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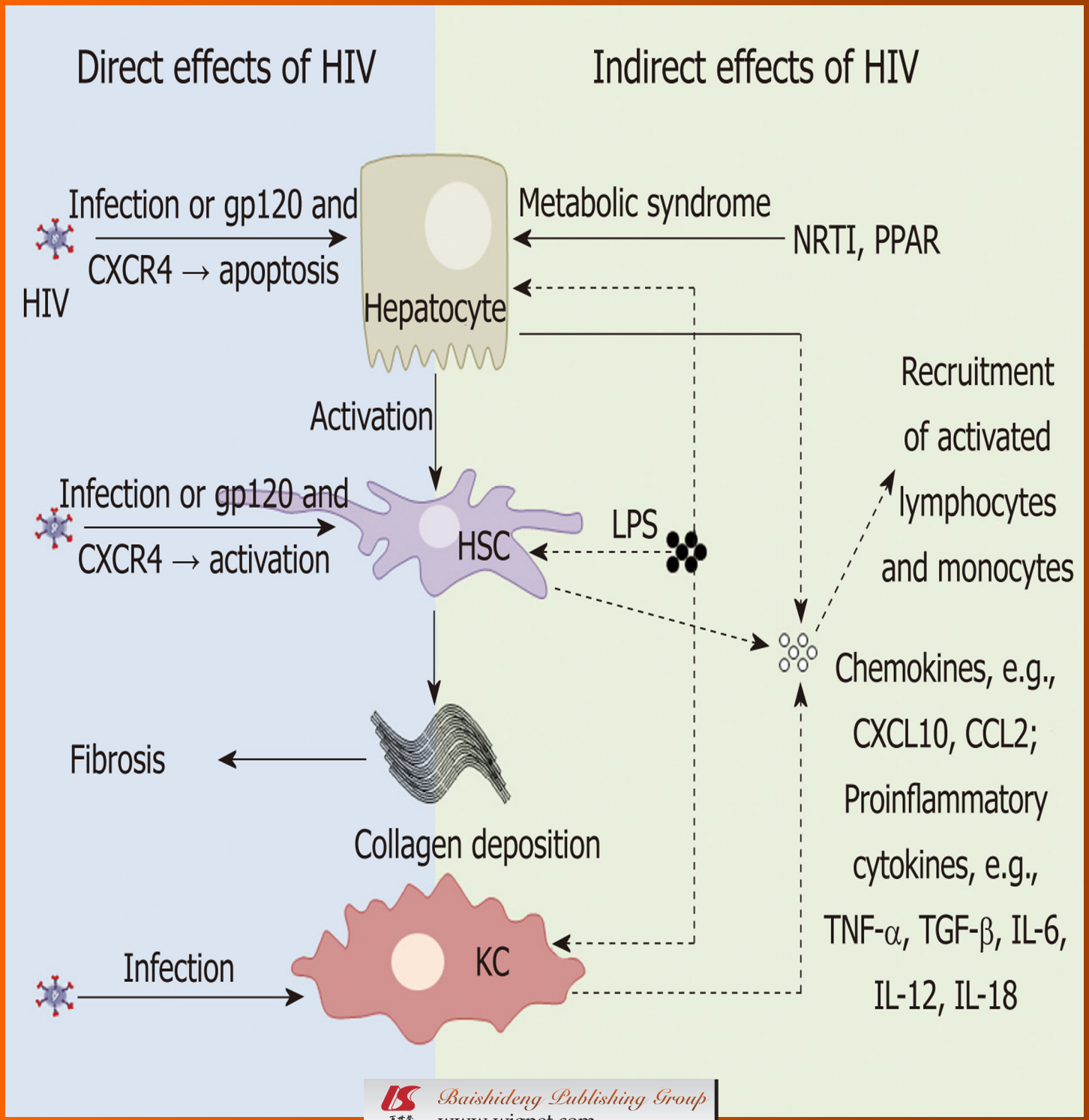

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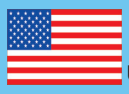

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EDITORIAL

Hepatic encephalopathy: An approach to its multiple pathophysiological features

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Abstract

Hepatic encephalopathy (HE) is a neuropsychiatric complex syndrome, ranging from subtle behavioral abnormalities to deep coma and death. Hepatic encephalopathy emerges as the major complication of acute or chronic liver failure. Multiplicity of factors are involved in its pathophysiology, such as central and neuromuscular neurotransmission disorder, alterations in sleep patterns and cognition, changes in energy metabolism leading to cell injury, an oxidative/nitrosative state and a neuroinflammatory condition. Moreover, in acute HE, a condition of imminent threat of death is present due to a deleterious astrocyte swelling. In chronic HE, changes in calcium signaling, mitochondrial membrane potential and long term potential expression, N-methyl-D-aspartate-cGMP and peripheral benzodiazepine receptors alterations, and changes in the mRNA and protein expression and redistribution in the cerebral blood flow can be observed. The main molecule indicated as responsible for all these changes in HE is ammonia. There is no doubt that ammonia, a neurotoxic molecule, triggers or at least facilitates most of these changes. Ammonia plasma levels are increased two- to three-fold in patients with mild to moderate cirrhotic HE and up to ten-fold in patients with acute liver failure. Hepatic and inter-organ trafficking of ammonia and its metabolite, glutamine (GLN), lead to hyperammonemic conditions. Removal of hepatic ammonia is a differentiated work that includes the hepatocyte, through the urea cycle, converting ammonia into GLN *via* glutamine synthetase. Under pathological conditions, such as liver damage or liver blood by-pass, the ammonia plasma level starts to rise and the risk of HE developing is high. Knowledge of the pathophysiology of HE is rapidly expanding and identification of focally localized triggers has led the development of new possibilities for HE to be considered. This editorial will focus on issues where, to the best of our knowledge, more research is needed in order to clarify, at least partially, controversial topics.

Key words: Liver failure; Hepatic encephalopathy; Ammonia and central nervous system; Hyperammonemia

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INTRODUCTION

Hepatic encephalopathy (HE) is a potentially reversible syndrome manifested by a wide spectrum of changes in consciousness, ranging from subtle behavioral abnormalities to deep coma and death. These neuropsychiatric conditions emerge as the major complication of acute or chronic liver disease.

The earliest known document to date on HE is the one in the Ancient Library of the University of Padova, Italy, authored by Giovanni Battista Morgagni in 1765. Morgagni's book of medicine described a case of liver cirrhosis associated with a possible HE complication[1]. Then and onwards, a long road has been traveled and much information has been compiled. There are many factors involved in HE, as a neurotransmission disorder in the central nervous system and in the neuromuscular system, that could lead to impairment of fine motor coordination, alterations in sleep patterns and cognition. Besides, in most cases only minor morphological changes were found in the brain; these are astrocyte swelling and Alzheimer type Ⅱ astrocyte[2]. Ammonium was initially indicated as the molecule responsible for the HE but may be incorrectly judged in advanced stages and may be only a scapegoat. Ammonia plasma levels are increased two- to three-fold in patients with mild to moderate cirrhotic HE and up to ten-fold in patients with acute liver failure (ALF)[3]. Ammonia is a neurotoxic molecule and its role has been widely studied. Hepatic and inter-organ trafficking of ammonia and its metabolite, glutamine (GLN), lead to hyperammonemic conditions. GLN metabolism *via* glutaminase is located in the intestinal epithelial cells and in the colonic epithelial cells[4]. Removal of hepatic ammonia is a differentiated work that includes the periportal hepatocyte, through the urea cycle and the perivenous hepatocytes converting ammonia into GLN *via* glutamine synthetase. When the liver is under pathological conditions, one or both types of hepatocytes could be damaged and the ammonia plasma level starts to rise. Minimal HE (formerly subclinical HE) is an initial clinical state with subtle abnormalities that can only be assessed by specific neuropsychometric and/or neurophysiological tests[5,6].

In 2002, a new classification of HE was proposed by the Working Party (World Congress of Gastroenterology)[7,8]. Basically, 3 types were proposed: type A, HE associated with acute liver failure; type B or HE associated with portal-systemic hepatocellular by-pass without intrinsic disease; and type C, or HE associated with cirrhosis and portal hypertension and portal systemic shunts. In this classification, minimal hepatic encephalopathy (MHE) was recognized as a subtype of C type HE. In 2009, the International Society for the Study of Hepatic Encephalopathy and Nitrogen Metabolism (ISHEN) completed this classification and added the experimental models associated with each type[9].

Although significant progress has been made in understanding the knowledge of molecular aspects involved in the development of HE, many questions remain unanswered and controversial issues need to be clarified. This editorial will focus on issues where, to the best of our knowledge, more research is needed in order to clarify, at least partially, controversial topics.

TOXIC SUBSTANCES

There are many neurotoxic substances and among them, related to HE, ammonia and manganese.

Ammonia in the central nervous system, muscle and kidney

It is well known that hyperammonemia (Figure 1), astrocyte changes and impairment in neurotransmission could lead to HE. Patients with liver injury concomitantly decrease the capability of detoxification of ammonia to urea, shifting this metabolic pathway to the muscular system and to the astrocyte in the central nervous system (CNS). In ALF, the progression of HE is associated with an increased risk of brain edema that could lead to brain herniation, a major cause of death. Therefore it is clear that when the ALF is complicated by HE, the risk to life is increased. The severity of HE is also associated with difference in survival[10]. The development of brain edema and ultimate death is a unique complication of ALF. There are factors that can be viewed with the ability to identify clinical situations with a higher possibility of developing brain edema, such as deep encephalopathy, hyperacute liver failure (acetaminophen-induced ALF), severe hyperammonemia, younger age and infection[11-13].

Currently, an area of controversy is to establish conclusively whether the percentages of deaths could be attributable to cerebral edema or to multiorgan failure. So, brain edema is a key for understanding the pathophysiology of ALF[14]. Chronic liver failure (CLF) spreads minimal to mild edema located surrounding the blood-brain barrier. The edema in CLF has very different consequences than those seen in acute liver failure[15].

In normal circumstances, the liver metabolizes all the ammonia coming from the small and large intestine. In the small intestine, the main source of energy in the enterocytes is glutamine, liberating ammonia[16].

Ammonia is sent to the liver through the portal vein. The liver metabolic stage of ammonia takes place in two major places, the periportal hepatocyte through the urea cycle takes care of the major part of the ammonia and the hepatocytes near the central vein transform the small quantities left into glutamine. Ammonia is also produced in healthy individuals by muscle and the kidney. These last two tissues have the ability to shift to ammonia detoxifying organs in the case of liver failure. Skeletal muscular tissue, due to its large size, becomes the main ammonia detoxifying organ in the case of chronic liver failure[17]. Muscular glutamine-synthase becomes important due to

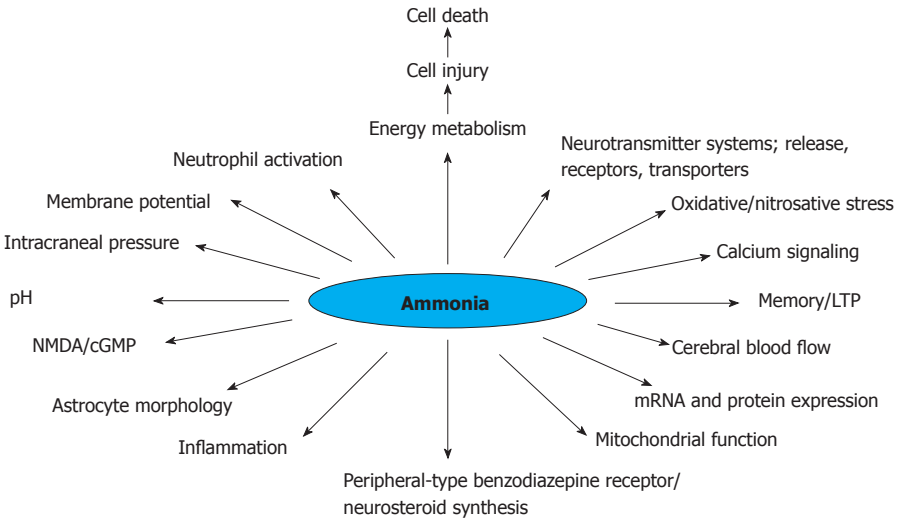

Figure 1 Changes induced by hyperammonemia (modified)[183]. NMDA: N-methyl-D-aspartate; LTP: Long term potentials.

the failing liver and brain metabolic activity[18]. Hypoproteic diet is a very common procedure in treating patients with liver failure, although a normal proteic diet may be metabolically more adequate and can be safely administered to the cirrhotic patient[19]. Hypoproteic diet may decrease the muscular mass and therefore the ammonia detoxifying ability. Recently, diet supplementation with branched chain amino acids has been shown to decrease minimal hepatic encephalopathy and to increase muscle mass[20].

The kidney is an organ capable of synthesizing and degrading ammonia. In normal conditions, the kidneys and liver interact closely to maintain the ammonia homeostasis. The kidneys are ammonia producers and only 30% of the ammonia produced is excreted with the urine. In liver failure and metabolic acidosis, the kidney has the ability to increase ammonia elimination to 70% of the produced ammonia[21,22]. Aquaporin 2 plays an important role in the regulation of water. Its expression is increased in the urine of cirrhotic patients, with a significant increase in patients with ascites, and is higher in compensated cirrhotic patients[23]. The Rh B and C glycoproteins group participates in the elimination of ammonia in the kidney[24,25]. Plasma ammonia concentration has been shown to be related to serum creatinine and the glomerular filtration rate. Renal dysfunction seems to increase cognitive impairment in patients with liver cirrhosis and might be implicated in the pathogenesis of hepatic encephalopathy[26]. Extra-hepatic ammonia metabolism appears to be the target of novel ways of treatment in chronic liver failure.

Manganese

Manganese is an essential trace metal that is involved in the metabolism of carbohydrates, lipids and proteins and has an important function as a cofactor for a number of enzymes[27]. It exists as divalent (Mn^{+2}) and trivalent forms in the plasma[28]. Both, divalent *via* an undefined transporter and trivalent Mn *via* the receptor-mediated endocytosis, may be transported into the brain, across the blood-brain and the blood-cerebrospinal fluid barriers and accumulate in the brain[29]. Manganese is neurotoxic, particularly affecting the actions of certain proteins (i.e., receptors) that interact with the neurotransmitter dopamine, probably *via* striatal dopamine depletion, N-methyl-D-aspartate (NMDA) excitotoxicity or oxidative/nitrosative stress. Moreover, three basic Mn cellular neurotoxicity mechanisms can be described: (1) mitochondrial dysfunction and disruption of energy metabolism; (2) the inflammatory activation of the glia; and (3) disruption of the synaptic transmission and neuronal-glial communication[30]. Furthermore, manganese has prooxidant activity and direct toxic effects have been observed in dopaminergic neurons. Manganese induces a decrease in the content of peroxidase and catalase in the substantia nigra. This metal produces active oxygen species, i.e., superoxide hydrogen peroxide and hydroxy radical, and also produces 6-hydroxydopamine or other toxic catecholamines. Manganese induces the autooxidation of dopamine followed by the formation of toxic (semi)quinones and dopamine depletion[30,31]. Besides this, apoptosis may play a role in the dopaminergic neurotoxicity associated with manganese, the first metal to be reported to induce this form of cell death[31,32]. The early biochemical events show the impairment of energy metabolism and the process may require new synthesis of proteins such as c-Fos and c-Jun. In addition, manganese induces phosphorylation of c-Jun and SEK1/MKK4 (c-Jun N-terminal kinase) and tyrosine phosphorylation of several proteins. Manganese activates specific signal cascades including the c-Jun N-terminal kinase pathway[32].

In chronic exposure with Mn^{+2}, a decrease of GABA concentration in discrete regions of the SNC as the globus pallidus, but not in substance nigra or hippocampus, was observed[33,34]. This effect on GABA levels could be due to the direct action of the Mn^{+2} on the expression of glutamic decarboxylase, an enzyme that regulates GABA synthesis[35].

Many authors suggest the participation of Mn^{+2} in the pathogenesis of the HE, in addition to ammonia[36-38].

The increased Mn^{+2} in plasma and as a deposit in the CNS is believed to be due to decreased elimination of Mn^{+2} *via* biliary excretion[39], and to increased systemic availability due to portal-systemic shunting associated with chronic liver disease[39,40]. Manganese (Mn^{+2}) brain deposits have also been demonstrated in a rat model of cirrhosis[41] and recently high plasma levels of Mn^{+2} and deposits of Mn^{+2} in the hippocampus of rats with MHE and altered integrity of the blood-brain barrier (BBB) (unpublished results) were demonstrated. These results also show that the sharp effects that Mn^{+2} produces on the aminoacidergic neurotransmitter can be opposite to the chronic effects. In this way, when the Mn^{+2} accumulates in the synapsis[34], it produces a consistent neuropathy with an excitotoxic effect, suggesting that the mechanism of glutamate is involved in the development of the pathology described by the Mn^{+2}. Manganese may operate at the same time on the hypothalamus GABAergic and glutamatergic neurons that integrate the self-regulation neuronal network.

Moreover, in patients it has been found that there is correlation between plasma levels of Mn^{+2} with deposits of Mn^{+2} in basal ganglia registered by MRI[37,38,41,42]. As Krieger *et al*[36] stated, in end-stage liver disease, the question is whether the increased hyperintensity of Mn^{+2} in the globus pallidus indicates chronic Mn^{+2} intoxication or is an adaptive process, leading to improved efficacy of astrocyte ammonia detoxification. Patients with chronic liver failure have shown increased plasma and brain levels of manganese, displaying many of the clinical and pathological features associated with manganese toxicity[37,38,43,44]. The divalent manganese and magnesium have some comparable important overlapping functions[45]. Therefore, the work of Bjerring *et al*[46] is an interesting approach that shows that hypermagnesemia does not prevent intracranial hypertension or affect the brain content of glutamate, glutamine, or aquaporin-4 expression. Interestingly, divalent manganese also offers fields of study to explore the pathogenesis of the HE more deeply.

ROLE OF ASTROCYTES

Cerebral edema is a response to injuries such as stroke and HE and it is well known that the initial step involves the swelling of astrocytes[47,48]. Mechanisms mediating the astrocyte swelling and the subsequent brain edema remain poorly understood[49].

Glutamine

In HE due to ALF, most frequently caused by drug toxicity and viral hepatitis, marked astrocyte swelling has been demonstrated by electron transmission microscopy in patients[50]. The main determinant molecule involved in astrocyte swelling, at least triggering this pathological condition, is ammonia. Astrocyte's glutamine synthetase (GS) plays a detoxifying role of ammonia, by amidation of glutamate (GLU) to GLN. In hyperammonemic conditions, GLN is increased in the astrocyte and astrocyte swelling occurs. In rats with induced hyperammonemia, astrocyte swelling was reduced when an inhibitor of GS, methionine sulphoximine, was administered[51]. This could explain why the edema is focused primarily on the astrocyte, because the neurons and capillaries and other membranes in general of the CNS have unusually low water permeability[52]. Glutamime could have a relevant role in oxidative stress/nitrosative as a critical factor in ammonia-induced cell injury[53-55].

Albrecht *et al*[56] proposed the theory of the trojan horse to explain the glutamine pathway that leads to astrocyte damage. In summary, glutamine is a "stealth" carrier of ammonia. Glutamine enters mitochondria *via* a histidine-sensitive glutamine carrier, which is potentiated by ammonia. Glutamine is then hydrolyzed by phosphate-activated glutaminase, located in the inner mitochondrial membrane, yielding glutamate and ammonia. Significant amounts of glutamine have been shown to be metabolized in astrocytes. Thus, the generation of mitochondrial ammonia may reach high levels, inducing the mitochondrial potential membrane, reactive oxygen species (ROS), with the potential consequence of morpho-functional oxidative damage of the mitochondria[57,58] and its respiratory chain[58].

Blood brain barrier

Astrocytes are important components of the BBB. Any change in astrocytes is also a potential change in the integrity of the BBB. Besides this, three major causes of astrocyte swelling are considered: (1) cellular edema; (2) vasogenic edema; and (3) aquaporins (AQP). Of these three, the latest are aquaporins which were described in 1992 as water channels and since then many isoforms have been identified[59]. So far seven AQPs: AQP1, AQP3, AQP4, AQP5, AQP8, AQP9 and AQP11 have been identified in various animal tissues in the CNS. *Via* real-time studies, including the above mentioned AQPs, we also observed the expression of AQP6 and AQP7 in mouse cortical neurons[60].

In CNS AQP1, 9 and 4 are mainly described and associated with brain edema. CNS AQP-4 and AQP-9 are mainly located in astrocytes, while AQP 1 is in the choroid plexus. AQP4 is the most important of the three mentioned in the development of cerebral edema observed in ALF or CLF. Two different isoforms of AQP4 have been shown to exist. Both isoforms are found in the brain[60]. AQP4 is highly expressed in the plasma membrane of astrocytes. It is plentiful in astrocyte cells bordering the subarachnoidal space, ventricles and blood vessels. High levels of AQP4 were noted in areas where astrocytes come into direct contact with capillaries, ependymal layer and pia. In addition, the basolateral membrane of the ependymal cells that line the subfornical organ is positive for AQP4. The sites of AQP expression in the brain suggest a role in the movement of water across the blood brain barrier and thus in cerebrospinal fluid dynamics and the formation of brain edema[60,61]. Importantly, the AQPs are also associated with apoptosis in the CNS. Considering the AQP localization in the astrocyte,

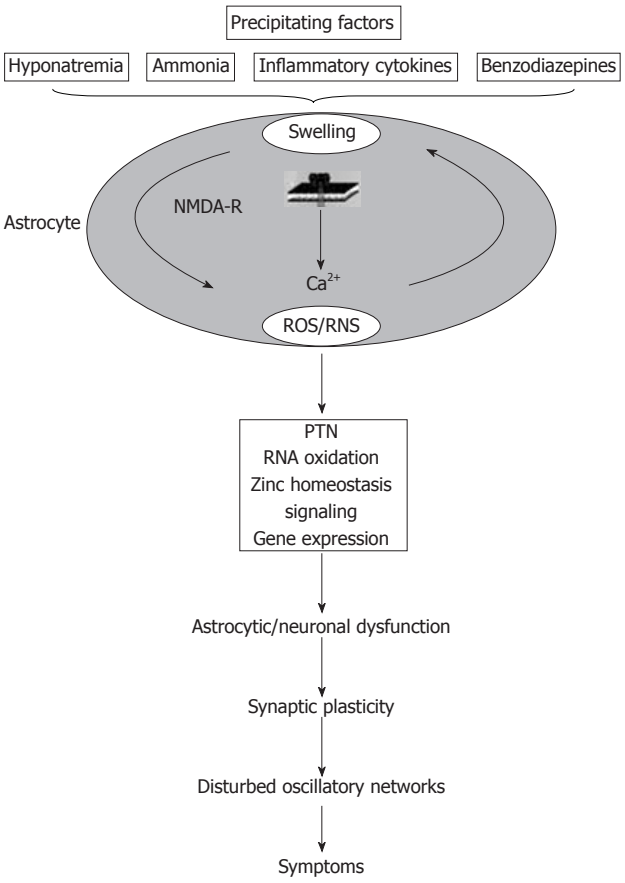

Figure 2 Interaction of oxidative stress, astrocyte swelling and cerebral ammonia toxicity. PTN: **Protein tyrosine nitration** (reproduced with permission)[64]. NMDA: N-methyl-D-aspartate; ROS: Reactive oxygen species.

the rectifying potassium channel Kir4.1 and the slow K^+ channel (Kslo), a dynamic view of Ast trafficking of fluid, can be constructed, likening it to ontogenetically modified epithelial cells[60-62].

Related to vasogenic edema, the ion channels, exchangers and transporters are important factors in cell volume regulation[59,60]. Changes in these systems may result in the loss of ion homeostasis[62] and the subsequent accumulation of intracellular water. These ion transporters and exchangers include the Na-K-Cl cotransporter-1 (NKCC1) that plays an important role in cell swelling/brain edema. Jayakumar *et al*[63] suggest that activation of NKCC1 is important in astrocyte swelling by ammonia and such activation is mediated by NKCC1 as well as by its oxidation/nitration and phosphorylation.

N-methyl-D-aspartate

Another point of view that should be included in the subject of swelling of astrocytes is the NMDA receptor pathway. As Häussinger *et al*[64] summarized, ammonia induces astrocyte swelling which is, in part, counteracted by volume-regulatory osmolyte depletion but leaves the astrocyte vulnerable towards swelling by a heterogeneous set of precipitating factors[64]. Astrocyte swelling involves NMDA receptor activation and the generation of ROS/RNS, which again favors astrocyte swelling. In this way, an autoamplificatory-signaling loop is generated. Consequences are PTN, oxidation of RNA, zinc mobilization and effects on gene transcription. This may impair glioneuronal communication and synaptic plasticity, resulting in disturbance of oscillatory networks, which finally accounts for the HE symptoms (Figure 2)[64].

Extracellular space

Another key component and little considered in HE is the extracellular space (ECS) diffusion parameters that may significantly affect communication between neurons as well as between neurons and glia. The diffusion of transmitters and other neuroactive substances through the ECS is also the underlying mechanism of extrasynaptic or so-called "volume transmission" in the brain[65,66]. Any decrease in the ability of neuroactive substances, ions or metabolites to diffuse through nervous tissue may represent a serious clinical problem due to the potential for disrupting brain function[67].

