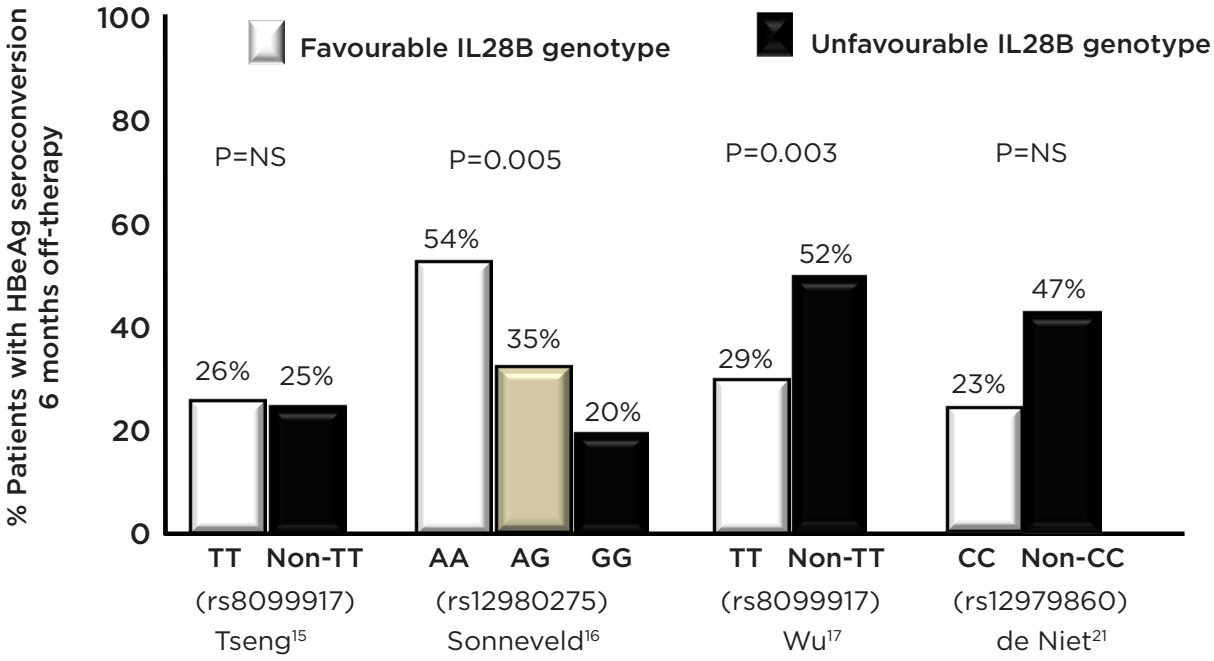

Figure 1. IL28B polymorphisms and interferon (IFN) response in HBeAg-positive chronic hepatitis B (CHB) patients

the best option in these patients. However, even this strategy has some disadvantages as 20% of patients receive Peg-IFN for three months without any chance of response, HBsAg quantification is not yet routinely performed in many centres, and good responders cannot be accurately identified.

As far as baseline predictors of response are concerned, a recent major breakthrough was the finding that variation in the region of the interleukin-28B (IL28B) gene is highly predictive of both sustained virological response (SVR) to Peg-INF and ribavirin in chronic hepatitis C (CHC) patients, and spontaneous HCV clearance in the acute hepatitis C setting.[10-14] These findings have also generated a lot of interest in CHB with the aim to select for only those patients with a high probability of success with interferon treatment.

IL28B AND INTERFERON RESPONSE IN HBEAG POSITIVE PATIENTS

In a Taiwanese study of 115 HBeAg-positive patients treated with Peg-IFNα-2a for six or twelve months; HBV genotype, major sequences of pre-core (PC) stop codon, basal core promoter (BCP), IL28B (rs8099917) in addition to two genetic variations of HLA-DPA1 (rs3077) and HLA-DPB1 (rs9277535), were genotyped.[15] At the sixth month, post-therapy, patients with the HLA-DPA1 rs3077 GG genotype had a higher HBeAg seroconversion rate than those with non-GG genotype (35% vs. 13%, p=0.007), whereas there were no differences between TT and non-TT IL28B rs8099917 and between GG and non-GG HLA-DPB1 rs9277535 genotypes (26% vs. 25%, p=0.92 and 33% vs. 19%, p=0.09, respectively) **(Figure 1)**. By multivariate analysis, excluding rs9277535 because it is in high linkage disequilibrium with rs3077, BCP mutation (OR 8.04, 95%CI 2.00-32.28) and the rs3077 GG genotype (OR 3.49, 95%CI 1.12-10.84) were independently associated with a higher HBeAg seroconversion rate. BCP mutation (OR 9.28, 95%CI 1.92-44.99), low baseline viral load (OR 4.78, 95%CI 1.37-16.69), genotype B (OR 5.74, 95%CI 1.06-31.00) and ALT levels >5 upper limit of normal (ULN) (OR 3.72, 95%CI 1.08-12.78) were independently associated with higher combined off-treatment response rates, defined as HBeAg seroconversion, HBV DNA <20,000 IU/mL and ALT normalisation.

In a multicentre study involving 11 Asian and European sites, 205 HBeAg-positive CHB patients treated with either Peg-IFN monotherapy (41%), Peg-IFN+lamivudine (LMV) (52%) or with standard interferon (7%) were retrospectively analysed for IL28B rs12980275 and rs12979860 genotypes.[16] At the end of treatment (EOT), 90 patients (44%) achieved HBeAg seroconversion, with significantly higher rates among rs12980275 AA genotype compared to AG and GG (51% vs. 26% vs. 10%, p<0.001), with similar rates observed for rs12979860 CC/CT/TT genotypes (50% vs. 29% vs. 10%,

p<0.001). IL28B genotypes were also independently associated with an increased probability of HBeAg clearance at six months post-treatment among the 182 patients who received no LMV or LMV for ≤52 weeks, with an adjusted OR of 3.54 (95%CI 1.33–9.41, p=0.008) for rs12980275 genotype AA versus AG/GG and OR of 3.24 (95%CI 1.21–8.69, p=0.016) for rs12979860 CC versus CT/TT, after adjustment for age, HBV genotype, HBV DNA, ALT levels and previous interferon exposure.

IL28B rs12980275 genotype AA and rs12979860 genotype CC appeared to be associated with a higher probability of HBeAg clearance with HBV DNA level <2,000 IU/mL, although associations were not significant (OR 2.09 95%CI 0.76–5.75, p <0.139 for AA vs. AG/GG; OR 1.92 95%CI 0.70–5.26, p<0.188 for CC vs. CT/TT). IL28B genotype was also independently associated with an increased probability of HBeAg seroconversion through long-term follow-up. HBeAg seroconversion rates were 54%, 35% and 20% in patients with rs12980275 genotype AA, AG and GG, respectively (p=0.005) **(Figure 1)**. In a Cox proportional hazards model, genotype AA was associated with a higher probability of HBeAg seroconversion (HR 2.14; 95%CI 1.14-4.31, p=0.018) when adjusting for HBV genotype and baseline HBV DNA and ALT levels. Moreover, after adjustment for HBV genotype A, rs12980275 AA genotype was associated with a higher probability of HBsAg clearance (HR 3.47 for AA vs. AG/GG; 95%CI 1.04-13.48, p=0.042).

A study in 512 Chinese HBeAg-positive patients with CHB, treated with Peg-IFNα-2a monotherapy (55%) or with NUC (45%) for twelve months, retrospectively demonstrated that six months after the EOT, the sustained response defined as normal ALT levels, serum HBV DNA <500 copies/mL and HBeAg seroconversion was lower among TT compared to non-TT IL28B rs8099917 SNPs (29% vs. 52%, p=0.003).[17] Interestingly, the frequency of the G allele of rs8099917 was significantly higher among patients with a sustained response than in the non-responders' group (8.3% vs. 3.9%, p=0.003, OR=0.44, 95%CI 0.25–0.79).

IL28B AND INTERFERON RESPONSE IN HBEAG NEGATIVE PATIENTS

One Italian study evaluated the role of IL28B in 101 HBeAg-negative patients treated with either standard interferon (68%) or Peg-IFN alfa-2a, for a median of twenty-three months and followed-up for eleven years after treatment.[18] Patients with IL28B

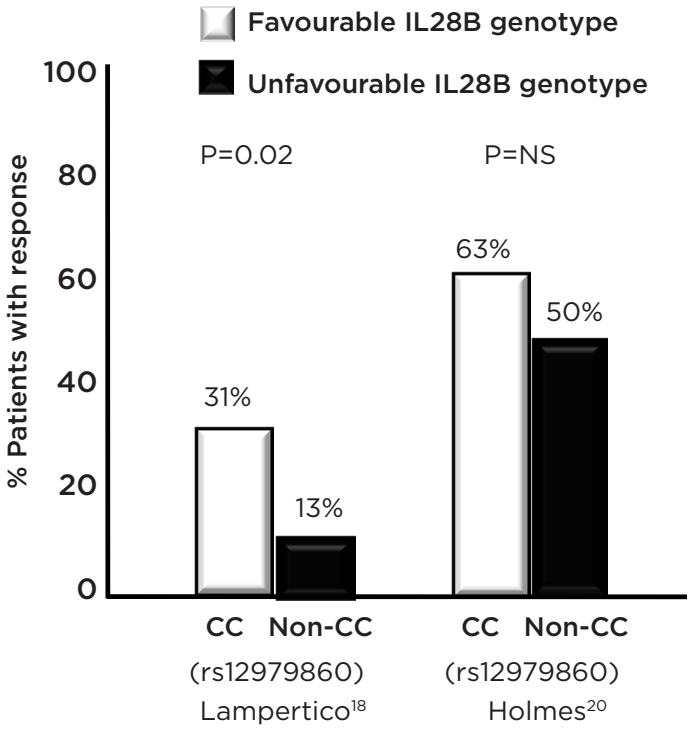

Figure 2. IL28B polymorphisms and interferon IFN response in HBeAg-negative chronic hepatitis B (CHB) patients

rs12979860 genotype CC were shown to have higher EOT (69% vs. 45%, p=0.01) and higher sustained virological response, defined as HBV DNA <2,000 IU/mL (31% vs. 13%, p=0.02), than non-CC patients **(Figure 2)**. Interestingly, CC patients had a higher cumulative probability of clearing HBsAg during long-term follow-up (29% vs. 13%, p=0.04). The IL28B CC genotype was shown to be an independent predictor of sustained virological response together with age and baseline HBV DNA levels, and of serological responses together with baseline HBV DNA and ALT levels, and with the duration of therapy.

To further identify any possible association between IL28B polymorphisms and HBV genotype, the IL28B re-analysis in 93 genotype D patients of the latter study, confirmed that the rates of EOT response, the sustained virological response and the HBsAg clearance were still significantly higher in CC than in non-CC carriers, i.e. 69% vs. 44% (p=0.014), 31% vs. 12% (p=0.028) and 29% vs. 12% (p=0.048). When the assessment of the IL28B polymorphism was extended to include rs8099917, which was shown to improve the prediction of a response to Peg-IFN and RBV therapy in CHC patients with the CT rs12979860 genotype, the favourable rs8099917 TT genotype was found in 100% of the rs12979860 CC patients, compared with only 31% of CT and 10% of

TT patients. The 42 rs12979860 CT patients with the rs8099917 TT genotype had a significantly higher rate of sustained virological response and HBsAg seroclearance (23% vs. 3%, p=0.045 and 23% vs. 0%, respectively p=0.007),[19] suggesting that multiple IL28B polymorphisms may be required to accurately define the pretreatment probability of a response at an individual level.

IL28B AND INTERFERON RESPONSE IN MIXED POPULATION STUDIES

Holmes et al. retrospectively analysed the role of IL28B rs12979860 in 96 Australian CHB patients (62% HBeAg-positive) treated with forty-eight weeks of Peg-IFN and followed up for thirty-nine months.[20] Among 60 HBeAg-positive patients, 27% achieved HBeAg seroconversion with HBV DNA <2,000 IU/mL, six months after EOT without significant difference in response according to IL28B genotype (25% in CC patients vs. 33% in non-CC patients, p=0.45) **(Figure 1)**. Also, the rate of patients having a serum HBV DNA level <2,000 IU/mL at twenty-four weeks after the EOT, among 36 HBeAg-negative patients, was not affected by IL28B genotype (63% in CC patients vs. 50% in non-CC patients, p=0.43). The overall long-term virological response of 39%, after thirty-three months of median follow-up, was not influenced by the IL28 genotype as the rate of HBsAg loss.

No association between IL28B rs12979860 genotypes and HBeAg seroconversion or HBsAg clearance was seen in a European cohort of 95 CHB patients (48% HBeAg-positive), who were treated with Peg-IFN and adefovir for one year and followed-up for twenty-four months.[21] 14 out of 46 (30%) HBeAg-positive patients achieved HBeAg seroconversion, without differences according to IL28B genotype (23% for CC compared to 47% of non-CC). 23 out of 49 (47%) HBeAg-negative patients attained HBV DNA levels ≤2,000 IU/mL in combination with normal ALT, twenty-four months after stopping therapy, without differences according to IL28B genotype (52% for CC compared to 42% of non-CC). Neither HBeAg-positive nor HBeAg-negative patients showed an association between the rs12979860 polymorphism and HBsAg seroconversion (14% for CC compared to 15% of non-CC).

CONCLUSION

Overall, the aforementioned studies which investigated the relationship between IL28B polymorphisms and the response to interferon in CHB patients have yielded conflicting results. Some identified that favourable genotype predicts therapeutic response both in HBeAg-negative and HBeAg-positive patients,[16-18,21] as well as the HBsAg clearance.[16,18] One study identified that the unfavourable IL2B-variant was more frequent in the subgroup of responders,[17] while other studies did not find significant differences in outcome between host genotypes.[15,20]

To justify these conflicting results in CHB and in comparison to CHC patients, consideration should be given to the heterogeneity between studies with respect to study populations, diversity of genetic backgrounds, sample size, treatment regimens, and duration of therapy, as well as length of follow-up. Therefore, to conclusively determine that IL28B effects are limited to certain subgroups or may be incidental in small sample-size studies, it is necessary to perform further studies in a large cohort of different ethnic groups to better understand the mechanisms underlying the beneficial effect of this single-nucleotide polymorphism (SNP) in response to interferon treatment. To date, the limited clinical utility for predicting interferon-treatment outcome for CHB patients, does not recommend its application for the selection of patients to start interferon treatment. For now, the success of interferon therapy in CHB patients depends on appropriate patient selection according to HBV DNA and ALT levels and also HBV genotype, but most importantly, on the monitoring of treatment-response based on early on-treatment HBsAg kinetics. Before the genetic test can be implemented into clinical practice, more studies will be needed; such as genome-wide association studies (GWAS), which allow the evaluation of a very large number of patients in informative cohorts, to overcome the previously reported limitations, or by the combination of multiple SNPs to define the pretreatment probability of a response at an individual level. Presently, the use of a genetic marker as a surrogate for the host's ability to clear the virus plays a major role in host immune response to HCV infection but not in HBV.

