

## Hepatitis

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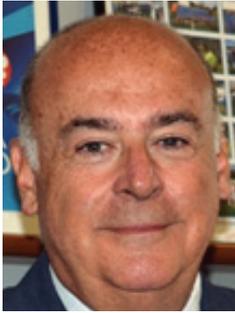
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## EDITORIAL

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# Natural history of HCV infection: what is the public health impact of untreated disease?



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“Hepatitis C virus replicates in the cytoplasm of hepatocytes within a structure, defined as membranous web which makes it easily curable with newly available direct acting antivirals.”

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HCV is a ssRNA virus which belongs to the large Flaviviridae family (reviewed in [1]). The RNA genome consists of a single 9600 bp open-reading frame encoding for a single polyprotein of about 3010 amino acids, which is processed by cellular and viral proteases into structural (core and envelope) and nonstructural proteins with enzymatic activity essential for the virus life cycle (p7, NS2, NS3/4A protease/helicase, NS4B, and NS5A and NS5B, the latter being the RNA-dependent RNA polymerase) [1]. HCV replicates in the cytoplasm of hepatocytes within a structure, defined as membranous web which makes it easily curable with newly available direct acting antivirals. The virus circulates in the host as a quasispecies of closely linked, yet sufficiently diverse, variants that cluster into seven different genotypes and several subtypes that are differentially distributed worldwide [2].

It is estimated that approximately 170 million people carry circulating antibodies to HCV and about one half are permanently infected [3]. Infection has

spread through iatrogenic transmission during massive parenteral treatment for schistosomiasis, leprosy, syphilis and yaws with reusable syringes [4], transfusion of blood and blood products, surgery, intravenous drug injection [5]. Heterosexual and mother-to-infant transmission is very low [6,7], whereas men who have sex with men (MSM) can more often be infected [8]. Infection is virtually always asymptomatic and becomes chronic in 50–80% of the cases, depending on certain polymorphisms of the *IFN-λ3* gene [9]. Reinfection with the same or other genotypes is possible in multiply exposed individuals, such as people who inject drugs [10], since adaptive immunity does not confer protection. Consequently, attempts at developing traditional vaccines have by and large been unsuccessful.

Once chronic infection is established, progression to chronic liver disease, cirrhosis and hepatocellular carcinoma (HCC) may take several years or may even not take place at all. Slow progressors usually acquire the infection in early life. Predictors

## KEYWORDS

• epidemiology • HCV • natural history

“Reinfection with the same or other genotypes is possible in multiply exposed individuals, such as people who inject drugs, since adaptive immunity does not confer protection.”

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“Chronic hepatitis C virus infection is a systemic disease which does not exclusively affect the liver.”

of fast progression include nonmodifiable (age, gender, ethnicity, degree of necroinflammation, HCV genotype) and modifiable or partially modifiable (obesity, metabolic syndrome, human immunodeficiency, HIV, HBV, alcohol, immunosuppression) variables. Several retrospective and prospective studies have addressed the theme of progression from mild to advanced fibrosis and cirrhosis. An old study by Poynard and coworkers [11] enrolled 2235 patients from three cohorts. All had liver biopsy assessed by the METAVIR scoring system. Fibrosis progression per year was defined as the ratio between fibrosis stage in METAVIR units and the duration of infection. Fast, intermediate and slow progressors were identified. About a third of the patients were predicted to never progress to cirrhosis in a period of 40 years, whereas another third had an estimated progression  $\leq 20$  years. Age at infection, male sex and alcohol intake  $>50$  g/daily were all associated with a faster progression. In a more recent systematic review and meta-analysis of 111 published prognostic studies including 33,121 HCV patients [12], annual stage-specific fibrosis progression rates and their determinants were estimated using Markovian maximum likelihood estimation method. Inclusion criteria were: presence of HCV infection determined by serological assays; available information about age at assessment of liver disease or HCV acquisition; duration of HCV infection; histological and/or clinical diagnosis of cirrhosis. Estimated annual mean (95% CI) stage-specific transition probabilities were: F0–F1 0.117 (0.104–0.130); F1–F2 0.085 (0.075–0.096); F2–F3 0.120 (0.109–0.133) and F3–F4 0.116 (0.104–0.129). This large systematic review provided increased precision in estimating fibrosis progression in chronic HCV infection supporting, however, a nonlinear disease progression. Discrepancies were noted depending on the clinical setting where data were retrieved.

Among independent predictors of fibrosis progression, genotype 3 has emerged as an important factor [13], which was also associated with an increased risk of HCC, twice more frequently than genotype 1 and four-times more frequently than genotype 2 [14]. The calculated risk of developing HCC in a cohort of cirrhotic patients with HCV nonresponders to IFN- $\alpha$ -based therapies was 13.2/1000 persons/year, reiterating the importance of achieving a virological cure in these patients. The need of halting fibrosis progression also in the broad view of

preventing HCC comes from a randomized clinical trial which, despite failing in its major end point (e.g., preventing progression), provided invaluable information on the natural history of chronic HCV infection. The HALT-C trial [15] of maintenance pegylated IFN- $\alpha$  for treatment of failure patients enrolled 1050 subjects, 60% with advanced fibrosis and 40% with cirrhosis. End points were: progression to cirrhosis at 4 years (biopsies at baseline, 1.5 and 3.5 years); clinical outcomes over 8 years, including cirrhosis progression, complications of portal hypertension, HCC and death. The rate of progression from advanced fibrosis to cirrhosis was a worrisome 10% per year and outcomes were maximal in the cirrhotic group as expected, but also rather frequent in the advanced fibrosis group. A platelet count  $\leq 100,000$  could discriminate patients at high risk of developing outcomes, including HCC.

Chronic HCV infection is a systemic disease which does not exclusively affect the liver. Thus, the impact of this infection is far higher than commonly appreciated. Established extrahepatic manifestations include mixed cryoglobulinemia, monoclonal gammopathies, and low-grade B-cell non-Hodgkin lymphoma, all emerging from inappropriate continuous B-cell stimulation resulting in terminal differentiation [16]. Other confirmed associations for which pathogenetic mechanisms are unclear are porphyria cutanea tarda and lichen planus [16]. Emerging associations include diabetes, cardiovascular disease and extrahepatic cancer. With respect to the latter, it is not well understood why extrahepatic cancer rates may be increased in patients with chronic HCV infection. There is evidence that patients with chronic HCV infection often have multiple cofactors such as alcohol and tobacco use, diabetes and obesity, which are also associated with an increased risk of cancer. Interestingly, data from a very large retrospective analysis of all new cancer cases reported between 2008 and 2012 in the Kaiser Permanente Southern California database were recently presented [17]. Cancer rates were calculated in patients with and without a diagnosis of chronic HCV infection. Data were stratified according to alcohol and tobacco use, diabetes and obesity. Unexpectedly, unadjusted cancer rates were two- to three-times higher in the HCV population for head and neck, lung, oesophageal, stomach, pancreatic, prostate and renal cancers, as well as myeloma and

non-Hodgkin lymphoma. Multivariate analysis showed a strong association between cirrhosis and an increased cancer risk. These data extend the potential public health implications of chronic HCV infection, even though that study had intrinsic limitations due to its retrospective nature and the fact that a causal relationship between HCV and extrahepatic cancer was not established, let alone the potentially inaccurate recording of confounders such as tobacco use and alcohol abuse.

As mentioned above, additional associations have become evident. A systematic review and meta-analysis showed that there was an increased risk for patients persistently infected with HCV to develop diabetes, although the CIs were quite broad [18]. Why HCV infection is associated with diabetes is unclear but it has been hypothesized that HCV may directly interfere with insulin signaling with consequent development of Type 2 diabetes. Moreover, chronic inflammation may be responsible for endothelial damage and ensuing cardiovascular or cerebrovascular outcomes. To this effect, a large cross-sectional study indicated that intima media thickening and carotid artery plaques were almost three-times and twice more frequently observed in patients with chronic HCV compared with controls, respectively [19]. A more recent study showed that carotid atherosclerosis was twice more frequent in patients with HCV compared with age- and sex-matched controls, and that older age and severe hepatic

fibrosis were associated with a high risk of early carotid atherosclerosis [20].

It is clear from the aforementioned that treatment of chronic HCV infection at an early stage is absolutely mandatory before the ominous complications of untreated infection eventually occur. This is particularly relevant in HCV for which no effective vaccine is on sight. However, disparity between HCV treatment efficacy and projected HCV treatment effectiveness is a major problem worldwide as most patients have no or highly restricted access to direct acting antivirals. Generic drugs cost a small fraction of brand medications and are equally efficacious [21]. They may represent a solution in places where access to care is difficult. In any case, lack of expanded testing will have no impact on unrecognized HCV infections. Therefore, healthcare providers should aim at optimizing population screening in an effort to treat and prevent liver-related and unrelated outcomes in chronic HCV.