So far, it is not known whether astrocyte swelling, proliferation and hypertrophy during physiological and pathological states lead to persistent and functionally significant changes in ECS diffusion parameters. Astrocyte swelling is an early event in numerous pathological states, such as ischemia, hyponatremia and hepatic encephalopathy, and likely results from reduced extracellular osmolarity, elevated extracellular K1 concentration and/or glutamate[68,69]. When rapid cellular, particularly astrocyte, swelling occurs, water moves from the extra to the intracellular compartment. This causes a decrease in ECS volume and changes in the geometry of the intercellular spaces, e.g., during repetitive neuronal activity, ischemia, X-irradiation and experimental autoimmune encephalomyelitis[70-72]. Several experimental conditions have been shown to result in ECS volume changes due to the movement of water from the extra to the intracellular space. In particular, these include a decrease in the extracellular osmotic pressure[73] and the accumulation of excitatory amino acids[74]. After the initial phase of swelling, cells, particularly astrocytes, actively down-regulate their volume by regulatory volume decrease (RVD) and by a net release of KCl, taurine and other amino acids[74,75]; in this way, ECS volume can return to normal values. Even later, when glia become reactive, astrogliosis may result in the formation of additional and persistent diffusion barriers formed, for example, by the hypertrophy of fine glial processes or by an accumulation of macromolecules in the ECS (e.g., extracellular matrix proteins and cytokines) produced by neurons and glia[76]. Syková[76] found that astrogliosis results in the formation of persistent diffusion barriers. By this mechanism, glial cells could significantly affect neuronal excitability synaptic as well as extrasynaptic transmission and be involved in plastic changes. Finally, the upstream sensors and transducers of cell volume changes, a number of integral membrane proteins, including integrins, growth factor receptors and cytokine receptors, have been assigned roles as sensors of cell volume perturbations. Direct evidence for roles in osmo-/volume sensing is so far limited with respect to cytokine

receptors and calcium-sensing receptors; hence, only the possible roles of integrins and growth factor receptors need further studies[77].

BLOOD BRAIN BARRIER

BBB is a diffusion barrier, a specialized system of brain microvasculature, essential for the normal function of the CNS. BBB is composed by the endothelial capillary cells, the capillary basement membrane, astrocyte end-feet ensheathing the vessels and pericytes embedded within the basement membrane[78]. The most relevant features of BBB endothelial cells which differentiate them from other endothelial cells are tight junctions and sparse pinocytic vesicular transport. BBB breakdown or alterations in transport systems play an important role in the pathogenesis of many CNS diseases, such as HIV-1 encephalitis, Alzheimer's disease, ischemia, tumors, multiple sclerosis and Parkinson's disease[79]. The pathophysiology of HE is closely linked to changes in the BBB. HE and the BBB is a field in which many controversies and many aspects not yet understood remain even today. It is well known that ammonia plasma levels correlate with CNS damage in liver failure[80]. Ammonia blood concentration is of major importance in the evaluation of patients with known or suspected HE[81]. Since Loockwood *et al*[82] published the first relevant data in this topic, many answers and new questions arose. Is arterial or venous ammonia concentration by itself an early marker and/or predictor in HE initiation or progression? Is BBB ammonia permeability increased in hyperammonemic states and/or in HE? Different views based on different data from different experiments or clinical work place this issue at a dynamic controversy. Ong *et al*[83] and Kramer *et al*[84] conclude that arterial, venous and partial pressure of blood ammonia in patients correlated with HE severity. On the other hand, Goldbecker *et al*[85] in a study with patients with liver fibrosis conclude that no increased BBB ammonia permeability was demonstrated or related to the development of HE. In a historical view, not many years ago, it was considered that ammonium did not pass from blood to brain tissue. Later, Stahl's work[86] demonstrates increased ammonia blood brain extraction in HE. Then, BBB permeability and integrity changes were demonstrated with PET, showing that brain ammonia concentration was significantly higher (80%) in HE, measured with the isotope ^{13}N by Lookwood *et al*[87]. A key data for this asseveration could be the permeability surface area product (PS), still not completely elucidated, in the framework of BBB. It is of interest that under their working conditions, Goldbecker *et al*[85] found no changes on PS. Maybe we could see a little further with a little help from some experimental data. In a model of MHE[57] that displays moderate hyperammonemia and portal hypertension, it was clearly demonstrated that BBB has morphofunctional changes. Transmission electron microscopy revealed tight junction disruption in BBB capillaries in the hippocampal area, with mild edema of astrocytes and ECS. Moreover, a hippocampal increased number of capillaries and capillaries area per field was also documented. That means that in an experimental MHE, increased permeability and angiogenesis in the hippocampal area was seen. Furthermore, energy failing, pathological changes in the endothelial mitochondria and decreased nitric oxide synthase (NOS) activity were documented[57]. Jensen *et al*[88] suggest that BBB leakage induce microglial reactions. These involve modulation of immunomolecules, cytokine and growth factor gene expression. In this sense, Rodrigo *et al*[89] conclude that chronic hyperammonemia is sufficient to induce microglial activation and neuroinflammation during HE. It may be important to start paying attention to other components of the BBB in HE, components that remain largely unknown as pericytes and the blood-cerebrospinal fluid barrier[90]. Pericytes are involved in angiogenesis and its recruitment to the developing blood vessels and attachment of pericytes to the abluminal surface require heparin sulphate proteoglycan N-sulfation to retain platelet derived growth factor-beta (PDGFRb) homodimers and to activate the receptor PDGFRb signaling[91]. Recently, new data helped to introduce this new player in HE development. Armulik *et al*[92] describes a novel and critical role for pericytes in the integration of endothelial and astrocyte functions at the neurovascular unit and in the regulation of the BBB. The increased permeability occurs by endothelial transcytosis, a process that is rapidly arrested by the drug imatinib. Besides, by employing a metabolic profiling of serum samples by high-field 1H-nuclear magnetic resonance spectroscopy based on metabonomics approach, Jimenez *et al*[93] explored a methodology that could enhance diagnosis and monitor disease progression and patient response to treatment during MHE. It is also important to keep in mind that BBB is a very dynamic structure and could be damaged in different stages of HE. Our laboratory has shown[94] that the altered integrity of BBB gets back to normal in a HE-PVL induced model after portal pressure returns to a normal value. Moreover, in a group of animals with portal hypertension (PVL-induced) plus acetaminophen, behavioral changes and increased the BBB alterations were observed[95]. Therefore it can be concluded that the BBB is an area where further research is needed to integrate all components and generate a view of the totality in HE. To understand the pathophysiology of BBB, the data contributed to its components conducted in isolation, e.g., culture of astrocytes, are important but do not provide a comprehensive vision of BBB as a network. BBB, as a key access to the CNS is, without any doubt, a special issue that needs further exploration in order to know the specific role of the carrier or receptors in HE.

CENTRAL NERVOUS SYSTEM CELL DEATH

Perhaps one of the areas that may present greater potential for conflict is the cell death in the CNS in HE. Beyond what can be considered "physiological replacement", there is a need to determine whether there is a mechanism of lethal cell injury as part of the patho-

physiology of HE. Mitochondria are a major target in hyperammonemic conditions and could trigger different pathways. Many of them (a-f) have an important overlap.

Mitochondria, NMDA and energy metabolism

Acute exposure of brain preparations to pathophysiologically relevant concentrations of ammonia has numerous metabolic and neurophysiological effects, including alterations of synaptic inhibition and excitation[96] effects on cerebral energy metabolism and modifications of neurotransmitter-related processes. The effects on cerebral energy metabolism are associated with alterations of mitochondrial function[97], such as in reductions of brain ATP concentrations[98]. Two possible mechanisms have been proposed to explain ammonia-induced reductions in brain ATP concentration: a mechanism involving NMDA receptors and inhibition of the tricarboxylic acid cycle[99]. NMDA receptor is a mechanism identified with the potential to cause neuronal cell death in liver failure mediated by excitotoxicity, lactic acidosis, oxidative/nitrosative stress and the presence of pro-inflammatory cytokines[100]. The role of NMDA receptor in the reduction of ATP levels can be explained by two mechanisms: increased consumption of ATP due to activation of Na^+-K^+-ATPase and decreased synthesis of ATP in mitochondria due to impairment of calcium homeostasis[99]. NMDA receptor activation also results in mitochondrial swelling[101]. Ammonia-induced depletion of ATP is prevented by the administration of antagonists of NMDA receptor[102].

Acute ammonia intoxication leads to a rapid increase in intramitochondrial calcium content in the brain, followed by a reduction in the calcium capacity and calcium uptake rate. Injection of ammonia results in increased spontaneous calcium efflux from rat brain mitochondria and in potent inhibition of Na^+-induced calcium efflux. NMDA receptors in rat brain *in vivo* alters mitochondrial calcium homeostasis at several distinct steps and independent of the mitochondrial permeability transition (PTP)[103]. Proliferation of astrocytic mitochondria has been reported in conditions of chronic hyperammonemia, attributed to increased energy requirements[50]. In addition, chronic hyperammonemia similar in magnitude to that observed in end-stage chronic liver failure leads to down-regulation of functional NMDA receptor and prevents loss of ATP[99].

Calcium

The calcium ion has important roles in cell growth, signal transduction[104-106] and HE[107]. Most cell calcium is sequestered in the endoplasmic/sarcoplasmic-reticulum (ER/SR) membrane system and this sequestration produces an ER-to-cytoplasm gradient difference of greater than three orders of magnitude. Up to 50% of the calcium so sequestered is mobilizable by inositol triphosphate (IP3), which is formed through the hydrolysis of inositol 4, 5-biphosphate by phosphoinositol-dependent phospholipase C[108]. Calcium stored in the ER is not inert; however, a draining of calcium ions from the ER can cause secretion of resident proteins[109]. The transduction of the ER/SR calcium filling/depleting signal may be mediated in part by the phenomenon of "capacitative calcium entry"[110,111] in which a sustained transsarcolemmal calcium entry is coupled to a depletion of intracellular Ca^{2+} storage that is itself initiated by IP3. In HE, glutamate neurotoxicity has been well documented and glutamate is mainly mediated by excessive activation of the NMDA type of glutamate receptors[112].

Mitochondria are implicated at multiple stages of glutamate neurotoxicity, including the sequestration of Ca entering *via* the NMDA receptor, the bioenergetic collapse that triggers the ischemic release of glutamate, the generation of reactive oxygen species and the triggering of an apoptotic cascade under certain circumstances[112,113]. Excessive activation of NMDA receptor leads to the opening of its ion channel, allowing the entry of Ca from the extracellular space into the cytoplasm of the post synaptic neuron[103]. Much of this Ca entering the cell is sequestered by mitochondria[113]. The uptake of calcium by the mitochondria is driven by the mitochondrial membrane potential and will compete with the mitochondrial ATP synthase for protons. The abnormal cell calcium regulation has been implicated in cell-cycle progression[114,115] and may participate or precipitate cell death[116,117]. Calcium ATPase located on the ER/SR membrane (SERCA)[117,118] and, to a lesser extent, both plasma-membrane calcium ATPase and the mitochondria contribute to cell-calcium sequestration[118].

In neurons, intracellular calcium levels are controlled by ion channels in the plasma membrane such as NMDA receptors, voltage-gated calcium channels and certain α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors, as well as by calcium exchange pathways between the cytosol and internal calcium stores, including the SERCA and mitochondria[118]. Synaptic activity and the subsequent opening of ligand and/or voltage-gated calcium channels can initiate cytosolic calcium transients which propagate towards the cell soma and enter the nucleus *via* its nuclear pore complexes embedded in the nuclear envelope. Recently, it was described that in hippocampal neurons the morphology of the nucleus affects the calcium dynamics within the nucleus[119-121].

The action of the calcium ion in the nucleus opens up new research possibilities in the interplay of calcium in HE with cellular structures, receptors, SERCA, mitochondria and nucleus[122]. The SERCA isogene group belongs to a family of ion-motive ATPase ("P" ATPase) whose reaction cycle involves the generation of an aspartyl-phosphate intermediate and is represented by three isogenes SERCA overexpression, produced through the use of an adenovirus vector or *via* a transgenic mouse model[123,124], which has suggested the existence of a phenotype consistent with facilitated intracellular calcium sequestration[123]. This finding supports the functional role of SERCA previously attributed to it through *in vitro* experimentation. The calcium compartmentalized in the SR may participate in cell-cycle progression. Moreover, the experimental SERCA blockade leads to a quiescent

G0-like state[115].

Altered bioenergetics and oxidative stress are also thought to play a major role in mitochondrial disorder inducing the PTP, involving the mechanism of ammonia neurotoxicity. The PTP is a Ca^{2+}-dependent, cyclosporin A sensitive process due to the opening of a pore in the inner mitochondrial membrane that leads to a collapse of ionic gradients and ultimately to mitochondrial dysfunction. Many of the factors that facilitate the induction of the MPT are also known to be implicated in the mechanism of HE, including free radicals, Ca^{2+}, nitric oxide, alkaline pH and glutamine[124]. Our laboratory has shown that oxidative stress was involved in hippocampal damage and curcumin protects against this oxidative stress in experimental MHE. These protective effects may be attributed to its antioxidant properties[125].

Peripheral-type benzodiazepine receptors

Another mitochondrial membrane receptor associated with alterations of cerebral energy metabolites and astrocytes swelling is the mitochondrial "peripheral-type" benzodiazepine receptors (PTBRs)[126]. PTBRs are 18 kDa mitochondrial membrane proteins and with a still elusive function in cell death. PTBRs are located in the mitochondrial membrane cells in the periphery and in astrocytes and they are not allosterically coupled to $GABA_A$ receptors[127]. Itzhak *el al*[128] described an increased PTBRs site in brain in mice with acute hyperammonemia resulting from toxic liver injury. Exposure of glioma cells in culture to PTBRs agonists results in proliferation and swelling of mitochondrial[129], phenomena which have been described following administration of ammonia resins to animals with chronic liver failure. PTBRs cause increases in state Ⅳ and decreases in state Ⅲ mitochondrial respiration rates, resulting in a significant decrease in the respiration control[130].

The peripheral-type benzodiazepine receptor is involved in control of Ca^{2+}-induced PTP opening in rat brain mitochondria. PTP opening is important in mitochondrial events leading to programmed cell death[113,131]. In addition to a specific anti-PBR antibody, delayed Ca^{2+}-induced dissipation of membrane potential (ψ_m) and diminished cyclosporine A-sensitive Ca^{2+} efflux, which are both indicative for the suppression of PTP opening[132]. Moreover, anti-PBR antibody caused partial retention of Ca^{2+} in the mitochondrial matrix in spite of ψ_m dissipation and reduced activation of respiratory rate at Ca^{2+}-induced PTP opening. A release of pro-apoptotic factors, apoptotic inducing factor (AIF) and cytochrome *c*, from rat brain mitochondria was shown at threshold Ca^{2+} load. Anti-PBR antibody blocks the release of AIF but does not affect the cytochrome *c* release. The endogenous PBR ligand, protoporphyrin Ⅸ, facilitated PTP opening and phosphorylation of the mitochondrial proteins, thus inducing effects opposite to anti-PBR antibody[132]. It is also interesting that in experimental ALF, moderate hypothermia prevents cerebral edema and reduces the up-regulation of astrocytic PTBRs[133].

Central nervous system cell death

A recent study from our laboratory has shown astrocyte death associated with mitochondrial dysfunction in hippocampal CNS in an experimental model of MHE[58]. The presence of DNA fragmentation with a higher ratio of the Bcl-2 family members Bax/Bcl-xL in the outer mitochondrial membrane and cytochrome c release indicate the presence of apoptosis. A marked decrease of cytochrome oxidase (complex Ⅳ of the electron transport chain) was also observed; mitochondria from these animals showed less ability to maintain the membrane potential ($\Delta\Psi_m$) stabilized[58].

Ammonia induced neuronal and oligodendroglial death, triggered apoptosis and activated caspases and calpain. Probably due to calpain activation, ammonia caused the cleavage of the cyclin-dependent kinase 5 activator, p35, to p25, the cdk5/p25 complex known to lead to neurodegeneration[134]. It is important to note that hyperammonemia induces different degrees of cellular damage and it mainly depends on the stage of development and whether the exposure is acute or chronic. Hyperammonemia during development is associated with neuronal cell loss and cerebral atrophy that leads to mental retardation and cerebral palsy in pediatric patients. Among the various pathogenic mechanisms involved, alterations in axonal and dendritic growth and cerebral energy have been demonstrated[135]. Acute hyperammonemia also results in decreased activities of free radical scavenging enzymes and again, free radical formation due to ammonia exposure is prevented by either NMDA receptor antagonists or NOS inhibitors[53,136].

Autophagy of mitochondria and/or selective mitophagy most likely play an important role in removing damaged organelles. Although cell death is often accompanied by autophagy, it is still controversial whether autophagy promotes or prevents cell death. When damaged mitochondria are removed by autophagy, this will prevent cytochrome c release and activation of caspases, whereas a block in autophagy would promote caspase-dependent cell death[137].

Studies containing opposite views have been reported. On one hand, Kosenko *et al*[138] did not observe apoptosis in an experimental model of acute intoxication with large ammonia doses. The animals (rats) were sacrificed 11 min after injection of ammonium acetate. This acute ammonia intoxication study did not affect caspase-9 or caspase-3 activities, the mitochondrial membrane potential remained unaltered in non-synaptic brain mitochondria, indicating that ammonia did not induce PTP formation in brain *in vivo*. Also, the nuclear level of p53 did not change, whereas its cytoplasmic level increased approximately two-fold. In agreement with the theory that translocation of the p53 from cytosol to nuclei is an essential step for induction of apoptosis, the authors did not find apoptotic nuclei in the brain. They conclude that this data supports the idea that ammonia neurotoxicity does not involve apoptosis and points to impaired p53 transfer from cytoplasm to nuclei as a possible preventer

of apoptosis. They also reported disturbances in the mitochondrial electron transport chain in brain mitochondria from rats injected with ammonia. On the other hand, in our laboratory, an opposite view in a chronic model of MHE PVL-induced cellular death was registered[58]. We found a 5 times higher expression of the proapoptotic member of the Bcl-2 family, Bax. Moreover, an increase of 2.3-times in the number of TUNEL-positive, simultaneously marked with GFAP was also observed in the hippocampal area.

A significant decrease in the mitochondrial respiratory control of MHE animals and a significant decrease (46%) of cytochrome oxidase (complex Ⅳ) were recorded. In addition, these mitochondria showed less ability to maintain membrane potential ($\Delta\psi_m$) (28% lower). The swelling experiments showed that mitochondria from MHE animals spontaneously tend to swell. This and the decreased $\Delta\psi_m$ could indicate an MPT mechanism. We conclude that the cellular death could be regarded as one of the earliest steps in the development of experimental MHE. Even although they are different experiments, e.g., an acute with animal sacrifice at 11 min after administration of ammonium and the other, chronic with sacrifice at 10 d of the PVL, the results, seemingly opposite, can be complementary with a broader view.

Programmed cell death

Cell swelling was shown to activate the release of excitatory amino acids, glutamate and aspartate, in astrocyte cultures[139-141]. In the brain, excitatory amino acids can promote neuronal cell damage *via* over-activation of glutamate receptors and could lead to cell death. One hypothetical route for astrocyte swelling-activated release of organic osmolytes is the ubiquitously expressed volume-regulated anion channel(s) (VRACs), which is activated by cell swelling and is permeable to a variety of inorganic and small organic anions, including the amino acids taurine, glutamate and aspartate. Cell shrinkage is a morphological feature of apoptosis, known as apoptotic volume decrease (AVD). AVD is an isosmotic cell shrinkage which is seen early after apoptotic stimuli and seems to be a prerequisite for apoptosis[142].

Under physiological conditions, brain cells, when subjected to osmotic fluctuations, will undergo regulatory volume increase/decrease (RVI/RVD) to achieve homeostatic balance with neurons in the brain being additionally protected by the BBB. However, during AVD following an apoptotic trigger, the cell undergoes anisotonic shrinkage that involves the loss of water and ions, particularly monovalent ions e.g., K^+, Na^+ and Cl^-. AVD results from a loss of KCl *via* $K^+$and Cl^- channels and concomitant loss of water[143]. VRAC seems to be the anion channel involved in AVD in several human and animal cells. Various K^+ channels, including inner membrane mitochondrial K^+, appear to be involved in AVD, depending on the cell type or stimulus used[144]. Among these are the two-pore K^+ channels that have been implicated in RVD in multiple cell types[145]. Inhibitors of swelling-activated K^+ and Cl^- channels attenuate AVD and several groups have suggested that the same channels are involved in RVD and in AVD[146]. Moreover, apoptotic cells exhibit an augmented RVD response that could reflect that volume-sensitive channels are more sensitive to cell swelling[147]. Whether the sensor and trigger mechanism for RVD and AVD have identical components with a different set point is still under discussion. In most cells, Cl^- conductance (gCl) is significantly lower than K^+ conductance (gK) under steady-state. Consequently, an increase in gK^+ alone results in K^+ loss and Na^+ uptake and not in KCl loss and cell shrinkage. This means that activation of VRAC is a necessity for initiation of AVD and in congruence with this, depolarization of the membrane potential during AVD has been demonstrated in several cell types[148-150]. Therefore, VRAC is a principal pathway for mediating organic osmolyte release in astrocyte swelling that could lead to cellular death[151].

Sharing pathways: Mitochondrial encephalopathy, lactic acidosis and stroke-like episodes

Although stroke is a pathological entity by itself, it shares some pathways or an important part of a pathway with HE. Mitochondrial encephalopathy, lactic acidosis and stroke-like episodes (MELAS) are a pathology that share many altered pathways with HE, such as cerebral edema, BBB abnormalities and alterations in brain mitochondria respiratory chain. MELAS is one of the most common and widely studied maternally inherited mitochondrial diseases that is frequently associated with the m.3243A > G point mutation in the mitochondrial tRNA LeuUUR gene[152]. The clinical phenotype is multisystemic but the triad of lactic acidosis, seizures and stroke-like episodes remains crucial to the diagnosis and reflects the complex and unique pathogenesis of this syndrome[153].

The levels of ventricular CSF lactate correlate with the severity of neurological impairment[154]. Besides, the increase in brain ROS activity was demonstrated in MELAS[155], as it was also clearly documented in HE[53]. MELAS experimental studies in cybrids showed that severe defects in protein synthesis and respiratory chain function segregate with the mutation, although the pathogenic threshold is high; more than 90% mutant mtDNAs are required to cause dysfunction[153]. However, most MELAS patients have well below 95% mutant mtDNA, suggesting that the data from cybrid studies may not be directly extrapolated to the clinical status. As in HE, the pathogenic mechanism of strokes and vasogenic edema cannot be explained by the available data. The same challenge for MELAS and HE is that without a better understanding of the pathogenesis, rational therapeutic intervention has not been possible.

The strokes, non-ischemic in origin and therefore called "stroke-like episodes", are at least partially reversible and do not conform to distribution of large cerebral arteries, but rather affect small arterioles and capillaries of the cortex while sparing the adjacent white matter[155]. The recurrent strokes are associated with vasogenic edema, as demonstrated by MR diffusion weighted imaging studies, suggesting that they may be due to increased

permeability in the BBB, perhaps caused by mitochondrial dysfunction in the endothelium of cerebral small vessels[156]. Furthermore, the accumulation of ventricular lactate indicates severe energy failure in the brain due to mitochondrial dysfunction and acute hypoxia during stroke-like episodes. Translational defects of the mitochondrial respiratory chain subunits and pathological alterations in the microvasculature and in BBB components have been documented in patients with MELAS, thus supporting the notion that BBB permeability may be increased due to mitochondrial respiratory failure in the cortical microvasculature[157], as it has also been demonstrated in MHE[57]. In MELAS, severe defects of respiratory chain complexes in immortalized endothelial cells and astrocytes as well as in primary astrocytes harboring the m.3243A > G mutation were associated[158,159]. Furthermore, the defects in EC cells with the MELAS mutation correlate with lower transendothelial electrical resistance, indicating increased permeability of the BBB endothelial cells[160] as it was also documented in experimental MHE[94].

NEUROENDOCRINE AXIS

Little is known about neuroendocrine changes that occur in portal-systemic hepatic encephalopathy and/or MHE. Scorticati *et al*[161] studied plasma prolactin (PRL) levels in our laboratory and the involvement of hyperammonemia, nitric oxide (NO) and dopaminergic and adrenergic systems in the control of this hormone secretion in a male rat model of mHE. The authors conducted *in vivo* studies to determine plasma ammonia and PRL levels, dopamine (DA), dihydroxyphenylacetic acid (DOPAC), epinephrine and norepinephrine content in medial basal hypothalamus (MBH) and anterior pituitary (APs). In addition, NOS activity and protein expression were evaluated in APs. In *in vitro* studies, the APs from intact rats were incubated with different doses of ammonia and PRL secretion was determined. In *ex vivo* studies, the APs from normal and PH rats were incubated in the presence of ammonia and/or a NOS inhibitor, NG-nitro-L-arginine-methyl ester (L-NAME), and PRL secretion was determined. It is well known that this model, PVL, has moderate hyperammonemia but the rest of the data obtained was a bit surprising, like a decrease in plasma PRL levels, a significant increase in norepinephrine content in both MBH and AP and an increase in NOS activity and NOS protein expression in APs. Also, *in vitro* the authors found reduced PRL secretion from APs and the presence of L-NAME, an inhibitor of NOS, abrogated the inhibitory effect of ammonia on PRL secretion from APs from control and MHE rats. Authors conclude that plasma PRL levels were decreased in MHE rats probably due to the high ammonia levels. The central noradrenergic system could also mediate this decrease. Also, the increase in NOS activity and/or content in AP induced NO production that directly inhibited PRL secretion from the AP, without the participation of the dopaminergic system. These results demonstrate the alteration of the neuroendocrine axis in a model of MHE and opens an interesting area of study that remains largely unexplored[161].

NEURONAL ENVIRONMENT, NEUROPLASTICITY, BEHAVIOR AND MEMORY

Pioneer investigations about memory processes began in 1957. Brenda Milner stated that certain forms of memory were stored in the hippocampus and the medial temporal lobe[162]. Since then, many works have described the relationship between the neuron and its environment in normal and pathological states. Investigations led by Kandel[163] have shown how the process of neuroplasticity modifies and adapts neuronal behavior in response to learning stimuli and transforms short term memory into long term memory. In the case of HE, although one of most important roles has been given to ammonia metabolism in astrocytes, many other mechanisms have been proposed such as gabaergic theory, manganese, receptor changes and modifications in permeability of BBB, among others. The NMDA receptor is the predominant molecular device for controlling synaptic plasticity and memory function[164]. Nonetheless, little is known about how these processes can modify neuronal behavior or how such behavior might be expressed by the neuron. In the early nineties, our workgroup found changes in norepinephrine uptake in CNS in portal vein of rats with experimental prehepatic portal hypertension induced by portal vein ligation (PVL)[165] and baro-reflex alteration[166]. These results triggered several other questions. Would changes in the peripheral nervous system be the expression of changes at the central nervous system level?