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MANAGEMENT OF HEPATITIS B VIRUS INFECTION IN LIVER TRANSPLANT RECIPIENTS

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Disclosure: No potential conflict of interest.
Citation: EMJ Hepatol. 2013;1:58-67.

ABSTRACT

Hepatitis B virus (HBV)-related liver disease is a common indication for liver transplantation (LT) in Asian countries.[1] When left untreated, the overall five-year survival rate in HBV-related cirrhosis is 71%, which in cases of decompensated cirrhosis decreases to 14%.[2] In the 1980s, hepatitis B-related acute liver failure and chronic liver disease (CLD) were considered contraindications for LT because of almost universal graft reinfection and high rates of graft and recipient failure (>50%).[3-4] These patients had severe and rapidly progressive liver disease with a two-year graft and patient survival of 50% compared to 80% in those transplanted for non-HBV-related CLD.[5] As a result, there were fewer LT for HBV liver disease for several years.[6] However, with the introduction of nucleoside and nucleotide analogues and the use of intra and post-operative hepatitis B immunoglobulin (HBIG), there was renewed interest in the application of LT in these patients. There was a significant decrease in post-operative HBV recurrence rates.[7-9] The current overall survival of patients transplanted for HBV-related cirrhosis has improved to 85% at one year, and 75% at five years.[7,10-12] The present review highlights issues pertaining to HBV reinfection and *de novo* infection in LT recipients with recommendations for its management.

Keywords: Hepatitis B virus (HBV), liver transplantion, cirrhosis, nucleoside, nucleotide.

A. HBV REINFECTION

Reinfection after liver transplant can be either early or delayed.

Early post-operative HBV reinfection in allograft: This is determined by HBV DNA levels in the pre-operative period. A high pre-LT HBV DNA level predisposes to high rates of early reinfection as compared to those with low or undetectable levels of HBV DNA. Marzona et al.,[13] in a study of 177 LT recipients receiving HBIG with or without lamivudine, noted HBV recurrence of 50%, 7.5%, and 0% when serum HBV DNA levels at the time of transplant were 100,000, 200-100,000, and less than 200 copies/mL respectively. The authors concluded that serum HBV DNA greater than 5 log10 copies/mL (>10^5copies/mL) at the time of transplant was associated with significantly higher risk of early HBV recurrence.

Delayed reinfection: Despite adequate pre-transplant antiviral therapy to suppress/eradicate HBV, a few potential reservoirs of the virus remain in the carriers. These include peripheral blood mononuclear cells (PBMC), bone marrow, spleen, gonads, thyroid gland, kidneys, pancreas and adrenal glands,[7] where the antivirals fail to achieve optimal drug levels. The introduction of steroids as part of immunosuppression in the post-transplant period reactivates the hitherto dormant virus, with steroids known to enhance HBV DNA gene expression (glucocorticoid-responsive transcriptional gene enhancer).[14] In the absence of immune-prophylaxis, the risk of reinfection with HBV is 33% to 78%.[15-19]

Factors predicting post-liver transplant reinfection

Pre-transplant factors

High risk factors:

i. Resistance/mutation to oral antiviral drugs: Nucleoside/nucleotide agents are given before LT with an intention to reduce replication and reduce the risk of HBV recurrence after LT. Lamivudine resistance shares resistance patterns with other oral antiviral drugs such as telbivudine as well as entecavir.

ii. HBV DNA mutation: Mutation in the HBV surface (S) and polymerase (P) gene before LT is associated with higher post-LT HBV recurrence.[20-22]

iii. LT recipients who have anti-HBc positive and HBsAg negative before LT: There are reports of reactivation of HBV virus if the recipient is anti-HBcore antibody positive, despite receiving non-HBsAg and anti-HBc negative liver graft. The virus in these patients is in the dormant state within non-liver sites, and in the presence of immunosuppressants, gets reactivated after transplant. In a study by Abdelmalek et al.,[23] of the 22 patients on the waiting list for LT who were HBsAg negative and anti-HBc positive patients, HBV DNA was detected in the liver in 5 (23%) before transplant, which persisted in 2 in the explanted liver.[23] However, none of the recipients became HBsAg positive, nor did they develop clinical hepatitis after transplant.

iv. Reinfection in the immune-compromised recipients: Associated HIV infection and an immune-compromised state significantly increases HBV recurrence in the post-operative period.[14,24]

v. Transmission via contaminated blood products or healthcare personnel in the peri-operative period: It is not uncommon if the blood products contain occult HBV infection (i.e: HBsAg negative, Total HBcore antibody positive with detectable HBV DNA level).

Low risk factors:

i. Fulminant hepatitis B: HBV reinfection rate in LT recipients following fulminant hepatic failure (FHF) is low.[9] As HBV-related hepatocyte injury is an immune mediated mechanism rather than due to direct viral pathogenecity, viral load in FHF is usually lesser than in HBV-related cirrhosis. In a study by Roche et al.,[12] no reinfection was seen in 8 patients who had LT for FHF during a follow up of ten years.

ii. Coinfection with Hepatitis Delta virus (HDV: Recurrence rates of HBV are low when there is superinfection with HDV, while HDV competitively inhibits the fixing of the HBV to hepatocytes. Roche et al.[12] noted that the ten-year actuarial risk of HBsAg recurrence in HBV and HDV-related cirrhosis was 15.3%, in comparison to 56.5% in non-Delta Hepatitis B-related cirrhosis.

Intraoperative factors

The use of parenteral corticosteroids in an anhepatic phase can induce resurgence of HBV secondary to the stimulatory effect of steroid therapy on the glucocorticoid-responsive enhancer region of the HBV genome.[24-25]

Post-transplant factors

i. Use of immunosuppressive drugs. (particularly steroid).

ii. Low anti HBs antibody level (i.e: Less than 100IU/mL): Despite the use of HBIG, the antibody to HBsAg may not be optimal, either due to poor compliance or failure of the immunoprophylaxis, resulting in recurrence of infection.

iii. Combination of HBIG and oral antiviral agents.

iv. Recipient's compliance.

B. *DE NOVO* HBV INFECTION

De novo post-transplant HBV infection is defined as the appearance of HBsAg in a transplant recipient not previously known to have a HBV infection. The clinical course in these transplant recipients is often relatively mild in comparison to recurrent HBV infections in the transplant population.[26-28] This may present itself as an asymptomatic elevation in serum transaminase levels.

The possible potential modes of acquiring a *de novo* infection are:

Emergence of occult HBV infection: This is of particular concern in regions with high endemicity of HBV infection and a high prevalence of anti-HBc positivity.[15] The source of occult infection may be the donor or the recipient himself.[29]

i. HBsAg positive or anti-HBc positive donor to a HBV negative recipient: Approximately, 10% of recipients would acquire *de novo* HBV infection, when a critically ill HBsAg negative end stage liver disease (ESLD) patient receives a liver allograft from a HBsAg positive donor[15,30] or a donor who is anti-HBc positive (total).[15,26,28-29,31-34] This may be related to a high viral load within the liver.[32] Dickson et al.[31] reported conversion to HBsAg positivity in 18 recipients (78%) when donors were anti-HBc positive as compared to

3 of 651 (0.5%) recipients when donors were anti-HBc negative.[35] The donor-transmitted *de novo* HBV infection was mild in most cases; 50% of patients had normal serum aminotransferases, while 85% had no or only mild inflammatory activity on post-LT liver biopsy at the end of one year. However, there was a decrease in the four-year survival rate (featuring an adjusted mortality hazard ratio of 2.4; 95% confidence interval, 1.4-4.0).

ii. Anti-HBs positive recipient: The recipient is not free from acquiring a *de novo* HBV infection, especially when the donor is anti-HBc positive.[29,36-37] The donor is likely to have an occult HBV infection (undetectable HBV DNA in the sera or extra hepatic tissue), which makes its appearance in presence of immunosuppressive therapy.

iii. Anti-HBc positive recipient: Even in the absence of serologic evidence of viral replication, HBV-DNA may be detectable in the livers of anti-HBc positive recipients.[38] Oral antiviral drugs should be continued in all anti-HBc negative recipients if receiving from an anti-HBc positive donor.

C. STRATEGIES TO PREVENT HBV REINFECTION

The natural history of HBV reinfection in post-LT has shown that not all patients get HBV infections. A small group of recipients even without post-LT HBV prophylaxis have survived without recurrence.[4]

In recent times, with the introduction of newer antiviral analogues for treatment of HBV positive patients with detectable serum HBV DNA, the post-operative recurrence rates are likely to be less than 10%. Hepatitis B vaccination to all non-HBV related liver cirrhosis will prevent occult HBV-related *de novo* infection.

Management of HBV positive patients during and after LT

During LT

In the anhepatic phase, a high dose of 10,000IU of HBIG is given intravenously. Nowadays, a low intramuscular dose (2,000IU) of HBIG has been proven to be as effective as a high dose of HBIG.[39-41]

After LT

The aims of treatment in the post-LT setting are to minimise the risk of HBV infection of the graft and to decrease the incidence of significant HBV-related liver disease over the long-term. The main agents available for the clinician are:

- Oral antiviral agents (Nucleoside/tide analogues).
- HBIG-nucleoside/tide combination therapy.

Combination therapy with HBIG and oral antiviral drugs are the current gold standard for post-LT HBV therapy. Recent studies have shown that newer oral antiviral drugs such as entecavir/tenofovir as monotherapy are as effective as combination therapy.[42-43]

i. Nucleoside/tide analogues

Nucleoside/nucleotide analogues as a single agent have been used as prophylaxis against HBV infection in post-LT recipients. In this case, oral antivirals are introduced in the pre transplant period and continued indefinitely post-operatively. As it is a HBV DNA reverse transcriptase inhibitor, it inhibits viral DNA synthesis.

Lamivudine monotherapy: A multicentre study by the Lamivudine North American Transplant Group, which enrolled 77 HBsAg positive (60% of them had detectable HBV DNA as well as HBeAg positive) subjects on transplant waiting lists, lamivudine as single therapy showed favourable results. 42 subjects underwent LT; 60% were HBsAg negative for more than 12 weeks after LT. However, a major drawback with lamivudine has been the emergence of a resistant hepatitis B virus (YMDD) mutant[39,44] even prior to transplant, causing problems in management of recipients in the post-transplant period.[45]

The incidence of post-transplant resistance ranges from 10% to 45% within one year of treatment[46,47] and almost 50% in six years. In the number of *de novo* HBV infections in 110 patients (i.e from livers of donors who were HBsAg negative and anti-HBc positive) given lamivudine prophylaxis, the rate of *de novo* infection was 3.6% after a mean follow-up of 25 months.[26] Hence, the drug is not considered the first-line of management in LT.

Adefovir has both *in vitro* and *in vivo*[48] efficacy against both wild-type and lamivudine-resistant HBV. Perillo and colleagues[49] enrolled 128 pre-LT and 196 post-LT patients all of whom had detectable HBV DNA despite ongoing lamivudine therapy. Median lamivudine exposure at the time of enrolment was 69 weeks in the pre-LT group (128 patients) and 56 weeks in the post-LT group (196 patients).

Adefovir (10mg/day or 5mg/day in presence of renal dysfunction) was added to the treatment schedule of lamivudine with/without HBIG therapy. Among subjects completing 48 weeks of adefovir therapy, the median HBV-DNA had decreased by 3.4 log copies/mL in the pre-LT group and 3.3 log copies/mL in the post-LT group. Majority of patients in both treatment groups experienced stabilisation or improvement in Child-Pugh scores. Survival following one year of adefovir therapy was 84% in the pre-LT group and 93% in the post-LT group. Extended data from this ongoing study demonstrated continued efficacy up to 144 weeks of therapy.[50]

A major drawback with adefovir is its nephrotoxicity. The concomitant use of nephrotoxic medications (i.e. calcineurin inhibitors) in post-LT patients or those with prior history of renal injury has limited its use among the transplant population. In the aforementioned study, adefovir had to be stopped in 2% of subjects in the post-LT group due to nephrotoxcity.[49] Adefovir resistance has been described in a post-LT patient with recurrence of HBV infection.[48]

Entecavir[51-53] is associated with lower rates of drug resistance, and is the recommended drug for life-long treatment. Its dual action against wild-type and lamivudine-resistant HBV, and its favourable toxicity profile, offers promise for its potential effectiveness in the post-LT population. The pilot study of Tenofovir post-LT shows that it is safe and efficacious.[43]

To summarise, entecavir is recommended as a first-line antiviral agent in nucleoside naïve transplant patients because of its greater potency, low rates of drug resistance, and nephrotoxicity.

ii. Hepatitis B immune globulin (HBIG)

This was first administered as passive immunoprophylaxis to a HBsAg positive LT recipient in 1978.[54] Samuel et al.[55] carried out a retrospective analysis of patients transplanted for HBV in 17 European centres to determine the role of HBIG immune-prophylaxis in HBsAg positive transplant recipients. HBIG is a polyclonal preparation of human anti-HBs purified from pooled donor plasma. It is given intravenously as a 10,000IU bolus dose during the anhepatic phase, followed by daily doses of 2,000IU during the first week. Subsequent doses are either given monthly or in accordance with anti-HBs titres. A trough anti-HBs titre of at least 150IU/ L is considered as protective. Several subsequent studies have further shown a reduction in reinfection, and improved patient and graft survival HBIG prophylaxis.[4,56-57] Early reinfection can be prevented by administration of HBIG in the intraoperative anhepatic phase and in the immediate post-operative period to maintain anti-HBs levels of more than 100-150IU/mL. Late reinfection can be prevented by continuing HBIG for a longer period of time in combination with antiviral medications. Overall, immunoprophylaxis strategy significantly reduces the actuarial ten-year risk recurrence rate to 25.4% and the ten-year survival rate increased to 74%.[12]

There are marked differences in the dose and duration of HBIG across various transplant centres. The serum HBV DNA level at the time of transplant is an important predictor of HBV reinfection.