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## REVIEW

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# Treatment of hepatitis B virus: an update

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Chronic hepatitis B virus infection is a global health concern as it affects over 240 million people worldwide and an estimated 686,000 people die annually as a result of complications of the disease. With the development of newer antiviral drugs, viral suppression of HBV is achievable, however elimination of HBV from infected individuals (functional cure) remains an issue. Due to persistence of HBV DNA (cccDNA) in infected cells, chronically infected patients who discontinue therapy prior to HBsAg loss or seroconversion are likely to relapse. Several novel therapeutic strategies are being researched and studied in clinical trials. Here we review these novel strategies to achieve sustained cure or elimination of HBV. These strategies include the targeting of the host or viral factors required for viral persistence as well as therapeutic vaccines.

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## Background

The long-term management of chronic hepatitis B (CHB) is a significant challenge. Approximately 240 million people have CHB worldwide [1], making it a major public health problem. HBV persistence is the result of a combination of viral factors and the inadequate immune system activation seen in patients with acute HBV, which naturally clears the HBV infection. CHB leads to liver cirrhosis and hepatocellular carcinoma (HCC), and is the major cause of noncirrhotic HCC worldwide [2]. These sequelae result in an estimated 686,000 deaths annually [3]. Therefore, it is important to successfully treat those who are infected in order to decrease the burden on health care systems worldwide. Current therapies suppress viral replication and liver inflammation with the goal of reducing liver-related morbidity and mortality [4]. Viral suppression is easily achieved in most patients using medication currently available; however, they do not lead to sustained remission, requiring indefinite treatment as viral load frequently rebounds once suppressive therapy is stopped [5].

This review will provide an overview of the basis of HBV persistence, current therapy and new therapeutic approaches.

### • Basis of HBV persistence

In order to design new treatment strategies to eradicate HBV, it is important to understand the factors that contribute to viral persistence in the liver. Viral factors and host immune factors interplay to create the complicated problem of persistence of HBV. The virus utilizes a replication system that uses covalently closed circular DNA (cccDNA) as a transcriptional

## KEYWORDS

• cccDNA • eradication • HBV  
• hepatitis B • Toll-like  
receptors • TLRs

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template. cccDNA is sequestered in the nucleus of infected cells and avoids detection by the DNA sensing cellular machinery (part of the innate immune response). Furthermore, the virus produces tolerogenic proteins (HBsAg, HBeAg) that lead to T-cell exhaustion [6]. Obtaining a more complete understanding of how these viral and immune factors lead to viral persistence has allowed for novel therapies that might lead to sustained viral response to be designed. The current therapeutics (reverse transcriptase inhibitors) prevent formation of new cccDNA, but have no effect on existing cccDNA [7].

#### • Viral factors

##### Error-prone replication

HBV is a small, enveloped virus with a unique genomic organization and replication mechanism. Its genome is only 3200bp long and has multiple overlapping open reading frames (ORFs). These are associated with constraints on variation of other viruses, such as HIV. In HBV, however, in spite of the ORFs replication is error prone because of the lack of proofreading mechanism on the HBV polymerase (error rate of  $10^{-4}$  to  $10^{-5}$ ). The high error rates allow for the accumulation of a heterogeneous viral population, or quasispecies [5,8].

##### Precore/core mutants

The major nucleocapsid protein and RNA dependent DNA polymerase are encoded by the viral core mRNA, which also acts as the pregenomic RNA – the template for reverse transcription [9]. Precore mRNA encodes the precore protein, which is processed in the endoplasmic reticulum to produce HBeAg. Mutations in HBV in the genome, in turn, influences HBeAg production. There are multiple reported precore and core mutants. Two well-studied precore mutations include: stop codon mutation at nt 1896, which results in cessation of HBeAg expression, and a mutation in the basal core promoter (BCP) at nt 1762 and nt 1764, which results in diminished production of HBeAg and an increased immune response as a result. These precore mutants are associated with increased pathogenicity, severe chronic liver disease, and acute liver failure. Double mutations of BCP at nt 1762 and nt 1764 are reported to be associated with severe liver disease, fulminant hepatitis, cirrhosis and HCC [9].

##### S mutants

The S gene codes for HBsAg. One particular region, the major hydrophilic region (amino acids 120–160), is especially prone to mutation. The most commonly transmitted mutation is G145R, which results in a transcriptionally competent virus even after vaccination [10]. The G145R variant is associated with high HBV DNA levels and viral persistence [11].

##### HBV genotypes

The ten known HBV genotypes (A–J) and multiple subtypes account for the heterogeneity seen in clinical manifestations and differential treatment response among patients with CHB in different parts of the world. Genotypes are associated with clinical outcomes of treatment, especially interferon treatment [12]. Furthermore, specific genotypes have different risks for disease progression. Having genotype A infection is an independent risk factor for progression to chronic infection after acute hepatitis B infection [13]. Acute infection with genotypes A and D are more likely to progress to chronicity than acute genotype B or C infection. HBV genotypes are also associated with precore and BCP mutations. For example, genotype C has higher frequency of double mutation in BCP A1762T/G1764A, pre-S deletion and is associated with higher viral load than genotype B. Genotypes C and D are also associated with more severe liver disease. Genotypes A and B respond better to interferon-based therapy than genotypes C and D, but there are limited consistent differences for nucleos(t)ide analogs (NAs) [14].

##### cccDNA

cccDNA is the main barrier to treating CHB due to its sequestration in the nucleus of infected cells. It avoids the cellular machinery that is part of the innate immune response by forming a supercoiled minichromosome, complete with histones and DNA chaperone proteins. These infected hepatocytes have a long half-life, which allows cccDNA to be maintained in the nucleus indefinitely. Thus, cccDNA acts as a reservoir for the reactivation of HBV genome replication after natural or treatment-induced silencing. Additionally, mutations that confer drug resistance may be maintained with the cccDNA found in the nucleus [15–17]. When reactivation of replication occurs, this drug resistance presents a problem for further treatment with antivirals. Thus, cccDNA serves as the latent

reservoir of CHB. Currently approved antiviral agents, including lamivudine (3TC), adefovir, telbivudine, entecavir and tenofovir, suppress viral replication, but not reverse transcription, allowing cccDNA persistence. Novel strategies for HBV eradication will need to eliminate or inactivate cccDNA.

#### • Host factors

##### Host genetics

Multiple host factors influence HBV persistence and the observed variability in outcomes. An individual's human leukocyte antigen (HLA) type is one of the important factors that determine variability in response to HBV. HLA DRB locus alleles DRB1\*1301/2 are consistently associated with spontaneous resolution upon initial infection with HBV [18]. HLA-DR7 (DRB\*07), HLA-DR3 (DRB\*0301) and HLA-DPA2 are associated with increased susceptibility to CHB [19–24]. Decreased expression of HLA-DPA1 is associated with HBV persistence [25]. Multiple other alleles are associated with increased susceptibility to chronic HBV infection in addition to failure to respond to HBsAg-based vaccine, including HLA-DRB1\*0701 and DRB1\*0301 [26,27]. Another study of the genomes of siblings with CHB has discovered that a region of linkage on chromosome 21 with single nucleotide polymorphisms spanning the IFN- $\alpha$  receptor II and IL-10 receptor II was associated with chronicity [28,29]. HLA-DP polymorphism might be a useful therapeutic predictor for PEG-IFN.

##### Host–viral interactions

HBV antigens have been shown to hinder signaling of Toll-like receptor (TLR) 2, 3, 7 and 9 molecules, which allows for diminished innate immune recognition of the virus. TLRs are vital in HBV immunity due to their role in generating effective innate adaptive crosstalk. In CHB subjects, innate immune responses are blunted and facilitate persistence, as described in **Figure 1**. TLRs are down regulated in hepatocytes and intrahepatic type-I interferon antiviral response is virtually absent [30]. This will in turn lead to inadequate priming of adaptive immune responses. Both humoral and cellular immunity to HBV antigens are impaired. Protective antibodies are not formed, since B cells are not optimally activated by CD4<sup>+</sup> T cells. Moreover, the T cells from these subjects with CHB have an exhausted phenotype and are less responsive

to HBV antigens [30]. Altogether, these lead to establishment of HBV persistence.