We found changes in norepinephrine´s metabolism in discrete diencephalic regions in PVL rats[167]. Moreover, ascending along the brain stem, we arrived at the hippocampus where we found changes in capillaries and astrocytes[168]. As described above, many molecule concentrations (in plasma, cerebrospinal fluid, *etc*) are modified or differentially expressed in portal hypertension acting on neurons, the glia, or both. It remains unclear whether BBB modifications selectively act on all those molecules or if these might modify the BBB integrity. Besides, inflammation and immunity have also been given general consideration regarding the pathophysiology of HE. In the words of Yirmiya[169], "in addition, immune-like processes are involved in tissue remodeling, which is a continuous process of dynamic alterations in a specific tissue or a whole organ that facilitates morphological and functional adaptations to the ever changing environmental demands". Therefore, immunomodulation of learning, memory, neural plasticity and neurogenesis are chapters worth considering in HE[169].

Reichenberg *et al*[170] found that experimental immune activation by endotoxin produced alterations in emotional states and decreased performance in memory tests. Moreover, endotoxin-induced changes in emotional parameters were found to have a complex time course,

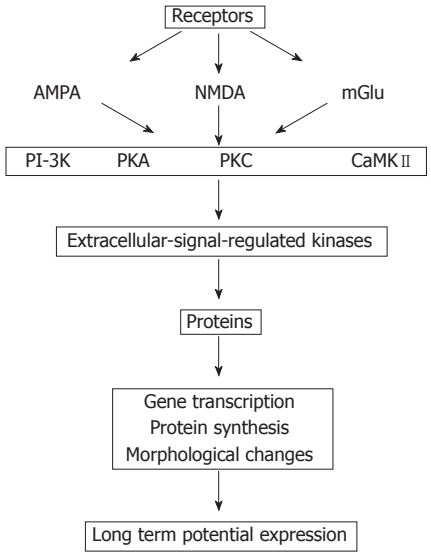

Figure 3 Proposed sequences that finally induce changes in the long term potentials, which is down regulated in hyperammonemia (modified from Lynch[182]). AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; NMDA: **N-metil D-**aspartato; mGlu: **Metabotropic glutamate receptors**; PI-3K: **Phosphatidylinositol 3-kinases; PKA and PKC: Protein kinase A and C**; CaMK Ⅱ: **Ca^{2+}**/calmodulin-dependent protein kinases Ⅱ.

characterized by an early elevation in anxiety levels followed by an increase in depressed mood[170]. Evidence suggests that glia and the soluble form of tumor necrosis factor-α (TNF-α) may be involved in a specific form of synaptic scaling[171,172]. Accumulating evidence suggests that inflammatory cytokines, such as TNF-alpha, interleukin (IL)-1 and IL-6, meditate these disturbances. In animals, sickness behavior can be induced by administration of cytokines. Antagonists or synthesis blockers of cytokines abolish sickness behavior in response to various immune challenges and a similar response was also demonstrated in humans[170].

Learning impairment is present in cirrhotic patients with neuropsychological abnormalities consistent with attention deficit secondary to MHE[173]. Patients with MHE score lower than controls in memory tasks, predominantly due to deficits in attention and visual perception[174]. Hilsabeck *et al*[175] found that cognitive impairment on a visuoconstruction task ranged from 9% to 38% of patients on a measure of complex attention, visual scanning and tracking, and psychomotor speed. The greater viral C hepatitis (HCV) disease severity indicated by liver fibrosis was associated with greater cognitive dysfunction. Objective cognitive impairment was not related to subjective cognitive complaints or psychiatric symptoms. These findings suggest that a significant number of patients with chronic HCV have experienced cognitive difficulties that may interfere with activities of daily living and quality of life[175]. Hepatocyte growth factor/scattered factor (HGF/SF) possess motogenic activity, which is essential for normal development of other organ systems and is a conserved mechanism that regulates trans-telencephalic migration of interneurons in embryonic state[176]. Porto-systemic encephalopathy (PSE) is often preceded by ascites and this condition is characterized by gastro-intestinal capillary increased permeability, endotoxin translocation, activation of cytokines and failure to endotoxin removal. Lipopolysaccharide is elevated in portal blood and activates Kupffer cells through the CD14/toll-like receptor-4 complex to produce ROS *via* NADPH oxidase[177,178].

Thus, it might be possible that the chain of events triggered by endotoxin could lead to a derangement of neuronal functioning. Some evidence supports the idea that both direct and indirect effects of lipopolysaccharides/endotokinemia might be related to neuron plasticity and memory changes, as well as mood alterations, as seen in PSE. Currently, there is no evidence that directly supports the relationship between HE, lipopolysaccharides and neuron derangements. HE is a neurological complication that affects attention and memory. Experimental animal models have been used to study HE, the most frequent being the porto-caval shunt (PCS). In order to investigate learning impairment and brain functional alterations in this model, Méndez *et al*[179] assessed reversal learning and neural metabolic activity in a PCS rat model.

PCS and sham-operated rats were tested for reversal learning in the Morris water maze. Brains were then processed for cytochrome oxidase histochemistry. The PCS group had reversal learning impairment and a reduction in cytochrome oxidase activity in the prefrontal cortex, ventral tegmental area and accumbens shell nucleus. These results suggest that this model of portosystemic HE shows learning impairments that could be linked to dysfunction in neural activity in the prefrontal cortex and regions involved in motivated behavior[179].

The memory consolidation at the cellular level is based on the facilitation or attenuation of transmission at specific synapses. Besides, antibodies against L1 glycoprotein and neural cell adhesion molecules (NCAM) can impair memory consolidation. Moreover, learning is followed by altered expression and glycosylation of L1 and in NCAM[180].

Addition of 8 Br-cGMP to slices treated with ammonia restores both phosphodiesterase activation and maintenance of long term potentials (LTP)[181]. Impairment of LTP in hyperammonemia may be involved in the impairment of the cognitive function in patients with HE (Figure 3)[182]. Although many theories have been proposed, the intimate mechanism whereby a liver pathology triggered HE remains to be known. There is insufficient evidence to determine if moderate hyperammonemia and/or portal hypertension, major complications in liver disease, or if one or more mediators (for instance, manganese, ammonia, HGF, TGFB, interleukins, reactive oxygen species, neurotransmitters, aquaporins, *etc*) are responsible or co-responsible for triggering MHE.

CONCLUSION

We have reviewed some of the mechanisms involved in the development of HE. We believe that these issues have areas of interest and potential controversy. Importantly, experimental models can study different

stages of HE. The earliest form of HE is MHE. We consider that a comprehensive study of MHE will be useful to identify the many complex and interrelated pathways and learn its sequence and the pathways that are activated from the beginning to the later stages.

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EDITORIAL

Liver diseases in developing countries

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Abstract

Liver diseases are an important and largely neglected health issue in low and middle income countries, which carry the highest burden. In this Topic Highlight, experts review hepatitis B and E, alcoholic liver disease, hepatic diseases in human immunodeficiency virus -infected individuals, hepatocellular carcinoma. Numerous gaps in our knowledge that need to be filled are outlined and feasible solutions to the several problems related to diagnosis and management of liver diseases in developing countries are suggested.

Key words: Liver diseases; Developing countries; Hepatitis; Hepatocellular carcinoma; Cirrhosis

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INTRODUCTION

Liver diseases are an important and largely neglected health issue in developing countries, which carry the highest burden but receive little attention. The scientific literature is engulfed with manuscripts describing the apparently high load of liver diseases in developed, high income countries, and one can get the misleading impression that liver diseases are prominent in those very countries where they are not.

The aim of this Topic Highlight is to raise awareness of the burden of liver diseases in low and middle income countries.

TOPIC HIGHLIGHT

Hepatitis B virus infection is a huge health problem in many low and middle income countries. Franco *et al*[1] (Department of Public Health, Tor Vergata University, Rome, Italy) outline that the most common route of infection remains vertical transmission from mother to child, and that screening of all pregnant women and passive immunization with human hepatitis B immunoglobulins are not affordable for many developing countries. Even anti-hepatitis B vaccination is not affordable for some countries. Abbas *et al*[2] (Department of Hepatogastroenterology, Sindh Institute of Urology and Transplantation, Karachi, Pakistan) advocate for a definition of the minimal requirements for delivery of care to patients with chronic hepatitis B and call for the establishment of research institutions that can ascertain the natural history and response to treatment in developing countries. They rightly outline that the mere extrapolation of treatment protocols used in developed countries is of little use due to lack of resources.

Teshale *et al*[3] from the Centers for Disease Control and Prevention, Division of Viral Hepatitis, Atlanta, United States, review hepatitis E epidemiology and draw attention to the fact that this virus is responsible for major outbreaks of acute hepatitis in developing countries, especially in Africa and Asia. Although more than one vaccine candidate has been shown to be safe and efficacious in clinical trials, none are currently available for use in the very countries where they are most needed. There is no specific therapy for acute hepatitis E, and the death

rate is considerable in pregnant women.

Alcohol consumption is high in developing countries and alcoholic liver disease is therefore obviously present. However, this form of liver disease receives little, if any, attention in low income countries. Bruha[4] from Charles University in Prague, Czech Republic, reminds us that severe acute alcoholic hepatitis is associated with a mortality rate of up to 50%, and that treatment of this form is extremely difficult. There is an absolute need for abstaining from alcohol of patients with chronic liver disease; the lack of policies to this effect is a serious concern that must be addressed.

Crane *et al*[5] (Monash University and The Alfred Hospital, Melbourne, Australia) describe liver disease in HIV-infected individuals, in the absence of co-infection with HBV or HCV, as an emerging issue. While antiretroviral therapy-related toxicities are an obvious cause, evidence is emerging that HIV infection may have a direct impact on the pathogenesis of liver fibrosis, non-alcoholic fatty liver disease and non-alcoholic steatohepatitis.

Professor Kew *et al*[6] (Department of Medicine, University of Cape Town and Groote Schuur Hospital, Cape Town, South Africa) describes a gloomy but realistic picture of hepatocellular carcinoma, a tumor that occurs commonly and with increasing frequency in developing countries, where it also carries an especially poor prognosis. Non implementation of hepatitis B vaccination and unavoidable dietary exposure to aflatoxin B1 play an important etiological role. Unfortunately, prevention of hepatocellular carcinoma in developing countries is unlikely in the foreseeable future.

CONCLUSION

I hope that the readers will enjoy this Topic Highlight and will be stimulated to conduct research that will fill the numerous gaps in our knowledge and will suggest feasible solutions to the several problems highlighted.

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TOPIC HIGHLIGHT

Francesca Cainelli, MD, *Series Editor*

Hepatitis A: Epidemiology and prevention in developing countries

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Abstract

Hepatitis A is the most common form of acute viral hepatitis in the world. Major geographical differences in endemicity of hepatitis A are closely related to hygienic and sanitary conditions and other indicators of the level of socioeconomic development. The anti-hepatitis A virus (HAV) seroprevalence rate is presently decreasing in many parts of the world, but in less developed regions and in several developing countries, HAV infection is still very common in the first years of life and seroprevalence rates approach 100%. In areas of intermediate endemicity, the delay in the exposure to the virus has generated a huge number of susceptible adolescents and adults and significantly increased the average age at infection. As the severity of disease increases with age, this has led to outbreaks of hepatitis A. Several factors contribute to the decline of the infection rate, including rising socioeconomic levels, increased access to clean water and the availability of a hepatitis A vaccine that was developed in the 1990s. For populations with a high proportion of susceptible adults, implementing vaccination programs may be considered. In this report, we review available epidemiological data and implementation of vaccination strategies, particularly focusing on developing countries.

Key words: Hepatitis A; Developing countries; Endemicity; Seroprevalence; Vaccine

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INTRODUCTION

Hepatitis A is the most common form of acute viral hepatitis worldwide. The first description of hepatitis (epidemic jaundice) is generally attributed to Hippocrates and outbreaks of hepatitis A have been recognized for centuries, affecting both military and civilian populations[1].

The hepatitis A virus (HAV) is a small non-enveloped single-stranded RNA virus. It is thermostable and acid-resistant. For some time after its identification, HAV was thought to be an enterovirus; in 1991, it was sub classified as a member of the Hepatovirus genus of the family Picornaviridae. HAV replicates in hepatocytes and interferes with liver function, sparking an immune response that causes liver inflammation. HAV is acquired by the fecal-oral route. Person-to-person transmission is com-

mon and generally limited to close contacts[2].

A typical symptomatic presentation includes non specific prodromal symptoms with variable combinations of fever, malaise, weakness, anorexia, nausea, vomiting, arthralgias and myalgias. Prodromal symptoms tend to decrease with the onset of jaundice, although anorexia, malaise and weakness may persist or increase transiently. Jaundice lasts for several weeks and is followed by a convalescent period. The peak infectivity occurs during the two weeks before the onset of jaundice or elevation of liver enzyme levels when the concentration of virus in the stool is highest. When jaundice appears, the viral concentration in the stool declines and most patients are noninfectious after one week[2,3].

The expression of clinical symptoms varies greatly with the age of the infected person. Approximately 50% of children with hepatitis A under the age of 6 years are asymptomatic, with most of the remaining having mild symptoms, often not recognized as hepatitis. Less than 5% of children below 4 years of age and less than 10% of children between ages 4 and 6 years with hepatitis A develop jaundice. Starting from 6 years of age to adulthood, more than 75% develop the characteristic illness traits with jaundice and dark urine[3,4]. Although rare, HAV infection can cause acute liver failure and death (in approximately 0.2% of clinical cases) and this risk increases with age and the presence of chronic liver disease[5].

Acute hepatitis A symptoms are similar to those of other viral hepatitis and serological testing for the detection of immunoglobulin M (IgM) antibodies to HAV (anti-HAV) is required to confirm the diagnosis. IgM anti-HAV is usually detectable when symptoms appear and concentrations decline to undetectable levels within 6 mo for most patients. However, cases of patients that test positive for IgM anti-HAV more than 1 year after infection have been reported. Immunoglobulin G (IgG) anti-HAV appears early in the course of the infection and remains detectable throughout the person's lifetime. Total anti-HAV tests are often used in epidemiological investigations or to detect susceptibility to HAV but they do not identify acute infection. Vaccines, available since the early 1990s, are not yet widely used, therefore most individuals with anti-HAV acquired immunity through infection[3].

GLOBAL EPIDEMIOLOGY OF HEPATITIS A

Approximately 1.5 million clinical cases of hepatitis A occur worldwide annually but the rate of infection is probably as much as ten times higher. The incidence rate is strongly related to socioeconomic indicators and access to safe drinking water: as incomes rise and access to clean water increases, the incidence of HAV infection decreases. The association of HAV infection risk with standards of hygiene and sanitation, the age-dependent clinical expression of the disease, and lifelong immunity determine the different patterns of HAV infection observed worldwide.

The HAV endemicity level for a population is defined by the results of age-seroprevalence surveys; a systematic review on the global prevalence of HAV infection was recently published by the World Health Organization (WHO)[6].

Areas of the world can be characterized as having high, intermediate and low endemicity for hepatitis A. In less developed countries with very poor sanitary and hygienic conditions, HAV infection is highly endemic and most persons become infected in early childhood. Because infection occurs at an early age when the disease is often asymptomatic, reported rates of the disease in these areas are relatively low and outbreaks are not common. Areas of high endemicity include most of Africa, Asia and Central and South America. Conditions which contribute to the propagation of the virus among young children in these areas include household crowding, poor levels of sanitation and inadequate water supplies[3,6,7].

In developing countries and some regions of developed countries, which include Eastern Europe, parts of Africa, Asia and America, sanitary and hygienic conditions vary and some children avoid infection during early childhood. Peak rates of infection commonly occur in later childhood or adolescence. Paradoxically, since HAV transmission occurs in these areas in older age groups, reported rates of hepatitis A can be higher than in less developed countries where HAV transmission is more highly endemic. Person-to-person transmission in large community-wide epidemics accounts for a significant amount of the disease in these areas. These outbreaks are very difficult to control with standard measures like hand washing and immune globulin administration to contacts of cases. Outbreaks are also observed in child care centers and schools and occasionally large food borne epidemics occur, such as in Shanghai in 1988 where the outbreak was associated with shellfish consumption[8]. In some areas, conditions are such that disease trends are cyclical; HAV is transmitted in community-wide outbreaks until the population is exhausted of susceptible persons, after which there is a period of several years until a new cohort of susceptible children reaches the age when clinical disease is more frequent[3,6,7].

In most developed countries, such as North America, Western Europe, Australia and Japan, sanitation and hygienic conditions are generally good and infection rates in children are generally low. Peak rates of infection and reported disease tend to be among adolescents and young adults. In these areas, large community-wide outbreaks with extended person-to-person transmission can still contribute significantly to the burden of hepatitis A disease. In addition, occasional outbreaks in child care centers or residential institutions and food borne or waterborne epidemics can occur. In some countries with very low prevalence (e.g. Northern Europe), disease predominates among specific adult risk groups: travelers to countries where hepatitis A is endemic; intravenous drug users; and men with a history of homosexual behavior. The prevalence of anti-HAV increases gradually with age, primarily reflecting declining incidence, changing endemicity and

a resultant lower childhood infection rate over time[2-4,6,9,10].

PREVALENCE OF HEPATITIS A: FOCUS ON DEVELOPING COUNTRIES

Africa

Information on HAV infection in Africa is limited. Available data shows that most of Africa remains a high endemicity region, with the exception of subpopulations in some areas, such as white people in South Africa. In the 1990s, almost all black children in South Africa were anti-HAV-positive by the age of 12 years and almost 100% of black adults had antibodies to HAV before the age of 20 years, while only 30%-40% of white adults were anti-HAV-positive by the age of 20 years, rising to about 60% by the age of 40-49 years[11,12]. North Africa has an intermediate level of anti-HAV seroprevalence. Studies from the 1980s showed nearly universal immunity in many countries; a 100% immunity rate by age 10 years was found in Algeria and nearly 100% of adults were anti-HAV positive in Morocco. More recent data shows that, in general, urban areas have experienced a decline in hepatitis A infection, while rates in rural areas remain high and the prevalence is generally lower in higher social classes[6].

The significant increase in the seroprevalence with older age and lower social class was confirmed in a 2008 study in which 296 Egyptian children aged 2.5-18 years of different social classes were tested to evaluate whether to give HAV vaccine early in life or to leave children to acquire natural immunity. Overall, 61.4% were seropositive; anti-HAV was detected in 27.3% of high and in 81% of low social class children aged < 6 years[13].

In Tunisia, child infection rates remain high with differences between urban and rural settings, depending on the development of the areas considered. In 2002, the overall seroprevalence in an area of central Tunisia was 60% among students aged 5-23 years (44% in children < 10 years old, 58% in those 10-15 years of age and 83% in those > 15 years of age). Regardless of age, seroprevalence rates for HAV were significantly lower in urban areas than in sub-urban and rural areas. At the age of 10, 21.3% of school children living in urban areas and 87.7% of those living in rural areas had antibodies to HAV[14]. In a larger study performed in three different regions of Tunisia in 2007, HAV seroprevalence was 84.0%, 90.5% and 91.7% in three groups with a mean age of 6.94, 12.84 and 20.71 years respectively[15].

In its recent report, WHO presents the information about HAV seroprevalence in North Africa and Middle East together because the trend of the infection is the same in both regions. In Yemen in the 1980s, a near 100% seroprevalence was detected, while in a recent study from Kuwait the prevalence of anti-HAV was 28% in adults. The highest prevalence was among the group with non educated parents, which reflects the relationship of the disease to low social background[6,16].

Sub-Saharan Africa has some of the highest anti-HAV prevalence rates in the world and nearly all older children and adults are naturally immunized. In Liberia in the late 1970s, more than 80% of 4-5 year olds had antibodies to HAV, which suggested an incidence rate of 45% per year between the first and fourth birthdays. In the same period in Senegal, nearly 100% of children had antibodies to HAV by age 5 years and in Nigeria more than 90% of adults had HAV antibodies. In the 1990s in Cameroon, adult prevalence was greater than 90% and in Sierra Leone a study of urban schoolchildren found a 97% prevalence rate. Although recent studies are not available, it is likely that Sub-Saharan Africa continues to have a very high HAV incidence rate and, correspondingly, a very high seroprevalence rate from childhood[6,7].

Asia

HAV seroprevalence rates vary considerably among countries in Asia, with some continuing to have high rates and others making a transition to moderate or low incidence.

Low endemicity areas include Japan and others countries such as Taiwan where the prevalence has decreased markedly in the last years. In fact, while in the 1970s the prevalence of anti-HAV in adults was more than 90%, later studies show that in the Taipei metropolitan area, the prevalence was nearly 0% and in the rural areas only very few adolescents and young adults showed signs of previous infection[17].

In the moderate endemicity countries, such as Korea, Indonesia, Thailand, Sri Lanka and Malaysia, the available data shows that the incidence rate may be decreasing, at least in urban areas, and the age at infection increases from very early to late childhood, which increases the risk of outbreaks. The number of cases of adult hepatitis A has progressively been increasing during the last several decades in Korea. In addition, the pattern of age-specific seroprevalence of anti-HAV has changed with economic growth. The prevalence of anti-HAV in the 10-50 year age range has declined rapidly during the last 3 decades. As a result, this age group has a high risk for HAV infection and clinically overt hepatitis A is increasing in adolescents and adults[6,18,19].

In China and India, the two most populous countries in the world that have shown a very rapid socio-economic development in the last years, many high endemicity areas for HAV infection coexist with others, making a transition to moderate incidence[6,7,20].

Hepatitis A has been a relevant public health problem in China. More than 300 000 cases of clinical hepatitis A were reported during a shellfish-associated outbreak in Shanghai in 1988; however, in the following two decades, the annual national incidence rate of hepatitis A dropped dramatically. In urban China in the early 1990s, although up to one-half of 10 year olds had antibodies to hepatitis A, the seroprevalence in most cities did not reach 100%, even for those aged 60 and above, leaving susceptible population groups and creating the possibility of outbreaks. In 2006, two major hepatitis A incidents occurred in China: one was an outbreak in a school in the

south and the other was an epidemic involving multiple HAV strains in the Autonomous Region of northwestern China[21,22].

In India, heterogeneous pockets of susceptible and exposed individuals may co-exist in different regions. About 15 years ago, the cord blood anti-HAV level in Indian newborns was almost 100%, which in turn reflected the maternal antibody prevalence. In recent studies, this level has come down to 50%-60%. Some studies also show that seroprevalence of HAV antibodies was lowest in the 6 mo to 2 year age group and maximum exposure to HAV occurred from 2 to 5 years of age. These observations may be the first indication of the epidemiological shift of the age of acquisition of the HAV infection in the community, even if the current available data does not confirm a consistent decline in childhood HAV seroprevalence rates and increased susceptibility to HAV in young adults[20,23,24].

Nowadays, in many other Asian countries, HAV infection is still highly endemic. Studies from Pakistan in the 1980s, 1990s and 2000s indicate that more than half of children acquire immunity by their preschool years and nearly all adolescents and adults are immune. Between the 1980s and 1990s in Nepal, nearly all adolescents were immune by age 15. In Bangladesh, more than half of 5 year olds and nearly all adolescents and adults are immune[6].

Central and South America

Latin American countries show many of the characteristics of developing countries, with migration from rural communities to cities leading to urban areas of low income and social deprivation. Improvements in public health programs and sanitary conditions have had an impact on the epidemiological patterns of HAV infection in developing economies and so previous studies showing Latin America to be an area of high endemicity with almost universal infection before the age of 10 years may no longer be valid. It is, nevertheless, difficult to estimate the exact incidence of hepatitis A because of the high proportion of subclinical infection and anicteric disease and the different surveillance programs. It has been estimated that the real incidence is 10 times higher than that reported[25-27].

The endemicity patterns continue to be high in several Latin America countries, such as the Central and the Caribbean areas, where studies performed between 1990 and 1999 showed a very high seroprevalence rate and found that more than half of the children had developed immunity by their second birthday and nearly all adults in both rural and urban areas were immune to HAV[6,7].

Data from recent studies has shown that the prevalence of anti-HAV is decreasing in several South American countries, including Argentina, Bolivia, Brazil, Venezuela, Chile and Uruguay where there has been a shift from high to medium endemicity. This shift was obtained with the improvements in public health programs and sanitary conditions in most parts of these areas[25,26].

A multicenter study carried out between 1996 and 1997 in six countries, including Mexico, the Dominican Republic, Chile, Brazil, Venezuela and Argentina, showed a general decrease in HAV seroprevalence rates compared to previous reports, except for the Dominican Republic where a total prevalence of 89.0% was detected and where the seroprevalence of HAV in children between 1 and 5 years of age was more than 50%. The seroprevalence rates were 81.0% in Mexico, 64.7% in Brazil, 58.1% in Chile, 55.7% in Venezuela and 55.0% in Argentina. In the 5-10 year old age group, seroprevalence rates have also decreased compared with previous reports. This suggests that the epidemiology is shifting from high to intermediate endemicity, with the population susceptible to HAV infection shifting from children to adolescents and adults. Even in Mexico, where anti-HAV prevalence remained high, it was shown that the average age at infection among children hospitalized with hepatitis increased from 6 years in 1991-1993 to 10 years in 2003-2005. Furthermore, data from Brazil, Argentina, Venezuela and Mexico shows that HAV seroprevalence is significantly lower in people living in medium and high socioeconomic conditions[6,7,26].