The mechanism of action of HBIG is unclear, but it is likely that it binds to HBV receptors on uninfected hepatocytes, and occupies potential viral entry sites. It undergoes endocytosis after binding to the hepatocyte receptor site and decreases the release of HBsAg from the cell.[58] The hepatocytes are subsequently protected from infection by HBV particles which are released from extrahepatic reservoirs[59] following immunosuppression in the post-transplant period. In circulation, HBIG also has a direct binding and neutralising effect on circulating virions. HBIG also induces antibody-dependent cell-mediated cytotoxicity.

iii. HBIG and nucleoside/nucleotide combination therapy

HBIG immunoprophylaxis has been less successful in preventing reinfection in HBeAg positive patient or those with significant detectable HBV DNA viral load i.e. >100,000 copies/mL (10^5).[4,13,60] Antiviral therapy with nucleoside/nucleotide analogues before transplant decreases the risk of reinfection by decreasing the amount of circulating virus at the time of transplant and prolonging the half-life of HBIG.[61]

Combination therapy of HBIG and nucleoside analogues reduces recurrence rates to less than 10%. The earliest published data, in this regard, was a 1998 study enrolling 14 HBsAg positive LT candidates.[44] Lamivudine was initiated pre-LT in 10 subjects, 4 of whom had detectable HBV DNA. HBIG therapy was initiated during the anhepatic phase of transplant, and all patients received combination HBIG and lamivudine therapy after LT. After a median follow-up of 387 days (range, 49-525) post-transplant,

	Hepatitis B Immunoglobulin	Nucleoside/Nucleotide Analogs
Mode of action	HBV DNA reverse transcriptase inhibitor, hence inhibits viral DNA synthesis	Unclear; it has direct binding and neutralising effect on circulating virions
Duration of therapy	Life-long	Life-long
Resistance	Vaccine escape mutant	Drug resistance (more with lamivudine; less with entecavir/ tenofovir)
Cost	Costly	Cheaper

Table 1. HBIG versus oral anti-viral drugs

HBsAg and HBV-DNA remained undetectable in 13 surviving subjects.

The efficacy of lamivudine/HBIG combination therapy in the prevention of HBV recurrence has been confirmed in subsequent series, including several protocols with low-dose HBIG,[20,40,62-64] with the combination also shown to be cost-effective.

A meta-analysis of two prospective and four retrospective studies where HBIG alone was compared with HBIG plus lamivudine concluded that combination therapy was associated with a significantly lower rate of HBV-related deaths and all-cause mortality.[65-66] The recurrence rate was also low in a select population.[66,67] Contradictory to this study, in a systematic review which included patients who received a liver from donors who were HBsAg negative and anti-HBc positive, the rate of *de novo* HBV infection in 73 patients who received HBIG and lamivudine prophylaxis was 2.7% after a mean follow-up of 31 months.[68] This was similar to that seen for lamivudine monotherapy (3.6%), suggesting that the addition of HBIG to the regimen did not provide any added benefit.

Combination of lamivudine plus adefovir in certain select population also prevents HBV recurrence.[69,70] In one study, 34 adults on HBIG and lamivudine prophylaxis followed up for at least 12 months after transplant showed no recurrent HBV in the graft.[71] In this study, at the end of 12 months, patients were divided into two groups, one group receiving adefovir (10mg) along with lamivudine and the other lamivudine in combination with HBIG. One patient in the adefovir group became transiently positive for HBsAg, one other had deterioration of renal function requiring dose adjustment and then cessation of adefovir after 15 months. The rest of the patients in this group had undetectable HBsAg and HBV DNA during a median follow-up of 21 months. The authors estimated that the adefovir plus lamivudine group was similarly effective and substantially less costly than the lamivudine plus HBIG approach ($8,290 versus $13,718 per treatment year).

The study concluded that antiviral therapy alone without HBIG was as effective in preventing HBV reinfection in a select group of patients. However, most patients in the study were at a relatively low risk; only 7 of 30 had pre-transplant detectable HBV DNA. Furthermore, HBIG was stopped after the most vulnerable period (the first 12 months). The results of the study cannot be generalised to high-risk patients i.e. those with initially high HBV DNA levels or patients in the first year after transplantation.

More recently, HBIG was substituted with tenofovir and emtricitabine combination to prevent recurrence of HBV infection.[72] The study showed prevention of HBV DNA recurrence in 100% (20/20) of patients who were compliant with the medication, and led to substantial cost savings over HBIG-containing regimens.

Issues pertaining to HBIG

HBIG is expensive. Long-term HBIG prophylaxis post-LT can significantly increase the cost of post-transplant care. Several studies have been undertaken to find alternative regimens that minimise the use of HBIG without sacrificing the benefit of low HBV recurrence. Fox et al.[73] has extensively reviewed the literature and has highlighted HBIG-free therapeutic options.

(iv) Alternatives to standard HBIG dose

This includes modification in method, frequency and duration of HBIG administration.

Modification in the method of HBIG administration (Use of low-dose intramuscular (IM) HBIG administration): Intramuscular administration of HBIG has been reported to be as effective as IV administration and also cheaper than IV administration. Several protocols have investigated the use of intramuscular HBIG either alone[39] or as an addition to an antiviral agent.[40] The Sawyer et al.[41] study included 147 patients who received low-dose HBIG intramuscularly (400-800IU daily for one week followed by monthly doses) plus lamivudine (100mg daily) following LT.[41] Before transplantation, patients with detectable HBV DNA received lamivudine (100 mg PO daily). Patient survival was 92% at one year and 88% at five years. The actuarial risk of HBV recurrence was only 1% at one year and 4% at five years. More than 50% of patients in this study had undetectable HBV DNA at the time of transplant, potentially contributing to the favourable results. Thus, low-dose HBIG can be recommended in combination with a nucleoside/nucleotide agent when HBV DNA viral load is low or undetectable in the pre-transplant period.

Modification in frequency of HBIG administration (On-demand administration of HBIG based on measurement of serum anti-HBs titres): McGory and colleagues[56] reported low rates of disease recurrence in HBsAg positive subjects undergoing LT following on-demand HBIG administration aimed at maintaining serum anti-HBs titres >500IU/L. 17 of 27 subjects (63%) were HBeAg-positive pre-LT, and disease recurrence was observed in only 2 subjects, neither of whom were able to maintain target anti-HBs titres. Low anti-HBs titre of 100-150IU/L has also shown low risk for disease recurrence.[55]

Modification in duration of HBIG administration (Maintenance with oral antiviral drugs): The withdrawal of HBIG after a defined course of combination HBIG and oral antiviral drugs has also been shown to be effective, particularly if combination antiviral therapy is used. Choloangitas et al.[42] stopped HBIG at least 12 months after LT. 40% of their patients received entecavir or tenofovir monoprophylaxis. The study confirmed that this protocol provided effective antiviral prophylaxis and only 3 (6.3%) of the 47 patients had HBV recurrence 24 months (range: 6 to 40 months) after HBIG withdrawal.

Newer formulations with longer lasting levels of circulating antibodies include OMRI-Hep-B preparation. This may require less frequent administration with a reduction in cost. The half-life of this preparation is significantly longer than standard therapy (22 versus 13 days).[74] Further studies are needed to confirm these results.

Combined passive immunoprophylaxis with active immunisation: HBV vaccination after HBIG therapy has been tried to prevent HBV reinfection in post-LT, but the results are conflicting.[60-61] The anti-HBs titres achieved in the responders were low despite the use of higher doses and multiple courses of vaccine.[21,75-76]

Sanchez-Fueyo et al.[61] selected 17 HBsAg positive LT recipients, 11 of whom had undergone LT for CLD and 6 for FHF. All subjects were HBV-DNA negative and HBeAg negative before LT, and all received HBIG for at least 18 months post-LT without evidence of HBV recurrence. HBV vaccination began several weeks after the final HBIG dose. 14 of 17 subjects (82%) responded to vaccination (6 after 3 vaccine doses, and 8 more after 3 additional vaccine doses) with detectable anti-HBs titres above a predefined threshold; there was no reported evidence of HBV recurrence for as long as 102 months of follow-up. More recent studies using new vaccines and adjuvants have been more encouraging but further studies are necessary to confirm the results.[77-78]

Use of hyperimmune plasma (HIP) transfusion: Fresh frozen plasma obtained from blood donors with high anti-HBs levels (hyperimmune plasma, HIP) containing at least 4,500IU anti-HBs has been used as an alternative treatment for HBV recurrence prophylaxis post-LT. In a study by Bihl et al.,[79] 21 HBV-related LT recipients received HIP starting at transplantation, followed by long-term combination treatment with nucleoside analogue. During a mean follow-up time of 4.5 years (range 0.5-12.6), each patient received on average 8.2 HIP per year (range 5.8-11.4). Anti-HBs terminal elimination kinetic after HIP administration was 20.6 days (range 13.8-30.9), which is comparable to values reported for commercial HBIG products. All 21 patients remained free of HBV recurrence during follow-up. There was no transfusion-transmitted infection. The cost for one HIP unit was US $140; the average yearly HBIG treatment cost was significantly low at US $148 per patient as compared to US $25,000 to 100,000 for commercial HBIG.

Subcutaneous HBIG injection: A recent study on weekly subcutaneous injections (500IU & 1000IU in

body weight of <75Kg & >75Kg respectively) of HBIG for 48 weeks in 135 HBV-related LT recipient shows that 97.8% of them had an anti-HBs level of more than 150IU/L (median of 232IU/L) with the least side effects[80] similar to previous studies.[81-82]

Oral antiviral drugs alone without HBIG: A study on a combination of HBIG with entecavir in the first five weeks after LT, followed by entecavir montherapy alone for one year, shows 96.6% and 96.4% disease-free survival in one year & two years follow-up respectively.[83] The Fung et al.[52] study on entecavir monotherapy without HBIG, with a median follow-up of 26 months on 80 post-LT recipients, shows HBV DNA to be undetectable in 98.8%, with HBsAg loss in 91% recipients. Similarly, tenofovir without HBIG also shows effective control on HBV recurrence in post-solid organ recipients.[43,72]

Drawbacks of HBIG

The major drawback with HBIG is its long-term use, the cost, and inconvenience of administration. In India, protocols that use high dose IV HBIG as described above are expensive i.e. Rs360,000 in the first year which includes the cost of IV infusion sets and monitoring. In the USA, the estimated cost is as high as $120,000 per year per patient.[84] Yet another problem is the failure to procure a constant supply of HBIG.[56] Adverse effects of HBIG, though rare, include:

i. Immune mediated reactions such as back pain, skin rash, and myalgia. Premedication is frequently not necessary.

ii. Mercury toxicity associated with intravenous administration of HBIG due to a thimerosal vehicle.

iii. Emergence of "a" determinant escape mutations,[21,85] which fails to get neutralised with HBIG administration. In a retrospective study, the estimated frequency of HBIG failure was 8% due to "a" determinant mutations at 28 months of therapy. Escape HBV mutations have occurred in recipients who have received anti-HBc positive liver grafts,[86] despite adequate dosing of HBIG.

Thus, at present, a rational approach to the prophylaxis of *de novo* HBV infection post-LT would be based on risk stratification i.e. pre-transplant HBV serology and viremic status which includes detectable anti-HBc, HBsAg or HBV-DNA in the donor and in the transplant recipient.[87-88]

SUMMARY

Detectable viremia and/or HBeAg positivity at the time of transplant are significant predictors of disease recurrence. Implementation of pre transplant treatment strategies will lower the burden of viral replication in transplant candidates. Newer oral anti-viral therapy, such as entecavir/tenofovir either as monotherapy or with HBIG, shows promising outcomes to prevent HBV reinfection in post-LT period. A safe time threshold for discontinuation of HBIG post-LT is not known, particularly among patients with a high risk for recurrence, i.e. those with detectable viremia at the time of transplant. Vaccination in the future will prevent HBV infection and reduce the disease burden worldwide. Future guidelines will help resolve some of these issues.

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VIRAL PROTEINS MEDIATE UPREGULATION OF NEGATIVE REGULATORY FACTORS CAUSING DOWN-MODULATED DENDRITIC CELL FUNCTIONS IN CHRONIC HEPATITIS C VIRUS INFECTION

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Disclosure: No potential conflict of interest
Citation: EMJ Hepatol. 2013:1,68-76.

ABSTRACT

Stunted cellular immune response against a narrow range of epitopes is the hallmark of chronic hepatitis C infection, but the underneath molecular mechanisms have not been well elucidated. Suboptimal antigen presentation through defective antigen presenting cells, have been suggested. The myeloid dendritic cells as professional antigen presenting cells have been found to be phenotypically and functionally defective in chronic hepatitis C-infected patients in our recently published study. In order to find out if the maturation defects in dendritic cells (DC) are induced by the persistence of virus, we tried to differentiate CD14+ monocytes isolated from the peripheral blood of a healthy volunteer in dendritic cell culture medium containing GM-CSF and IL-4 supplemented with hepatitis C virus (HCV) proteins, core and NS5. The results indicated that a lesser number of monocytes differentiated to DC in presence of HCV proteins. Moreover, the differentiated cells depicted immature phenotype, which will not respond to the TLR-4 mediated stimulation *ex vivo* with significantly lesser upregulation of activation markers, HLA-DR, CD83, CD80 and CD86 as compared to cells differentiated in the absence of HCV proteins. Besides, these immature cells showed characteristics of defective antigen presentation, with significantly lower allostimulatory capacity towards lymphocytes from a healthy donor. Semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR) showed upregulated expression of negative regulatory genes SOCS3, PDL1 and IDO in cells grown in presence of HCV proteins, suggesting the role of HCV and associated antigens in functional down-modulation of dendritic cells. This may correlate with the antigen persistence and maturation-defective status of dendritic cells in chronic HCV infection.

Keywords: Dendritic cells, hepatitis C virus, SOCS3, IDO, PDL1, HCV-NS5 protein.

INTRODUCTION

Hepatitis C Virus (HCV) poses a major global health predicament. The virus can be transmitted parenterally, sexually and through blood transfusion.[1] Infection with this morbid virus is associated with serious consequences. Chronic hepatitis in about 70–80% of cases further results in severe liver necrosis and an increased risk of cirrhosis and hepatocellular carcinoma in a smaller proportion of these individuals.[2-4] It is important to understand the mechanisms by which HCV establishes chronic infection so that better therapeutic interventions can be devised. Whilst the involvement of host genetic factors has been a major focus of research, the role of the immune system in the outcome of disease also cannot be marginalised. It has been proposed that a high rate of viral replication leads to exhaustion of the immune system through the production of overwhelming quantities of viral antigens and the production of immunomodulatory proteins by HCV.[5] Furthermore,

1	Positive for anti-HCV antibodies
2	Negative for HBV & HIV by serology
3	No prior history of any treatment for HCV
4	Negative for auto-antibodies (ANA, SMA, LKM, AMA, PCA and GBM) to exclude autoimmune hepatitis
5	Other non-viral factors responsible for liver damage like alcoholism, inherited metabolic disorders.