#### • Current therapy for HBV

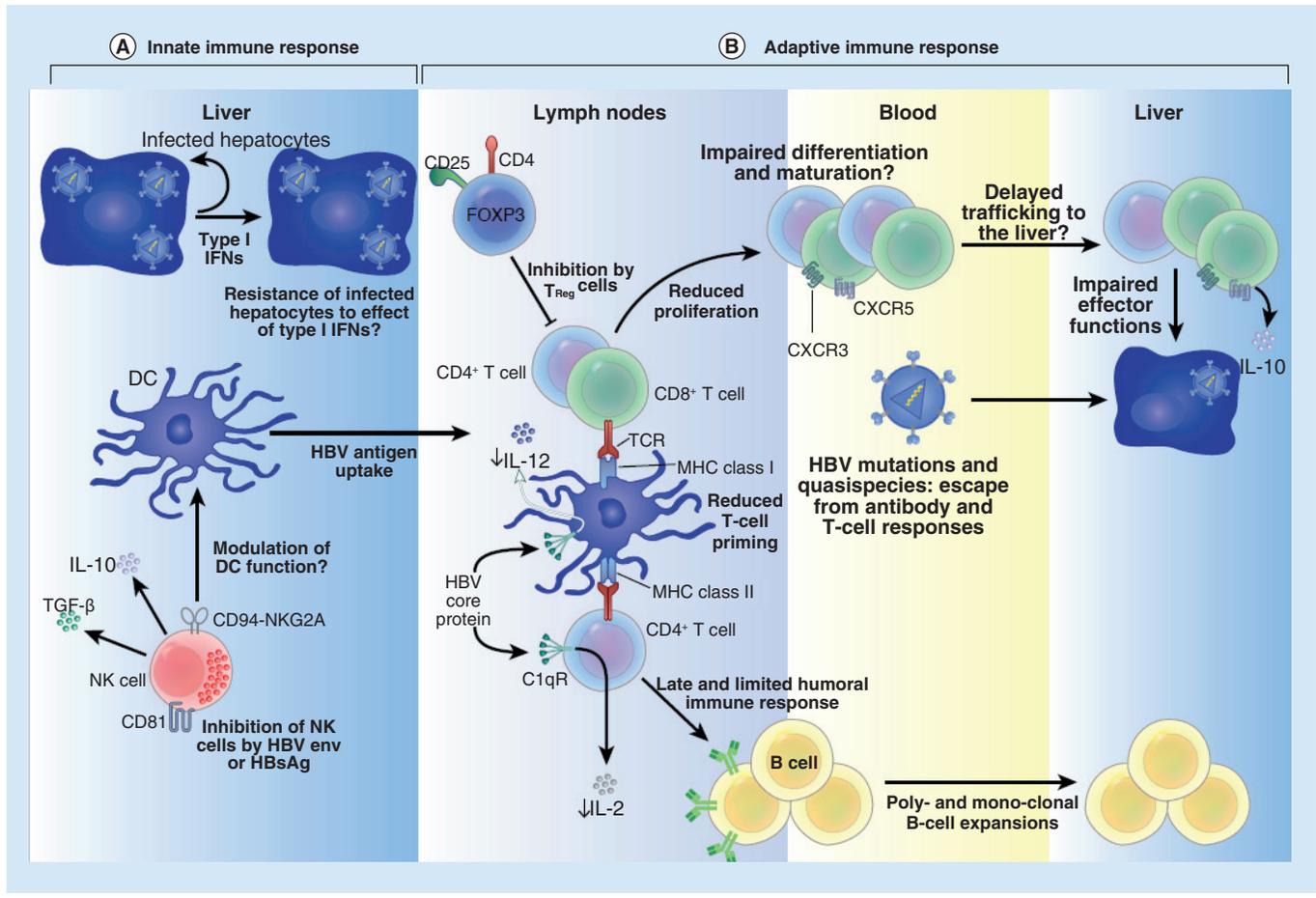
Increased HBV DNA level is associated with poorer outcomes, including cirrhosis and HCC [31–33]. Therefore, current therapy suppresses HBV DNA levels (by inhibiting viral replication but not affecting cccDNA) with the goal of slowing the progression to liver fibrosis and cirrhosis and preventing HCC, which are the primary complications of the disease. Currently, interferon or pegylated-IFN- $\alpha$  and/or long-term use of NAs are the primary treatment strategies. The newer NAs, tenofovir (TDF) and entecavir, have a higher barrier to resistance and are well tolerated, thus are prescribed as the first line of treatment more often than older NAs, including lamivudine (3TC), adefovir and telbivudine. Additionally, telbivudine has been shown to cause myopathy and neuropathy with long-term use [34].

In CHB patients that are HBeAg positive, PEG-IFN- $\alpha$  with or without 3TC may be used to achieve durable viral suppression, increased rate of HBsAg seroconversion, and improved fibrosis and cirrhosis, as demonstrated by long-term follow-up studies [35–37]. Genotypes A and B demonstrated increased response rates to this treatment compared with genotypes C and D [38–40]. Additionally, favorable outcomes are more likely in CHB patients with lower levels of HBV DNA and higher alanine aminotransferase (ALT) levels [38,40].

While PEG-IFN is effective in some patient populations, its use is limited greatly by intolerance, thus NAs are likely to be used to achieve durable viral suppression.

One problem with the earlier NAs (3TC, adefovir and telbivudine) is the increased likelihood to develop resistance mutations. Tenofovir and entecavir overcome this problem with high barriers to resistance, as well as having increased potency and good tolerability for CHB patients. Patients show durable viral suppression and improvements in both histological and biochemical signs of disease while taking tenofovir or entecavir, according to long-term follow-up studies [41,42]. However, it has been demonstrated that persistence of HBeAg and high ALT remain risk factors for HCC despite patients receiving suppressive therapy [2].

There remain several limitations to current therapy. As stated earlier, despite being effective at suppressing viral replication, for the majority



**Figure 1. Interactions between hepatitis B virus proteins and the immune system. (A) Innate immune system:** HBV-infected hepatocytes trigger a type 1 interferon response in the liver. This in turn primes DCs to antigen uptake, processing and initiation of an effective adaptive immune response. This interferon response also activates NK cells to lyse target (HBV-infected) cells and promote maturation of DCs. HBsAg can inhibit NK cells thereby downmodulating intrahepatic innate immunity. **(B) Adaptive immune system:** appropriate priming of T cells results in maturation of an effective HBV response that leads to secretion of protective antibodies and/or cytotoxic T cells in the lymph node. These HBV-specific T and B cells circulate to the liver via blood, targeted by CXCR3 and CXCR5 ligands. Chronic HBV infection is associated with enhanced  $T_{reg}$  activity (inhibits T-cell maturation and proliferation), impaired humoral response (low protective antibodies), emergent escape mutants (ineffective cytotoxic T-cell response) and enhanced T-cell exhaustion. Collectively these effects contribute to HBV persistence. DC: Dendritic cell; HBV: Hepatitis B virus; NK: Natural killer.

of patients, current therapy will not result in cure as they do not target cccDNA. Furthermore, long-term use of current antiviral agents is hindered by the emergence of drug resistance as changes within the viral polymerase – the target of NAs – generates HBV surface protein variants due to the overlapping nature of the viral genome [5,43,44], which may influence treatment response [45].

• **Current end points of HBV treatment**

Currently, biochemical, serological, virological and histological endpoints defined below and measured during treatment course are used in HBV

treatment [46]. Percentage of participants who normalize elevated levels of ALT after initiation of therapy constitute biochemical endpoint [46]. Similarly, proportion of patients with undetectable HBV DNA after initiation of therapy is used for the virological endpoint for HBV treatment. Since sustained suppression of HBV DNA levels after cessation of therapy seldom happens, this is not a completely accurate measure of HBV elimination. For patients with CHB who are HBeAg positive, serological response is defined as loss of HBeAg and seroconversion to anti-HBe antibodies [46]. For CHB patients who are HBsAg positive, serological response is defined as the loss of HBsAg

and seroconversion to anti-HBs antibodies [46]. Loss of HBsAg is associated with a diminished likelihood of developing complications such as cirrhosis and HCC; therefore it is the most valuable clinical outcome marker for treatment endpoint [47–49]. Finally, the histological treatment endpoint is defined by lowering in necroinflammatory activity by two points or more  $\geq 2$  points in hepatic activity index (HAI) or Ishak's system without worsening fibrosis compared with baseline histology [46]. According to a long-term follow-up study of patients treated with TDF, suppression of HBV over time can reduce fibrosis and cirrhosis [50]. While many patients achieve viral suppression on current therapy, only 10–25% of patients seroconvert [51]. It is because numbers remain so small that novel therapeutics are necessary.