In the same six countries, a seroepidemiological study was undertaken in 2002-2003 to determine whether this pattern has changed. Analysis of the different age groups showed that at age 6-10 years, 30% of children in Chile and 54%-55% in Brazil, Venezuela and Argentina had been infected, compared with almost 70% in Mexico and 80% in the Dominican Republic. At age 11-15 years, nearly 90% in Mexico and 91% in the Dominican Republic had been infected, compared with 54% in Argentina, 62% in Venezuela, 60% in Brazil and 70% in Chile. By age 31-40 years, over 80% of the populations in all six countries had been exposed to HAV. In all of the countries except Brazil and Venezuela, the seroprevalence of anti-HAV was significantly higher in females than in males. In Mexico, Argentina and Brazil, anti-HAV seroprevalence was significantly higher in the low than in the middle/high socioeconomic groups. Seroprevalence rates in American Indian and Amazonian populations tend to be higher, except for some extremely isolated villages. The results show that there has been a shift from high to medium endemicity of HAV infection in a large part of Latin America, which may result in more clinical cases in adolescents and adults and a greater potential for outbreaks[25,28].

In Bolivia, studies performed in the same rural area in 1987 and ten years later showed a significant decrease in the seroprevalence rates from 86.9% to 28.4% in children less than 5 years of age, although rates in older children and adults remained very high in both study years. Most seroprevalence studies from Mexico and Venezuela performed between the 1990s and 2000s showed that about 50% of 10 year olds were immune but significant differences in seroprevalence were related to socioeconomic status[6].

In Argentina, a sharp reduction in the infection rate was reached by the introduction of a universal HAV vac-

cination program in 2005 and other countries, like Brazil and Chile, are evaluating the possibility of introducing a specific prevention policy[29-31].

PREVENTION OF HEPATITIS A INFECTION

Adequate supplies of safe drinking water and proper disposal of sewage within communities, combined with personal hygiene practices, such as regular hand washing, reduce the spread of HAV[32]. There was a marked reduction in virus transmission in most developed countries several decades ago due to improvements in living standards, better sanitation and environmental conditions. The same trend was observed during the 1990s in several developing countries with increasing economic prosperity. These changes occurred without a specific vaccination strategy, underscoring the critical importance of environmental and personal hygiene and sanitation to prevent fecal-oral transmission of pathogens[17].

Safe and effective inactivated hepatitis A vaccines have been available since 1992 worldwide and are generally used in developed countries to protect risk groups and stop outbreaks. The different vaccines are similar in terms of efficacy and side-effects, highly immunogenic, inducing antibodies to HAV that persist for at least 15 years. Based on current scientific evidence, protection is considered to be life long after a complete hepatitis A vaccination schedule (two doses). Long-term protection after a single dose needs to be further surveyed. The vaccines can be delivered alone or in combination and administered with flexible schedules[33,34].

Vaccination policies range from being part of national universal immunization programs for children to targeting at risk groups.

National immunization programs have been successful, with good coverage rates and declines in incidence up to 90%. Countries or regions that have implemented universal immunization, e.g. Israel, Italy (Puglia), Spain (Catalonia) and the United States, have demonstrated a successful impact on the incidence of hepatitis A; the data for the United States is particularly striking, with evidence of a two-thirds decrease in admissions to hospital and markedly lower medical expenditures between 1996 and 2004. Targeted policies, especially for travelers, have also been shown to be effective and are adopted by different countries and vaccination is included as post-exposure prophylaxis of contacts[35].

In some rapidly developing countries, a new approach to control and prevention of HAV epidemics using a vaccine is being considered. In South America, several trials to evaluate the immunogenicity and safety of inactivated HAV vaccine were performed among Argentinean and Chilean children[36,37], while cost-benefit studies were performed in Brazil, where a hypothetical vaccination strategy was developed to eliminate hepatitis A[28].

In 2005 in Argentina, a universal hepatitis vaccination with a single dose at 12 mo of age was implemented. Argentina's Ministry of Health was established to monitor the impact and follow up the strategy in order to evaluate the need for a second dose. Surveillance data showed an important decline in hepatitis A incidence rates in 2007, when the rate recorded was the lowest in the last 12 years. It is important to consider that these declines since 2005 have been unprecedented in magnitude and have been observed in all age groups and regions, showing a marked herd immunity effect. Brazil and Chile reassessed their immunization policy after cost-effectiveness studies and looking at the successful results of the areas where vaccines were introduced[29].

In China, at the same time when lifestyles began changing and the country's economy boomed, hepatitis A vaccines were introduced. A safe and immunogenic live attenuated HAV vaccine based on the H2 strain has been developed and licensed by the Chinese State Drug Administration. The vaccine meets the requirements of China and WHO for the manufacture of biological substances and is now widely applied in the immunization program to prevent HAV epidemics in China and other countries, such as India[38]. Recent Indian studies with this vaccine have confirmed its high immunogenicity and excellent safety profile[39,40].

Recommendations for the use of the hepatitis A vaccine vary considerably among countries. Guidance from WHO on hepatitis A vaccines emphasizes the need to consider the cost-benefit and sustainability of various prevention strategies in the context of the epidemiological characteristics of the setting where vaccination is being considered. In more developed countries, hepatitis A vaccine is primarily being used to protect persons at increased risk, such as travelers to areas where hepatitis A is endemic, men who have sex with men, or persons with chronic liver disease. Hepatitis A vaccination currently has few indications in the areas of the world where the infection is highly endemic and where most of the population is already immune. In areas of intermediate or high endemicity that are transitioning to a lower level of transmission, shifts in the age-specific patterns of the disease result in an increasing proportion of susceptible adolescents and adults, often in urban areas or higher socioeconomic classes, among whom outbreaks may occur[3,32-40,41].

In these settings, HAV vaccination may be considered on the basis on epidemiological and cost-effectiveness studies.

CONCLUSION

Hepatitis A virus is still a major cause of infection and disease in the world and heterogeneous pockets of susceptible and exposed individuals may co-exist in rapidly developing societies. Thereafter, small localized or large outbreaks of HAV infection will remain a threat in such areas. The situation demands that conclusive guidelines be produced for HAV vaccination in these communities after characterizing them appropriately. WHO is in the process of revising its position paper on hepatitis A, issued in 2000, with a view to: update and evaluate the data

on disease burden, epidemiology, vaccine products and availability and immunization protection; review the use of the vaccine in outbreaks and for contacts of cases; and issue guidance to countries where the prevalence rates are declining from high levels. In determining national policies, the results of appropriate epidemiological and cost-benefit studies need to be carefully considered and the public health impact weighed[18,32].

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TOPIC HIGHLIGHT

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Hepatitis B: Epidemiology and prevention in developing countries

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Abstract

Hepatitis B virus (HBV) infection is a serious global public health problem. The infection may be transmitted through sexual intercourse, parenteral contact or from an infected mother to the baby at birth and, if contracted early in life, may lead to chronic liver disease, including cirrhosis and hepatocellular carcinoma. On the basis of the HBV carrier rate, the world can be divided in 3 regions of high, medium and low endemicity. The major concern is about high endemicity countries, where the most common route of infection remains vertical transmission from mother to child. Screening of all pregnant women and passive immunization with human hepatitis B immunoglobulin are not affordable for many developing countries. **The infection rate can** be reduced by modifying behavior, improving individual education, testing all blood donations, assuring asepsis in clinical practice and screening all pregnant women. However, **availability of a safe and efficacious vaccine** and adoption of appropriate immunization strategies are the most effective means to prevent HBV infection and its consequences. The unsolved problem for poorest countries, where the number of people currently infected is high, is the cost of the vaccine. A future challenge is to overcome the social and economic hurdles of maintaining and improving a prevention policy worldwide to reduce the global burden of the disease.

Key words: Hepatitis B; Developing countries; Endemicity; Seroprevalence; Vaccine

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INTRODUCTION

Viral hepatitis type B is a common, serious disease caused by the hepatitis B virus (HBV), a partially double-stranded DNA virus of the Hepadnaviridae family. Four major serotypes (adw, ayw, adr and ayr) and nine minor subtypes have been serologically identified at the hepatitis B surface antigen (HBsAg) level. The complete sequencing of DNA from HBV isolates worldwide has led to the identification of eight genotypes (from A to H) and a number of subgenotypes, showing different ethno/geographic distributions. HBV genotypes have also been associated with different clinical outcomes and response to interferon therapy. HBV is one of the main causes of hepatic

decompensation, cirrhosis and hepatocellular carcinoma (HCC). Acute disease usually occurs when the immune response is well preserved, while patients with an immunodeficiency are more likely to develop a chronic disease, then becoming a source for new infections. The likelihood that an HBV infection become chronic depends upon the age at which a person is infected, infants and young children being the most likely to develop chronic infection[1,2].

HBV is carried in blood and other body fluids, including saliva, tears, semen and vaginal secretions. Depending on the epidemiological pattern within a geographic area, the main ways of transmission are sexual intercourse, parenteral contact or infection of the baby at birth from an infected mother. Globally, about one third of the population has been infected with HBV; six percent are chronic carriers and over 600 000 people die each year from acute disease or chronic sequelae secondary to HBV infection. On the basis of the HBV carrier rate, the world can be divided in to high, medium and low endemicity regions. The major concern is about high endemicity countries, especially in Asia and Africa, where the most common routes of infection remain vertical transmission from mother to child and horizontal transmission between children[1].

Vaccination is the most effective measure to reduce the global incidence of hepatitis B. Compared to other healthcare interventions, vaccination is an economically advantageous option, both in terms of cost-effectiveness and benefit-cost ratios. In 1991, the World Health Organization (WHO) recommended that all countries introduce a policy of universal hepatitis B vaccination to prevent and control HBV infection and its long term sequelae on a global scale. By the end of 2008, hepatitis B vaccine for infants was introduced nationwide in 177 countries. To date, global hepatitis B vaccine coverage is estimated at 69%[2,3].

GLOBAL PREVALENCE AND EPIDEMIOLOGY OF HEPATITIS B: FOCUS ON DEVELOPING COUNTRIES

HBV infection occurs all over the world. The WHO has estimated that there are more than 2 billion HBV infected people and about 378 million chronic carriers worldwide. There are approximately 620 000 HBV related deaths each year. In addition, approximately 4.5 million new HBV infections occur worldwide each year, of which a quarter progresses to liver disease. In high endemic areas, like central Asian republics, Southeast Asia, Sub-Saharan Africa and the Amazon basin, the HBV carrier rate is over 8%. In low endemic regions, like the United States, Northern Europe, Australia and parts of South America, HBsAg prevalence is less than 2%. The Middle East, some Eastern European countries and the Mediterranean basin are considered areas of intermediate endemicity with a carrier rate between 2% and 8%[1].

In many countries, after the introduction of mass immunization campaigns, the prevalence of HBV notably changed, resulting in a decrease of the HBsAg carrier rate and HCC incidence[2]. It was estimated that liver cancer represents approximately 4% of all new cancer cases diagnosed worldwide and that more than 50% of liver cancers were attributable to HBV. The highest age-adjusted incidence rate (> 20 per 100 000) was reported from Southeast Asian and Sub-Saharan African countries that are endemic for HBV infection. Up to 90% of infants infected during the first year of life and 30%-50% of children infected between one to four years of age develop chronic infections and about 25% of adults who become chronically infected during childhood die from HBV-related liver cancer or cirrhosis[4].

HBV continues to be the major HCC risk factor worldwide, although its importance will continue to decrease during the next decades due to the widespread use of the HBV vaccine in newborns[5-13].

In the last few years, more and more data have been produced in developing countries and areas with high/intermediate endemicity where the most common route of infection is still vertical transmission from mother to child and horizontal transmission between children, particularly siblings[2,14-20].

Globally, perinatal HBV transmission accounts for an estimated 21% of HBV-related deaths, while regionally it ranges from 13% in the Eastern Mediterranean region to 26% in the Western Pacific region. Recent studies in Africa confirm the relatively high HBsAg seroprevalence in pregnant women, irrespective of age, parity, gestational age, residence, history of blood transfusion, dental manipulation, tattooing and circumcision[14]. The maternal-neonatal transmission was studied in Libya where HBsAg positivity was 1.5% and transmission 60.9% and in Ghana with a HBsAg prevalence of 16% but a materno-fetal transmission only in 8.4% of neonates[15,19].

In high endemic areas, other important modes of HBV transmission concern some high-risk groups such as health care workers (HCWs)[21-25], but also sexual contacts[26] and intravenous drug use[27-32]. The predominant ways of infection in areas of low endemicity play a role. Parenteral or percutaneous routes of HBV transmission, such as needle stick injury and mucus membrane splash in healthcare setting, as well as tattooing, piercing, sharing razors or toothbrushes, are also important in spreading the virus[33-35]. Surgery and dental care may be a source of infection; transfusion-related infections have currently become very rare in developed countries thanks to the improved serology and advances in molecular blood screening but can be an important source of infection in the poorest countries[36-45].

The prevalence of the infection in HCWs, a high risk group for acquiring infection with blood born pathogens due to occupational contact with infected body fluids, depends upon HBV prevalence in the general population. In India, an intermediate endemic zone where the estimated prevalence rate of HBV in the healthy general

population is around 4.7%, a recent study showed a 5% HBsAg positivity in HCWs, but a highest seropositivity of around 40% among laboratory technicians[23]. In Taiwan, among HCWs who were exposed to high risk patients, nearly 16% had HBV[24]. In north-west Turkey between 2002 and 2003, the occupational hazard of exposure to HBV was evaluated among 595 nurses. In total, 18.7% had been exposed to HBV infection and 2.7% were HBsAg positive. This result was in accordance with findings of several other studies, showing the level of prevalence for exposure to HBV among nurses to be between 16%-20%. In this study, 28% of nurses working in high risk departments were not vaccinated. Education plays a role in exposure to infection, with a decreasing trend from nurses that had received a normal high-school or equivalent education and those who had been educated to university standard[23].

Transfusion-related infections are an important source of HBV transmission, especially in the poorest countries. In countries with advanced medical, diagnostic and laboratory services, a large proportion of blood is used in sophisticated treatments requiring a high level of transfusion support, including chemotherapy, open heart surgery, organ transplantation and the management of hematological disorders such as leukemia, thalassemia and hemophilia. The pattern of blood usage is very different in countries where diagnostic and treatment options are more limited, with a much greater proportion of transfusions being given to women with obstetric emergencies and children suffering from severe anemia, often resulting from malaria and malnutrition. Data from WHO shows that, of the estimated 80 million units of blood donated annually worldwide, only 38% is collected in the developing world where 82% of the world's population live[44]. In 2007, 162 countries provided data to WHO on 85.4 million blood donations. The data comes from countries that account for a total of 5.9 billion people, representing 92% of the global population. About one fourth of the countries are not able to screen all blood donations for one or more of the transfusion-transmissible infections, including HIV, hepatitis B, hepatitis C and syphilis; only 48% of blood donations in developing countries are screened following basic quality assurance procedures; only 25% of the hospitals performing transfusions in developing countries and 33% of the hospitals in transitional countries have a transfusion committee to monitor transfusion practices, compared to 88% of the hospitals in developed countries[37].

In Sub-Saharan Africa, the risk of transfusion-transmitted infections is thought to be substantial because of the high prevalence of these infections, the frequent use of paid or replacement donors and incomplete screening coverage[41]. In most Latin American and Asian countries, blood and blood products are now regularly screened for HBsAg; for example, in Brazil where the screening of blood became mandatory in 1993, the prevalence of HBsAg decreased significantly from 0.36% in 1998 to 0.14% in 2005 due to the better control of blood donors and the decreasing infection rate in the general population[36].

In the majority of the Latin American countries where endemicity is intermediate/low, sexual intercourse is thought to be the most common route by which HBV infection is transmitted. In Brazil, a study from Victoria showed a HBV prevalence of 3.8% in HIV patients, while in other settings the prevalence rates ranged from 5.7% to 24.3%. While chronic HBV infection in the setting of HIV/AIDS is not considered an opportunistic infection, it is a common co-existing infection seen in HIV-infected individuals because of the shared modes of transmission[26].

Drug use is another important route of transmission of HBV. In Latin America, in low endemicity areas, injectable drug-related and sexual transmission of hepatitis viruses are a significant problem among young, HIV-infected and heterosexual individuals, even if the use of parenteral drugs is not common in these regions[27].

PREVENTION OF HEPATITIS B INFECTION

Prevention of HBV infection is a public health priority, especially for those groups at major risk of becoming chronic carriers.

Infection rate can be reduced through a modification of behavior and improving individual education. Testing of all blood donations and assuring asepsis in clinical practice reduce the risk of contracting HBV. Moreover, screening of all pregnant women helps to avoid mother to child transmission at birth. Administration of human hepatitis B immunoglobulin contributes to preventing neonatal infection and can be used after exposure to HBV as prophylaxis. Vaccination is the most effective means of preventing hepatitis B, cirrhosis and hepatocellular carcinoma worldwide[46-51].

The first vaccines, available between 1981 and 1982, were produced by harvesting the hepatitis B surface antigen from plasma of chronic HBsAg carriers and contained highly purified 22 nm HBsAg particles inactivated through a combination of urea, pepsin, formaldehyde and heat. These immunogenic plasma-derived vaccines have been used with success in several hundred million individuals and are still produced in Asia and used in a number of countries. Concern about the safety of these vaccines regarding transmission of blood-borne pathogens has proved to be unfounded. In the mid 1980s, recombinant DNA hepatitis B vaccines containing HBsAg expressed in HBV transfected yeasts (i.e. *Saccharomyces cerevisiae*), the so-called "second" generation hepatitis B vaccine, were commercialized. This new technology offered the potential of unlimited production, which allowed the hepatitis B vaccine to become one of the most widely used in the world. Several hundred million doses of hepatitis B vaccine have been administered worldwide with an excellent record of safety and efficacy. Similar results were obtained in India, where a new recombinant DNA HBV vaccine was produced[52,53].

Following a full course of vaccination (3 doses given at 0, 1 and 6 mo), seroprotection rates of antibodies against HBsAg (anti-HBs) are close to 100% in children

and almost 95% in healthy young adults. People who are elderly, obese, heavy smokers or immunocompromised, including those infected with HIV, may have suboptimal responses when vaccinated. Immunodeficient patients, such as those undergoing hemodialysis or immunosuppressant therapy, require higher doses of vaccine and more injections (i.e. at months 0, 1, 2 and 6) to achieve an adequate immune response. Rapid protection (i.e. for health care workers exposed to HBV or a susceptible sexual partner of an acute hepatitis B patient) can be achieved through the adoption of an accelerated schedule, including 3 doses of vaccine administered at 0, 1 and 2 mo, followed by a booster dose given at 12 mo. The site of injection and mode of administration are critical factors in achieving an optimal response. The vaccine should be given intramuscularly into the deltoid region in children (≥1 year of age) and adults or into the anterolateral thigh in newborns and infants (< 1 year of age). The intradermal route and buttock administration are not recommended. Hepatitis B vaccines are well tolerated. Side effects are generally mild, transient and confined to the site of injection (erythema, swelling, induration). Systemic reactions (fatigue, slight fever, headache, nausea, abdominal pain) are uncommon. However, in recent years, the safety of the hepatitis B vaccine has been questioned, but extensive studies concluded that there is no reason to change the current policies of vaccination. Hepatitis B vaccination is not contraindicated in pregnant or lactating women. The only absolute contraindications are known hypersensitivity to any component of the vaccine or a history of anaphylaxis to a previous dose.

Follow-up studies have shown that the vaccine-induced antibody persists over periods of at least 10-15 years and that the duration of anti-HBs is related to the antibody peak level achieved after primary vaccination. Follow-up of those vaccinated has shown that the antibody concentrations usually decline over time but clinically significant breakthrough infections are rare. Evidence indicates that successfully vaccinated individuals who have lost their antibodies over time usually show a rapid anamnestic response when boosted with an additional dose of vaccine or when exposed to the HBV. This means that the immunological memory for HBsAg can outlast the anti-HBs detection, providing long-term protection against acute disease and the development of an HBsAg carrier state. For immunocompromised patients, regular testing and booster administrations when anti-HBs antibody level falls below 10 mIU/mL are recommended instead.

Antibodies to the hepatitis B surface antigen are mainly targeted to bind the amino acid hydrophilic region, referred to as a determinant of HBsAg. This provides protection against infection with all HBV genotypes and is responsible for the broad immunity afforded by hepatitis B vaccination. The emergence of HBV S-gene mutants possibly able to escape the vaccine-induced response was suggested. However, at least at present, the overall impact of such mutants remains low and they do not pose a public health threat or a need to modify the established hepatitis B vaccination programs[1,2].

STRATEGIES FOR PREVENTION OF HEPATITIS B INFECTION SUITABLE FOR DEVELOPING COUNTRIES

In 1991, WHO recommended that all countries with a high hepatitis B disease burden should introduce the hepatitis B vaccine in their routine immunization programs by 1995 and all other countries by 1997. However, uptake of the vaccine was slow and the targets were not met. Even when the initial high price of the vaccine came down substantially, most low-income countries were unable to secure the funds needed to introduce the vaccine. Before the launch of GAVI (Global Alliance for Vaccines and Immunization) in 2000, less than 10% of the world's poorest countries were using hepatitis B vaccine in their routine immunization programs.

With support from GAVI through its financing arm, The Vaccine Fund, by December 2003, over 42 million children in low-income countries had been immunized with hepatitis B vaccine; as a result, over 500 000 premature deaths from hepatitis B have been prevented among children born in 2001-2003[54].

The most recent available data show global hepatitis B vaccine coverage at 69% with the following regional distribution: 67% in the African region; 76% in the European region; 81% in the Eastern Mediterranean region; 88% in the Americas; and 89% in the Western Pacific. Coverage in the South-East Asia region increased from 29% to 41% from 2007 to 2008. The immunization coverage with the 3rd dose of Hepatitis B vaccines in infants, in 2009, for country is: ≥ 90% (108 countries or 56%, most of them in low endemic areas); 80%-89% (31 countries or 16%); 50%-79% (31 countries or 16%); < 50% (7 countries or 4%); Hepatitis B not on schedule (16 countries or 8%)[55].

In 2005, the World Health Assembly approved and the United Nations Children's Fund (UNICEF) Executive Board endorsed the Global Immunization Vision and Strategy (GIVS). The primary objective of GIVS was to reduce vaccine-preventable disease mortality and morbidity by two-thirds by 2015 compared to 2000.

A mathematical model was developed to estimate the cost required to reach this goal in 117 low- and lower-middle-income countries and the study conclusions were that, in the 72 poorest countries, up to 40% of the overall resource needs were unmet[56].

WHO recommends that all countries introduce the hepatitis B vaccine into routine national infant immunization programs. Furthermore, in countries where a high proportion of infections with HBV are acquired perinatally (specifically in countries where the prevalence of chronic HBV infection in the general population is ≥ 8%), WHO recommends the first dose of hepatitis B vaccine be given as soon as possible after birth (< 24 h) to prevent perinatal HBV transmission.

In 2006, birth-dose coverage varied widely by region,

from 3% to 71%. Birth-dose coverage for states with ≥ 8% prevalence of chronic HBV infection was 36% (range by region, 1%-92%), and for countries with < 8% prevalence, it was 20%. Among the 81 states with immunization schedules that include a birth dose of hepatitis B vaccine, 22 (27%) did not report coverage data on the birth dose. It is likely that the reporting of the coverage of newborns with hepatitis B vaccine through the reporting form could be improved[57].

When introducing the hepatitis B vaccine into infant immunization programs, national policy-makers must decide when to begin the vaccine series: (1) at birth for all infants; (2) at birth but targeted only at newborns of HBV-infected women; or (3) at the same time in the immunization schedule as other vaccines are administered for all infants (for example, at 6 wk when other vaccines in the Expanded Program on Immunization are initiated), which is too late to prevent perinatal HBV infection. Administering a birth dose of hepatitis B vaccine only to newborns of HBV-infected women is usually not feasible in developing countries where the endemicity is high; this strategy is prone to error and misses post-exposure prophylaxis of infants, even in countries where testing and identifying infected women during pregnancy are well established. Additionally, it fails to provide early pre-exposure protection to babies born to uninfected women who may live with infected household contacts. Administering hepatitis B vaccine to infants within 24 h after birth is logistically challenging for several reasons. Firstly, many infants, especially in remote or poor areas, are born at home without the services of skilled attendants and therefore no trained providers are present to administer immunizations. Secondly, infant vaccines are usually given by specialized providers in well-baby clinics or other outpatient health settings, or during outreach immunization sessions in the community, but care of mothers during delivery and of infants immediately after birth is often provided by maternal health workers; so administering a birth dose of hepatitis B vaccine requires the coordination of these two types of workers. Thirdly, in many parts of the world, vaccines are delivered from central stores to peripheral clinics at monthly or at even longer intervals; these are primarily intended for use during periodic immunization sessions. Thus, the hepatitis B vaccine needed for the birth dose may not be available every day for administration to newborns.

Interventions that could improve birth-dose coverage include: increasing the number of infants born in facilities or attended by skilled health staff; improving coordination between immunization staff and maternal health staff; integrating delivery of the birth dose of hepatitis B vaccine into part of essential newborn care; improving the reach of the cold chain; exploring options for delivering the vaccine to infants born and residing in areas beyond the cold chain; and conducting health promotion and training to improve awareness among providers and parents of the importance of administering hepatitis B vaccine within 1 d after birth[2, 14-20].

Immunizing newborns with the hepatitis B vaccine should be the highest priority in highly endemic areas where the contribution of perinatal transmission to the overall disease burden is greatest. Nevertheless, even in countries with < 8% prevalence of chronic HBV infection, vaccinating newborns may be an important control strategy. Disease modeling suggests that the implementation of a birth dose of hepatitis B vaccine in WHO regions with a relatively low prevalence of chronic HBV infection, such as the Americas or Europe, will result in an additional 10%-20% reduction in HBV mortality in those regions compared with a hepatitis B vaccination schedule without a birth dose[57,58].

Post-exposure prophylaxis was thoroughly studied in infants born to HBeAg-positive HBsAg carrier mothers. The efficacy of protecting from chronic HBsAg carriage with passive-active immunoprophylaxis in these infants is more than 90%. In the case of hepatitis B immune globulin (HBIG) being skipped, active immunization is still effective, but the effectiveness of protecting from chronic HBV infection decreases slightly to more than 83%. The other means to increase the effectiveness of immunization against perinatal mother-to-infant HBV infection is to give HBIG to newborns of all HBsAg carrier mothers, as is done in the United States and in most European countries, but the costs rises considerably.