Table 1. Inclusion criteria for recruiting CHC patients

Parameters	CHC (n=35)
Median Age (years)	39 (19-67)
Gender M/F	31/4
Genotype 1/3/4	6/28/1
Median Viral Load (copies/ml)	$1.91x10^6$
Median ALT/AST	80/95

Table 2. Demographic and clinical features of patients recruited

the inability of the innate immune response to promote timely and appropriate T-cell priming has not been well understood yet. It has been suggested that viral proteins such as NS3, NS4, E1 and core protein play an important role in impairing the generation of effective immune response against the virus.[5-7] The status of dendritic cells in chronic HCV infection(CHC), has been the focus of research for many investigators around the world. Although there are a few studies supporting the theory that dendritic cells in CHC remain functional,[8-11] there are a few who argue that during HCV infection, the primary crosstalk between virus or virus-derived proteins and the DCs may render the DCs functionally defective, and further contribute to impaired T-cell responses leading to viral persistence.[12-16] Moreover, it is not yet clear whether the establishment of a chronic carrier state is due to dysfunctional DCs, which causes inefficient priming and maintenance of HCV-specific T-cells, or whether it is a possible outcome of persistent and active HCV infection. It could very well be possible that the host-mediated immunosuppressive mechanisms might be activated in the scenario of persistent antigenic exposure. Certain negative regulatory genes have been implicated as key factors for sustaining an immunosuppressive and tolerogenic microenvironment in different viral infections and even some cancers.[17-19] We have recently demonstrated that DCs from therapy-naïve CHC patients are dysfunctional and fail to mature.[20] The present study was designed to answer the pertinent question: whether the early interactions of virus proteins with DC render them immunosuppressive instead of being immunostimulatory. We report here that the monocytes continuously exposed to viral proteins *ex vivo* during differentiation to immature DCs render these cells maturation-defective, as they fail to respond to external stimulation and we also show that this maturation and functional defect coincides with upregulation of the expression of certain immunosuppressive genes such as SOCS3, PDL1 and IDO in these cells.

MATERIALS AND METHODS

Study Subjects

This study was approved by the Institute Ethics Committee. All the patients recruited in this study were registered with the Hepatology Clinic based on the inclusion criteria **(Table 1)** after an informed consent. A cohort of 35 therapy-naïve patients with CHC infection, were recruited for the study. A total of 14 age-matched normal healthy volunteers (HC) were also recruited as controls. Major demographic and clinical features of all the patients included in this study are detailed in **Table 2**.

Generation of Monocyte Derived Dendritic Cells (mo-DCs)

The peripheral blood mononuclear cells (PBMCs) were isolated from venous blood drawn in heparinised vacutainer tubes, of each subject from CHC and HC groups by Ficoll-Hypaque density gradient centrifugation using Histopaque (Sigma Aldrich). Subsequently, CD14+ monocytes were separated from PBMCs using anti-human-CD14 magnetic particles [(DM-(MφP9); BD Imag TM, BD Biosciences Pharmingen, USA] according to the manufacturer's instructions. Cell suspension enriched with CD14+ monocytes (purity 95% by flow cytometry, data not shown) was cultured in Dendritic Cells Culture Medium (DCCM), consisting of RPMI 1640 supplemented with: 2mM L-glutamine, 5mM HEPES buffer, 100IU/ml penicillin and 100µg/ml streptomycin, 10% fetal bovine serum (Sigma Aldrich), along with 20ng/ml recombinant human GM-CSF and 20ng/ml recombinant human IL-4 (both from PeproTech,

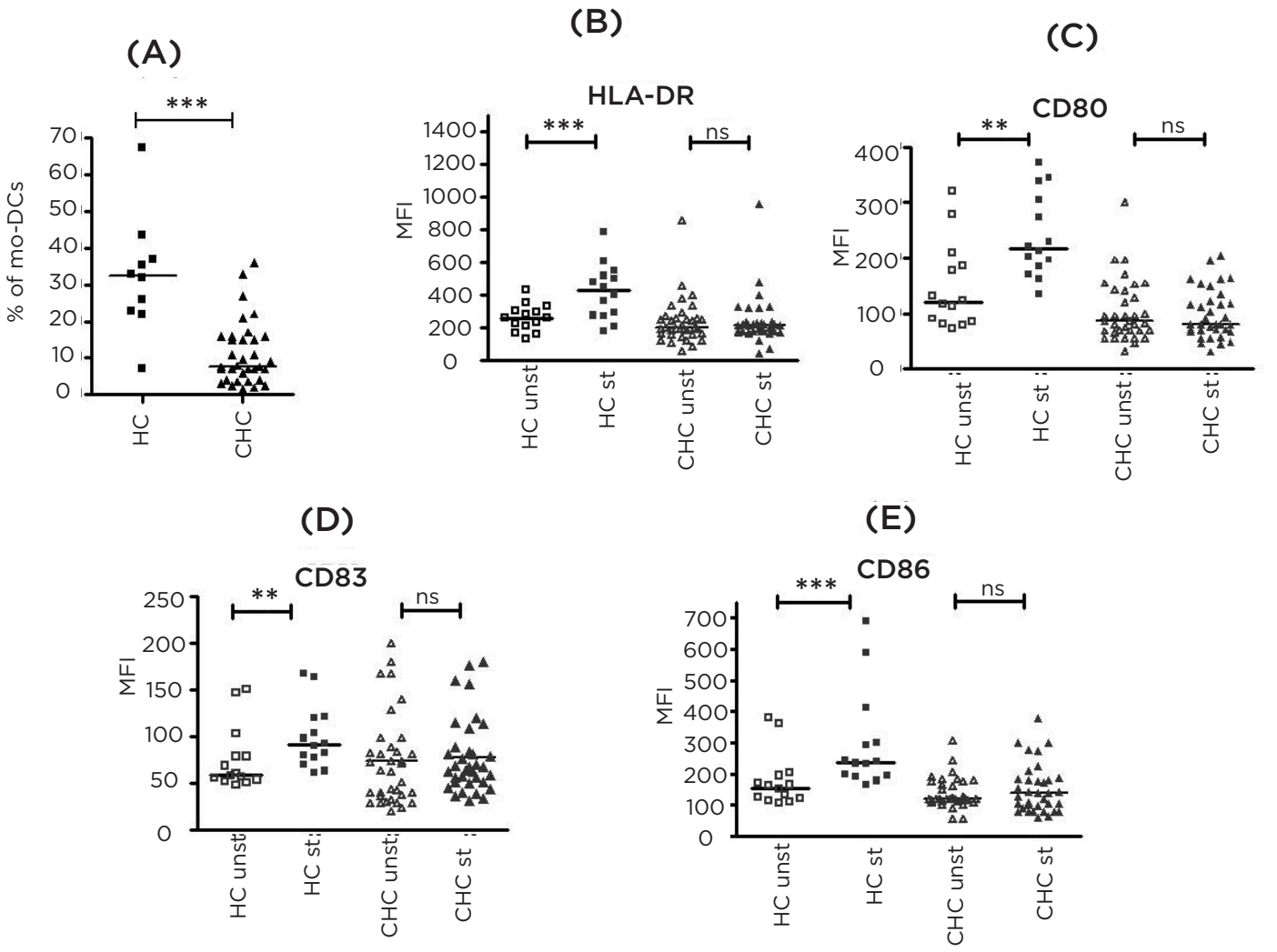

Figure 1. Frequency and expression of activation markers on mo-DCs. Cells with phenotype CD14-HLA-DR+CD11c+ were considered to be monocyte-derived dendritic cells (mo-DCs).
(A) Comparative frequency of mo-DCs in CHC and HC. Frequency of mo-DCs in CHC (n = 35) was significantly lower than HC (n = 14). Surface expression of (B) HLA-DR, (C) CD83, (D) CD80 and (E) CD86 on mo-DCs, pre and postlipopolysaccharide (LPS) stimulation. Mo-DCs from CHC, were able to upregulate expression of CD83, CD80 and CD86 upon LPS stimulation. Data presented as mean ± SD. Horizontal line represents the median value.

Israel), for six days in a humidified incubator (Thermo Forma, USA) at 37°C with 5% CO_2. Half medium exchange was performed on alternate days with fresh DCCM. DC viability on day six was >95% (trypan blue exclusion). To minimise the variations, we started with an equal number of monocytes in all sets of experiments. Toll-like receptor-4 (TLR-4) mediated maturation of DC was induced ex vivo by adding bacterial lipo-polysaccharide (LPS, 500ng/ml) to the culture medium on the sixth day for 48 hours. Simultaneously cells were also maintained without LPS.

Phenotyping of mo-DC

Analysis of cell surface markers on immature (iDCs) and mature DCs was performed on day eight of cell culture. The mo-DCs were stained with FITC-CD14, PE-HLA-DR/CD83/CD80/CD86 and PECy5-CD11c (all antibody-fluorochrome conjugates from BD Biosciences, USA) to characterise the phenotype of mo-DC in different conditions. Briefly, the cells were washed in staining buffer and were stained with respective fluorochrome-labelled antibodies for 20 minutes at room temperature in the dark. The cells were then washed, fixed and acquired using a three colour flow cytometer (FACS Calibur, Becton Dickinson, USA). In each case, unstained cells were also acquired to evaluate non-specific binding. Analysis was performed on gated population that included CD14 negative cells. The percentage positive cells and geometric mean fluorescence intensity (MFI) for each marker on mo-DC was

evaluated by analysis of dot plots or histograms generated by acquiring at least 100,000 cells using CellQuest software (BD Biosciences, USA). Fold increase in MFI was expressed as the ratio of MFI of stimulated cells and unstimulated cells.

Endocytosis Assay

To assess the endocytosing capacity, LPS-stimulated mo-DCs were incubated in PBS, 2% FCS, with 1mg/ml FITC-dextran (Sigma Aldrich) at 37°C to determine specific uptake, or at 4°C to determine background non-specific binding. After one hour of incubation, cells were analysed using flow cytometry. Uptake of FITC-dextran was measured in terms of fold increase in mean fluorescence intensity [MFI (i.e. ratio of MFI at 37°C to MFI at 4°C)].

Mixed Leucocyte Reaction

Allostimulatory capacity of LPS-treated mo-DCs was assessed by mixed leucocyte reaction (MLR). Briefly, LPS-stimulated DCs were treated with mitomycin (50µg/ml; Sigma Aldrich) for 30 minutes followed by co-culturing with allogenic PBMCs from a healthy, HCV negative donor at a ratio of 1:20 in triplicate wells at 37°C, with 5% CO_2 in a humidified incubator. Different healthy donor PBMCs were used in at least three different MLR experiments for mo-DC from each CHC patient. After five days of co-culture, 1µCi/well of [methyl-3H] thymidine (Bhaba Atomic Research Centre, Mumbai, India) was added for 12–16 hours. The incorporation of [methyl-3H] thymidine in proliferating PBMCs was measured using a beta-counter (Beckman-Coulter, USA) and expressed as counts per minute (cpm).

Expression of IDO, SOCS3 and PD-L1 in mo-DCs Differentiated in Presence of Viral Proteins

Peripheral blood samples were obtained from healthy uninfected donors after informed consent. CD14+ monocytes isolated from PBMCs were cultured in presence or absence of HCV proteins, core and NS5 (both 1µg/ml) of HCV genotype 3 (ProSpec-Tany TechnoGene Ltd, USA) from day zero, along with DCCM for seven days at 37°C and 5% CO_2 in a humidified incubator. On the seventh day LPS was added to the cultured mo-DCs for 48 hours for *ex vivo* stimulation through TLR4. At the end of the ninth day, flow cytometry was carried out to check for the surface expression of activation markers and co-stimulatory molecules like HLA-DR, CD83, CD80 and CD86. The expression of IDO, SOCS3 and PD-L1 genes was measured using semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR). Total RNA was extracted from mo-DCs (cultured in presence or absence of HCV proteins) using Trizol reagent (Sigma, USA). RNA was reverse transcribed to make complimentary DNA copies (MBI Fermentas, European Union), which were then further amplified by normal PCR reaction. The first strand cDNA was used in PCR reactions for the amplification of SOCS3, IDO and PD-L1 genes. The estimation of constitutively expressed β-actin gene was taken as control and used to normalise the values for semi-quantitative estimation of other genes (SOCS3, IDO and PD-L1) under study.

The sequences (5'-3') of the primers (custom synthesised from Sigma Genosys, Bangalore, India) used in the study were as follows:

β-actin F β-actin R	AGCACAGAGCCTCGCCTTTGC GCCGTGCTCGATGGGGTACTT
IDO F IDO R	GGCACACGCTATGGAAAAC ATGCATCCCAGAACTAGACG
SOCS3 F SOCS3 R	TCCGGAGGAGCCAGCTGTCC TTTCCTTCGCCAGCCCGCAG
PD-L1 F PD-L1R	TTCTGTCCGCCTGCAGGGCA ACAGCCGGGCCCTCTGTCTG

RESULTS

Phenotypic Characterisation of mo-DC

From both patients as well as controls, mo-DCs were obtained after seven days of culture. Phenotypic characterisation of differentiated immature mo-DCs was performed using flow cytometry. A significantly lesser number of monocytes from CHC patients could differentiate to DCs in presence of GM-CSF and IL-4 in CHC as compared to that of HC ($p<0.005$, **Figure 1A**). The difference observed in number of mo-DCs was not due to decreased leukocyte counts in patients, as there was no difference in total leukocyte count of CHC vs. HC (data not shown).

The surface expression of HLA-DR, CD80, CD86 and CD83 molecules was used as a hallmark of activation and maturation of DCs. While none of the molecules got upregulated significantly on the surface of mo-DCs from CHC in response to LPS stimulation, a uniformly significant upregulation in the surface expression of HLA-DR ($p<0.005$), CD80 ($p<0.005$), CD86 ($p<0.005$)and CD83 ($p<0.05$) was seen on the mo-DCs from HC **(Figure 1 B,C,D,E)**. These data clearly suggest that the mo-DCs from CHC patients were functionally defective and were incapable of

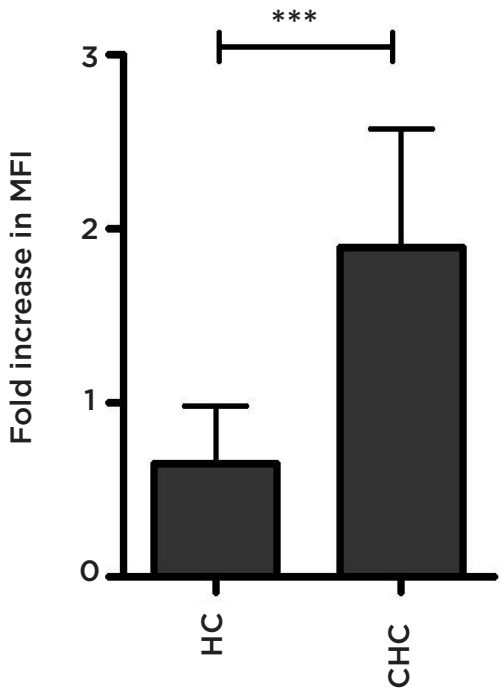

Figure 2. Endocytosing capacity of lipopolysaccharide-stimulated mo-DCs. Uptake of FITC-dextran by mo-DCs from CHC was significantly higher than HC.