Ultimately, HBV elimination can be defined by complete suppression of HBV DNA levels, the loss of HBsAg and seroconversion to anti-HBs antibodies after stopping antiviral therapy. Loss of HBsAg levels is critical since HBsAg levels are surrogate markers for levels of transcriptionally active cccDNA, meaning that if HBsAg is eliminated, the virus is most likely inactivated [52,53].

#### • Novel treatment strategies

The purpose of this review is to highlight potential newer treatment for HBV aimed at achieving a functional cure of sustained virologic remission. This is defined as absence of HBV DNA levels and HBsAg with or without HBsAg seroconversion after cessation of therapy.

#### • Improved nucleos(t)ide analogs

In order to overcome concerns about long-term safety of entecavir and tenofovir, which are the current first-line treatments for CHB, besifovir and tenofovir alafenamide are being studied. Besifovir has been shown to be safe and efficacious over 96 weeks, with potency similar to that of entecavir. The only problem found was the depletion of L-carnitine, which was easily rectified with a carnitine supplement [54]. Tenofovir alafenamide is a phosphonate prodrug of tenofovir that optimizes potency and safety [55]. These will be valuable in conjugation with other treatments in development.

#### • Targeting the virus

##### Targeting viral entry

HBV viral entry is mediated through interactions between viral envelope proteins and

cellular receptors. This provides a new target for treatment with receptor antagonists that would prevent entry of the virus into the cell. Yan *et al.* reported that sodium taurocholate cotransporting polypeptide (NTCP) is a functional receptor for HBV *in vitro* [56]. NTCP is expressed at the basolateral membrane of hepatocytes and responsible for most of the sodium-dependent bile acid uptake. The synthetic lipopeptide, Myrcludex-B, specifically targets the NTCP and has been shown to efficiently block HBV infection *in vitro* [57,58] and in mouse models by efficaciously blocking entry of the viral particles into the uninfected hepatocytes [59,60]. In the upA/SCID human mouse model, administration of Myrcludex-B results in suppression of not only HBV DNA levels but HBsAg levels as well [60]. These results are promising that Myrcludex-B could be an adjunct to antiviral therapy for CHB.

#### Targeting cccDNA

Current NA-based therapies can prevent the formation of new cccDNA, but do not affect the existing cccDNA in already infected cells, which has a long half-life (33–50 days) and is dependent on several host factors. The viral cccDNA in the nucleus serves as a store of viral escape variants, which can cause a viral rebound once treatment is stopped. cccDNA also serves as a reservoir of escape mutants that confer drug resistance.

#### Epigenetic silencing of cccDNA

Recent studies have demonstrated that cccDNA can be epigenetically silenced so that they remain transcriptionally inactive even after cessation of antiviral therapy. Using uPA-SCID mouse model with HBV-infected human hepatocytes, IFN- $\alpha$  was shown to reduce pgRNA and pre-S1 RNA even though cccDNA levels were not affected. Hence, it is possible to suppress HBV replication by targeting the epigenetic control of cccDNA function and transcription [61]. A mapping of post-translational histone modifications in cccDNA from infected liver revealed chromatin targets for epigenetic manipulation as potential therapy [62].

#### Transcriptional silencing of cccDNA

One of the recently developed therapeutic approaches involves using zinc-finger nucleases (ZFNs) that directly target HBV cccDNA. Zinc finger proteins (ZFP) can be used to block

transcription of cccDNA and have two components. One is a restriction endonuclease enzyme that is capable of introducing DNA breaks and the other (zinc finger motifs) provides DNA binding specificity. ZFPs can be used specifically to target the cccDNA of duck HBV infection and inhibit viral transcription and replication *in vitro* [63]. Expression of ZFPs in LMH cells undergoing the DHBV viral lifecycle resulted in decreased expression of viral RNA and decreased protein expression compared with the empty vector control. No toxicity effects were observed. Additionally, the viral particle production decreased in the presence of the expressed ZFPs [63].

While it has been shown that ZFNs can cleave HBV DNA *in vitro*, there are concerns about off target effects *in vivo* and the challenge of delivering ZFNs specifically to the patients' infected liver [64]. To overcome this challenge, a vector platform may be applied for delivery. Adeno-associated virus vectors are being investigated as delivery vehicles for designer nucleases to cells. These vectors were found to be safe in clinical and preclinical applications [65].

Transcription activator-like effector nucleases (TALENs) can cleave sequence specific DNA targets, making them an ideal treatment strategy for silencing of cccDNA [66]. In cells transfected with monomeric linear full-length HBV DNA, expression of TALENs decreased production of HBeAg, HBsAg, HBcAg and pgRNA. Furthermore, it suppressed the cccDNA level and misrepaired cccDNA without any apparent cytotoxic effects. In a mouse model, it was shown that TALENs can be used in combination with IFN- $\alpha$  to specifically target and inactivate the sequestered cccDNA, potentially providing a new treatment option for patients with CHB [66].

#### Disruption of cccDNA

Two disubstituted sulfonamides, CCC-0975 and CCC-0346 were recently shown to inhibit cccDNA production [67]. Mechanistic studies demonstrated the reduction of both cccDNA and its precursor, deproteinized relaxed circular DNA (DP-rcDNA) without inhibition of HBV DNA replication or reduction of viral polymerase activity. Furthermore, it was shown that the disubstituted sulfonamide compounds did not promote intracellular decay of DP-rcDNA and cccDNA, which suggests that these compounds interfere with the conversion of rcDNA into cccDNA [67].

Recent studies have also shown that IFN- $\alpha$  and lymphotoxin- $\beta$  receptor activation in primary human hepatocytes and differentiated HepaRG (dHepaRG) cells can induce degradation of cccDNA via the induction of the APOBEC3 family of proteins, which restrict foreign DNAs. HBV core protein facilitates interaction between nuclear cccDNA and APOBEC3A and APOBEC3B cytidine deaminases resulting in cytidine deamination, apurinic/aprimidinic site formation. This culminates in the degradation of cccDNA and prevents further HBV reactivation [68].

Additionally, clustered regularly interspaced short palindromic repeat (CRISPR)/CAS-9 can be used to target the HBV genome and inhibit HBV infection by cleaving the cccDNA of HBV transfected cells. This method has been tested in a mouse model and provides a promising novel technique for treating humans [69].

#### Targeting viral assembly/encapsidation

Viral assembly is a critical step in the viral life cycle, thus is an attractive target for therapeutics. The assembled core particle of the virus is composed of a capsid protein, polymerase and pregenomic RNA [7]. Proper assembly is vital to the ability of the virus to persist in humans. Heteroaryldihydropyrimidines are known to inhibit HBV virion encapsidation both *in vitro* and *in vivo*, thus preventing assembly [70,71]. Bay 41–4109 is a heteroaryldihydropyrimidine that both inhibits capsid formation and also reduces the half-life of the core protein. Furthermore, these drugs misdirect viral assembly when in excess, which decreases the stability of normal capsids [72–74]. Furthermore, these compounds are active against HBV mutants resistant to NAs, making them an even more attractive therapeutic agent [75].

Phenylpropenamides also inhibit viral encapsidation by inducing changes in tertiary and quaternary structures of HBV capsid proteins. Phenylpropenamides are active against 3TC-resistant strains [75–77]. A phenylpropenamide derivative, AT-130, acts by binding to a promiscuous pocket at the dimer–dimer interface and decreases viral production by initiating virion assembly at the wrong time, resulting in normal capsids that are empty and noninfectious [78,79]. While these are promising therapeutic agents, their clinical efficacy still needs to be studied. It is unclear whether capsid inhibitors have any effect on HBsAg levels, which could

determine their utility in achieving sustained viral remission.

NVR 3-778, a core inhibitor, is another newly discovered drug that may be used for the treatment of CHB. In the humanized mouse model, NVR 3-778 demonstrated high antiviral activity. In combination with PEG-IFN- $\alpha$ , antiviral activity was higher than with NVR 3-778 or PEG-IFN- $\alpha$  alone [80]. This may be a beneficial treatment in conjunction with currently approved therapies.

### Targeting envelopment

The HBV genome contains four ORFs that code for three envelope proteins, the viral polymerase and capsid-forming core protein. The three envelope proteins: large (LHBs), middle (MHBs) and small hepatitis B surface antigens share the same S-domain and contain N-linked glycosylation at amino acid 146 within that domain. Within the S-domain are the pre-S1 and pre-S2 domains. MHBs contain the pre-S1 domain, while LHBs contain both of the pre-S domains [81]. The interaction between non-glycosylated pre-S sequences with specific regions on the core particle leads to envelopment of viable hepatitis B capsids. There is evidence that the transport of the enveloped virus and subviral empty envelope particles (SVPs) depends on glycosylation and the processing of the MHBs glycoprotein [82,83]. Glucosidase inhibitors suppress viral infectivity and morphogenesis, likely by inhibition of the glycosylation of the envelope protein in endoplasmic reticulum [84–88].