Although immunizing with universal vaccination is the only way to control HBV infection, recent advances in the specific treatment have enabled suppression of the chronic viral infection. Because humans are the only reservoir of the virus, if HBV could be eradicated or strongly and effectively suppressed in human carriers, the spread of HBV would be prevented.

Chronic hepatitis B can be treated by α-interferon (IFN- α; regular or pegylated) or nucleoside analogs. In properly chosen patients with chronic hepatitis B, 30%-40% will have a sustained virological response 6-12 mo after IFN-α treatment. More importantly, 30%-70% of the initial virological responders will clear serum HBsAg on follow up. The wide range of HBsAg clearance may be due to different durations of follow up, different treatment regimens, different distributions of HBV genotypes and different ethnic background of the patients. Seronegativity of HBsAg has very important implications: it signifies a better prognosis for patients and a much lower infectivity of the previous HBsAg carrier. The oral nucleoside analogs are effective and very well-tolerated. At present, these treatments are indicated for HBV carriers with disease activities; nevertheless, there may be exceptions: because high maternal viral load of HBV is the most critical factor in perinatal HBV transmission, even after on-schedule immunoprophylaxis, lowering the maternal viral load by antiviral therapy may reduce the perinatal HBV infection[59].

CONCLUSION

In spite of the decrease of the burden related to HBV

infection due to the adoption of mass immunization campaigns all over the world, the number of people still currently infected, especially in developing countries, will represent a public health concern in the foreseeable future. Improving prevention policy worldwide is mandatory in order to reduce the global burden of the disease.

A substantial number of WHO member states in areas with low or intermediate hepatitis B endemicity have implemented vaccination of newborns with the hepatitis B vaccine. Consideration should be given to implementing routine vaccination of newborns against HBV infection globally to prevent mortality and morbidity due to infection acquired perinatally.

Unfortunately, screening of all pregnant women and the use of human hepatitis B immunoglobulin as passive immunization is not affordable for many developing countries, so child vaccination remains the only means to prevent HBV spreading.

With its relatively modest costs and high benefits, HBV immunization continues to be one of the best values for public health investment today.

Moreover, enforced testing for HBsAg of blood donations in those countries where it is not a universal requirement yet could be an important measure to prevent infections in clinical settings, as well as maintaining asepsis in invasive techniques and vaccination for high risk groups.

A future challenge is to overcome the social and economic hurdles to maintain and improve prevention policy worldwide to reduce the global burden of the disease.

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TOPIC HIGHLIGHT

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Alcoholic liver disease

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Abstract

Alcohol use disorders affect millions of individuals worldwide. Alcohol consumption is directly associated with liver disease mortality and accounts for elevated social and economic costs. Alcoholic liver disease (ALD) may take the form of acute involvement (alcoholic hepatitis) or chronic liver disease (steatosis, steatohepatitis, fibrosis and cirrhosis). The severity and prognosis of alcohol-induced liver disease depends on the amount, pattern and duration of alcohol consumption, as well as on the presence of liver inflammation, diet, nutritional status and genetic predisposition of an individual. While steatosis is an almost completely benign disease, liver cirrhosis is associated with marked morbidity, mortality and life expectancy shortening. The median survival of patients with advanced cirrhosis is 1-2 years. Severe acute alcoholic hepatitis (AH) is associated with mortality as high as 50%. It has been managed with corticoids, pentoxifylline and enteral nutrition, although evidence based data are still conflicting. Some author suggest that pentoxifylline could be a better first-line treatment in patients with severe AH. Absolute abstinence is a basic condition for any treatment of acute or chronic ALD, the other therapeutical procedure being of a supportive nature and questionable significance. Acamprosate appears to be an effective treatment strategy for supporting continuous abstinence in alcohol dependent patients. Patients with advanced liver cirrhosis who demonstrably abstain can be considered for liver transplantation, which leads to a markedly prolonged life expectancy. The crucial step in ALD prevention is in the prevention of alcohol abuse, whereas the prevention of liver injury in active alcohol abusers is not clinically applicable.

Key words: Alcohol; Alcoholic liver disease; Liver cirrhosis; Liver fibrosis; Steatohepatitis; Steatosis

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INTRODUCTION

Alcohol is a most frequent cause of liver disease in western countries[1]. Mortality due to liver cirrhosis in those countries is in direct proportion to absolute alcohol consumption per capita-the highest in France and Spain (over

30 deaths per a population of 100 000 per year), the lowest in the northern European countries (up to 5 deaths per 100 000 inhabitants per year). In Central Europe, the figure is 15 deaths due to cirrhosis per 100 000. The highest mortality is in men aged 35-64 years, lower in women (Figure 1)[2]. The past two to three decades have seen a stabilization if not a drop in the intake of alcohol in western countries, while a very adverse trend is reported from Eastern Europe and developing countries[3].

In what is an alarming development, alcohol abuse also afflicts societies and nations without any "drinking tradition", such as in Asia. For example, in a cross-sectional study of two rural communities in China (in which almost 10 000 inhabitants were interviewed for current and lifetime alcohol use)[4], the age-standardized prevalence of lifetime alcohol dependence ranged from 4.8% to 11.8% in different regions. Unlike most western reports, alcohol dependence shows a higher prevalence than the abuse itself.

Coincidence with HIV infection is another attribute of alcohol abuse. This was described in India for example, where the recent increase in alcohol consumption in many sectors of the general population is coupled with strong evidence of the role of alcohol in the spread of HIV infection and other health risks[5]. An even more critical situation appears to have developed in Africa. Pithey *et al*[6] performed a systematic review of sub-Saharan African studies concerning the association between alcohol abuse and HIV infection. Their findings strongly support an association between the two factors. A Fisher *et al*[7] study of high-risk African women showed, even after adjustment for demographic and employment variables, that drinkers were more likely to be HIV positive than non-drinkers (relative risk 2.1). Problem drinkers were also more likely to have engaged in several types of high-risk sexual behavior and to have other sexually transmitted infections, including HSV-2.

Many studies have shown that the amount of undiluted ("pure") alcohol consumed and the duration of that consumption are closely related to cirrhosis. According to some reports, cirrhosis does not develop below a lifetime alcohol consumption of 100 kg of undiluted alcohol[8]. This amount corresponds to an average daily intake of 30 grams of undiluted alcohol for 10 years. Heavy alcoholics consuming at least 80 g of alcohol per day for more than 10 years will develop liver disease at a rate of nearly 100%. A detailed study of 256 heavy drinkers admitted to hospital not because of liver complaints, found steatosis at a rate of 45%, steatohepatitis at 34%, steatohepatitis with cirrhosis at 10% and cirrhosis alone at 10% in their liver biopsies[9]. Formerly, 40-60 g of undiluted alcohol (i.e., 2-3 beers) per day used to be reported as a safe limit for men, less (20 g/d) for women. Data from the "Dionysos" study show, however, that consumption of more than 30 g of pure alcohol daily, regardless of sex, already increases the risk of liver disease[10].

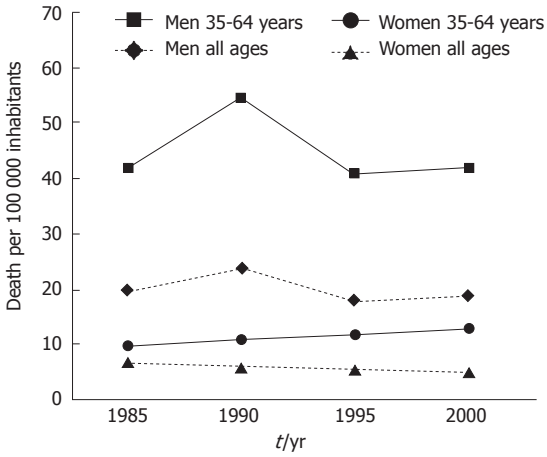

Figure 1 Mortality from cirrhosis in Czech Republic[2].

For practical purposes, alcohol intake is rated by the count of "drinks". The National Institute on Alcohol Abuse and Alcoholism defines a standard drink as 11-14 g of alcohol, which corresponds to approximately one drink of 40% spirit, one glass of wine or one 0.33 l (12-oz) beer. Hence, a "safe" daily intake of alcohol should not be more than two "drinks". On the contrary, moderate ethanol consumption (mainly wine) may mean a reduced cardiovascular risk[11], especially in women[12].

Much the same applies to Asians. For example, in the Chinese population, the ethanol risk threshold for developing alcoholic liver disease (ALD) is 20 g per day with the risk increasing in proportion to the daily intake[13]. Those drinking 20 g of ethanol per day and for less than 5 years are safe from ALD. In this study of 1270 alcohol drinkers, obesity also increases the risk. Abstinence and weight reduction will directly improve the prognosis of ALD.

As for liver injury, it has been postulated for many years that the type of alcoholic beverage makes little, if any difference. Nevertheless, some authors have proposed that mortality from cirrhosis is associated with the consumption of spirits more strongly than with other alcoholic beverages[14]. It is not clear whether this effect can be put down to the drinkers' socio-behavioral characteristics or to increased toxicity of alcoholic beverages[15].

ALD may take the form of acute involvement (alcoholic hepatitis) or chronic liver disease (steatosis, steatohepatitis, fibrosis and cirrhosis). Their progression also depends on the pattern of alcohol intake-drinking alcohol at mealtimes results in a lower risk of liver disease than consumption at other times; fitful, intermittent drinking is more sparing for the liver than a continuous supply of alcohol[16].

Although ALD is a disease that displays an absolute requirement for a voluntary environmental exposure (the consumption of alcohol), many other factors, including genetic host system attributes, are involved in the ALD evolution and progression.

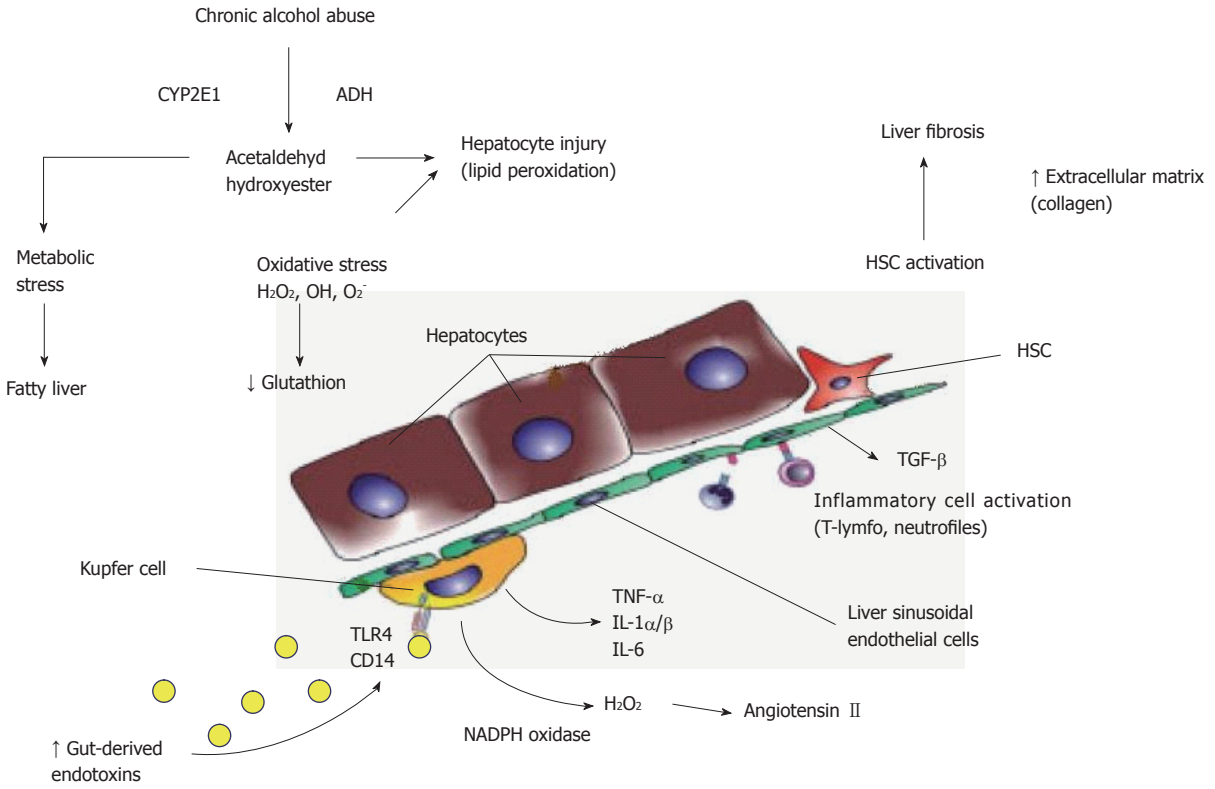

Figure 2 Pathogenesis of inflammatory changes in alcoholic liver disease[56]. ADH: Alcohol dehydrogenase; HSC: Hepatic stellate cell.

ETIOLOGY, PATHOGENESIS, NATURAL COURSE AND PROGNOSIS OF ALCOHOLIC LIVER DISEASE

The liver is the main organ of alcohol metabolism. Alcohol is metabolized in the liver in three ways: (1) by the enzyme alcohol dehydrogenase (ADH); (2) by cytochrome P-4502E1 (CYP2E1); and (3) by mitochondrial catalase. Only the first two pathways are of practical significance-ADH finds use in the degradation of limited quantities of alcohol, while alcohol-induced CYP2E1 takes place in excessive alcohol intake. Apart from the liver, ADH is also present in the gastric mucosa and the assumption is that individuals with low gastric ADH activity are more susceptible to alcoholic liver disease. This may also help to explain why women who have decreased gastric ADH activity[17] are more susceptible to developing alcoholic liver disease.

Both enzymes convert alcohol to acetaldehyde, which is in part responsible for the liver injury too. However, the process of liver injury is much more complex (Figure 2)-resulting from biochemical, genetic, cellular, immunological and humoral disorders in connection with the intake and metabolism of excessive quantities of alcohol. A major role is played there by oxidative stress (which is mainly due to alcohol-induced CYP2E1), by simultaneous shortage of antioxidants in the hepatocytes and, last but not least, by acetaldehyde alone and altered balance of many cytokines-mainly tumor necrosis factor (TNF)-α[18]. Changes in lipid metabolism and in adipose tissue also enhance the process of liver injury[19]. All above mentioned changes result in the injury of cell membranes and organelles (especially mitochondria). The mechanisms of hepatocytic damage due to excessive intake of alcohol show some similarity to changes seen in non-alcoholic steatohepatitis, except that the primary insult is different[20].

Individual susceptibility is another factor to take into account; moreover, any other liver involvement such as viral hepatitis[21] or metabolic disease adds to the risks of alcoholism, as does obesity and metabolic syndrome[22].

In fact, alcoholics were clearly shown to have an increased prevalence of HCV when compared with non-alcoholics and this combination synergistically accelerates liver injury[23]. As for alcohol influence on the liver, the caloric intake should also be taken in account. Increased caloric intake leads to excessive fat deposition and obesity in some patients and can aggravate the liver injury[24].

Of late, there has been an influx of information on correlations between genetic polymorphisms of alcohol-metabolizing enzymes and alcoholic liver disease[25]. The genetics of ALD development involves an inherited predisposition to alcohol dependence, as well as the resulting development of liver injury[26]. Family studies have established an important role of genetics in alcohol dependence. To date, only two genes, which are involved in alcohol metabolism, have shown significant involvement. The alcohol dehydrogenase ADH1B*1 allele was found to be associated with an approximately threefold increase in alcohol dependence and the aldehyde dehy-

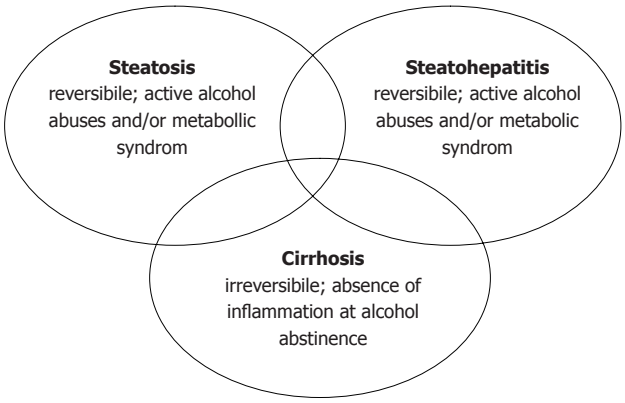

Figure 3 Spectrum of alcoholic liver disease.

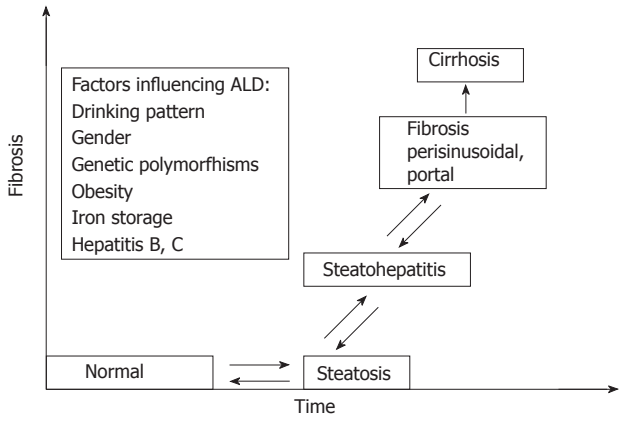

Figure 4 Dynamic process of alcoholic liver disease. ALD: alcoholic liver disease.

drogenase ALDH2*2 allele was found to be instrumental in a 10-fold reduction of the alcohol dependence risk[27]. This association was described in Asian populations[28]. Also reported have been links between alcohol dependence and certain genetic polymorphisms of genes for the GABA receptor or some other neuropeptides[29].

Although most heavy drinkers do develop fatty liver, only a minority progress to liver cirrhosis, suggesting that some other genetic or environmental factors are important for the disease progression. Evidence of genetic involvement in the progression of alcoholic fatty liver to advanced ALD comes from a twin study. The rate of alcoholic cirrhosis was described to be significantly higher in monozygotic twins than in dizygotic twins (16.9% *vs* 5.3%, respectively)[30]. A study of genes involved in alcohol metabolism (e.g., alcohol and aldehyde dehydrogenase and cytochrome P450 2E1) and genes associated with inflammation (e.g., TNF-α and interleukin-10) proved to be inconclusive, with several allelic associations detected but not verified in follow-up studies[31]. The Asian population's hypersensitivity to alcohol could be put down to polymorphisms of genes for the enzymes ADH and CYP2E1. Perhaps the most compelling genetic finding for advanced ALD risk involves the immune regulatory cytotoxic T lymphocyte antigen-4 gene, in which homozygosity for the A49G polymorphism was found to confer a significant risk of alcoholic cirrhosis (odds ratio 3.5) in Italians[32]. However, this finding has yet to be confirmed in follow-up studies.

Polymorphisms for TNF-α co-responsible for an increased risk of liver disease have been discovered in a similar way[33]. For the time being, though, we do not know how to make use of this new knowledge in routine practice.

Malnutrition is another clinical situation with an impact on the evolution of ALD. Heavy alcohol drinkers often lack proper diets or consume diets which are compromised in various nutrients, such as proteins, polyunsaturated fatty acids and vitamins[34].

Liver steatosis is the most frequent primary change in chronic alcohol abuse. Changes associated with alcohol metabolism may subsequently trigger an inflammatory reaction, resulting in alcoholic hepatitis or chronic liver disease (Figure 3).

Liver disease in alcohol abusers is more likely to take the form of chronic changes (steato-hepatitis and fibrosis), leading to cirrhosis later in life. The spectrum of histological findings can be described as a dynamic process[35] (Figure 4). Simple steatosis is reversible after a number of weeks of abstinence; steatohepatitis, a condition seen in only some alcoholics, is a fibrogenic process which can induce changes leading to cirrhosis. Steatohepatitis is also reversible, although a certain degree of fibrosis may persist. The reversibility of steatohepatitis or even fibrosis in humans is well documented by trials on the treatment of chronic hepatitis C[36] and experimentally on NASH models[37]. Steatohepatitis, in particular, often coincides with liver cirrhosis in active alcoholics and is a frequent cause of decompensation of cirrhosis[38].

Simple steatosis is regarded as a benign condition; nevertheless, given continued abuse, it too, can induce fibrogenesis[39]; in any case, up to 20% of the patients with simple steatosis are likely to develop fibrosis or cirrhosis within a period of ten years[40]. The prognosis of a patient with cirrhosis depends mainly on the presence of complications because of portal hypertension and continued abuse of alcohol. Abstainers with decompensated cirrhosis have a five year survival at a rate of 60% against the 30% survival rate in those who continue in the abuse[41].

Severe alcoholic hepatitis, although relatively rare, has a death rate of up to 50%. Identifying individuals with a high mortality risk is crucial in the management of acute alcoholic hepatitis. Multiple prognostic factors were studied over the last decade, including Child-Pugh classification (CTP), Maddrey score (bilirubin mg/dL + 4.6 × prothrombin time)[42] and others. The MELD score was found a more valuable model than CTP or the Maddrey score in the detection of high risk patients admitted with alcoholic hepatitis[43]. Alternatively, the more recent Glasgow alcoholic hepatitis score could be used[44]. A Glasgow score exceeding 9 points is associated with poor prognosis (Table 1).

Table 1 Glasgow alcoholic hepatitis score[44]

Parameter/score	1	2	3
Age (yr)	< 50	⩾ 50	-
Leucocytes (10^9/L)	< 15	⩾ 15	-
Urea (mmol/L)	< 5	⩾ 5	-
INR	< 1.5	1.5-2	> 2
Bilirubin (μmol/L)	< 125	125-250	> 250

The score is to be added to each parameter, the sum total being between 5 and 12 points. The value of 9 and higher implies poor prognosis in alcoholic hepatitis. **INR: International normalised ratio.**

CLINICAL MANIFESTATION AND LABORATORY FINDINGS

Patients with steatosis are usually symptom-free; they may have slightly elevated liver function tests and enlarged liver (both are often discovered accidentally during examination for other reasons).

In the stage of acute alcoholic hepatitis, there may be nausea, loss of appetite, gradual loss of weight, icterus and other symptoms of liver dysfunction (prolonged prothrombin time, hypoalbuminemia, ascites, and hepatic encephalopathy). Patients with alcoholic hepatitis usually show increased liver test results, including gamma-glutamyl transferase (GGT), hypergammaglobulinemia and enlarged liver.

Sonography is the basic imaging technique for liver examination. Liver biopsy, while not always necessary, can help to differentiate simple steatosis from steatohepatitis, fibrosis or incipient cirrhosis. Precise definition of the liver fibrosis stage is essential for management and prognosis in clinical practice. Recently, blood markers and instrumental methods have been proposed for non-invasive assessment of liver fibrosis[45]. However, there are still some doubts as to their implementation in clinical use. Non-invasive examination with transient elastography takes advantage of the fibrotic liver tissue ability to change the velocity of ultrasound propagation. The results of this method correlate well with the bioptically proved degree of fibrosis[46]. Similar results could be obtained from a combination of biochemical and clinical parameters of fibrosis. As for the clinical picture, the state of alcoholic liver cirrhosis shows no difference from cirrhosis of other etiology[38].

ASSESSMENT OF ACTIVE ALCOHOL ABUSE

Assessment of continued alcohol abuse in patients with alcoholic liver disease is essential for their treatment as well as prognosis. Those with alcoholic cirrhosis also make up a significant part of patients indicated for liver transplantation (30%-50%), bearing in mind that abstinence is an essential condition for considering this treatment. Continued alcohol abuse is evaluated on the basis of clinical history, psychological examination and laboratory testing. Thorough clinical and psychological examination is the crucial condition for alcohol abuse diagnosis. Regarding the clinical history, the diagnosis of alcohol abuse and dependence was substantially improved by implementation of simple methods such as a single question inquiring how often the maximum daily alcohol limit has been exceeded[47]. Other clinical screening tools such as the need to cut down, annoyed by criticism, guilty about drinking need for an eye-opener in the morning (CAGE), and the alcohol use disorders identification test (AUDIT-C) are also very easy to apply[48]. With the CAGE questionnaire, two positive answers indicate alcohol dependence with a sensitivity of more than 70% and specificity of more than 90%. The AUDIT-C screening thresholds for the detection of alcohol abuse are ⩾ 4 points for men (sensitivity 86%, specificity 89%) and ⩾ 3 points for women (sensitivity 73%, specificity 91%).

As for laboratory tests, continued abuse can be read from higher GGT values, increased AST/ALT ratio or an increased volume of red blood cells (MCV). In advanced liver cirrhosis, however, the values of hepatic enzymes fall short of sufficient sensitivity or specificity levels. More information about the actual abuse of alcohol can be derived from the percentage of carboxy-deficient transferrin estimation (%CDT) in serum or ethyl glucuronide in urine or hair[49]. A CDT value greater than 2.8% has a 79% sensitivity and 92% specificity for active alcohol abuse detection in patients with advanced cirrhosis[50].

PREVENTION OF ALD

Prevention of or treatment for alcohol abuse are crucial steps in the prevention of ALD[51]. Alcohol dependence is a chronic relapsing medical disorder which is treatable when efficacious medicines are added to enhance the effects of psychosocial treatment. Medication with, e.g., naltrexone and acamprosate showed mixed results in previous clinical trials[52]. Rősner *et al*[53] recently performed a meta-analysis to determine the efficacy and tolerability of acamprosate in comparison with placebo and other pharmacological agents. Almost 7000 patients in 24 double-blind randomised controlled trials were evaluated. Compared to placebo, acamprosate was shown to significantly reduce the risk of any drinking (RR 0.86) and to significantly increase the cumulative abstinence duration. The only side effect that was more frequently reported under acamprosate than with placebo was diarrhea. The authors of this Cochrane review conclude that acamprosate appears to be an effective and safe treatment strategy for supporting continuous abstinence after detoxification in alcohol dependent patients. Indeed, without a pharmacological adjunct to psychosocial therapy, the clinical outcome is poor, with up to 70% of patients resuming drinking within one year[54].