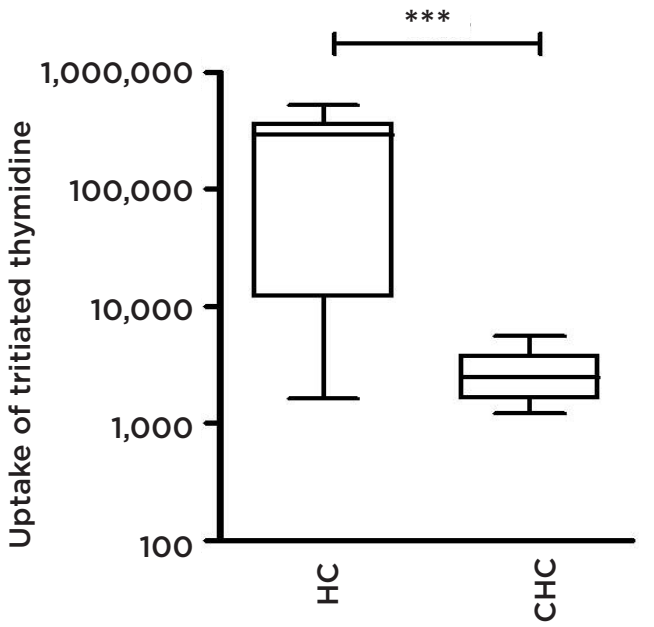

Figure 3. Comparative allostimulatory capacity of mo-DCs. Allostimulatory capacity of mo-DCs from CHC is significantly impaired as compared to HC.

maturing in the presence of an external stimulus.

Antigen Uptake Efficiency of Differentiated mo-DC

Immature dendritic cells are characterised by their marked capability of phagocytosing the invading foreign antigen. As they mature and become efficient, antigen-presenting cells they tend to lose this property. To evaluate antigen uptake efficiency of LPS-stimulated DCs, endocytosis assay was performed using FITC-conjugated dextran particles. Flowcytometric analysis revealed that the amount of FITC-dextran endocytosed by mo-DCs from CHC was significantly higher ($p<0.005$) than those of HC **(Figure 2)**, suggesting that mo-DCs from CHC did not respond to maturation stimulus and remained functionally immature.

Impaired Allostimulatory Capacity of mo-DCs from CHC

The allostimulatory capacity of mo-DCs from different groups was measured by performing Mixed Lymphocyte Reaction (MLR) using mitomycin treated mo-DCs from patients/controls and PBMCs from a single healthy donor. The degree of stimulation of allogeneic lymphocytes induced by mo-DCs from CHC was significantly lower than that of HC ($p<0.005$) **(Figure 3)**. This data is in concordance with the low surface expression of activation and maturation markers on CHC indicating that DCs remained phenotypically as well as functionally immature even upon *ex vivo* stimulation. These data also suggest that viral persistence could be having a negative effect on the allostimulatory potential of DCs.

Assessing the Role of HCV Core and NS5 Proteins on Differentiation of Monocytes to Dendritic Cells

To ascertain whether the defect in dendritic cells is virus mediated, an *in vitro* experiment was performed by exposing monocytes from healthy individuals to HCV Core or NS5 antigen in DCCM during their differentiation to dendritic cells. Simultaneously, a control experiment was run in parallel in which only DCCM was added to mo-DC culture without the addition of viral antigens. The differentiated mo-DCs were then stimulated with LPS for 48 hours, after which surface expression of HLA-DR, CD83, CD80 and CD86 was evaluated. It was observed that mo-DCs exposed to NS5 and core antigens during differentiation, showed characteristics of maturation defect as they expressed lesser amounts of HLA-DR, CD83, CD80 and CD86 on their surface as compared to cells grown in medium without viral proteins, and there was no upregulation of these molecules even after

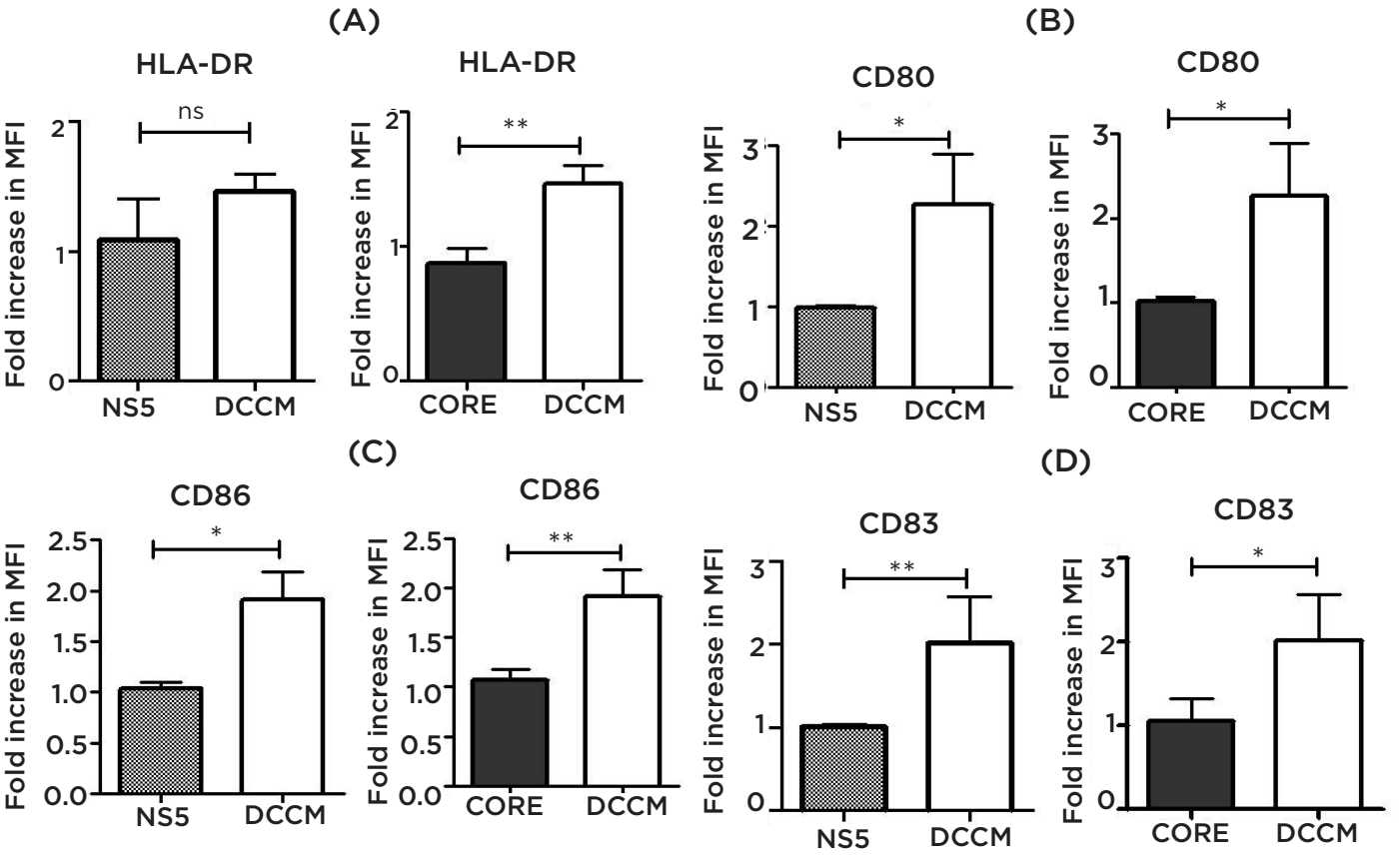

Figure 4. Effect of LPS stimulation on expression of (A) HLA-DR, (B) CD83, (C) CD80 and (D) CD86 on mo-DCs exposed to HCV-NS5 and Core. Each figure represents the fold increase in MFI as compared to mo-DCs raised in absence of LPS stimulation denoted as DCCM only. Both NS5 and Core antigens have deleterious effect on maturation of mo-DCs.

stimulation with LPS. After LPS stimulation, the upregulation of CD83, CD80 and CD86 on mo-DCs exposed to NS5 antigen was significantly lower ($p<0.05$ in all three) as compared to that of medium alone. Similarly, after LPS stimulation, the upregulation of HLA-DR, CD83, CD80 and CD86 on mo-DCs exposed to core antigen was significantly lower ($p<0.05$ in all four, **Figure 4**) as compared to that of DCCM only.

Expression of SOCS3, IDO and PD-L1 in mo-DCs Differentiated in Presence of HCV Core and NS5

To check whether the exposure to HCV antigens caused an increase in expression of down-modulatory genes such as SOCS3, PD-L1 and IDO in mo-DCs, semi-quantitative RT-PCR revealed that in presence of NS5 and Core proteins, the expression of PD-L1 and IDO genes got significantly upregulated ($p<0.05$ for all) in the mo-DC, while only NS5, and not Core, caused a significant increase in the expression of SOCS3 ($p<0.05$) **(Figure 5)**.

DISCUSSION

Viruses are known to devise different strategies to counteract antiviral immunity by manipulating the functions of various components of the immune system. It has been demonstrated that infection of DCs by measles virus resulted in diminished IL-12 production and inhibition of DC maturation,[21,22] while HIV-infected subjects have been reported to have defects in the number and functions of circulating DC subsets.[23,24] CMV infection has also been shown to cause inhibition of DC maturation and T-cell activation, as well as increased apoptosis in T-cells and downregulation of MHC class I molecules.[25, 26]

In recent years there have been some reports describing the role of DCs in HBV or HCV-induced hepatitis.[27,28] However, these reports have been diverse and conflicting. While some studies have reported that HBV or HCV-related chronic hepatitis displayed a phenotype defect and functional deficiency of DCs,[29,30] others reported no observable defect in DCs from CHC subjects as well as chimpanzee models.[8,9,31]

This has brought in a state of ambiguity to the scenario of CHC infection as far as functional status of DCs is concerned and justified for a systematically designed study to further evaluate this aspect. Our results from the present study regarding status of mo-DC from CHC patients clearly demonstrate that there is a defect in terms of number and function in this population during the chronic HCV infection. We found that lesser number of monocytes from CHC patients' blood could be differentiated to DCs in presence of IL-4 and GM-CSF as compared to HC, which further fail to mature upon *ex vivo* stimulation. This observation is coherent with our observation (data not shown) and other studies where reduced number of DCs has been reported in CHC patients.[20,32-35] Moreover the DCs also displayed immature phenotype which was demonstrated by their low allostimulatory potential and high phagocytosing capacity after LPS stimulation *ex vivo*. This could be attributed to the viral persistence in these patients as we have earlier reported that the DCs from patients who cleared virus after therapeutic intervention showed a marked improvement in their numerical as well as functional status.[20]

By exposing the monocytes from healthy individuals to HCV-3 specific core and NS5 antigens during differentiation to immature DCs *in vitro*, and by further inducing maturation in presence of LPS, we found that both core and NS5 antigens induced maturation and activation defects in healthy mo-DCs as they failed to upregulate surface expression of HLA-DR, CD83, CD80 and CD86 upon *ex vivo* LPS stimulation. These results clearly elucidate the direct evidence of virus-mediated downmodulation of DC functions in chronic viral hepatitis. Cell culture grown HCV (HCVcc) when incubated with immature mo-DCs has been shown to have deleterious effects on their ability to present the antigen.[15] Recently Landi et al. found that HCV core, when transfected in immature mo-DCs, does not have any inhibitory effect on human DC maturation,[36] while monocytes exposed to high concentrations of HCV proteins during differentiation to dendritic cells had diminished capacity to present the antigen.[37] These observations corroborate our findings, indicating virus-driven mechanisms causing maturation and functional defects in dendritic cells. Moreover, this approach seems to be convincingly closer to the actual *in vivo* situation in which the precursor cells in chronically infected patients would be exposed to various HCV proteins present in the milieu over a long period of time while differentiating, leading to development of defective dendritic cells. Our study, as well as the findings of Krishnadas et al. supports this hypothesis.[37]

Trying to elucidate the possible mechanism, we further investigated the effect of these viral proteins on expression of certain down-modulatory genes such as SOCS3, IDO and PD-L1, which might be playing a significant role in rendering the DCs tolerogenic or maturation defective. We found that in the presence of NS5 and core antigens, the expression of PD-L1 and IDO got upregulated while only NS5, and not core, caused the increase in expression of SOCS3 in the mo-DC that were differentiated in the presence of these viral proteins right from day one.