### Targeting HBsAg levels

Typically, antiviral response starts with the production of type I interferons (IFN- $\alpha$  and IFN- $\beta$ ), signaled by the innate immune response. In HBV-infected chimpanzees, it has been shown that type I interferons are not produced at all during the early stages of infection. Furthermore, type I interferon response is completely lacking in human patients with HBV as well [89,90]. In the early stages of acute HBV infection, IL-10 is produced rather than type I interferon, accompanied by a temporary attenuation of natural killer (NK) cell and T-cell responses [91].

Patients with HBV experience a suppressed immune response to the virus that is likely caused by direct interference of HBV antigens with host cells. HBV-infected patients have high levels of HBsAg [81,92,93], which are thought to suppress

immune activation via direct action on dendritic cells' production of cytokines. Reduction of HBsAg production can be achieved with several classes of drugs [94]. Specifically, it has been demonstrated that nitazoxanide and its active metabolite, tizoxanide reduce not only extracellular HBsAg, but also HBeAg and intracellular HBcAg in a dose-dependent manner *in vitro*. In combination with lamivudine or adefovir, nitazoxanide has synergistic activity and inhibits HBV replication [94].

Triazolo-pyrimidine inhibitors of HBsAg secretion were recently identified using high-throughput screening of the HBV-expressing HepG2.2.15 cell line [93]. The parent compound does not inhibit viral replication, but does selectively inhibit HBV envelope secretion. The triazolo-pyrimidine derivatives were active in inhibiting HBsAg secretion of HBV variants that are resistant to current NAs [92,93]. It is still unclear whether these compounds would be able to enhance HBV specific immunity *in vivo* or impact secretion of important host translated proteins of significance.

HBV produces two other types of particles in addition to infectious virion: SVPs and naked capsid particles. Noninfectious subviral particles share antigenic features of the virus envelope and are thought to act as a decoy for the immune system. Recently, it has been found that SVPs and infectious virions do not share a pathway to production, as it was originally hypothesized [82].

Nucleic acid polymers are amphipathic oligonucleotides shown to have both entry and postentry antiviral activity in the duck hepatitis B virus model and can be engineered to remove the secondary proinflammatory and immunostimulatory effects associated with single-strand nucleic acids [95,96]. In the same *in vivo* duck model, the NAP REP 2055 resulted in rapid clearance of duck HBsAg with an increase in antiduck HBsAg antibodies with no viral antigens detected in the liver, and only trace amounts of intrahepatic cccDNA post-treatment [97]. Two recent proof-of-concept studies evaluated the safety and antiviral response to REP 2055 (and its calcium chelate formulation, REP 2139-Ca) monotherapy and in combination with immunotherapy in patients with CHB infection. HBsAg and HBV DNA levels were reduced in participants treated with REP 2055, accompanied by detection of HBsAb. However, HBsAb titers were not consistently maintained with REP 2055 monotherapy. The majority of

participants experience virologic rebound at follow-up after monotherapy. Immunotherapy with either PEG-IFN or Thymosin- $\alpha$  (compound with immunomodulatory activity) was added on to nine other participants who had HBsAg reductions but persistent low grade viremia after REP 2139-Ca monotherapy. With the addition of immunotherapy, HBV DNA levels declined to below the lower limit of detection in seven of the of the nine participants, and eight participants had HBsAg loss by the end of initial follow-up; however, the majority did eventually experience virologic rebound with reappearance of HBsAg and loss of HBsAB [98,99]. A follow-up trial evaluating the safety and efficacy of triple combination therapy with nucleic acid polymers, PEG-IFN and tenofovir in patients with HBeAg negative CHB is currently underway and we await the results (NCT02565719).

#### Targeting viral mRNA

Antisense oligonucleotides, ribozymes and RNAi can be used to directly target viral mRNA. According to *in vitro* data, the following can be used to decrease HBV transcript levels: antisense oligonucleotides [100], hairpin ribozymes [101], or hammerhead ribozymes using a lentiviral vector for delivery [102].

RNAi uses small interfering RNA molecules to induce gene silencing post-transcriptionally, which effectively knocks down genes of interest. In mammalian cells, it can be used to specifically target the degradation of mRNA [103]. HBV is particularly vulnerable to RNAi because of the use of multiple ORFs in the HBV genome [104]. Multiple studies have shown that in cell cultures and mice, viral mRNA and HBV replication are inhibited through the use of RNAi [105–108]. Furthermore, a recent study has shown that RNAi can be applied in cell culture and in immunocompetent and immunodeficient mice transfected with an HBV plasmid to inhibit the production of HBV replicative intermediates [106]. RNAi inhibited all steps of replication both in culture and in mice. In mice RNAi reduced HBsAg in serum and HBV RNA in the liver. Furthermore, HBV genomic DNA was undetectable in the liver, and fewer cells showed any presence of HBcAg [106].

ARC-520 has recently been shown to significantly decrease HBsAg in CHB patients in a single-dose Phase IIa clinical trial [109]. This drug utilizes a dynamic polyconjugate method of delivery to the infected hepatocytes. In dynamic

polyconjugates the RNAi trigger is conjugated to cholesterol and is coinjected with a hepatocyte-targeted membrane-active peptide, which limits off-target effects of the drug. Based on the results of this Phase II trial, ARC-520 may be a useful agent for treating CHB.

#### • Targeting the host

HBV clearance requires robust immune responses from both the adaptive and innate immune systems. The innate immune response results in the production of type I interferon, which leads to suppression of viral replication, mediation of NK cell-mediated killing of viral infected cells and supports the efficient maturation and site recruitment of adaptive immunity through production of proinflammatory cytokines and chemokines [30,110]. Interferon modulates both innate and adaptive immune cells [91,111]. Plasmacytoid dendritic cells (pDC) are the primary producers of type I interferon. pDC respond to viruses and other pathogens via recognition of pathogen-associated molecular patterns by two intracellular TLRs: TLR7 and TLR9 [91,112]. In addition to the increased production of interferon upon activation of pDC, other cytokines are also released, including TNF- $\alpha$ , IL-6 and cell surface costimulatory molecules. pDC also activate NK cells and T lymphocytes, allowing further priming and regulation of antiviral immunity [111,113,114]. Priming of the adaptive immune system causes the maturation of B- and T-cell clones, which recognize specific infectious agents, leading to infection control and memory responses for future infections with the recognizable pathogen [30].

As described above, in patients with CHB, HBV is associated with diminished adaptive and innate immune responses. Therefore, augmenting the innate immune response may aid in reconstitution of HBV-specific immunity by enhancing intrahepatic innate immunity and priming effective adaptive anti-HBV immunity. TLR 2, 3, 4, 7 and 9 play important roles in controlling infections and eliminating virally infected cells. Studies have shown that HBV interferes with their signaling, and that expression is decreased in CHB [115–118].

#### TLR agonists

Studies in which HBV transgenic mice were injected with ligands specific for TLR 2–9 have shown that the livers of these mice produce IFN- $\alpha$ , - $\beta$  and - $\gamma$  to inhibit HBV replication [119]. This suggests that the activation of the innate immune

response in the liver can suppress HBV replication [119]. Inhibition of HBV replication is accomplished post-transcriptionally by suppressing the assembly or stability of HBV RNA-containing capsids [119,120]. Thus, TLR activation directly inhibits HBV replication [121,122]. However, HBV somehow evades detection by TLRs as a strategy to escape innate immune response [121,122]. Expression of TLRs in hepatocytes is down-regulated in the presence of multiple HBV viral products [116,123–127].

Although HBV circumvents endogenous type I interferon pathways, the use of a TLR7 agonist could plausibly induce the IFN- $\alpha$  response. When combined with current NA therapy for viral suppression, the use of TLR agonists may allow for the development of protective immunity via induction of exogenous interferon. Various studies have shown that NA therapy results in long term suppression of HBV and partial reconstitution of adaptive immunity [128]. Thus, an adjuvant therapy using TLR agonist may be able to accelerate the process of immune reconstitution and HBV clearance.

TLR agonists are available as oral compounds and could potentially be combined with other NAs as a single pill, making treatment regimens simple for patients to follow. Finally, similar to injected interferon, TLR agonists induce interferon production, triggering the production of cytokines to facilitate intracellular communication and cellular trafficking [128]. While injected interferon causes many adverse events, selective TLR agonists would act specifically in the liver, limiting the systemic innate immune activation by bypassing TLR8 stimulation.