The prevention of liver injury in active alcohol abusers is not clinically applicable. For example, in an experiment, the addition to the diet of polyunsaturated fatty acids prevented alcohol-induced fatty liver and mitochon-

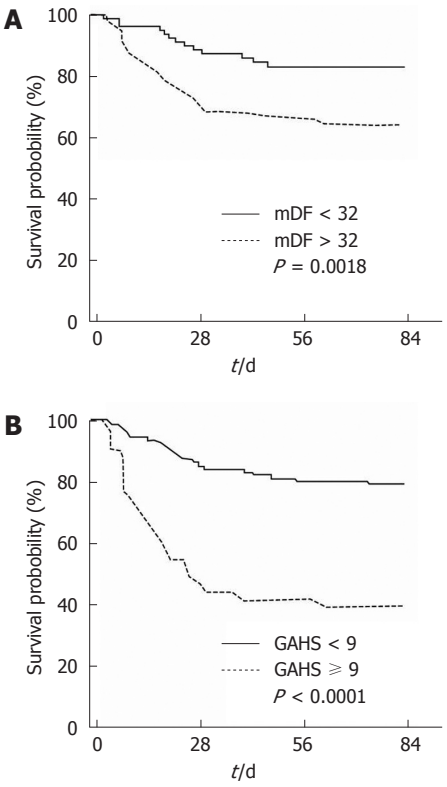

Figure 5 Kaplan-Meier survival analysis relative to the modified Maddrey discriminant function (mDF) (A) and the Glasgow alcoholic hepatitis score (GAHS) (B). The Glasgow score was developed on 241 patients and validated on 195 separate patients[44].

drial dysfunction in an animal model of ALD by protecting various mitochondrial enzymes, most likely through reducing oxidative/nitrosative stress[55]. The clinical use of similar medicaments would probably be always hampered by alcohol abusers´ failure to comply.

TREATMENT

Absolute abstinence is essential to consider any treatment for alcoholic liver disease. Even major changes, including cirrhotic restructuring, may show partial regression during total abstinence[56]. Portal hypertension declines and even regression of esophageal varices have been reported in abstainers. This, however, appears to have resulted from the remission of inflammatory changes and steatosis rather than from regressing fibrosis or cirrhosis. Sustained abstinence markedly improves the patient's prognosis in any phase of the liver disease[57], prevents the progression of the disease and fibrosis and, probably, also the development of hepatocellular carcinoma[58].

Pharmacotherapy of liver disease has but a supportive and rather dubious relevance. Treatment with silymarin, essential phospholipids or vitamin preparations was very popular in the past. Since an oxidative stress has been implicated in the pathophysiology of hepatic insult, the use of natural compounds with anti-oxidant properties represents an extremely popular therapeutic option for the treatment of liver disease. One such phytochemical, resveratrol, is remarkable as it is known as a major constituent of an alcoholic beverage, red wine. Resveratrol was shown to prevent liver injury by means of scavenging free radicals and inflammatory cytokines in experimental studies[59]. Its clinical utilization, though, is still far away. There are no conclusive data to prove the efficacy of any antioxidant medicaments for longer survival time or improved clinical conditions in the treatment of ALD. These are mostly cases of rather costly placebo. In contrast, dietary readjustment in the sense of sufficient energy intake and adequate supply of proteins is of value because malnutrition is a very poor prognostic factor in liver diseases[60]. What has been described as "liver diet" with increased supply of saccharides at the restriction of proteins and fats has no substantiation. Appropriate caloric intake with sufficient supply of proteins and polyunsaturated fats is important[34,61].

Severe alcoholic hepatitis has been treated with corticoids in many trials, with the best results in patients with hepatic encephalopathy, Maddrey score > 32 or Glasgow score > 9[62]. The Glasgow score is very simple to evaluate and its prognostic value is also greater than that of any other classification (Figure 5). The corticoid dose in that case is 40 mg prednisolone per day. The side effects of glucocorticosteroids must be also taken into consideration, as some patients on glucocorticosteroids experience adverse effects, mainly in the form hyperglycemia, Cushing's syndrome and increased risk of infection[63]. Despite the fact that the available trials are rather heterogeneous and some authors do not recommend the use of steroids in alcoholic hepatitis, recently published data emphasize the effect of corticosteroids on short-term survival of patients with severe alcoholic hepatitis[64], particularly in those with Maddrey score > 32.

Some trials and reviews of pentoxifylline (PTX) have shown a better risk/benefit profile than that of steroids and suggested that PTX could be a better first-line treatment in patients with severe AH. The efficacy of PTX in severe AH was first demonstrated by Akrividais *et al*[65] in 2000 on a group of 101 patients with severe AH. 24.5% of the patients who received PTX died during their index hospitalization, compared to a 46.1% mortality in the placebo group ($P = 0.037$). Remarkably, hepatorenal syndrome was the cause of death in 50% of patients on PTX compared to 91.7% of the HRS-related deaths in the placebo group ($P = 0.009$). According to the authors, the benefit appears to be related to a significant decrease in the risk of developing hepatorenal syndrome. In fact, renal dysfunction is frequent in patients with severe alcoholic hepatitis and, it seems, could be prevented with PTX[66].

Even in direct comparison with corticosteroids in a randomized trial, pentoxifylline was found to be superior to prednisolone for the management of severe alcoholic hepatitis regarding reduced mortality, improved risk-benefit profile and renoprotective effect[67]. Nevertheless, this observation should be confirmed on a larger cohort of patients[68]. A recent study by Lebrec *et al*[69] stopped short of confirming the effect of PTX on better survival but,

unlike a previous study, only Child-Pugh class C patients were included. However, the study did confirm a reduced risk of complications, such as bacterial infection, renal insufficiency, hepatic encephalopathy or gastrointestinal hemorrhage in patients treated with PTX compared to placebo.

Some centers recommend the use of PTX as the routine first line treatment of severe alcoholic hepatitis at a dose of 400 mg orally 3 times daily for a period of at least 4 wk[70]. They point to its safety, low cost and scope for long-term treatment. Significantly enough, the sweeping use of PTX as a first-line option is not generally recommended[71] and steroids should be used in patients with severe alcoholic hepatitis. Pentoxifylline could be used in patients with ineffectiveness or contraindications to steroids. The combination of pentoxifylline and steroids waits for clinical evaluation.

Biological treatment with anti- TNF-α antibodies fell short of expectations[72,73] so it can no longer be recommended for the management of alcoholic hepatitis[74].

Many studies with diverse conclusions have been published on the subject of nutrition and alcoholic hepatitis. In general, patients with alcoholic liver disease are frequently malnourished, a condition which worsens the prognosis[75]. However, the situation is not all that easy, as the spectrum of nutritional status in these patients may range from severe malnutrition to morbid obesity. The nutritional intervention on an outpatient basis depends on the degree of malnutrition, obesity and cooperation. In general, supplementation of multivitamins, folic acid and thiamine could be of value in chronic alcohol abuse, but data in the relevant literature are limited. Night-time nutritional supplements (approximately 700 kcal/d) may prevent muscle wasting and improve lean muscle mass in patients with liver cirrhosis[76] and should be considered, also relative to alcoholic hepatitis in patients with evidence of liver cirrhosis.

More data are available regarding the treatment of severe alcoholic hepatitis by enteral nutrition. The benefit of tube-feeding over the regular diet was demonstrated previously[77]. Patients on tube-fed nutrition had improved PSE scores, bilirubin and antipyrine clearance.

Many reviews and recommendations refer to a study by Cabre *et al*[78], which clearly demonstrated the efficacy of tube-fed nutrition. In their multi-center study, 71 patients with severe alcoholic hepatitis were randomized to receive 40 mg/d prednisolone or enteral tube feeding for 28 d and were followed up for 1 year. Mortality during the treatment was similar in both groups but during the follow-up significantly higher with steroids (37% *vs* 8%; $P = 0.04$), mainly because of infections with steroid treatment. The authors concluded that, unlike steroids, enteral nutrition had similar short-term mortality rates, improved 1 year mortality rates and reduced infectious complications. While some studies refrain from confirming any favorable effect of enteral feeding on survival, the implementation of tube-feeding in the treatment of acute alcoholic hepatitis is generally accepted[79]. There are only inconsistent data concerning the use of parenteral nutrition.

Despite the progress in the treatment of severe acute alcoholic hepatitis, the prognosis is still poor.

Alcoholic cirrhosis as such is treated in the same way as cirrhosis of other etiology; in particular, with adequate nutrition, bone disease prevention and prevention or treatment of liver cirrhosis complications (e.g., bleeding from esophageal varices, ascites, spontaneous bacterial peritonitis, hepatic encephalopathy)[80].

Quite a few medicinal products were tested for the treatment of alcoholic cirrhosis: antiphlogistics/propylthiuracil[81], colchicine[82], antioxidants/silymarin[83,84] and also phosphatidylcholine[85]. However, none of these were found to have a favorable effect on survival time and none are recommended for this particular indication any longer. Medicaments with a direct antifibrotic effect are still under evaluation[86].

Patients with advanced cirrhosis can be considered for liver transplantation, provided they are total abstainers[87]. In such cases, a five year post-transplantation survival can reach anything up to 85%[88].

CONCLUSION

Long-term intake of more than 30 g of absolute alcohol per day increases the risk of alcoholic liver disease; liver disease is nearly certain in long-term consumption in excess of 80 g of absolute alcohol per day. Alcoholic liver disease may take the chronic form (steatosis, steatohepatitis, fibrosis, cirrhosis) or that of acute hepatitis. Steatosis is fully reversible, which does not apply to the other conditions; cirrhosis is associated with a markedly shortened life expectancy. The results of laboratory testing in alcoholic liver disease usually include: increased GGT, AST/ALT ratio greater than 2 and increased MCV. Sonography will reveal enlarged liver and signs of steatosis. Absolute abstinence is an essential therapeutic precaution; no hepatoprotective treatment has been shown to improve the course of the disease. Likewise, there is no medicine that would demonstrably "protect" from the effects of alcohol.

The clinical course of severe alcoholic hepatitis could be improved with corticoids, enteral nutrition and pentoxifylline, although more clinical data are necessary to standardize or combine this treatment.

Patients with advanced cirrhosis should be considered for liver transplantation, provided they are verifiable abstainers.

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TOPIC HIGHLIGHT

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Human immunodeficiency virus infection and the liver

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Abstract

Liver disease in human immunodeficiency virus (HIV)-infected individuals encompasses the spectrum from abnormal liver function tests, liver decompensation, with and without evidence of cirrhosis on biopsy, to non-alcoholic liver disease and its more severe form, non-alcoholic steatohepatitis and hepatocellular cancer. HIV can infect multiple cells in the liver, leading to enhanced intrahepatic apoptosis, activation and fibrosis. HIV can also alter gastro-intestinal tract permeability, leading to increased levels of circulating lipopolysaccharide that may have an impact on liver function. This review focuses on recent changes in the epidemiology, pathogenesis and clinical presentation of liver disease in HIV-infected patients, in the absence of co-infection with hepatitis B virus or hepatitis C virus, with a specific focus on issues relevant to low and middle income countries.

Key words: Human immunodeficiency virus; Liver disease; Low-middle income settings

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INTRODUCTION

There are 33 million people infected with human immunodeficiency virus (HIV) globally with the greatest burden of disease in low and middle income countries. With the increased availability of antiretroviral therapy (ART), the number of people surviving with HIV and presenting with liver disease is increasing[1]. Most clinical trials and cohort studies have studied liver disease in HIV-infected individuals living in high income countries but liver disease is likely to emerge as an important co-morbidity in HIV-infected patients in low and middle income countries.

In the absence of co-infection with either hepatitis B virus (HBV) or hepatitis C virus (HCV), liver disease in HIV-infected individuals encompasses the spectrum from abnormal liver function tests, liver decompensation, with and without evidence of cirrhosis on biopsy, to non-alcoholic liver disease (NAFLD) and its more severe form, non-alcoholic steatohepatitis (NASH) and hepatocellular cancer (HCC)[2-6]. This review focuses on recent changes in the epidemiology, pathogenesis and clinical presentation of

liver disease in HIV-infected patients with a specific focus on issues relevant to low and middle income countries.

EPIDEMIOLOGY OF HIV AND LIVER DISEASE

Liver disease, including liver-related mortality secondary to chronic liver disease and HCC, is an emerging management problem in HIV-infected individuals in high income countries, where people are surviving longer on ART[1]. In the most recent data collection on adverse events of anti-HIV drugs (D:A:D) study of over 33 000 individuals, liver related deaths were the commonest cause of non-acquired immunodeficiency syndrome (AIDS) mortality amongst HIV-infected individuals[1]. While liver related deaths were strongly associated with viral hepatitis (84% of liver related deaths, 11% of total deaths), 16% of the liver related deaths (or 2.3% of the total deaths) in this population occurred in the absence of viral hepatitis[1].

Liver-related mortality in HIV-infected patients not co-infected with HBV or HCV has not been widely studied in low and middle income countries. In a retrospective South American study of serious non-AIDS events in the LATINA cohort (comprising over 6000 HIV-infected individuals on ART from Brazil, Mexico, Peru and Argentina), terminal liver failure or cirrhosis was the leading cause of death with 54/130 (42%) confirmed or probable cases based on clinical, laboratory and histological findings[7]. In this study, co-infection with HBV or HCV and low CD4 count were the major risk factors[7]. Similarly, a post-mortem study of 86 HIV-infected individuals undergoing autopsy in rural South Africa demonstrated that 10% had liver related conditions at the time of death[8]; however, it is likely that co-infection with viral hepatitis was a contributing factor.

Chronic liver disease, as measured by raised alanine aminotransferase (ALT), has been widely studied in HIV-infected individuals, particularly in high income settings. In a Swiss cohort of 2365 HIV-infected individuals not co-infected with either HBV or HCV, 385 (16%) had chronically elevated ALT (defined as > 2 × upper limit of normal)[9]. Risk factors associated with elevated ALT were high HIV RNA and prolonged ART exposure as well as high body mass index (BMI), alcohol abuse and increasing age[9]. A number of studies have now shown a link between BMI, high cholesterol levels, diabetes mellitus and liver disease in HIV mono-infection in high income countries[9-11], indicating lifestyle may play a significant role in the development of liver disease amongst HIV-infected individuals. Of concern, high BMI and diabetes mellitus are increasingly reported in low and middle income countries[12]. Ocama *et al*[13] recently reported that following 36 mo of ART in a cohort of 546 individuals in Kampala, only 1.5% had grade 3 aspartate aminotransferase (AST) elevations. In this study, a subset (n = 470) of patients were tested for HBV surface antigen and 9% were positive. A study of 59 individuals in Mexico showed a moderately strong positive correlation between elevated transaminases and HIV RNA in individuals not receiving ART and without viral hepatitis co-infection[14], consistent with reports from North America[11].

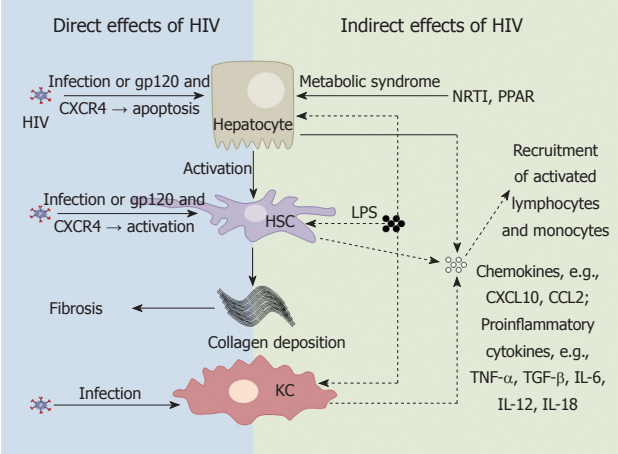

Figure 1 Human immunodeficiency infection and the liver. Mechanisms by which human immunodeficiency virus (HIV) infection of liver cells can contribute to liver disease progression by either direct (left panel) or indirect (right panel) mechanisms. HIV can directly infect hepatocytes, hepatic stellate cells (HSCs) and Kupffer cells (KCs). In the absence of productive infection, gp120 binding to CXCR4 may induce hepatocyte apoptosis and activation of HSCs, both contributing to fibrosis. Nucleoside reverse transcriptase inhibitors (NRTIs) and HIV itself [*via* peroxisome proliferator-activated receptor (PPAR) effects] may also contribute to liver disease by inducing the metabolic syndrome. HIV infection of the gastrointestinal tract leads to an increase in lipopolysaccharide (LPS) which can stimulate hepatocytes, KCs and HSCs to produce pro-inflammatory cytokines and chemokines which attract activated lymphocytes and monocytes to the liver which may further drive fibrosis. TNF-α: Tumor necrosis factor-α; TGF-β: Transforming growth factor-β; IL: Interleukin.

Data on HCC in HIV mono-infection in both low and high income countries is limited. In a study of over 3500 HIV-infected Chinese individuals, HCC was one of the leading causes of morbidity and mortality; however, this was primarily due to co-infection with viral hepatitis[15]. In a French prospective database study, a low CD4 count was a major risk factor for HCC with the rate ratio, or risk, increasing from 2 to 7.6 when comparing patients with a high CD4 count (> 500 cells/mL) to those with a low CD4 count (< 50 cells/mL) respectively[16]. Importantly for low and middle income countries, it has been demonstrated that the use of ART in individuals with low CD4 count (< 350 cells/mL) was associated with a reduced risk of hospitalization for liver related complications[17].

PATHOGENESIS OF HIV AND LIVER DISEASE

HIV may alter liver function by either direct or indirect mechanisms. HIV predominantly infects CD4+ T-cells, monocyte/macrophages and dendritic cells. However, there are multiple studies now showing HIV infection of a wide range of non hemapoietic cells, including cells in the liver. In addition, changes in gastrointestinal (GI) tract permeability *via* massive depletion of GI tract associated

CD4+ T-cells by HIV may have indirect consequences on immune activation and liver disease (Figure 1).

Direct effects of HIV on the liver

There are numerous studies demonstrating HIV infection of hepatic cells. Kupffer cells, differentiated tissue macrophages that reside in the liver, can be infected by HIV *in vivo*[18-20]. *In vitro* studies suggest that HIV infection of primary Kupffer cells leads to productive infection[21,22].

HIV RNA has also been detected in sinusoidal cells and hepatocytes *in vivo*[18,19]. Primary human sinusoidal cells have also been shown to be permissive to HIV infection *in vitro*[23]. A number of studies have demonstrated HIV infection of hepatocyte cell lines[24]. Infection of hepatocyte cell lines is thought to be CD4-independent as most hepatocyte cell lines, as well as primary hepatocytes, do not express CD4[24]. HIV infection of hepatocytes cells may therefore occur *via* receptor-mediated endocytosis or alternative co-receptors[25]. Hepatocytes may act as a transient HIV reservoir and promote CD4+ T cell infection by cell-cell contact[26].

HIV can also induce hepatocyte apoptosis *in vitro via* gp120 signalling through CXCR4 in the absence of infection[27]. Hepatocyte apoptosis can trigger pro-fibrotic activity of hepatic stellate cells (HSC) activity, as has been demonstrated in both HIV-HBV co-infection and HIV-HCV co-infection[28,29]. Further work is needed to determine the precise role of HIV-induced hepatic cell apoptosis and liver disease in HIV mono-infection.

HSCs are lipid storing cells and the main cells responsible for fibrogenesis in the liver. HIV infection of HSCs, including primary HSCs and the LX-2 stellate cell line, has recently been reported[30]. While HSCs express the HIV co-receptors CCR5 and CXCR4, HIV infection of HSCs appeared to be CD4-independent[30]. However, gp120 has also recently been shown to activate HSC *via* ligation of CXCR4[31]. HSCs infected with HIV or exposed to gp120 showed increased activation and fibrogenesis, as measured by alpha-smooth muscle actin and collagen production and increased levels of monocyte chemotactic protein-1 (MCP-1 or CCL-2). CCL-2 binds to CCR2 which is primarily expressed on activated pro-inflammatory monocytes and migration of these cells into the liver could potentially contribute to hepatic fibrosis and the accelerated progression to liver disease observed in HIV-HCV co-infected individuals[30,31].

Indirect effects of HIV on the liver

HIV infection of GI tract associated CD4+ T-cells leads to increased permeability to bacterial endotoxins such as lipopolysaccharide (LPS). Increased systemic levels of LPS are hypothesised to contribute to chronic immune activation in HIV-infected patients *via* activation of monocytes[32]. Kupffer cells are the main cell type in the liver that responds to LPS. When stimulated through ligation of the LPS receptor, toll like receptor (TLR)-4, Kupffer cells produce pro-inflammatory cytokines including tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), interleukin-6 (IL-6), IL-12 and IL-18[33]. Under normal physiological conditions, Kupffer cells remain tolerant, or refractory, to repeated LPS stimulation[33]. Elevated LPS has been shown to contribute to liver disease progression in alcoholic liver disease[34], as well as in NAFLD and NASH[35-37], chronic HCV[38] and HIV-HCV co-infection[39]. In addition to activation of Kupffer cells, LPS also directly activates HSC to produce CCL-2[40], and *in vitro* following co-culture with monocytes, induces hepatocytes to produce chemokines CXCL9, 10 and 11[41]. These chemokines will induce chemotaxis of both T-cells and monocytes to the liver. In HIV-infected individuals, an increase in systemic LPS levels may therefore potentially contribute to liver disease progression, although to date this has not been demonstrated in the setting of HIV alone (Figure 1).

Finally, HIV may also contribute to the metabolic syndrome associated with NAFLD or NASH *via* several mechanisms. ART and chronic inflammation can promote insulin resistance[42], where free fatty acids are released from adipose tissue, leading to increased intrahepatic triglyceride droplets (macrosteatosis) or *via* mitochondrial toxicity (microsteatosis) and oxidative stress[43,44]. HIV has also been shown to modify the activity of peroxisome proliferator-activated receptors (PPAR)[45]. PPARs are a family of nuclear receptor transcription factors that regulate insulin sensitivity, glucose and lipid metabolism, as well as inflammation, tissue repair, carcinogenesis and fibrosis, and are expressed by hepatocytes, HSCs and Kupffer cells[46-48]. Two HIV accessory proteins, Vpr and Nef, have been shown to suppress PPAR-γ subtype activity *in vitro*[49,50]. One small study in HIV-infected individuals also showed a reduction in the mRNA levels of PPAR-γ and a correlation with increased liver fibrosis[51]. Suppression of PPAR-γ activity by HIV may therefore represent another mechanism by which HIV contributes to liver disease progression.

CLINICAL PRESENTATIONS OF LIVER DISEASE IN HIV MONO-INFECTION

Noncirrhotic portal hypertension

Portal hypertension has been described recently in HIV mono-infected individuals without other known risk factors for liver disease. Individuals may present with decompensated portal hypertension, such as ascites or bleeding esophageal varices, without histological cirrhosis on liver biopsy[2-5]. This clinical scenario has been variably termed "noncirrhotic portal hypertension" (NCPH)[52], idiopathic portal hypertension[3] or "cryptogenic pseudocirrhosis"[4] to differentiate it from cirrhosis from other etiologies.

Numerous case reports and series of individuals with NCPH in HIV have been described[2,52-60], including those undergoing liver transplantation[61]. NCPH has not yet been described in low and middle income countries. It is possible that NCPH has not been recognized due to limited availability of liver biopsy. Alternatively, the

Table 1 Potential causes for liver disease in human immunodeficiency virus infection[43]

Viral hepatitis	HBV, HCV, (HDV)	Co-infection common (up to 10%)
	HAV, HEV	Self-limited acute increase in ALT
Drug hepatotoxicity	Alcohol	Limited data in low and middle income countries
ART[1]	Nevirapine	Hypersensitivity, usually early (< 12 wk)
	Efavirenz	Direct liver cell stress or hypersensitivity
	Abacavir	Hypersensitivity, (predominantly in HLA B57 carriers)
	ddI, d4T	Mitochondrial toxicity with long-term use
	Ritonavir	Steatosis, metabolic disturbance
	Darunavir	Hypersensitivity
	Tipranavir	Hepatic failure reported with ritonavir 200 mg
	Maraviroc	Hypersensitivity with liver involvement
Anti-TB therapy[2]	Rifampicin	Drug interactions with ART and direct hepatotoxicity
	Isoniazid	Hepatotoxicity may be increased in HIV
	Pyrazinamide	Dose-related hepatotoxicity
Hepatotropic infections	Schistosomiasis	Leads to portal hypertension
	Leishmaniasis	Fever +/- hepatosplenomegaly
	Herpes viruses inc EBV CMV HHV6 HSV	Often cause raised transaminases, occasionally symptomatic hepatitis
	Liver abscess	Unlikely to cause chronic liver disease
HIV cholangiopathy		Usually when CD4 < 200 cells/μL
NAFLD		ART-related, prevalence unknown

[1]Raltegravir rarely causes hepatitis; [2]Ethambutol rarely associated with hepatitis, and may be due to concurrent therapy. HBV: Hepatitis B virus; HCV: Hepatitis C virus; HDV: Hepatitis D virus; HAV: Hepatitis A virus; HEV: Hepatitis E virus; ART: Anti-retroviral therapy; ddI: Didanosine; d4T: Stavudine; TB: Tuberculosis; NAFLD: Non-alcoholic fatty liver disease; EBV: Epstein barr virus; CMV: Cytomegalovirus; HHV: Human herpesvirus; HSV: Herpes simplex virus; HIV: Human immunodeficiency virus; ALT: Alanine aminotransferase; HLA: Human leukocyte antigen.

impact of NCPH may be overshadowed by more common diseases such as co-infection with viral hepatitis, pyogenic infections or tuberculosis[8,62,63]. NCPH has been described in adolescence[64] but the lead time prior to clinical presentation of NCPH may mean it remains a future problem for low and middle income countries.

Liver histology from individuals with HIV mono-infection and NCPH is variable and includes periportal or perisinusoidal fibrosis, low grade inflammation and steatosis[65]. However, a common pattern appears to be portal vein occlusion and focal fibrous obliteration of small portal veins, so-called "hepatic venopathy", often in the setting of nodular regenerative hyperplasia (NRH)[52,57].