The HCV protein has earlier been shown to cause upregulation of SOCS3[38] and it has recently been proposed that non-responsiveness to antiviral therapy

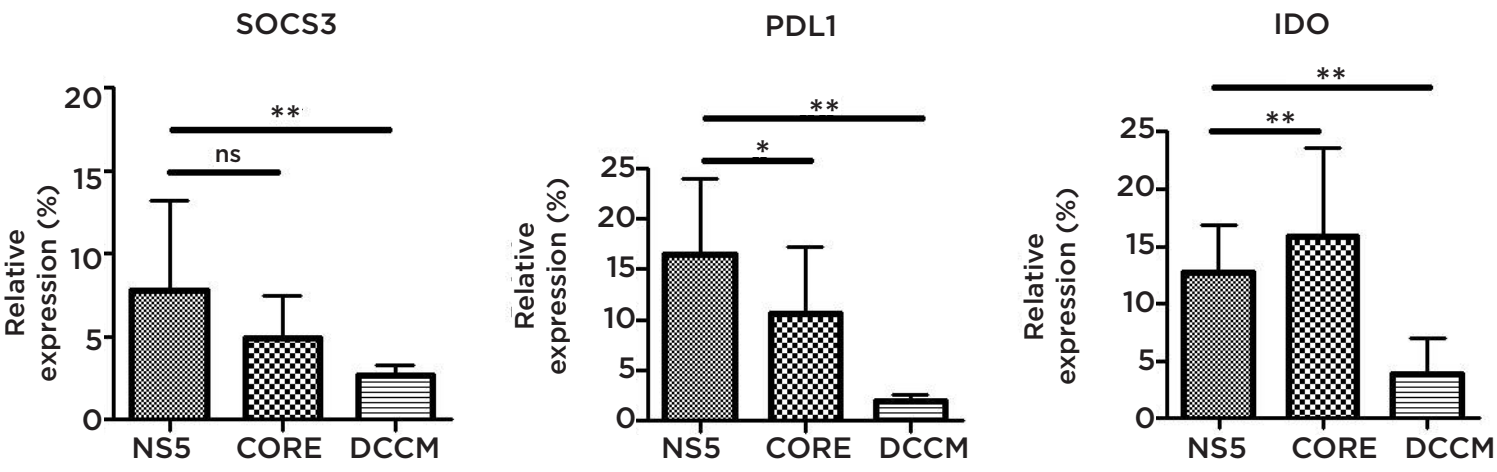

Figure 5. Relative gene expression of SOCS3, IDO and PD-L1 in mo-DCs exposed to HCV-NS5 and Core antigen.

might be related to up-regulation of SOCS3.[39] These reports have used human hepatoma cell line HepG2 or Epstein-Barr virus–transformed lymphoblastoid cell lines derived from peripheral lymphocytes from patients. None of these studies have been executed on human blood derived dendritic cells so far. In addition, a recent study has suggested that increased PD-L1 expression and PD-L1/CD86 ratio may be responsible for the dysfunction of DCs in chronic HCV infection, as it might be correlated with exhaustion of T-cell function.[40] Our results confirm the same hypothesis. Moreover, another recent study highlights the dual nature of dendritic cells in ovarian cancers, where there is strong expression of PD-L1 by the immunosuppressive DCs.[19] The tumour microenvironment resembles the chronic disease as the continuous presence of antigen leads to immune exhaustion in chronic disease as well as cancer. Although IDO has been implicated in causing tolerogencity in DCs elsewhere it has not been linked so far with HCV.[41] Nevertheless, it cannot be denied that HCV might upregulate this gene for its own survival. We have demonstrated for the first time that this gene gets upregulated in mo-DCs raised in presence of HCV proteins suggesting that IDO might also be involved in causing dysfunction in DCs. These results also suggest that various HCV proteins may coordinate their negative modulatory action on DCs to impair T-cell stimulation and facilitate the establishment of chronic HCV infections. It would be really interesting to conduct similar studies on DCs from patients recovering from acute HCV infection and to find whether they have an immunostimulatory profile which might be instrumental in resolving the infection. Nevertheless, the findings elucidate the mechanisms of immune dysfunction in chronic HCV infection, which may be helpful in designing newer strategies of intervention in future.

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TOWARDS A STANDARDISED METHOD TO ACQUIRE AND STORE LIVER SAMPLES AND GUIDELINES TO IMPROVE QUALITY CONTROL AND EXCHANGE OF RELATIVE EXPRESSION DATA

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Disclosure: No potential conflict of interest
Citation: EMJ Hepatol. 2013;1:78-84.

ABSTRACT

The current 'state-of-the-art' molecular techniques are extremely sensitive and consequently prone to false results. Even more so than in the past, today's hepatology research depends on high quality samples, especially for the molecular analyses. In all steps, starting with specimen sampling, fixation, storage, molecular processing and finally data calculation, variations in procedures between research laboratories may have a profound effect on the final conclusions. At the end of the day, this is an enormous drawback once data from different research institutes need to be reproduced, compared and/or combined. To improve standardisation, the so-called MIQE guidelines (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) were presented for quantitative PCR (qPCR) studies.[1,2] Furthermore, around the same time, recommendations were presented regarding human biospecimen collection, storage and processing, the so-called BRISQ-guidelines (Biospecimen Reporting for Improved Study Quality).[3] Finally, the editors of *The Journal of Pathology* as well as *Histopathology* required in the December 2012 issue of *The Journal of Pathology* that researchers needed to follow the BRISQ guidelines in their papers in order to improve the sample quality in biomedical research.[4]

These initiatives hold great promise to improve the comparison and independent reproduction of data acquired in different research centres. Pancreas, gall bladder and liver research will especially benefit from the standardisation protocols since these organ systems are highly vulnerable to post-biopsy autolytic degradation. This comment illustrates that standardisation in molecular liver research is not yet at the point where experiments can be easily replicated, and data can be compared and combined.

Keywords: Quantitative PCR, MIQE-precise, normalisation, reference genes.

INTRODUCTION

Molecular expression studies on biospecimen can gain insight into the etiology of a disease, and may lead to information on therapeutic effects and potentially facilitate biomarker studies. These samples need to be acquired, stored and processed in such a way that laboratory-to-laboratory comparison is possible and independent reproduction can be achieved. Standardisation of protocols in all three steps mentioned above is a way to come to meaningful comparisons. Cost-effective scientific progress can be achieved by different means, for instance, by combining data and data-comparisons of different research groups. High quality data is crucial in this respect. Space limitations often hamper detailed description of materials and methods, and consequently comparisons between laboratories, not to mention meta-analyses, are often flawed. For biopsies the BRISQ guidelines exist and there are guidelines to standardise quantitative PCR (qPCR) expression studies (MIQE-precise guidelines).[1-3] The MIQE guidelines are summarised in a checklist format and assist in experimental design, facilitate accurate data analysis, relieve the job of a manuscript reviewer, and make data interpretation easier for the readers of the scientific paper. Altogether they are beneficial in all steps from experimental design and biospecimen sampling

to acceptance and implementation in the scientific community. This chapter is an initiative to raise awareness of the cost-effective progress molecular liver research can make once data are calculated and presented in such a way that experiments can be easily repeated and data can be combined and compared.

Scientists prefer their biopsies, taken at surgery not under time pressure or other forms of stress, to be fixed specifically for their individual research questions which can be either at tissue, cellular or molecular level. However, these separate research questions require different fixation and storage methods. Such a complexity of tissue handling is clearly prone to the introduction of mistakes, leading to biospecimen of potentially lesser quality for a specific analysis. Although RNA is far less stable then DNA some studies indicate that the RNA integrity is not largely influenced even up to 48 hours on ice.[5,6] The last study included tonsil and liver samples. In contrast, two studies exemplified the effects of variations in liver tissue sampling on subsequent mRNA expression studies.[7,8] One study described the influence of the biopsy needle size in rat liver biopsies on the RNA quality in a subsequent micro-array expression study.[7] The second study assessed different sampling techniques, fixation methods, and storage procedures for canine liver tissue to optimise the use of a single liver biopsy for histological and molecular (qPCR) measurements.[8]

Not only can total RNA be subject to degradation (usually measured as a RNA Integrity Number (RIN) based on 18S and 28S) during the sampling, storage and processing, but mRNA (only 2-5% of total RNA, but most often the compound of interest) can also be degraded. One way to correct for mRNA degradation, and other steps in mRNA expression studies is the inclusion of so-called reference genes (previously erroneously called housekeeping genes) to normalise for mRNA input and PCR efficiency. The assumption here is that the expression of reference genes is always constant, irrespective of variations in samples, experimental conditions etc. In fact this assumption has been debated for about one decade now.[9] Obviously data comparison in molecular liver research faces an enormous hurdle if reference gene stability is either not evaluated nor are other parts of the sample and data processing are not described in detail. Whether this molecular deficit indeed exists in molecular liver research was not reported previously, and is investigated in this chapter. As it turned out, based on a PubMed search (http://www.ncbi.nlm.nih.gov/), the crucial step in expression studies, viz, evaluation in reference gene stability, was often omitted in molecular liver studies. This book chapter therefore is a clear advocacy to implement MIQE-precise guidelines as soon as possible.

Variation	Reference gene used	Ref.
Colorectal liver metastases	18S rRNA	21
HCC and HCA	18S rRNA	23
HCC	18S rRNA	25
Hepatocellular adenoma	18S rRNA	30
HepG2 cell line	18S rRNA	31
Nonalcoholic fatty liver disease	18S rRNA	32
Hepatocellular adenoma	18S rRNA	39
Gallstone disease	18S rRNA	41
HCC progression in mice	18S rRNA	25
Fibrogenesis in ABCb4 /- mice	18S rRNA	45
PHX in ob/ob mice	18S rRNA	58
BDL-induced fibrosis	Beta-2-Microglobulin	46
BDL- or TAA-induced fibrosis	Beta-2-Microglobulin	50
HCC patients	Beta-Actin	17
HBV-related HCC	Beta-Actin	18
HCC	Beta-Actin	19
HEV	Beta-Actin	28
HBV-related HCC	Beta-Actin	29
Freshly isolated hepatocytes	Beta-Actin	34
HCC	Beta-Actin	35
Hepatoma cell line	Beta-Actin	42
HBx-transgenic mice	Beta-Actin	18
Lipid accumulation in mice	Beta-Actin	49
Hepatosteatosis in mice	Beta-Actin	55
Iron-dextran overload	Beta-Actin	56
HCC and cell lines	Beta-Actin (HCC) GAPDH(cellline)	10
HCC	Beta-Globin	40
Rosiglitazone and LPS treatment	cyclophilin	52
LPS injections	cyclophilin	53
Rats	Cyclophilin A	47
Primary malignant liver tumor	GAPDH	33
NASH	GAPDH	36
HCC	GAPDH	37
HCC	GAPDH	38
Mouse model for NAFLD	GAPDH	43
DEN-treated cyclD -/- mice	GAPDH	44
Lithogenic diet	GAPDH	51
Doxorubicin mdr trangenenic mice	GAPDH	57
Alcohol liver disease	HPRT	26
HepG2 cell line	POLR2A	22
HCV + HIV	RPL0	27
HCV-induced dysplasia and HCC	RPL41 and SFRS4	11
CBS +/+, CBS +/-, CBS -/- mice	SOD-1	54
Mouse primary hepatocytes	TBP	22
Biliary atresia	Unspecified "housekeeping gene"	24
C57Bl6 mice	Unspecified commercial	48
Primary biliary cirrhosis	Villin	20

Table 1. Papers reporting on quantitative PCR in human and murine liver samples and cell lines, with emphasis on the reference gene included to normalise expression data.

MATERIAL AND METHODS

A PubMed search was performed via http://www.ncbi.nlm.nih.gov/ on Tuesday March 19th 11am CET on the terms 'human AND quantitative PCR AND expression AND hepatology'. The search was limited to *Hepatology* and the *Journal of Hepatology* only, the two highest top-ranked journals in the ISI-field of 'Gasteroenterology and Hepatology' specifically for hepatology. Moreover both are official journals of the American Association for the Study of Liver Diseases (AASLD) and the European Association for the Study of the Liver (EASL) respectively. A similar search was performed on '(murine OR mouse) AND quantitative PCR AND expression AND hepatology'.

Finally, a PubMed search on papers evaluating reference expression stability in liver samples from human and other mammalian species was performed to reveal which reference genes were evaluated under what kind of research samples, and which freeware was used to indicate expression stability and consequently which were most reliable reference gene under that specific condition.

RESULTS

Approximately the first 50 hits on the combined terms 'human AND quantitative PCR AND expression AND hepatology' and '(mouse OR murine) AND quantitative PCR AND expression AND hepatology' were screened to establish which presumed stable reference gene was used **(Table 1)**. The preference for the classical reference genes, *viz*, beta-Actin, GAPDH or 18S rRNA, was obvious. Thirteen times was normalised against beta-actin, eleven times with 18S rRNA, and nine times with GAPDH. In one paper for the clinical samples normalisation was performed with beta-actin, whereas in cell lines GAPDH was used.[10]

None of these papers provided information on whether or not the indicated reference gene was expressed at a stable level. Most surprising was the observation that in all papers analysed, except for one, only one reference gene was used for normalisation. The exception included two independent reference genes: SFRS4 and RPL41.[11] Even worse, in view of data comparison, was the number of other reference genes used, including beta-2-microglobulin, beta-globin, cyclophilin A, villin, POLR2A, RPLP0, SOD-1, cyclophilin, TBP, or HPRT. There were no calculations on the expression stability of the reference genes included in any of the papers summarised in **Table 1**.

Six papers described the evaluation of reference gene expression stability in human samples as depicted in **Table 2**. GAPDH, beta-actin and HPRT, were included in five out of six studies, TBP was used three times, SFRS4, GUSB, 18S rRNA and B2M were include twice. RPL13A, HMBS, SDHA, RPL41, CYCC, RPS0, UBC, PMM1 and POLR2L were evaluated once. GeNorm analysis[12] and Normfinder[13] were used to evaluate expression levels and depending on the paper, either GUSB (twice), HPRT (twice) or TBP (twice) performed the best, exhibiting the highest stability of expression. SFRS4, HMBS, RPL41 and PMM1 turned out to be the best only once. The three most frequently used reference genes (beta-Actin, 18S rRNA or GAPDH) never ranked as most stably expressed reference genes **(Table 2)**. The GeNorm algorithm allows us to calculate the set of reference genes minimally required to normalise the expression of genes of interest. This analysis ('pairwise variation') has been included in as little as two of the six papers described above. Romanowski et al.[14] concluded that two reference genes, *viz* GUSB and PMM1, were sufficient to obtain a pairwise variation below 0.15, the recommended threshold to calculate the number

Variation	Reference genes	Software	Best reference gene(s)	Pairwise variation	Reference
HCV patients	18S rRNA, Beta-Actin, GAPDH, GUSB, HPRT, SFRS4	G, N, B	SFRS4 GUSB	Not tested	16
HBV-induced HCC	18S rRNA, Beta-Actin, GAPDH, HPRT, RPL13A, TBP	G, N	TBP HPRT	Not tested	59
HCC patients	B2M, GAPDH, HMBS, HPRT, SDHA, UBC	G, N	HMBS	Paired samples: V3/V4<0.15	15
HBV-induced HCC	B2M, Beta-Actin, GAPDH, HPRT, TBP	G, N	HPRT TBP	Not tested	60
HCV-induced HCC	Beta-Actin, GAPDH, RPL41, RPS20, SFRS4, TBP	G, N	RPL41 SFRS4	Not tested	61
HCV and HBV patients	Beta-Actin, CycC, GUSB, HPRT, PMM1, POLR2L	G, N	GUSB PMM1	V2/V3<0.15	14

Table 2. Papers reporting on the evaluation of expression stability of potential reference genes in human liver samples. Abbreviations in the software column: G=GeNorm, N=NormFinder, B=Bestkeeper.