GS-9620 is one of the TLR7 agonists currently being studied for CHB treatment. Oral TLR7 has demonstrated significant reductions in both HBV DNA and HBsAg levels in HBV-infected chimpanzees, although the scope of the study is limited because of a short dosing duration and only three animals were studied [129]. In humans, it has been shown to be safe and tolerable in healthy volunteers [130] as well as in virally suppressed and treatment naive patients with CHB [131]. No reduction in HBsAg or HBV DNA, however, were observed, likely because of the short dosing period [131].

### Cytokine therapy

Several cytokines have been studied extensively in relation to immunomodulation of chronic viral infections. The most promising of the

compounds that have been studied are IL-7 and IL-21 [132–134].

IL-7 is essential for primary T-cell development and likely plays a role in B-cell development as well [135,136]. It also plays a role in the development of some dendritic cell (DC) subsets. Regulation of peripheral CD4<sup>+</sup> T-cell homeostasis is accomplished via IL-7-mediated signaling [137]. In patients with CHB, IL-7 therapy may enhance, rejuvenate and restore immune response to HBV. In addition to its immune restorative effects, IL-7 has vaccine adjuvant effects, as well as beneficial effects in adoptive cell therapy.

IL-21 mediates the induction of maintenance of effector CD8<sup>+</sup> T cells. In mice, decreased IL-21 or IL-21R causes a progressive decline in the number of virus-specific effector CD8<sup>+</sup> T cells. This correlates with poor viral control [138–140]. In mice induced with CHB, IL-21 is important for promoting infection-controlling immune responses [141]. Patients with CHB who were treated with antivirals and show complete viral suppression also demonstrated significantly higher levels of serum IL-21 than those who did not achieve viral suppression [142]. Furthermore, increased levels of serum IL-21 is associated with better viral control due to HBeAg seroconversion. An option for IL-21 therapy is recombinant IL-21 (rIL-21), which is an immune modulator undergoing Phase I and II trials in cancer patients. In CHB patients receiving NA-based therapy, rIL-21 is a promising new approach to therapy [143].

### • Therapeutic vaccines

Development of an efficacious therapeutic vaccine for HBV is a promising avenue for the eradication of the disease. Control of CHB may be accomplished through inducing helper and cytotoxic T-cell response, counteracting immune tolerance and the activating the humoral immune response. Several promising options currently being explored are: vaccines based on recombinant proteins, HBV-envelope subviral particles, naked DNA combined with viral vectors and vaccines, dendritic cell-based vaccines (tarmogens) and those based on T-cell peptide epitopes derived from HBV proteins [144–146]. However, T cells of patients with CHB are associated with an exhausted immunophenotype, with reduced response to HBV proteins. This may contribute to why HBsAg-based vaccines have unsuccessful so far [147].

### Therapeutic vaccination based on recombinant HBV proteins or HBV-envelope subviral particles

#### Immunogenic complexes

Immunogenic complex vaccines, using anti-gen-antibody complexes, target dendritic cells in order to efficiently prime HBV-specific CD8<sup>+</sup> cytotoxic T-cells responses [148]. In a double-blind placebo-controlled Phase IIb trial using HBsAg and anti-HBs immunoglobulin complexes there was initial evidence suggesting clinical and virological efficacy [149]. However the Phase II trial failed to show therapeutic efficacy of the immune-based complex vaccine [150].

#### HBsAg & HBcAg combination

HBsAg and HBcAg are used in the nasal vaccine candidate. This vaccine complex has been shown to have a good immunogenicity and safety profile in preclinical studies. In a Phase I clinical trial, recombinant HBcAg acted as a potent Th1 adjuvant to HBsAg and also as a strong immunogen [151,152]. A pilot study of nasal and subcutaneous HBsAg/HBcAg combination-based vaccination monotherapy reported good tolerability and safety profile among 18 patients with CHB. HBV DNA levels were suppressed in seven participants during the vaccination period. HBV DNA remained undetectable for all seven participants, and a further two participants at 48-week follow-up. This included two HBeAg positive participants, both of whom seroconverted to HBeAg negativity and HBeAb positivity [153]. Furthermore, production of pro-inflammatory cytokines (IL-1 $\beta$ , TNF- $\alpha$  and IL-12) by dendritic cells from vaccinated participants were higher than unvaccinated controls. This pilot is followed by a Phase III trial and results are awaited (ClinicalTrials.gov identifier: NCT01374308).

#### Whole recombinant yeast-based therapeutic vaccine

Tarmogens (targeted molecular immunogen), a yeast-based immunotherapy platform, are currently under development. Tarmogen incorporates multiple viral antigens, expressing HBV (X), surface (S) and core Ags (X-S-Core). Tarmogens can be engineered to express HBV antigens by genetically modifying whole, heat-killed yeast. *Saccharomyces cerevisiae* (yeast) facilitates preferential uptake and processing by dendritic cells in order to effectively prime adaptive immune responses to inserted HBV

antigens. This platform favors induction of cellular effector responses (T cells) over humoral immunity (B cells) that arises from the use of subunit vaccines.

In mice studies, it has been shown that GS-4774, a tarmogen currently undergoing clinical trials in humans, protects mice from tumors specifically engineered to express HBV antigens [154]. Additionally, human studies using peripheral blood mononuclear cells collected from healthy donors and patients with CHB to show that tarmogens can elicit HBV-specific T-cell responses *ex vivo* [154]. GS-4774 has been shown to be safe and tolerable in healthy subjects [154]. The safety and efficacy of GS-4774 was recently evaluated in a Phase II study. In this study, 178 patients with CHB who were already suppressed on antiviral therapy were randomized to continue oral antiviral alone or receive GS-4774 2, 10, or 40 yeast units subcutaneously every 4 weeks until week 20 in addition to oral antiviral therapy. The vaccine was safe and well tolerated; however, no clinical benefit was observed, with no significant reduction in HBsAg levels [155].

#### Adeno-virus based therapeutic vaccination

TG1050 is a therapeutic vaccination based on a recombinant nonreplicative human adenovirus serotype 5, expressing multiple specific HBV antigens (core, polymerase and envelope) from genotype D. It has been designed to prime *de novo* and/or stimulate functional T cells expected to control the HBV replication and to clear HBV. In naive mice, TG1050 induced high levels of T cells targeting core, polymerase and HBsAg domains. High frequencies of cytolytic HBV-specific T cells were detected in spleens and livers of immunized mice. Further, induced HBV-specific T cells were detected in the blood of injected mice up to 10 months postimmunization [156].

#### Therapeutic vaccination based on DNA & T-cell peptide epitope

Based on the results of a woodchuck hepatitis virus (WHV) model study, a T-cell vaccine may be a viable option for treatment of HBV in humans. WHV-transgenic mice were vaccinated with a DNA prime-adenovirus boost vaccine in order to determine whether the vaccinated mice would control the WHV infection and induce WHcAg-specific T-cell response. In 70% of immunized mice, a strong WHcAg-specific

CD8<sup>+</sup> T-cell response that resulted in reduction of viral load beneath the limit of detection was demonstrated [157]. Combining the vaccine with entecavir resulted in WHsAg- and WHcAg-specific CD4<sup>+</sup> and CD8<sup>+</sup> T-cell response. These animals demonstrated prolonged response with two out of four chronic carriers developing anti-WHs antibodies in the process [157]. These results show that a combination of antivirals and a vaccine may be a useful approach to a functional cure for HBV.

#### DNA vaccine

DNA vaccines may be used to induce a T-cell response from CHB patients. The DNA vaccine has been shown to induce both CD8<sup>+</sup> and CD4<sup>+</sup> cells, with the helper cells producing IFN- $\gamma$  [158]. This particular vaccine activates HBV- and NK-cell specific T-cell responses [159].

In a proof of concept study, 12 CHB carriers treated with 3TC were vaccinated with a combination of HBV genes and genetically engineered IL-12. Type 1 T-cell responses correlated with viral suppression, and showed a 50% virological response rate. Moreover, T-cell responses were well-tolerated and maintained for at least 40 weeks after cessation of treatment [160].

#### T-cell peptide epitope vaccine

In a recent Phase I trial the safety of a multi-epitope-based (HBV derived) vaccine presented with HLA molecules was tested in healthy volunteers. This vaccine was found to be safe and tolerable in healthy volunteers and future studies are awaited [161].