A hypercoagulable state may contribute to the hepatic venopathy and NRH, as described in the antiphospholipid syndrome associated with rheumatoid arthritis[66]. Hypercoagulable states were identified in 8 out of 10 individuals with NCPH in one case series[57]. In a recent case-control study, NRH was seen in all 5 out of 11 individuals with NCPH where histology was available, and protein C and S activity was lower in cases than controls[67]. In another small case-control study including 15 individuals with confirmed esophageal varices and absence of cirrhosis on liver biopsy, periportal fibrosis was the most common hepatic lesion described and NRH was only seen in 1 patient[65]. A strong association between prolonged exposure to didanosine (ddI) and the development of NCPH was found in this study. Two cross sectional studies looking at associations between multiple clinical factors and NCPH have also demonstrated an association with prior and current use of ddI[2], as well as NASH[5]. ddI is now almost never used in high income countries but still frequently used in low and middle income countries, and therefore NCPH may soon be seen more frequently in these settings[68].

ART and liver disease

Hepatotoxicity due to ART may be related to agents from a number of classes, including nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors[43] (Table 1). The severity of hepatotoxicity may range from transient elevations in transaminase levels to hepatic failure and death, *via* a variety of mechanisms. NNRTI such as nevirapine and efavirenz may cause hypersensitivity[69-71]. NRTI, primarily ddI, may cause direct mitochondrial toxicity leading to abnormal liver function[72]. Other mechanisms by which ART causes liver-related toxicity include direct cell stress and disturbances in lipid/sugar metabolism and steatosis, as seen with protease inhibitors[43]. The protease inhibitors ritonavir, tipranavir and darunavir have all been associated with elevations in ALT[43].

Fatty liver disease in HIV

NAFLD is now commonly reported in many high income countries in the presence and absence of HIV infection. The spectrum of NAFLD ranges from mild steatosis to NASH, which may progress to severe fibrosis and cirrhosis. NAFLD may be associated with other features of the metabolic syndrome, including central obesity, insulin resistance, diabetes mellitus and dyslipidemia, such as raised triglycerides, low high-density lipoproteins and raised low-density lipoproteins. NAFLD has been identified in up to 30% of HIV mono-infected Americans, although this study was unable to demonstrate

whether the prevalence of NAFLD was different to HIV-negative individuals[6]. While risk factors for NAFLD may be similar in people with or without HIV[73], a small study has suggested that people with HIV and NAFLD may be more physically active and less obese than HIV-negative individuals with NAFLD, and that other factors such as HIV itself or ART may contribute to NAFLD[74]. NAFLD is frequently described in the setting of HIV-HCV co-infection[75-77] where ART including ddI or stavudine may also be implicated[75]. Insulin resistance and NASH have been described in individuals with liver disease in HIV mono-infection[5,52]. Insulin resistance and exposure to ddI and/or stavudine were factors associated with advanced fibrosis in 681 HIV-HCV co-infected individuals in Spain[78]. Few studies of NAFLD have been published from low and middle income countries.

Co-infections other than HBV or HCV

Co-infection with *Mycobacterium tuberculosis* and its treatment are also important causes of liver disease in low and middle income countries and are summarised in Table 1. Hepatitis related to isoniazid therapy is common[79], and HIV or ART may increase the risk of isoniazid-related hepatotoxicity, as demonstrated in a small Ethiopian study[80]. Concomitant anti-tuberculosis therapy was associated with a 5-fold increased risk of abnormal aminotransferase levels in Ugandan individuals commencing NNRTI-based ART[81].

Schistosomiasis may also cause liver disease but was an uncommon cause of liver disease in HIV-infected individuals in Uganda in one study of 77 patients[62]. In a recent study using transient elastography (TE, FibroScan®) in 1000 individuals in Uganda, 14% of the 500 HIV-infected individuals had positive schistosomiasis serology[82]. Significant fibrosis (≥ F2, liver stiffness measurement ≥ 9.3 kPa) was detected in 17% of HIV-infected individuals. Positive schistosomiasis serology was not a significant predictor of cirrhosis [odds ratio 1.7, (0.9-3.3); $P = 0.10$] in this study but the authors concluded that schistosomiasis may play a role in the burden of liver disease in HIV-infected individuals in Uganda[82]. Further work is required to better understand the true relationship between HIV, schistosomiasis and liver disease.

The impact of other infections, including visceral leishmaniasis (also known as Kala-Azar), on liver disease in HIV infection is unclear but has been reported[83] and may cause chronic disease[84]. Amebic liver abscesses are frequently described in HIV infection[85] but are usually treatable and are unlikely to contribute significantly to ongoing liver disease[62]. HIV cholangiopathy in individuals with CD4 T-cell counts < 200 cells/mL may be due to infections with cytomegalovirus, cryptosporidium or microsporidium[63], although prevalence in low and middle income countries has not been reported.

Alcohol

Alcohol is a common cause of liver disease world-wide and is likely to be as important in low and middle income countries, as it is in HIV-infected individuals in high income countries[86]. Alcohol is responsible for significant morbidity and mortality in South Africa[87] and may also contribute to significant fibrosis detected by TE in Uganda[62]. However, data on alcohol consumption and its impact on liver disease in the setting of HIV in low and middle income countries are limited.

NON-INVASIVE ASSESSMENT OF LIVER DISEASE IN HIV MONO-INFECTION

The availability of liver biopsy is limited in low and middle income countries and non-invasive measures of liver fibrosis, such as TE, have been studied in many liver conditions but largely in high income countries[88]. Algorithms to diagnose liver disease severity based on a range of biochemical and hematological indices, such as FibroTest®, Hepascore or AST to platelet ratio index, have also been used[89]. Most data regarding TE come from studies of individuals with HCV[88]. TE is most reliable at detecting cirrhosis (F4) or severe fibrosis (F3/4) but is less reliable at differentiating absent or mild fibrosis (F0/1) from significant fibrosis or greater (F2/3/4)[88]. In many studies considered in a recent meta-analysis, the sensitivity for detecting at least significant fibrosis was less than 70%[88]. The mean area under the receiver operator characteristic curve (AUROC) was 0.84 (95% CI: 0.82-0.86), where a diagnostic tool is considered good if the AUROC is greater than 80% and excellent if the AUROC is greater than 90%[88]. The AUROC for the detection of cirrhosis was 0.94 (95% CI: 0.93-0.95).

Non-invasive measures like TE have been used to detect severe fibrosis in HIV-infected individuals with persistently elevated transaminase levels[2,5,90]. Few studies in low and middle income countries have been reported to date[82]. However, the convenience of TE and or biochemical or hematological indices offers great potential for screening and monitoring liver disease in large cohorts of HIV-infected individuals in low and middle income countries and there is a great need for further work in this area. A proposed algorithm for the use of TE in evaluating HIV-infected individuals is presented in Figure 2[91].

CONCLUSION

Liver disease in HIV-infected individuals, in the absence of co-infection with HBV or HCV, is an emerging issue in all settings. While ART related toxicities are an obvious cause, there is emerging evidence that HIV infection may have a direct impact on the pathogenesis of liver fibrosis, NAFLD and NASH and subsequent progression to liver disease. Further research is needed to determine the causal link between HIV infection and liver disease progression. Two potentially important risk factors for low and middle income countries in the development of liver disease are prolonged exposure to high HIV RNA and low CD4 count, providing further support for earlier initiation of ART. One of the major obstacles to

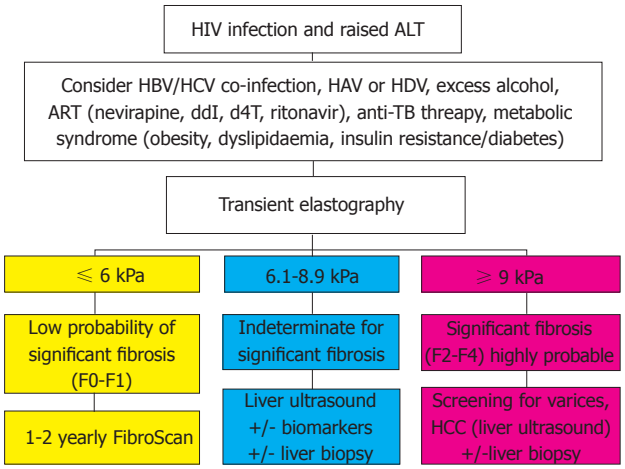

Figure 2 Proposed use of transient elastography (FibroScan) in human immunodeficiency virus infected individuals with raised liver enzymes (adapted from Ref.[91]). HIV: Human immunodeficiency virus; ALT: Alanine aminotransferase; HAV: Hepatitis A virus; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HDV: Hepatitis delta; ART: Anti-retroviral therapy; ddI: Didanosine; d4T: Stavudine; TB: Tuberculosis; HCC: Hepatocellular carcinoma.

research into the epidemiology of the true incidence of liver disease in both high income and low and middle income countries is the lack of suitable non-invasive methods of determining liver disease progression. However, with the advent of newer convenient technologies, such as TE and non invasive plasma markers, we may see this change in the near future.

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TOPIC HIGHLIGHT

Francesca Cainelli, MD, *Series Editor*

Hepatocellular carcinoma in developing countries: Prevention, diagnosis and treatment

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Abstract

Hepatocellular carcinoma (HCC) occurs commonly and with increasing frequency in developing countries, where it also carries an especially grave prognosis. The major risk factor for HCC in these regions is chronic hepatitis B virus (HBV) infection, although dietary exposure to aflatoxin B1 also plays an important etiological role. Prevention of HCC in developing regions is unlikely in the foreseeable future. Although an effective vaccine against HBV is available, the percentage of babies born in developing countries that receive the full course of immunization remains low. Moreover, the usually long interval between infection with HBV and the development of HCC means that 30 to 50 years will elapse before the full effect of the vaccine will be realized. Practical measures to prevent aflatoxin B1 exposure are not in place. Serum α-fetoprotein levels are a useful pointer to the diagnosis of HCC in low-income countries, but definitive diagnosis is hampered both by the lack of the sophisticated imaging equipment now available in developed countries and by obstacles to obtaining histological proof. In the majority of patients in low-income regions, the tumor is inoperable by the time the patient presents. Hepatic resection is seldom possible in sub-Saharan Africa, although the tumor is successfully resected in a larger number of patients in China. Liver transplantation for HCC is rarely performed in either region. Sophisticated new radiotherapy techniques are not available in developing countries. The beneficial effects of the multikinase inhibitor, sorafenib, are encouraging, although financial considerations may restrict its use in low-income countries.

Key words: Hepatitis B virus infection; Aflatoxin B_1; α-fetoprotein; Hepatic resection; Hepatic transplantation; Sorafenib

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common human cancer, with approximately 750 000 new cases occurring worldwide each year[1]. Eighty percent of global HCCs occur in developing countries, and in these countries HCC is one of the three most common tumors. Eastern and south-eastern Asia (with the exception of Hong Kong, Japan, South Korea, Singapore, Taiwan and Macau) and almost all of sub-Saharan Africa are low-income regions with high incidences of the tumor[1].

HCC ranks third in annual global cancer mortality rates and has the shortest survival time of any cancer in

both males and females[2]. The prognosis is even poorer in patients in low-income countries. Among the reasons for the poor prognosis are the often advanced stage of the tumor when the patients are first seen and that optimal management of HCC requires resources that are seldom available in developing countries.

The reason for the high incidence of HCC in resource-poor countries is that two of the three major environmental causes of the tumor worldwide, namely, chronic hepatitis B virus (HBV) infection and dietary exposure to the fungal toxin, aflatoxin B_1, occur far more often in these countries. Chronic HBV infection occurs in more than 8% (and in as many as 15%) of the population and, of those infected, one-quarter or more will develop the tumor[2]. The virus is responsible for 80% to 89% of HCCs in these regions, where HBV infection is almost always acquired in infancy or early childhood, as a result of either perinatal or horizontal transmission of the virus. Ninety percent of those infected in the first year of life, decreasing to 50% those infected in the fifth year and far fewer of those infected in the next few years, will become chronically infected with the virus and face a life-time relative risk of developing the tumor as high as 100%[3]. With chronic HBV infection being acquired predominantly in infancy or early childhood, the resultant HCC presents before or in mid-adulthood, the most productive years of life, causing a drain on productive capacity in addition to a substantial burden on the healthcare system. The reasons for the poorer prognosis of HCC in low-income countries are the generally longer interval before the patients receive medical attention and hence the more advanced stage of the tumor at the time of diagnosis, the fewer doctors and the scarcity and inferior quality of the diagnostic facilities available, and the limited treatment that can be offered. In addition, financial and logistical constraints in resource-poor regions compromise attempts to prevent the tumor.

Aflatoxins are difuranocoumarin derivatives of Aspergillus flavus and A.parasiticus. These fungi contaminate crops, particularly maize, ground nuts and fermented soy beans, in tropical and sub-tropical countries with warm, humid climates and, more especially, in subsistence farming communities in these countries. Contamination occurs both during growth of the crops and as a result of their improper storage. Sub-Saharan Africa and the Asia-Pacific region have high levels of exposure to the fungal toxin. Aflatoxin B_1 (AFB_1) is the aflatoxin most often found in contaminated human foodstuffs and is the most potent hepatocarcinogen[4]. The hepatocarcinogenic effects of AFB_1 and HBV are synergistic, with a multiplicative relative risk for HCC development[5].

With few exceptions, chronic hepatitis C virus (HCV) and HCV-induced HCC are significantly less common in developing than in developed countries. One exception is Somalia, where chronic HCV infection is as common as chronic HBV infection[6].

In most rural regions of China, the population drinks primarily pond, ditch or river water. High concentrations of tumor-promoting microcystins, derived from blue-green algae, have been identified in these waters and high microcystin contents correlate with a high incidence of HCC[7,8].

PREVENTION

Because of the high incidence, inadequate treatment and graver prognosis of HCC in low-income countries, prevention of the tumor is an urgent priority in these countries.

Prevention of hepatitis B virus-induced hepatocellular carcinoma

An effective and safe vaccine against HBV has been available for a number of years. Based on data in 2008, HBV vaccine was included in the expanded Program of Immunization in 177 countries and an estimated 69% of the birth cohort that year received 3 doses of the vaccine[9]. Unfortunately, the percentage of babies born in resource-poor countries that receive the full course of immunization is far lower that that in developed countries. In sub-Saharan African countries, between 10% (in Chad) and 99% (in The Gambia) of babies receive the full course of the vaccine[10]. In 12 countries, less than 70% of infants receive the full course[10]. The coverage is even worse in the Asia-Pacific region.

In Taiwan (not a low-income country), where immunization of babies against HBV began in 1984 and universal coverage was achieved by 1986, coverage of all preschool children by 1987 and extension to older children and adults by 1990, the vaccination program has already resulted, in the age groups that have been immunized, in a decrease from 90 to 15% in the number of infected babies born to highly infectious carrier mothers and a ten-fold or more decrease in the rate of chronic HBV carriage in these babies, as well as in those infected slightly later by horizontal infection[11]. The prevalence of HCC among recipients of the vaccine has already decreased by 70% in comparison with those in the non-vaccinated age groups[12]. After adjustment for age and sex, the relative risk for HCC was 0.31 among children aged 6 to 19 years in the vaccinated cohort, compared with similar aged children in unvaccinated cohorts[11]. These encouraging results give promise that the universal incorporation of a full program of HBV vaccination into the Expanded Program of Immunization in developing countries in which HBV infection is endemic will, in the future, prevent thousands of deaths from cirrhosis and HCC and that HBV-induced HCC could ultimately be completely prevented.

In the early years after immunization was introduced, some cases of chronic HBV infection and HCC were still seen in Taiwanese children and adolescents who had received a full course of the vaccine. This led to the realization of the importance of administering hyperimmune γ-globulin (HBIG) at the time of birth (in spite of its high cost) in addition to the first dose of the vaccine

to babies born to highly infectious (HBeAg-positive) carrier mothers. Addition of HBIG to the vaccine schedule has resulted in a further reduction in the occurrence of chronic HBV infection and HBV-induced HCC[11]. Regrettably, the high cost of HBIG will have important implications in low-income countries. In the few individuals still developing "break-through" infections and tumors in Taiwan, failure to complete the full course of the vaccination program has been shown to be responsible[11].

Because of the usually long interval between infection with HBV and the development of HCC, it will take 30 to 50 years for the full effect of the vaccine on the incidence of HBV-induced HCC to be realized in these countries. In the interim, the incidence of HBV-induced HCC continues to increase.

Prevention of aflatoxin B_1-induced hepatocellular carcinoma

Contamination of staple foodstuffs by AFB_1 does not occur in high-income countries because those food stuffs that might be infected are screened for their aflatoxin content by governmental agencies and do not enter the commercial market if unacceptably high levels are found. In low-income countries, regulations to control dietary exposure to AFB_1 are either non-existent or unenforceable in practice. Contaminated crops are consumed by the subsistence farmer's family, neighbors, friends and relatives, and are sold locally or regionally without ever coming under the scrutiny of a governmental agency. It is estimated that about 45 million of the world's population are exposed to aflatoxins.

Because contamination by Aspergillus species takes place both during growth of the crops and as a result of their improper storage, attempts at primary prevention must be directed at minimizing both sources of fungal contamination[4,13-15]. One possible intervention is to alter the agricultural practices in regions of high dietary AFB_1 intake by replacing crops that are highly susceptible to fungal contamination with others at lower risk. This approach has been successfully used in one limited study in China when a change to a rice-based diet resulted in an appreciable decrease in AFB_1 intake[13,14]. However, for most communities in resource-poor countries, a change in agricultural practices leading to a change in diet is not feasible. Because damaged plants are more susceptible to fungal contamination, relatively simple and inexpensive pre-harvest prevention could be achieved by adequate irrigation and spraying of the crops with fungicides[13-15]. A study confirming the effectiveness of combating post-harvest contamination by simple interventions such as sun-drying the crops on cloth rather than the earth, hand sorting to remove mouldy crops, and better storage practices has been reported[15]. For such interventions to be successful on a wide scale in resource-poor countries will require training of the farmers in practical ways to prevent contamination of susceptible crops and the provision of appropriate storage facilities[15].

Prevention of hepatitis C virus-induced hepatocellular carcinoma

Despite considerable research over many years into the development of a vaccine against HCV, there appears to be little likelihood that a vaccine will become available in the near future. In both developed and developing countries, attempts at prevention of HCV infection (which also apply to HBV infection) should include encouraging the avoidance of the high risk behaviors of illicit drug injection and promiscuous sexual activity, insisting on the use of needles and syringes on a single occasion only, or if this is not possible, careful sterilization before their reuse, and on sterilization of surgical instruments[16]. Equally important is encouraging the screening of donated blood for the presence of these viruses[16].

Despite recent advances in treating patients with chronic HCV infection with anti-viral drugs, the overall impact of therapy is small because the majority of individuals chronically infected with the virus are unaware that they are infected. The high cost of the anti-viral agents is an additional impediment in developing countries. Efforts to prevent infection should include identifying persons at increased risk of HCV infection and providing them with appropriate counseling.

Prevention of microcistin-induced hepatocellular carcinoma

Since 1973, the Chinese government has been urging the rural population to drink deep-well water[17]. This has resulted, for example in Qidong county, in 80% of the population now drinking deep-well water compared with only 20% in earlier years. However, the effect of this intervention on the occurrence of HCC has yet to be published.

DIAGNOSIS

Because of the higher incidence of HCC in developing countries, medical practitioners in these countries are more likely than those in low incidence regions to be mindful of this tumor. Moreover, history taking and physical examination can be performed equally well in resource-poor as in resource-rich regions and, in all but the most impoverished or remote regions, the least expensive of organ imaging techniques, ultrasonography, may be available. It should therefore be possible in low-income countries to make a provisional diagnosis of HCC or, at least, to include the tumor in the differential diagnosis. Moreover, the diagnostic laboratories should be able to measure the serum α-fetoprotein (AFP) concentration. The serum AFP concentration is particularly useful in the diagnosis of HCC in low-income regions[18,19]. The level is raised in 90% of sub-Saharan black Africans with HCC and in 75% of the patients it is raised to a level higher than that which may be present in benign hepatic diseases (usually given as greater than 400 or 500 ng/mL)[18,19]. In Chinese patients, approximately 75% have a raised serum

AFP level, with about 50% having a level above 400 ng/mL[20,21]. In contrast, the value is raised in only approximately 55% of patients in developed countries[22].

Definitive diagnosis of HCC depends upon either histological examination of the hepatic mass or the finding with a dynamic imaging technique of one or more mass-lesions in the liver showing a typical vascular profile in the form of early arterial phase enhancement, followed by washout with loss of the enhancement. Micro-bubble contrast ultrasonography, three-dimensional contrast-enhanced computerized spiral tomography or magnetic resonance imaging enhanced with gadolinium or gadoxetic acid will not be available in the rural areas and may not even be available in the cities of resource-poor countries.

In developing countries, fewer medical (or para-medical) practitioners than their counterparts in high-income countries are trained to perform percutaneous liver biopsies. Moreover, it is necessary to send the biopsy material to a central diagnostic laboratory to have the histological examination performed. In practice therefore, particularly in rural regions of developing countries, the tumor is often diagnosed on the basis of the clinical features and either a raised serum AFP concentration or, less often, the finding of a mass-lesion on ultrasonography of the liver, without obtaining histological confirmation. Furthermore, because of the poor results of treating HCC and dismal prognosis of the patients in developing countries, many medical practitioners, particularly those in the rural areas, develop a nihilistic attitude to definitive diagnosis and treatment of the tumor and the patient is often sent home to die on the basis of a provisional diagnosis only.

HCC is frequently diagnosed only when the tumor has reached an advanced stage. This applies in all geographical regions but more so in developing countries. A further difficulty in resource-poor countries is the insufficient number of hospital beds available.

In all geographical regions, HCC commonly co-exists with cirrhosis[23]. However, the effect of the associated disease on the diagnosis of the tumor differs between developed and developing regions. In the former, HCC often presents as a complication of long-standing symptomatic cirrhosis and the patient may have few, if any, symptoms attributable to the tumor, whereas in the latter, the presence of cirrhosis is typically overshadowed by the symptoms and signs of the tumor[23].

Although HCC generally presents clinically in a sufficiently characteristic way to allow the presence of the tumor to be suspected by an experienced clinician, it may manifest in any of a number of unusual ways, at least some of which occur more commonly in patients in developing countries. Unusual presentations of HCC may result from a number of complications of the tumor, such as tumor rupture causing an acute hemoperiteum, Budd-Chiari syndrome and inferior venal caval obstruction from invasion of the hepatic venous system and inferior vena cava by the tumor, and obstructive jaundice from spread of the tumor into the biliary tree. Atypical presentations may also result from paraneoplastic manifestations of the tumor, the more common of which are hypoglycemia, polycythemia, and hypercalcemia[24]. A physical sign that is a useful pointer to the diagnosis of HCC but is often missed is the presence of a hepatic arterial bruit. The systolic bruit, which is heard in as many as 27% of patients with HCC[25], is focal and its detection requires thorough auscultation over the liver.

The finding of an elevated right hemidiaphragm (or rarely left hemidiaphragm) on chest X-ray is a useful pointer to the presence of HCC in developing countries[26], provided that an amebic liver abscess, another common space-occupying hepatic lesion in these countries and one that can present clinically in a manner not dissimilar to HCC, can be excluded. Multiple pulmonary metastases are often evident in patients with HCC in resource-poor regions at the time of first admission[26,27] and this finding in association with a pathologically raised right hemidiaphragm on chest X-ray is virtually diagnostic of HCC.

Serum markers of HCC, reactivity of AFP with Lens culinaris agglutinin (AFP-L3), des-γ-carboxy prothrombin and α -fucosidase, have not proved to be more useful than AFP as indicators of HCC in resource-poor countries.

The use of a 6-month surveillance program consisting of a physical examination, measurement of the serum AFP level and ultrasonographic examination of the liver aimed at the early detection of HCC is beyond the means of resource-poor countries, even if the ultrasonographic facilities are available.

TREATMENT

The first decision that needs to be taken when a definitive diagnosis of HCC has been made is whether or not the tumor is resectable.

Hepatic resection

Unfortunately, in resource - poor regions, HCC is very often inoperable at the time the diagnosis is made. Apart from the advanced stage of the tumor resulting from the difficulty and delay in obtaining medical advice, in many patients in these regions, HCC runs a particularly fulminant course and has reached an advanced stage by the time the patient seeks medical attention. Little information has been published on the rate of growth of the tumor in these patients, but in one study, based on serial estimations of serum AFP levels in black African patients, the doubling time of HCC was estimated to be as short as 10 d[28]. This time contrasts with an average tumor doubling time as long as 136 d in patients in resource-rich countries[29,30].

Even in those patients in developing countries in which the tumor runs a less rapid course and who come under medical attention timeously, early detection may be difficult. Because of the large size of the liver, the tumor must grow to an appreciable size before it can be felt or before it invades adjacent structures. Moreover, the considerable functional reserves of the liver ensure that jaundice and other evidence of hepatic dysfunction appear only when a

large part of the organ has been replaced by tumor.

Only 8% of Ugandan[31] and 1% of rural southern African black patients[32] were found to have resectable tumors, at a time that resectability rates of up to 37% were being recorded in some western countries with low or intermediate incidences of HCC[33] and up to 20% in Japanese patients[34]. These resection rates have not changed significantly in sub-Saharan Africa in more recent times, whereas resectability rates in industrialized countries are now appreciably higher. However, hepatic resections have been performed more often in some of the larger hospitals in China.

Further evidence for the advanced stage of HCC when patients from developing countries first present is the often large size of a single tumor or the extent of the tumor burden when more than one tumor mass is present in the liver. This too has important implications when considering the operability of the tumor. The average weight of the cancerous liver at necropsy in African black patients with HCC ranges in different studies from 3045 to 3914 grams (with a largest size of 8780 grams) (the average weight of a normal liver in an adult black male is 1750 grams)[35-37]. This contrasts with average weights of 2036 grams in Japanese[38], 2615 grams in North American[36] and 2477 grams in South African Caucasian patients[35-37]. The tumors are generally even larger in non-cirrhotic livers (average weights of the tumorous liver in Ugandan patients without cirrhosis is 4134 grams compared with 2768 grams in those with cirrhosis[38]) and the same is true in southern African blacks (3918 and 3085 grams, respectively)[35]. Multiple tumor masses throughout the liver, irrespective of their size, obviously preclude hepatic resection. Moreover, resection of one or a few small tumor masses may be precluded by its or their position in the liver.