Variation	Reference genes used	Software	Best reference gene(s)	Reference
Steatotic mice	B2M, Beta-Actin, GAPDH, HMBS, HPRT, RPL13A, RPLPO, TBP, TFRC, TuBP	G, N, B	HPRT GAPDH	62
Bos Taurus, cattle	Beta-Actin, GAPDH, HPRT, SDHA, TBP, YWHAZ	G	TBP Beta-Actin	63
Specific liver cells after Phx in rats	18S rRNA, B2M, Beta-Actin, GAPDH, HK1, UBC	G	Cell type dependent	64
90% PHx in rats	Alb, GAPDH, HPRT, UBC, YWHAZ		HPRT	65
Sus scrofa, pig	B2M, Beta-Actin, GAPDH, HPRT, HTPAP, RPL13A	G, N	GAPDH HPRT	66
Felis catus, cat	B2M, GAPDH, GUSB, HMBS, HPRT, RPL17, RPL30, RPS19, RPS5, YWHAZ	G	RPL17 HMBS	67
Canis lupus familiaris, dog	B2M, Beta-Actin, GAPDH, HMBS, HPRT, RPL13A, RPL32, RPS18, SDHA, TBP, YWHAZ	G	B2M Beta-Actin GAPDH	68
Canis lupus familiaris, dog	B2M, GAPDH, GUSB, hnRNPH, HPRT, RPL8, RPS19, RPS5	G	RPS5 HPRT B2M	69

Table 3. Papers reporting on the evaluation of expression stability of potential reference genes in mammalian non-human liver samples. Abbreviations in the software column: G=GeNorm, N=NormFinder.

of reference genes minimally required.[12] Combining tumourous and non-tumourous tissues revealed that at least four reference genes were needed.[15] The paper by Congiu et al.[16] clearly showed that a different set of reference genes were most stably expressed if the groups were arranged according to the levels of inflammation, or the levels of steatosis or fibrosis. Unfortunately, it was not indicated by pair-wise variation which number of reference genes was optimal for each specific condition. The situation is similarly disturbing once the expression stability is evaluated in liver samples from other mammalian species like mice, rats, pigs, cats, dogs and cattle **(Table 3)**. Again, a large list of potentially stably-expressed reference genes evaluated for their respective expression stability, including the favourable, but not necessarily the most stably expressed, human reference genes beta-actin, GAPDH and HPRT.

DISCUSSION

For relative expression levels of gene products, normalisation is needed. The expression of reference genes, of which the expression is to be stable amongst different conditions, is then used to standardise. The stability of their expression is tacitly presumed to be high. Analysis of the expression stability, by the inclusion of several independent reference genes, showed that this assumption does not always hold true. The few calculations on the minimal number of reference genes needed to properly normalise relative mRNA expression levels showed that, depending on the experimental comparison, at least two and sometimes more reference genes are needed. The plethora of various reference genes and the variable outcome in the papers evaluating reference gene expression stability, made one point clear: there are no standardised descriptions incorporated in the papers, nor are relevant details for data comparison, experimental repetition or data combination provided in most liver-related expression studies. Is this a purely academic fine-tuning issue? This is a rhetorical question. What are the cost-benefits for the inclusions of more reference genes? Imagine a simple *in vivo* experiment, two groups of six mice, six weeks of age, one group treated with a fibrotic agent and the other group as control. After six weeks (cost of animal housing around $500, 42 days 12 mice $1 per day per mouse), histology, slicing of slides, HE staining and one specific staining with an antibody (altogether costing $350). Molecular assays including one reference gene and three genes of interest ($200, qPCR for one gene around $50). So in total this imaginative experiment does cost around $1000, not taking into account the working hours. Histology and immunohistochemistry, once proper negative and positive controls are included, will be clear and open to comparisons and replications. Expression data can be replicated, however, they might not be comparable with other studies which use another reference gene to normalise expression. Even worse, the relative expression is potentially miscalculated, since it is unknown whether the reference gene was indeed expressed at a stable level throughout the two experimental conditions. For as little as $100 (two additional reference genes) the expression

data will be much more reliable, and since reference gene expression stability was evaluated and recorded a comparison of these expression data with other reports becomes feasible. The investment of just $100 will save a multitude of this amount once one can avoid a repetition of the experiment due to a lack of proper information on the stability of the included reference genes.

BRISQ-guided standardisation for histological research and biobanking is obligatory in leading pathological journals at present. Liver research can make great progress if an improved standardisation can be accomplished for molecular investigations. The proposed MQIE guidelines and MQIE-precise guidelines, including proper reference gene expression stability evaluation, offer an easy way to make the presented data easy to repeat, allow data comparison, and facilitate manuscript reviewing.[1,2]

ABBREVIATIONS

Alb, albumin
BDL, bile duct ligation
B2M, beta-2-microglobulin
CycC, cyclophillin C
GAPDH, glyceraldehyde-3 phosphate dehydrogenase
GUSB, beta-Glucoronidase
HBC, hepatitis B virus
HCC, hepatocellularcarcinoma
HCV, hepatitis C virus
HEV, hepatitis E virus
HIV, human immunedeficinecy virus
HMBS, hydroxymethyl-bilane synthase
HPRT, hypoxanthine phosphoribosyl-transferase
HTPAP, PPAP2 domain-containing protein 1B
LPS, lipopolysaccaride
NAFLD,nonalcoholic fatty liver disease
NASH, nonalcoholic steatohepatitis
PHx, partial hepatectomy
PMM1, Phosphomannomutase 1
POLR2, polymerase (RNA) II polypeptide L
RPL0, Ribosomal Protein Large0
RPL17, Ribosomal Protein Large17
RPL31A, Ribosomal Protein Large13A
RPL41, Ribosomal Protein Large41
RPS5, Ribosomal Protein Small
SDHA, succinate dehydrogenase complex, subunit A
SFRS4, splicing factor serine/arginine-rich 4
SOD1, Super Oxide Dismutase-1
TBP, TATA Box Binding Protein
TFRC, transferrin receptor
TuBP, tubulin alpha 4a
UBC, Ubiquitin C
YWHAZ, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide

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Robust protection against recurrent episodes of hepatic encephalopathy[1]

Significant reductions in episodes† of hepatic encephalopathy and hospitalisation rates‡ have been demonstrated with XIFAXAN® 550 b.d. and concomitant lactulose*[1]. XIFAXAN® 550 b.d. provides a cost-effective treatment option[2] that enhances quality of life for patients.[3]

† p<0.001 ‡ p=0.01
* >90% were receiving concurrent lactulose in both treatment arms

NEW

Xifaxan®550
Targaxan®550▼
Rifaximin-α

INTERNATIONAL ABBREVIATED PRESCRIBING INFORMATION: XIFAXAN®/ TARGAXAN® 550 mg (rifaximin)
Presentation: Blister pack containing 14 film-coated, pink tablets of 550 mg rifaximin for oral administration. **Indication:** Reduction in recurrence of episodes of overt hepatic encephalopathy in patients ≥ 18 years of age. **Dosage and administration:** Recommended dose: 550 mg twice a day orally with a glass of water, with or without food. No specific dosing adjustment is necessary for patients with hepatic insufficiency or for the elderly. **Contraindications:** Hypersensitivity to rifaximin, any rifamycin antimicrobial agents or any of the excipients. **Warnings and precautions:** The safety and effectiveness of XIFAXAN® for the prevention of recurrence of hepatic encephalopathy have not been established in patients under 18 years of age. *Clostridium difficile*-associated diarrhoea (CDAD) has been reported with use of nearly all antibacterial agents, including rifaximin. The potential association of rifaximin treatment with CDAD and pseudomembranous colitis (PMC) cannot be ruled out. Caution is advised in patients with impaired renal function. Concomitant administration of rifaximin with other rifamycins is not recommended. Caution should be exercised when administering XIFAXAN® to patients with severe hepatic impairment (Child-Pugh C) and in patients with MELD (Model for End-Stage Liver Disease) score >25. **Interactions:** Due to the negligible gastrointestinal absorption of orally administered rifaximin, the systemic drug interaction potential is low. *In vitro* studies have shown that rifaximin did not inhibit cytochrome P450 isozymes 1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1 and CYP3A4 at concentrations up to 200 ng/mL (at least 10 times the clinical C_{max}). Rifaximin is not expected to inhibit these enzymes in clinical use. The effectiveness of oral oestrogenic contraceptives could decrease after rifaximin administration. Additional contraceptive precautions are recommended, in particular if the oestrogen content is less than 50 μg. **Pregnancy and lactation:** Nonclinical studies of placental transfer of rifaximin/metabolites have not been conducted. There was no evidence of teratogenicity in pregnant rats or rabbits treated with rifaximin during the period of organogenesis. It is unknown whether rifaximin/metabolites are excreted in human milk. A risk to the child cannot be excluded. A decision must be made whether to discontinue breast-feeding or to discontinue/abstain from rifaximin therapy. Use of rifaximin during pregnancy is not recommended. **Undesirable effects:** The adverse effects identified from the pivotal clinical trial most likely to be associated with rifaximin treatment (incidence ≥10%) are: nausea, dizziness, ascites, oedema peripheral. The following adverse reactions have been identified during post approval use of rifaximin. Common (≥1/100 to <1/10): Depression, dizziness, headache, dyspnoea, abdominal pain upper, abdominal distension, diarrhoea, nausea, vomiting, ascites, rashes, pruritus, muscle spasms, arthralgia. Prescribers should consult country approved prescribing information for further information in relation to undesirable effects. **Overdose:** No case of overdose has been reported. In patients with normal bacterial flora, rifaximin in dosages of up to 2,400 mg/day for 7 days did not result in any relevant clinical symptoms related to the high dosage. In case of accidental overdosage, symptomatic treatments and supportive care are suggested. **Price and pack sizes:** PVC-PE-PVDC/Aluminium foil blisters in cartons of 28 or 56 tablets. Contact local distributor for price. **Legal category:** POM. **Prescribing information:** Medicinal product subject to medical prescription. **Marketing authorisation holder:** Norgine Pharmaceuticals Ltd. Norgine House, Widewater Place, Moorhall Road, Harefield, Middlesex UB9 6NS, UK. **Product licence number:** PL20011/0020. **ATC code:** A07AA11. **Date International Prescribing Information prepared:** 10 December 2012. **Company reference:** INT/XIF/1212/0160.

XIFAXAN® has varying availability and licensing internationally. Before prescribing, consult your country approved prescribing information, available from your local distributor or Norgine Ltd.

Adverse events should be reported to your regulatory agency. Adverse events should also be reported to your local distributor or Norgine Limited, Norgine House, Moorhall Road, Harefield, Uxbridge, Middlesex UB9 6NS, United Kingdom. Email: globalmedinfo@norgine.com

References: 1. Bass, N.M., *et al.* N Engl J Med, 2010; 362(12): 1071-81. 2. Norgine data on file. 3. Sanyal, A., *et al.* Aliment Pharmacol Ther, 2011; 34(8): 853-61. 4. XIFAXAN® 550 Summary of Product Characteristics, Dec 2012.

XIFAXAN® 550 is indicated for the reduction in recurrence of episodes of overt hepatic encephalopathy in patients ≥ 18 years of age.[4]

Rifaximin-α is licensed under the Trade Names of XIFAXAN®, TARGAXAN®, and others. Please note that Trade Names and licensed indications may vary throughout Europe and between countries.

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INT/XIF/0313/0182
Date of preparation: March 2013.

NORGINE

NEW DRUG STIMULATES IMMUNE SYSTEM TO KILL CELLS INFECTED WITH HEPATITIS B VIRUS IN ANIMAL TESTING

HEPATITIS B virus infection is able to be suppressed by stimulating the immune system and inducing the loss of infected cells, achieved through a novel drug recently tested in an animal model.

It was found that the immune modulator GS-9620, which targets a receptor on immune cells, reduced both virus level and the number of infected liver cells in chimpanzees chronically infected with the hepatitis B virus (HBV), according to the study tested at Texas Biomedical Research Institute's Southwest National Primate Research Center.

"This is an important proof-of-concept study, demonstrating that the therapy stimulates the immune system to suppress the virus and eliminate infected liver cells," said co-author Dr. Robert E. Lanford of Texas Biomed's Department of Virology and Immunology and SNPRC.

The report, appearing in the May issue of *Gastroenterology* and co-authored by scientists from Texas BioMed and Gilead Sciences, adds to the drug company's list of achievements in this specific field, having previously demonstrated that the same therapeutic techniques could induce a hepatitis infection cure in woodchucks infected with a virus similar to human HBV.

"This GS-9620 therapy represents the first conceptually new treatment for HBV in more than a decade, and combining it with the existing antiviral therapy could be transformative in dealing with this disease," Dr. Lanford added.

The drug binds a receptor named Toll-Like Receptor 7, which is present in immune cells. The receptor recognises invading viruses, and triggers the immune system to subdue viral replication and kill infected cells by the innate immune response and adaptive immune response respectively.

As current HBV infection treatment suppresses the replication of the virus while delaying the progression of liver disease, the fact that it is a lifelong therapy drives researchers to discover a full cure, with 1.4 million Americans alone chronically infected with HBV.

Two billion people have been infected with HBV, causing 620,000 deaths and 240 million chronic infections annually.

- World Health Organization

OPENING OF HCV SHELTER FOR TREATING HOMELESS

THE homeless of Montreal have gained access to a new resource, after The Old Brewery Mission has unveiled new facilities, which up to 14 men and women may use as a sanctuary while they undergo year-long Hepatitis C virus (HCV) treatment.

The initiative, called Pause-Santé, is Canada's first treatment centre dedicated to homeless patients, and is the result of a two-year long collaboration between the Old Brewery Mission and the Center and Association for People Living with Hepatitis C (CAPAHC), funded by the federal government and individual donations. The initiative's treatment is shown to boast a success rate of 70%, potentially slowing the rate of infection and helping those whose health is overlooked.

"It is impossible for someone who is homeless to receive effective treatment for Hepatitis C while living on the street," Old Brewery Mission's CEO and spokesperson, Matthew Pearce, said.

Mr. Pearce estimates that over half of Montreal's homeless population suffer from Hepatitis C. He added: "Pause-Santé enables patients to follow the prescribed treatment plan, which requires a strict treatment regimen that can last for up to twelve months. They can now imagine being cured of something they thought they had to endure."

According to the last count of homeless people by the Institut de la statistique du Québec, there were 28,214 people who had been to a shelter, soup kitchen or a day centre in Montréal, 12,666 of whom being without a place of residence for the past 12 months.

19-YEAR-OLD WOMAN NEARING FULL PREGNANCY UNDERGOES SUCCESSFUL LIVER TRANSPLANT, BABY DELIVERED SAFELY

A 19-YEAR-OLD girl, suffering from acute liver failure due to a hepatitis E (HEV) infection while in her 38th week of pregnancy, was saved following a 12-hour transplant surgery. Defying the odds, her child was also delivered safely, the surgery taking place at Sir Ganga Ram hospital, New Delhi.

The patient, Swati Kumari, went into spontaneous labour on the second day of her admission, falling into a potentially fatal coma after giving birth to her child. She required urgent liver transplantation as a life-saving procedure, however, this could have put the unborn child at risk of anaesthesia and death.