#### Therapies involving T-cell response in HBV

T cells control HBV through direct cytolytic action; in addition, TNF- $\alpha$  and IFN- $\gamma$  released by T cells was recently shown to eliminate HBV replication and reduce cccDNA in infected hepatocytes. This required activation of nuclear APOBEC3 deaminase by these cytokines [162]. Hepatocytes from acute but not CHB patients or healthy controls expressed APOBEC3A/3B. Strategies to restore T-cell response in chronic HBV – such as use of checkpoint inhibitors or adoptive T-cell therapy – might achieve sustained viral control.

#### PD-1 inhibition

Most T cells specific to HBV are found to be exhausted and expressing PD-1 receptors. These T cells are energized when they interact with

PD-1L1/2 on antigen presenting cells *in vivo*. Ability to reverse this effect by interfering with PD-1/PD-L1/2 interaction presents an opportunity to rejuvenate T-cell responses and aid in HBV clearance as shown in a recent study using the woodchuck model [163]. Studies using PBMCs from CHB patients when stimulated with HBV genome overlapping peptides in the presence of PD-1/L1/2 blockade results in increase in IFN- $\gamma$  secreting HBV-specific CD8<sup>+</sup> T cells [164]. It is unclear if such approaches can be reproduced *in vivo*. Recently, both anti-PD-1 and PD-L1 monoclonal antibodies have been studied in patients with cancer. Whether such strategy can be tried for CHB patients is not clear.

#### Chimeric antigenic receptor

The above strategies of stimulating or rejuvenating HBV-specific T cells *in vivo* would only work in the presence of T cells specific to HBV. In some cases, T-cell deletion may occur, depleting the frequency of HBV specific T cells *in vivo*. In such cases, passive transfer of engineered chimeric T cells (CARs), may be an effective strategy. At least one patient with CHB and HCC has received CARs and much more are awaited [165].

#### • Combination approach

Using an antiviral therapy prior to treatment of CHB with a vaccine may lower the HBV DNA enough that there is significantly improved efficacy of the therapeutic vaccination. Multiple studies demonstrate correlations between low HBV DNA in serum at the start of vaccine therapy and increased treatment efficacy [166,167]. Therefore, development of a more effective therapeutic vaccine may hinge on using NAs to lower the HBV DNA levels.

#### Future perspective

The ultimate goal for HBV treatment is to be able to completely eliminate the virus from the body. By using combinations of the therapies mentioned above, achieving a cure may soon be possible. For example, TALENs, which disrupts cccDNA, has an increased antiviral effect when used in combination with IFN- $\alpha$ , thus may allow the immune system to eliminate the virus when used in combination. Additionally, through the use of an entry inhibitor, such as Myrcludex-B, and a potent antiviral, the body may be able to eventually eradicate the virus due to the lack of production of new cccDNA. Combination therapy using both viral

and host targeting approaches offer the best hope for accomplishing a functional cure for CHB, which may be a reality in the near future.

*financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.*

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## EXECUTIVE SUMMARY

- Chronic hepatitis B (CHB) affects a large number of people worldwide and remains a leading cause of hepatocellular carcinoma, liver failure and mortality.
- Current antiviral therapy is effective in suppressing hepatitis B virus (HBV) DNA levels and normalizing toll-like receptor levels, but cessation of therapy is associated with rebound of HBV DNA levels.
- Viral and host factors play a major role in establishment of persistence of chronic disease.
- Presence of covalently closed circular DNA and secretion of HBsAg and HBeAg subviral particles remain the two major viral causes for HBV persistence.
- Blunting of intrahepatic innate immunity and presence of exhausted HBV-specific T cells remain major host immune factors of HBV persistence.
- Absence of HBV DNA levels and HBsAg after cessation of therapy is the goal for sustained viral remission for CHB patients.
- Targeting various aspects of HBV lifecycle offers unique perspective for combination therapy that may be able to achieve sustained viral response.
- Blocking HBV entry, nucleocapsid formation, secretion of subunits and destabilizing or deactivating covalently closed circular DNA are some of the novel strategies to achieve sustained remission.
- Toll-like receptor agonism, therapeutic vaccinations, PD-1 blockade and CARs are novel strategies to target host immunity and aid in HBV clearance.

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## SYSTEMATIC REVIEW

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# Prevalence of hepatitis E infection in the general population of Iran: a systematic review and meta-analysis

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**Aim:** Hepatitis E is a form of acute viral hepatitis, which is caused by hepatitis E virus (HEV). The study objective was to evaluate the prevalence of HEV to provide an overall relative frequency in Iran. **Methods:** In this study, databases such as PubMed and Scopus were searched using the following keywords according to MeSH, "Hepatitis E virus", "Epidemiology", "Enzyme-linked immunosorbent assay (ELISA)" and "Iran". Then studies which had reported prevalence of HEV were selected. **Results:** We selected 36 researches out of 9246 for our study. From all the samples, the pooled estimated prevalence of HEV positive was 9.7%. It should be noted that HEV infection's relative frequency varied from 1.1% in Tehran to 46.1% in Ahvaz. **Conclusion:** HEV is common in Iran, although comparing to some neighboring countries, its prevalence is lower.

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Viral hepatitis is a public health challenge in several regions of the world [1]. Hepatitis E virus (HEV) is a small nonenveloped single-strand RNA virus which belongs to the Hepeviridae family and is a causative agent of acute hepatitis [2]. The main route of HEV transmission is considered to be the fecal-oral route and human-to-human, parenteral and perinatal transmission are three additional routes [3,4]. Recent studies have indicated that zoonosis is involved in the transmission of HEV, especially in industrialized countries where hepatitis E is believed to be nonendemic [5]. It has also been reported that blood donors are potentially able to cause transfusion-associated hepatitis E in high-endemic areas, thus providing safer blood and blood products is a major concern of blood banks in the world [6]. HEV infection, also called enterically transmitted non-A non-B hepatitis, is a main agent of epidemic hepatitis (in developing countries) and acute sporadic outbreaks in many areas of Asia, Africa and Mexico, where HEV is considered endemic [7–9]. In countries where the virus is endemic, HEV is associated with greater than 50% of cases of sporadic acute hepatitis [10]. HEV appears to be the second most frequent cause of enteric hepatitis after hepatitis A virus infection [1]. HEV infection is self-limiting, however, sometimes it has severe complications and a high case-fatality rate among

**KEYWORDS**

- ELISA • epidemiology
- Hepatitis E virus • Iran

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pregnant women (from about 20–30%) in the form of fulminant liver disease [11,12]. While high attack rates occur in adults aged 15–40 years, low attack rates usually occur in susceptible household contacts (0.7–2.2%) which is a result of the lack of person-to-person transmission [13]. HEV isolates are classified into five major genotypes, all of them belonging to one serotype. Four genotypes of HEVs are capable of causing human infection. Genotypes 1 and 2 exclusively infect humans and are endemic in many parts of Asia, Africa and South America and also are often associated with outbreaks and epidemics in developing countries. Genotypes 3 and 4 infect humans, pigs and other animal species and have been responsible for sporadic cases of the disease. Genotypes 3 have a worldwide distribution and genotype 4 has been often reported in Asian countries [14–17].

A major public health challenge in developing countries of Asia is HEV-mediated hepatitis, so that deserves the attention from world agency [8]. In most Asian countries, HEV is the single most frequent cause of acute hepatitis in adults. Also in parts of the Middle East and North Africa, it is second only to hepatitis B. In the word, endemicity of HEV is currently under investigation and a comprehensive picture of the seroprevalence of HEV infections and worldwide distribution are emerging. HEV infections occur predominantly in young adults [18]. Worldwide distribution of HEV is affected by predominating factors such as inadequate sanitation, poor personal hygiene and tropical climates. Also overcrowding, floods and rainy seasons are affecting factors on outbreaks of HEV [2]. So far, no commercial vaccine is made for HEV and hence travelers to endemic areas must take a series of precautions, including avoiding eating uncooked shellfish, vegetables or fruits and drinking water from sources of unknown purity to prevent the infection [19,20].

Iran was accounted as an endemic country for hepatitis E and its seroprevalence increased significantly with age from 3.3 to 37.5% in 30–50 years age groups. Although, to date, several studies have presented local information about HEV rates in different parts of Iran [21], the average rate of HEV prevalence in Iran is still

unknown [22]. Thus, the present study aimed to systematically review published data about the prevalence rate of HEV in different parts of Iran and provide an overall relative frequency (RF) for the country through meta-analysis.