Another reason for the low resectabilty rates of HCC when patients from resource-poor countries are first seen is the frequency with which the tumor has already spread beyond the liver. For example, 19% of southern African blacks with HCC have radiologically-evident pulmonary metastases at this time[26,27] (compared, for instance, with 7% in patients in the United Kingdom[22]). Moreover, in a further 27% of these patients, pulmonary metastases too small to have been seen radiologically during life were present at necropsy, performed on average only 6 wk later[26].

Cirrhosis is present in about 70% of patients with HCC in developing countries (although in some regions of sub-Saharan Africa it is present less often than in patients in other parts of the world) and this too greatly influences the decision whether or not surgical resection will be possible. The resulting liver dysfunction may make surviving the operation impossible or unlikely. In addition, the presence of cirrhosis precludes post-operative regeneration of the remaining liver tissue. In all populations, the requirements for hepatic resection in a patient in whom HCC has arisen in a cirrhotic liver are that the serum bilirubin level should be normal, significant portal hypertension and esophageal varices should be absent, and the platelet count should be above 100 000/mm[39].

Careful follow-up of patients from all regions after successful surgical resection of HCC is mandatory because of the high recurrence rate of the tumor, which may be as high as 50% at 3 years and 70% at 5 years[39]. Apart from incomplete resection of the original tumor, the reasons for HCC recurrence are that new tumor foci develop subsequently because the cause of the malignant transformation is still present in the remaining liver tissue. In addition, the possibility exists that malignant cells in the circulation, increased in number by the handling of the tumorous liver during the operation[40,41], may seed the liver post-operatively. Tumors caused by chronic HBV infection are often multifocal and small tumors missed at the time of the original surgery may become clinically evident at any time thereafter. Those patients in whom the tumor has been resected but who are still actively infected with HBV or HCV must be treated with appropriate anti-viral drugs with the aim of curing the infection or, if this is not possible in the case of HBV, of seroconverting HBV e antigen-positive patients to an anti-HB e-positive status and keeping the viral loads at as low a level as can be achieved.

Hepatic transplantation

With the generally far advanced stage of HCC at the time that patients in resource-poor countries seek medical attention, very few meet the criteria required for liver transplantation (no spread of the tumor beyond the liver, a single tumor less than 5 cm in diameter or fewer than three tumor masses, each less than 3 cm in diameter and no macrovascular invasion (Milan criteria)[26]). Few, if any, hepatic transplants have been performed for the treatment of HCC in developing countries.

Other treatments

In patients with inoperable HCC, improved results are now being obtained with the use of 3-dimensional high-dose photon beam radiotherapy and stereotactic radiotherapy. However, the sophisticated equipment necessary for these therapies are not available in resource-poor countries. Similarly, forms of local treatment or palliation of HCC, such as radio-frequency ablation, ablation with alcohol or trans-arterial chemoembolization, which are available in high-income countries, require equipment and expertise that are unlikely to be available in low-income countries.

For those patients in whom surgery or palliation cannot be performed, drug therapy needs to be considered. Up until recently, no form of chemotherapy has been found to be effective in the treatment of HCC[42]. The reasons for the poor results are the late presentation with very large tumor burdens and perhaps the frequency of multi-drug resistance genes. However, recent studies have shown the beneficial effects on survival time of the oral multikinase inhibitor, sorafenib. Trials with this and other multikinase inhibitors have and are being conducted in a number of centers in different parts of the world but, as

far as I am aware, no results have yet been published in patients in resource-poor countries. In addition, the cost of the drug may be an important issue in low-income countries.

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BRIEF ARTICLE

Relationship between interleukin 18 polymorphisms and susceptibility to chronic hepatitis B virus infection

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Abstract

AIM: To identify the relationship between the tagging single nucleotide polymorphism sites (tagSNPs) of the Interleukin-18 (IL-18) gene and genetic susceptibility to chronic hepatitis B virus infection in Chinese patients.

METHODS: Five hundred and one cases of chronic hepatitis B virus (HBV) infection and 301 HBV natural clearance controls were studied. Two tagSNPs in the IL-18 gene (rs1946518A/C and rs574424C/G) were genotyped by the Multiplex Snapshot technique. The genotype and allele frequencies were calculated and analyzed.

RESULTS: In the genotypes of rs1946518, the AA type was present at a higher frequency in the patients compared to those in the controls. Odds ratio (OR) of the AA genotype for the comparison with that of the AC and the CC genotype was 1.537 (95% confidence intervals (CI): 1.116-2.218, $P = 0.009 < 0.025$). In phenotypes, the allele C at rs1946518 was of a significantly lower frequency in the patients with chronic hepatitis B than that in the controls ($P = 0.017 < 0.025$). OR of the allele A for the comparison with that of the allele C was 1.279 (95% CI: 1.045-1.567). As for the rs574424 genotypes, no significant difference in this genotype distribution or in this allele frequency between the patients and the control subjects was observed. No significant difference in the haplotype frequencies between the patients with chronic hepatitis B and HBV natural clearance individuals was displayed.

CONCLUSION: The data suggest that genotype AA and the allele A of the IL-18 at position rs1946518 are closely associated with the resistance to chronic hepatitis B and may be the dangerous gene. However, no statistical association was found between polymorphisms of rs574424 for IL-18 and hepatitis B.

Key words: Hepatitis B virus; Interleukin 18; tagSNP; Genetic susceptibility

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INTRODUCTION

The outcome of HBV infection is mainly influenced by the virus, immune response and genetic diversity[1-3]. The clinical course of chronic hepatitis B virus (HBV) infection varies from spontaneous recovery after acute hepatitis to a chronic persistent infection that may progress to chronic hepatitis B virus carrier, chronic hepatitis B, cirrhosis or hepatocellular carcinoma. Many studies strongly support the role of host genetic components in determining the outcome of HBV infection[4-6]. Genetic susceptibility of HBV infection is considered to be determined at different functional levels, such as cytokine production, antigen presentation and receptor recognition, and the single nucleotide polymorphisms (SNPs) of cytokine genes involved in the immune response after HBV infection become the emphasis of gene susceptibility, which may highlight the genetic background of HBV infection[7].

Interleukin-18 (IL-18), which was first described as an interferon-γ (IFN-γ) producing factor, has multiple functions, including the activation of cytotoxic T lymphocytes, natural killer cells and the promotion of T-helper type 1 (Th1) immune responses[8]. IL-18 leads to activities against pathogens, activates effector cells involved in the cellular interactions that occur during inflammation, is part of the acute and chronic stages of viral hepatitis and induces target-cell apoptosis. Migita *et al*[9] reported that a strong virus-specific CD4+ and CD8+ T lymphocyte response to hepatitis B virus was associated with IL-18's production decided by the IL-18 gene.

IL-18 gene polymorphisms have been reported to be implicated in susceptibility to chronic hepatitis B, in its pathogenesis[10] or in disease evolvement[11]. Many SNPs in the IL-18 gene region were predicted to be involved in clearing hepatitis B virus, such as -607C/A and -137G/C in the IL-18 promoter regions[9], 148G/C[12] and 105A/C[13] in regulatory gene sequences, and so on. Even although the role of the IL-18 polymorphism in the outcome of HBV infection has been examined in many different nations, no firm conclusion has been reached for the Chinese Han population. The purpose of this report was to investigate the association between the tagSNPs of the IL-18 gene and the genetic susceptibility to HBV infection.

MATERIALS AND METHODS

Patients

Eight hundred and two irrelevant Han Chinese with HBV infection were enrolled in this study. They were recruited with their informed consent for genetic analysis. They had no abnormalities, based on physical examination, chest radiography, electrocardiogram, urinalysis and routine laboratory blood testing. Liver, renal, endocrine and cardiovascular disorders were excluded. Five hundred and one were chronic HBV infection patients (221 males and 280 females). The remaining 301 were HBV natural clearance individuals and served as the control group (143 females and 158 males). The average age was 44.2 years for HBV chronic carriers and 44.9 years for controls. All patients with chronic HBV infection fulfilled the diagnostic criteria of the Proposal of Prevention and Treatment of Viral Hepatitis, 2005, issued by the Chinese Society of Infectious Diseases and Parasitology and the Chinese Society of Hepatology of the Chinese Medical Association[14]. Clinical criteria of self-limiting HBV infection patients were positive for HBsAb and HbcAb but negative for HBsAg, plus without a history of HBV vaccination. Controls were age and sex-matched subjects with cases ($P > 0.1$). All cases and controls were followed for more than 6 mo. No anti-HBV therapy had been given to the patients.

Isolation of DNA from whole blood

Genomic DNA was isolated from whole blood of all the subjects, using phenol/chloroform with MaXtract high-density tubes. Genomic DNA was extracted from the peripheral blood leucocytes pellet using a DNA extraction kit (Yuan Ping-Hao Biotechnology Co., Ltd. Tianjin, China), according to the manufacturer's instructions. The DNA samples were stored at -80 °C with a concentration of 100 ng/μL.

tag SNP selection

We selected SNPs on the basis of the following principal criteria: tag SNPs (tagSNP) were identified using genotype data from the panel (Han Chinese in Beijing) of the phase II HapMap Project. The criteria for tagSNPs were $r^2 > 0.8$, minor allele frequency MAF > 0.1, functional relevance and importance, and SNPs significantly associated with diseases in previous studies. A total of two tagSNPs in IL-18 gene (rs1946518A/C and rs574424C/G, $r^2 = 0.981$) were selected, which captured 100% of common SNPs (minor allele frequency > 0.1) in the HapMap Chinese database at $r^2 > 0.8$.

Determination of the IL-18 genotypes

The two SNPs of IL-18 were genotyped using the Multiplex Snapshot technique. The primers and probes used were (5'to3'): for the rs1946518: forward primer: 5'-CCCTCTCCCCAAGCTTACTTTC-3', reverse primer: 5'-CCCCTTCCTCCCAAGCTCAATA-3', and extended primer: 5'-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCTGTTGCAGAAAGTGTAAAAATTATTA-3'; and those for the rs5744247: forward primer: 5'-CACCCTGCCTGTACCCTCAGAT-3', reverse primer: 5'-CACCTGAGGATGCCATAAACACA-3', and extended primer: 5'-TTTTTTTTTTTTTTTTTTTTTTTTTTCGTGCCCTTTAGGAAGGACT-3'.

The PCR amplification conditions were: a 15-μL final volume containing 10 μL × 1.5 μL buffer, 0.3 μL dNTPs (10 mmol/L), 0.9 μL $MgCl_2$ (25 mmol/L), 0.1 μL HotstarR Taq DNA polymerase, 0.5 μL each primer (10 pmol/L) and 1μL DNA template (20 mg/L). Conditions for the multiplex PCR reaction using touch-down PCR response procedures included initial denaturation at 95 °C for 15 min, denaturation at 94 °C for 40 s, annealing at 63 °C for 1 min, and recursive-descent 0.5 °C, followed

Table 1 Comparison of IL-18 gene promoter polymorphism between patients with chronic hepatitis B and controls

Position	Polymorphism	Control n = 301 (%)	Patient n = 501 (%)	χ^2	P value	OR (95% CI)
rs1946518	AA	60 (0.199)	141 (0.281)	6.742	0.009	1.573 (1.116-2.218)
	AC	156 (0.518)	239 (0.477)	1.279	0.258	0.848 (0.637-1.129)
	CC	85 (0.269)	121 (0.242)	1.437	0.200	0.809 (0.585-1.119)
	A	276 (0.279)	521 (0.619)	10.84	0.017	1.279 (1.045-1.567)
	C	326 (0.622)	481 (0.381)			
rs574424	CC	127 (0.422)	198 (0.395)	0.557	0.456	0.895 (0.670-1.197)
	GC	134 (0.445)	232 (0.463)	0.243	0.622	1.075 (0.806-1.433)
	GG	40 (0.133)	71 (0.142)	0.123	0.751	1.070 (0.705-1.623)
	C	388 (0.645)	628 (0.627)	0.512	0.475	0.926 (0.750-1.149)
	G	214 (0.355)	374 (0.373)			

by extension at 72 °C for 1.5 min for a total of 15 cycles. This was followed by 25 cycles of denaturation at 94 °C for 40 s, annealing at 56 °C for 40 s, extension at 72 °C for 1.5 min, with a final extension at 72 °C for 8 min. Amplified samples were stored at 4 °C. After amplification, 1.5 μL PCR product was examined on an agarose gel to test for successful amplification.

SNaPshot reaction: Take the purified PCR product, each concentration of 0.2 μmol/L SnaPshot primer mixture, SNaPshot fluorescent mixtures (containing Ampli Taq DNA polymerase and different fluorescently labeled ddNTP) consisting of an PCR reaction system. SNaPshot response procedures: (1) initial denaturation at 96 °C for 10 s; (2) denaturation at 96 °C for 10 s; (3) annealing at 53 °C for 5 s; (4) extension at 60 °C for 30 s; and (5) for a total of 25 cycles. Finally, keep extension at 60 °C for 30 s. Amplified samples were stored at 4 °C. SNaPshot PCR products using SAP purification in 10 μL the SNaPshot PCR product with 1 U SAP or 1 U CIP, mixed, insulated at 37 °C for 1 h, 75 ℃ for 15 min to inactivate the enzyme. The samples can be stored at 4 °C for 24 h or -20 °C for long term.

DNA sequencing: The Snapshot product was diluted 20-fold. In a total volume of 10 μL, we mixed 8.6 μL HiDiFormamide (high-purity formamide), 0.9 μL GeneScan-120 LIZ Size Standard and 0.5 μL Snapshot purification product. Samples were incubated at 95 °C for 5 min, chilled quickly for 4 min and then loaded on an ABI 3730XL DNA sequence detector for capillary electrophoresis, running GeneMapper4.0 software analysis of experimental results.

Statistical analyses

Allele and genotype frequencies were obtained by direct counting and the χ^2 test was used to compare allele and genotype distributions. We assessed the quality of the genotype data by testing for Hardy-Weinberg equilibrium in the case and control samples using Fisher's exact test ($P > 0.05$). We adjusted the significant threshold to $P < 0.05/2 = 0.025$ after Bonferroni's correction. Odds ratio (OR) and 95% confidence intervals (CI) were calculated according to Woolf's method.

RESULTS

Polymorphisms at the position rs1946518 and rs574424 in the IL-18 gene were analyzed by SNaPSHOT reaction. In every polymorphic site, a common reverse primer and two sequence-specific forward primers were used and two SNaPSHOT reactions were performed for every individual DNA. In total, 802 Chinese subjects were studied for IL-18 polymorphisms. All the genotypes of IL-18 gene polymorphisms were in Hardy-Weinberg equilibrium in both the case and control subjects. As shown in Table 1, there were AA, AC and CC genotypes at position rs1946518, and CC, GC and GG genotypes at position rs574424.

Genotype and allele frequencies for IL-18 polymorphisms are summarized in Table 1. The genotype frequencies were in agreement with the Hardy-Weinberg ($P > 0.05$ for all analyses). As the rs1946518 genotypes, of 501 patients with chronic hepatitis B, 141 had the AA type (28.1%), 239 the AC type (47.7%) and 121 the CC type (24.2%). Of the 301 control subjects, 60 had the AA type (19.9%), 156 the AC type (51.8%) and 85 the CC (28.2%). In genotypes, the AA type at position rs1946518 was present at a higher frequency in patients with chronic hepatitis B compared to those in the controls. OR of the AA genotype for the comparison with that of the AC and the CC genotype was 1.573 (95% CI: 1.116-2.218, $P = 0.009 < 0.025$). In phenotypes, the allele C at rs1946518 was of a significantly lower frequency in patients with chronic hepatitis B than that in the controls ($\chi^2 = 10.84$, $P = 0.017 < 0.025$). As for the rs574424 genotypes, 198 of the 301 patients with chronic hepatitis B had the CC type (39.5%), 232 the GC type (46.3%) and 71 the GG type (14.2%). 127 of the 301 control subjects were type CC (42.2%), 134 were GC (44.5%) and 40 were CC (13.3%). No significant difference in the genotype distribution or in the allele frequency between the patients with chronic hepatitis B and the control subjects was observed.

Haplotype analysis

We also estimated the IL-18 haplotype frequencies and evaluated the association among these variants and HBV infection. We observed three haplotype combinations, but non-significant association was found in the distribu-

Table 2 Haplotype frequencies of two interleukin-18 bi-allelic polymorphisms in chronic hepatitis B and healthy controls

Haplotypes	rs1946518	rs574424	Controls (%)	Patients (%)	χ^2	*P* value
Ⅰ	A	C	62 (10.3)	147 (14.7)	2.02	0.155
Ⅱ	A	G	214 (35.5)	374 (37.3)	0.511	0.475
Ⅲ	C	C	326 (54.2)	481 (48.0)	2.623	0.101

tion of the haplotype frequencies between cases and controls ($P > 0.025$). Haplotype frequency less than 0.03 will be ignored in analysis (Table 2).

Three haplotypes of the IL-18 at position rs1946518 and rs574424 were present in both patients and controls (haplotypes Ⅰ, Ⅱ and Ⅲ in Table 2). The frequencies of haplotype I, Ⅱ and Ⅲ in the controls were 10.3%, 35.5% and 54.2%, respectively. The frequencies of haplotype Ⅰ, Ⅱ and Ⅲ in the patients with chronic hepatitis B were 14.7%, 37.3% and 48.0% respectively. The frequencies of haplotype Ⅰ and Ⅱ, which bear A at rs1946518, in the patients were little higher than that in the healthy control subjects, but no significant difference in the haplotype frequencies between the patients and HBV natural clearance individuals was displayed.

DISCUSSION

The human IL-18 gene is located on chromosome 11q22.2-q22.3 and is composed of six exons and five introns[15]. Sugiura *et al*[16]described that there were some SNPs at position -607C/A, -137G/C, -656G/T and 105A/C within IL-18 gene exons. Cloning and gene expression analysis showed that the SNPs of the promoter of IL-18 gene at position -607 and -137 were suggested to cause the differences in transcription factor binding and have a critical impact on IL-18 gene activity and potentially also to IFN-gamma[17]. Further studies showed that the people with allele C at position -137 in the promoter of IL-18 gene may be protected against HBV infection; moreover, AA genotype at position -607 may be closely linked to inhibiting HBV-DNA replication. Meanwhile, haplotype frequencies' distributions suggested that the frequencies of -607C/-137C and -607A/-137C haplotypes in the chronic hepatitis B groups were significantly lower than that in normal controls[18]. But a recent study found that the polymorphisms at position -148, +8925 and +13925 could play a main role in the expression of IL-18 and had a clear correlation between IL-18 and IFN-gamma mRNA expression[19]. Because IFN-gamma, mainly mediated by IL-18, could limit the hepatitis B virus by activating the immune cells, high levels of expression of IL-18 caused by the above genotypes might explain the mechanism of viral clearance in HBV infection. But not all studies had the same view and with scientific and technological progress, the susceptibility genes in IL-18 will be discovered more and more.

To identify the relationship between the SNPs of IL-18 gene and genetic susceptibility to chronic hepatitis B virus infection in Chinese patients, we selected two SNPs in IL-18 (rs1946518and rs574424) using genotype data from the panel (Han Chinese in Beijing) of the phase II HapMap Project. The two tagSNPs captured 100% of common SNPs (minor allele frequency > 0.1) in the HapMap Chinese database at $r^2 = 0.981$. We analyzed the associations of the two SNP alleles with HBV-infected patients compared to spontaneously cleared HBV controls.

In the present study, we found that the allele frequencies of rs1946518A in the chronic hepatitis B group were markedly higher than those in the control group and there was a significant correlation between them (Table 1). These findings suggest that rs1946518 (A > C) is closely associated with the susceptibility to chronic hepatitis B and may be the susceptible gene. We analyzed the rs574424C/G genotype in a series of patients with chronic hepatitis B and acute hepatitis B and it was not associated with HBV infection. But Zhang[18] confirmed that rs574424C alleles were associated with the clearance of HBV infection and protected people against chronic hepatitis B. Those conclusions were rather contradictory. It is likely that the contrary results may be related to the size of samples or the criteria of inclusion.

With regard to the haplotypes, Giedraitis V[20] found that patients with acute hepatitis B carrying haplotype of rs1946518A/ rs187238C had a more vigorous CD8+ T cell response to HBV core than patients not carrying rs1946518A/rs187238C, suggesting that rs1946518A/ rs187238C was associated with a self-limited course of HBV infection. Unfortunately, no haplotype (rs1946518A/574424C or G) was detected to have association with HBV infection in our study. The reason for different results is likely due to different gene, races and sample sizes.

The results of the present study suggest that the genotypes rs1946518AA (OR = 1.573) and the allele A (OR = 1.279) are closely associated with chronic hepatitis B and may be the dangerous gene. IL-18 gene is an important factor that determines the outcome of HBV infection, which will give some new clues in the study of the pathogenesis of chronic hepatitis B. In fact, responses to HBV infection and HBV antigens (vaccines) and treatment are connected with genetic traits. Such correlations are still not clear, especially with regard to different populations, age and course of disease. These investigations should be continued, especially in patients treated with interferon, still the most important means of treatment. This could be useful in typing patients for this very expensive therapy.

COMMENTS

Background

Persistent hepatitis B virus (HBV) infection is considered a multifactorial and polygenic disorder with viral, environmental and genetic components, as well as contribu-

tions from HBV genomic variability, host age, gender, concurrent infection with the hepatitis C virus, hepatitis D virus and human immune deficiency virus. Interleukin-18 (IL-18) plays an important role in the response of the innate immune system to viral infection. High levels of expression of IL-18 caused by the above genotypes might affect induction of IFN-γ expression.

Research frontiers

This study is the first to investigate the association between two tagSNPs (rs1946518/ rs574424) of IL-18 and the genetic susceptibility to chronic HBV infection in Chinese patients using the Multiplex Snapshot technique.

Innovations and breakthroughs

The genotype AA and the allele A of the IL-18 at position rs1946518 are closely associated with the susceptibility to chronic hepatitis B and may be the dangerous gene. But the tagSNPs of IL-18 at rs574424 position are not associated with HBV infection in Chinese patients.

Applications

Based on the results of our study, further genetic studies are needed to examine the roles of other IL-18 SNPs and their association with disease progress in chronic HBV infection.

Terminology

The human IL-18 gene is located on chromosome 11q22.2-q22.3 and is composed of six exons and five introns. Interleukin-18 (IL-18) was first described as an interferon-g (IFN-g)–producing factor and has multiple functions, including the activation of cytotoxic T lymphocytes or natural killer cells and the promotion of T-helper type 1 (Th1)-type immune responses, which limits virus infection.

Peer review

This manuscript investigates the association between 2 SNPs claimed to be TaqSNP for common haplotype in the Chinese population and chronic hepatitis B. It is well written and interesting.

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Events Calendar 2012

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AHPBA Sponsored Consensus Conference on the Multidisciplinary Treatment of Colorectal Cancer Liver Metastases
San Francisco, CA, United States

January 20-21, 2012
AGA Clinical Congress of Gastroenterology and Hepatology: Practice, Evidence and Quality in 2012
Miami, FL, United States

January 27-28, 2012
28th Annual Meeting of the German Association for the Study of the Liver
Hamburg, Germany

January 30-31, 2012
5th International Conference on the Management of Patients with Viral Hepatitis
Paris, France

February 8-10, 2012
Stockholm Liver Week 2012
Stockholm, Sweden

February 16-19, 2012
22nd Conference of the Asian Pacific Association for the Study of the Liver
Taipei, Taiwan, China

March 16 -17, 2012
Hepatitis Single Topic Conference
Atlanta, GA, United States

March 16-17, 2012
ESGE - Workshop on Advanced Endoscopy with Live Demonstrations
Vienna, Austria

March 31-April 1, 2012
27th Annual New Treatments in Chronic Liver Disease
San Diego, CA, United States

April 18-22, 2012
The International Liver Congress by EASL
Barcelona, Spain

April 27-28, 2012
The European Society for Paediatric Gastroenterology, Hepatology and Nutrition
Stockholm, Sweden

May 16-19, 2012
International Liver Transplant Society 18th Annual International Congress 2012
San Francisco, CA, United States

May 19-22, 2012
Digestive Disease Week 2012
San Diego, CA, United States

June 22-23, 2012
EASL Monothematic Conference: Vascular Liver Diseases
Tallin, Estonia

July 1-5, 2012
10th World Congress of the International Hepato-Pancreato-Biliary Association 2012
Paris, France

September 5-8, 2012
International Congress of Pediatric Hepatology, Gastroenterology and Nutrition
Sharm El-Sheikh, Egypt

September 7-9, 2012
Viral Hepatitis Congress 2012
Macclesfield, United Kingdom

September 7-9, 2012
The Viral Hepatitis Congress
Frankfurt, Germany

September 14-16, 2012
The International Liver Cancer Association's 6th Annual Conference
Berlin, Germany

September 20-22, 2012
Prague Hepatology Meeting 2012
Prague, Czech Republic

September 20-22, 2012
1st World Congress on Controversies in the Management of Viral Hepatitis
Prague, Czech Republic

October 18-20, 2012
2nd World Congress on Controversies in the Management of Viral Hepatitis
Berlin, Germany

November 9-13, 2012
AASLD - The Liver Meeting 2012
Boston, MA, United States

November 9-13, 2012
The Liver Meeting - 63rd Annual Meeting and Postgraduate Course of the American Association for the Study of Liver Diseases
Boston, MA, United States

November 14-18, 2012
4th World Congress of Pediatric Gastroenterology, Hepatology and Nutrition
Taipei, Taiwan, China

December 26-28, 2012
International Conference on Gastroenterology, Hepatology and Nutrition
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3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

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5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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