"If we had decided to conduct the delivery then the patient would have been at risk of severe uncontrolled bleeding because of thinning blood," Dr. Naimish Mehta, the liver transplant surgeon in question, said.

According to a Bi-Index Spectrography, a healthy person's brain activity is rated at over 90, while a patient's under anaesthesia may be reduced to between 40-60. Swati's reading was 17-18, a reading where brain death tests are advised.

"We were in a quandry whether to perform a transplant to save her or not," Dr. Mehta added. "Finally, on the family's permission, a 12-hour long transplant procedure was conducted. The girl's mother donated part of her liver."

HEV is the most common cause of liver failure during pregnancy. Considered dangerous for both the mother and unborn child, the mortality rate of such cases as Swati's ranges between 60-100%.

> "The first thing she asked after gaining consciousness was about the child. It's her first baby."
>
> *- Reena Devi, the child's grandmother*

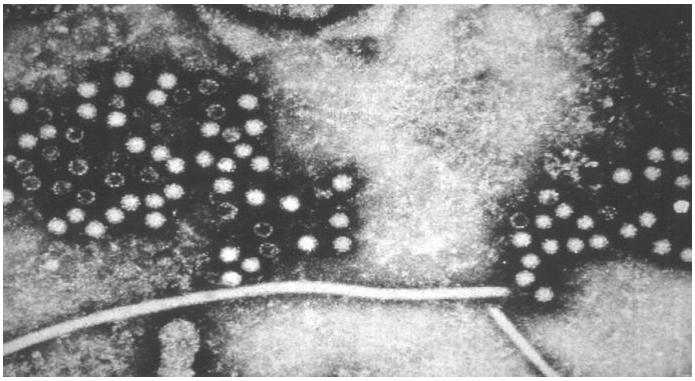
The girl's mother, Reena Devi, was next to her daughter when Swati regained consciousness within 48 hours of her surgery being completed, Mrs. Devi saying: "The first thing she asked after gaining consciousness was about the child. It's her first baby."

Most people with Hepatitis E recover completely, with overall case-fatality rate is about 1%. However, for pregnant women, Hepatitis E can be a serious illness with mortality reaching 10%–30% among pregnant women in their third trimester of pregnancy.

Pregnancy itself will not hasten the disease process or make it worse, although if the liver is already burdened and scarred with cirrhosis, the extra demands of pregnancy may predispose the expectant mother to a condition called acute fatty liver of pregnancy.

Although taking such precautions as drinking clean water, avoiding fruit grown outside, and not eating fruits without washing them thoroughly can help avoid infection, the body's immunity levels lower themselves during pregnancy, thereby increasing the risk of infection.

MISSION: OBM supplies healthcare to the homeless



HEPATITIS E: HEV is transmitted via food and water

"GAME-CHANGING" HEPATITIS C VIRUS DRUG DEVELOPMENT CONTINUES TO ACCELERATE AT BREAKNECK SPEEDS

AN avalanche of Hepatitis C (HCV) cures are around the corner, with three antivirals in different combinations both with or without interferon, as highlighted at the 2013 Conference on Retroviruses and Opportunistic Infections (CROI) in Atlanta, USA. The most prominent news is an astonishing game-changing treatment, which fully cures HCV within 12 to 16 weeks.

According to several studies on direct-acting antivirals (DAAs) for HCV monoinfection and HIV coinfection, several interferon alfa-sparing, all-oral regimes demonstrated cure rates of over 90% within 12 weeks of treatment for HCV monoinfected, including patients classed as "hard to treat."

Cure rates of 75% were also attained for HIV/HCV coinfected patients with the addition of an investigational HCV protease inhibitor (PI) simeprevir to peginterferon alfa and ribavirin. With the news that the U.S. Food and Drug Administration (FDA) has granted Priority Review to the New Drug Application (NDA) by Janssen for simeprevir (TMC435), it is likely that treatment will be available to the public within one to two years.

"This is a very important step bringing simeprevir closer to the market, making this therapy available to hepatitis C patients," Charlotte Edenius, EVP Development of Medivir said.

The study, from the University of California and the San Fransisco General Hospital, USA, is hoped to provide foundations for other breakthroughs, as the study presented at the conference bolstered growing evidence that that 12-week, interferon alfa-free regimens that are highly effective in curing HCV should soon be a reality.

In the same conference, there were a continued emphasis on pathogenesis, management, and prevention of long-term complications of the HIV disease and its therapies, disease, renal disease and vitamin D deficiency.

SWINE CELLS HOLD POTENTIAL TO POWER ARTIFICIAL LIVERS

A RESEARCH scientist has stated his belief that a line of pig liver cells called PICM-19 could perform many of the same functions as a human liver, in an interview with the *American Society of Animal Science*.

"There is no effective way at the moment to deal with the toxins that build up in your body," Dr. Neil Talbot, a Research Animal Scientist for the U.S. Department of Agriculture's Agricultural Research Service (ARS) said. "Their only option now is to transplant a liver."

Back in 1991, Dr. Talbot created PICM-19 from the cells of an 8-day-old pig embryo, finding the cell line to be "immortal", meaning it is able to divide an infinite amount of times. What seperates PICM-19 from the majority of immortal cell lines is that, rather than being derived from cancer cells, it is derived from epiblast cells. From this, Dr. Talbot can study the differentiations between the two.

Hepatocytes from the PICM-19 lines, which secretes bile, stores glycogen, controls blood glucose, processes vitamin D, metabolises cholesterol and fat, and cleans toxins from the blood. Potentially, PICM-19 cells could do the exact same thing inside an artificial liver.

> "There is no effective way at the moment to deal with the toxins that build up in your body... their only option is to transplant a liver,"
>
> *- Dr. Neil Talbot*

Though artificial livers are still in development, there are other applications for PICM-19 cells, having been used to study malaria, toxoplasmosis, and hepititis viruses. Meanwhile, several *in vitro* tests of artificial livers have been committed, as scientists in the ARS are working on ways to grow the PICM-19 without the need for "feeder cells", which holds PICM-19 in place and provides cell growth and maintenance.

LIFETIME COPIOUS COFFEE CONSUMPTION NOW LINKED TO REDUCED RISK OF RARE PRIMARY SCLEROSING CHOLANGITIS

DRINKING coffee may protect your body against primary sclerosing cholangitis (PSC), but not against primary biliary cirrhosis (PBC), according to data presented at Digestive Disease Week in Orlando, Florida, USA.

"Our data showed that coffee consumption over a lifetime was associated with reduced risk of PSC, but not PBC," said the study's author, Dr. Craig Lammert, a Mayo Clinic gastroenterologist.

"We think these diseases are much more different than we originally thought. Moving forward, we can look at what this finding may tell us about the causes of these diseases, and how to better treat them, and also give some feedback to patients as potentially modifiable risk factors."

Patients with PSC were both more likely to have never consumed coffee and significantly less likely to be current coffee drinkers compared to the control group (21% to 13% and 67% to 78% respectively). While PSC patients consumed an average of 50 cups monthly and had drunk coffee for 50% of their lives, controls reported to drink 78 cups per month and have actively consumed for 67% of their lives.

Researchers mailed a coffee questionnaire to 530 PBC patients, 348 PSC patients and 456 controls, including questions concerning the participants' current and lifetime coffee consumption.

PSC is an autoimmune disease of the bile ducts in the liver. The disease damages the liver to the point of liver failure, and can cause cancer of the bile duct, with a liver transplant currently the only known cure.

"While rare, PSC has extremely detrimental effects," Dr. Lammert said: "We're always looking for ways to mitigate risk, and our first-time finding points to a novel environmental factor that also might help us to determine the cause of this and other devastating autoimmune diseases."

Dubbed the world's most popular "psychoactive drug" by *New Scientist* magazine, the wide-ranging benefits and risks of drinking coffee is well-documented. Several studies comparing moderate coffee drinkers (defined as 3–5 cups per day) with light coffee drinkers (defined as 0–2 cups per day) found those who drank more coffee were significantly less likely to develop Alzheimer's disease later in life, as well as reducing the risk of cardiovascular disease.

CROHN'S DISEASE MEDICATION LINK TO ACUTE LIVER DAMAGE

BIOLOGICAL medications for those patients with Crohn's disease were found to be linked with acute liver damage by researchers from the University of California, USA.

According to the NHS website, Crohn's disease is a long-term condition that causes inflammation of the lining of the digestive system. Over time, inflammation can damage sections of the digestive system, resulting in additional complications, such as narrowing of the colon.

Medications called tumor necrosis factor-alpha antagonists, which treat such inflammatory conditions as Crohn's disease, ulcerative colitis, and joint and skin disorders, modifies patient immunity, potentially causing elevated liver enzymes and acute liver injury.

The tumor necrosis factor-alpha drugs are the newest type of medical treatment for Crohn's, controlling the inflammation of the gut by targeting the tumour necrosis factor. Patients can receive some intravenously while others can be self-injected.

Though no deaths were linked to the biologics, through reviewing the U.S. Drug-Induced Liver Injury Network, the California researchers located six clear-cut cases of drug-induced liver injury (DILI) during tumor necrosis factor-alpha antagonist treatment.

Twenty-eight more cases were analysed, all of which found reflected acute liver injury. The scientists concluded that doctors should monitor patient enzyme levels if any feelings of nausea, abdominal pain or fatigue whilst on the medication are experienced.

The data, published in *Clinical Gastroenterology and Hepatology*, tied DILI to treatments with the drugs infliximab, etanercept, and adalimumab.

FEATURED SUPPLIERS

Norgine is a specialty pharmaceutical company with an extensive pan-European presence, committed to developing products not only to treat life-threatening conditions, but importantly, to improve the quality of life for patients with a variety of acute and chronic illnesses.

The Philips healthcare sector is dedicated to providing solutions designed around the needs of clinical care teams and patients. We believe we can make a difference by removing boundaries in healthcare - with innovative and affordable technology solutions throughout the entire care cycle.

SOCIETIES

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AASLD - AMERICAN ASSOCIATION FOR THE STUDY OF LIVER DISEASES

ALEH - LATIN AMERICAN ASSOCIATION FOR THE STUDY OF LIVER

APASL - ASIAN PACIFIC ASSOCIATION FOR THE STUDY OF THE LIVER

CHBG - COCHANE HEPATO-BILAIRY GROUP

CLDF - CHRONIC LIVER DISEASE FOUNDATION

CSH - CHINESE SOCIETY OF HEPATOLOGY

DDW - DIGESTIVE DISEASE WEEK

DG RESEARCH

EAHC - EUROPEAN COMMISSION

EFSUMB - EUROPEAN FEDERATION OF SOCIETIES FOR ULTRASOUND IN MEDICINE AND BIOLOGY

ELPA - EUROPEAN LIVER PATIENTS ASSOCIATION

IASL - INTERNATIONAL ASSOCIATION FOR THE STUDY OF THE LIVER

ILCA - INTERNATIONAL LIVER CONGRESS

RUSSIAN NATIONAL INTERNET SOCIETY FOR INTERNISTS

UEGW - UNITED EUROPEAN GASTROENTEROLOGY WEEK

VIRTUAL LIVER NETWORK

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HEPATOLOGY

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EVENTS

APASL Liver Week

June 6-10, 2013

Singapore

2013 is the year that APASL returns to the place where it all began 35 years ago: Singapore. Since being formed in 1978, the APASL Conference has grown dramatically to become the largest hepatology congress in the Asia Pacific. Accessing APASL will enable you to improve your practice. Learn about the latest developments, state-of-the-art pioneering research updates and new technological advancements in the field. The Conference is also a unique opportunity for you to learn from the experts and network with colleagues from across the region.

British Association for the Study of the Liver Annual Meeting

September 4-6, 2013

London, UK

The British Association for the Study of the Liver is dedicated to the knowledge and understanding of the biology and pathology of the liver for the optimal care of patients. BASL members are composed of both scientists and clinicians, including GPs, and members from the pharmaceutical industry interested in liver physiology and pathophysiology. The BASL annual meeting consists of both clinical and basic science symposia, two state-of-the-art talks, update lectures on contentious issues as well as selected oral and poster presentations.

Viral Hepatitis Congress 2013

September 26-28,2013

Frankfurt, Germany

Building on the success of the 2012 Congress, this years congress will provide a relevant, meaningful and topical Scientific Programme reflecting the latest progress and innovations in therapeutic strategies and research in the management of viral hepatitis. The Congress will have a particular focus on developments in the practical aspects of disease management – an important consideration as we draw closer to the era of wider treatment choices and more individualised clinical decisions.

The Congress will continue to use the single session format, allowing delegates to attend all plenary presentations, and will also feature keynote lectures, clinical case studies, and industry symposia.

The 64th Annual Meeting of the American Association for the Study of Liver Diseases (AASLD)

November 1-5, 2013

Washington DC, USA

During The Liver Meeting 2013, over 8,000 hepatologists and hepatology health professionals from across the nation and around the world will gather in Washington, DC to exchange the latest liver disease research, discuss treatment outcomes, and interact with colleagues at the annual, must-attend event in the science and practice of hepatology.

COURSES

EASL Clinical School of Hepatology Course 20: Clinico-Pathological

June 6-7, 2013

Leuven, Belgium

The course is divided into a balanced blend of lectures on theoretical, practical and clinical case-based discussions presented during a residential course with limited attendance.

Each school allows time for:

Intense interaction

Time for personal discussion

Exchanges with a distinguished faculty

The schools contribute to the training of new generations of hepatologists and are a major element of the association, and is aimed at young fellows enrolled in hepatology-oriented departments or more experienced clinicians who want to be exposed to the newest trends in hepatology.

EASL Masterclass

November 15-16, 2013

Bordeaux, France

This 2-day Masterclass is the first event of its kind ever organised by EASL, with the course dedicated to "Hot Topics in Hepatology".

A combination of scientific content with training in other areas (i.e. presentation skills presented by a professional coach).

Workshop-like atmosphere.

Possibility for scientific/clinical presentations given by the participants.

Sessions organised as "pro & con – open discussions".

Networking opportunities between Young Investigators.

A unique occasion to meet senior EASL experts, Key Opinion Leaders (KOLs) in the field of Hepatology.

EASL Clinical School of Hepatology Course 21: Clinico-Pathological

December 6-7, 2013

Bern, Switzerland

This course is for physicians who are at the beginning of their specialisation in hepatology.

Attendees will review of the most important areas in the management of liver diseases.

Specific questions in the treatment of common liver diseases will be highlighted.

Discussion of cases with the faculty.

Application is open to young fellows under the age of 35 and/or still in training.

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July 2013 • emjreviews.com

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