## Methods

### • Search strategy

We searched in the PubMed, EMBASE, web of science ISI, Google scholar and Scopus (up to January 2015) databases using the following keywords: “Hepatitis E virus,” “Epidemiology,” “Prevalence,” “ELISA” and “Iran” along with their Persian equivalents with “OR” and “AND” and “NOT” Boolean Operators in the Title/Abstract/Keywords fields (Box 1). In addition to English articles, three Persian scientific search engines including The Iranian Scientific Information Database [23], Magiran [24] and IranMedex [25] were searched as well for relevant articles. No limitation was used while searching databases. Also, reference lists of all related studies were reviewed for any other related publications. We also reviewed nonelectronic evidences and interviewed with relevant experts and research centers to identify any gray literatures. Our search was restricted to the original articles/abstracts published in English and Persian which reported the prevalence of HEV by enzyme immunoassay in Iran.

### • Inclusion criteria

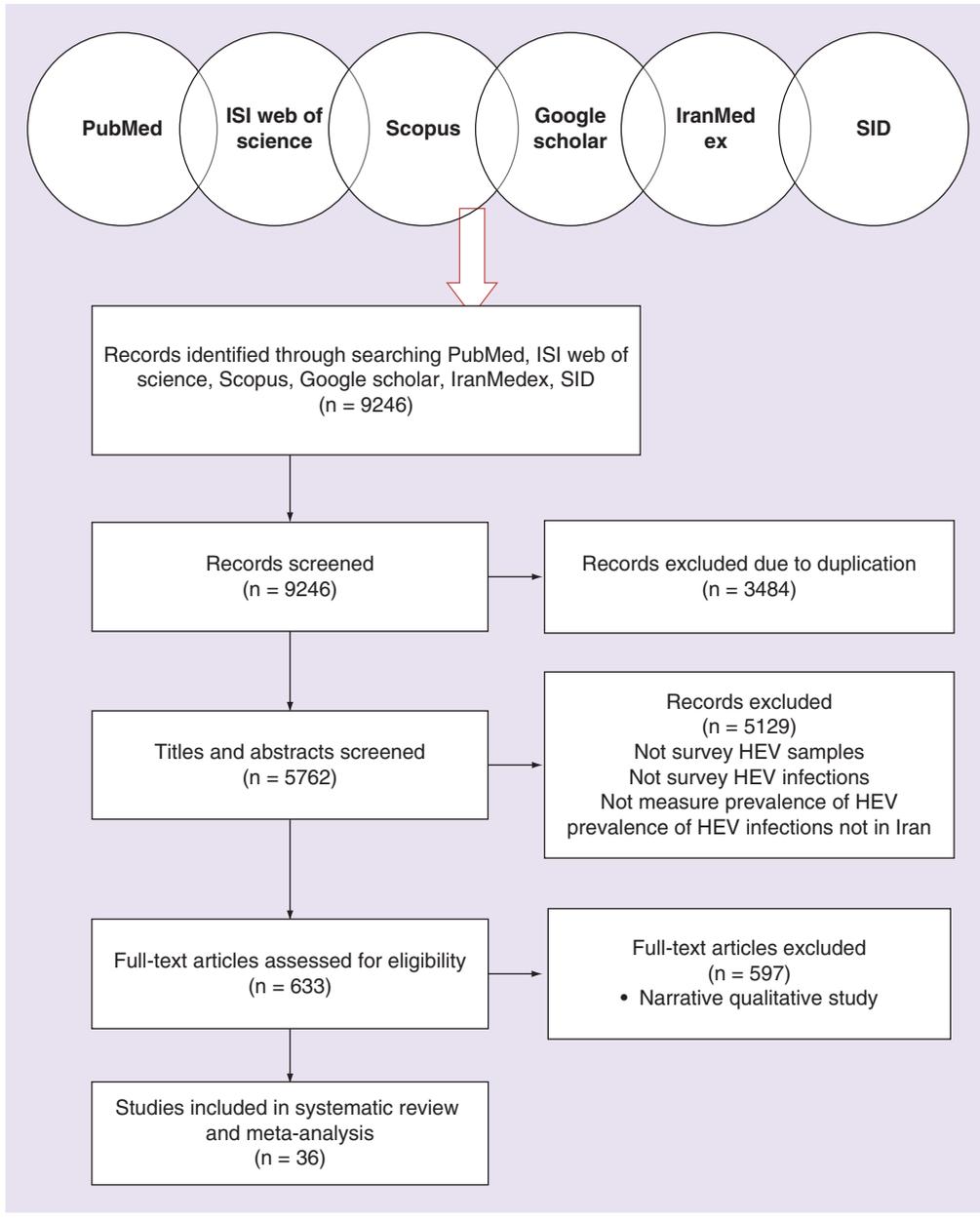
The inclusion criteria for both English and Persian study was: since this review study is limited to Iranian population and aimed to measure the HEV prevalence, samples (Serum [blood]) were collected exclusively from the Iranians; assays such as enzyme immunoassay, which are approved and accepted laboratory methods for HEV; researches that studied general population and studies that reported HEV IgG.

### • Exclusion criteria

Articles were excluded from this review if: samples were archived before; studies which used other methods instead of standard tests to identify HEV; laboratory studies on animals; congress abstracts, review articles, studies reported in languages other than English or Persian,

#### Box 1. Search strategy.

- (“Hepatitis E virus” [MeSH Terms]) AND (“Epidemiology” [MeSH Terms]) AND (“Prevalence” [MeSH Terms]) AND (“ELISA” [MeSH Terms]) AND (“Iran” [MeSH Terms]) AND (Limited to [LANGUAGE, “English” or “Persian”]) AND (Limited to [DOCUMENT TYPE, “original article”]) (Filters: Publication date from 1980/01/01 to 2015/12/30).



**Figure 1. Flowchart of literature search and study selection.**

HEV: Hepatitis E virus.

meta-analysis or systematic reviews and duplicate publication of the same study (or published both in English and Persian) were also excluded, with the exception of duplicate studies in which more sample size and more detailed results were provided.

#### • Data collection

From all of the studies, the author's name, publication date, sample size, study setting, mean age, the RF of HEV, research location and risk

factors were extracted. Data were found from studies based on title, abstract and keywords by two blinding researchers, separately. Disagreements were resolved by consensus of the whole team in both phases.

#### • Assessment of quality studies

We used checklist and diagram of the PRISMA, then critical appraisal has been done with STROB form. PRISMA and STROB can also be used as a basis for reporting system-

**Table 1. Characteristics of studies included in the systematic review and meta-analysis after full evaluation.**

Study	Year	City of data collection	Total sample size	Total number of cases	Techniques for HEV identification	IgM/IgG detection	Ref.
Aminiafshar	2004	Tehran	90	7	ELISA	IgG and IgM	[29]
Taremi	2004	Tabriz, northwestern part of Iran	324	24	ELISA	IgG	[30]
Somi	2006	East Azerbaijan	200	55	ELISA	IgG	[21]
Ataei	2005	Isfahan	816	31	ELISA	IgG and IgM	[1]
Alavi	2006	Ahvaz	228	35	ELISA	IgG	[31]
Rezazadeh	2005	Hamadan	280	36	ELISA	IgG	[32]
Assarehzadegan	2005	Khuzestan	400	46	ELISA	IgG	[33]
Ghorbani	2006	Tehran	800	9	ELISA	IgG and IgM	[2]
Shamsizadeh	2007	Ahvaz	566	48	ELISA	IgG	[34]
Mohebbi	2006	Tehran	493	39	ELISA	IgG	[35]
Mohebbi	2007	Tehran	551	51	ELISA	IgG	[36]
Rasti	2010	Ahvaz	418	22	ELISA	IgG and IgM	[37]
Taremi	2007	Tabriz	399	31	ELISA	IgG	[38]
Pourahmad	2007	Jahrom	43	3	ELISA	IgG	[39]
Taremi	2008	Nahavand	1824	170	ELISA	IgG	[40]
Saffar	2009	Mazandaran	1080	25	ELISA	IgG	[10]
Raofi	2009	Khorramabad	400	31	ELISA	IgG and IgM	[41]
Sepanlou	2010	Iran	1423	105	ELISA	Not specified	[22]
Amanat	2010	Southwest of Iran	80	5	ELISA	IgG	[32]
Eini	2010	Hamadan	153	29	ELISA	IgG	[42]
Zekavat	2010	Jahrom and Shiraz	80	5	ELISA	IgG	[43]
Mamani	2011	Hamadan	131	8	ELISA	IgG	[44]
Mamani	2011	Hamadan	1050	77	ELISA	IgG	[45]
Tabarraei	2011	Gorgan, North East of Caspian Sea	394	29	ELISA	Not specified	[46]
Rostamzadeh Khameneh	2011	Urmia	91	28	ELISA	IgG	[47]
Sharif	2011	Kashan	558	21	ELISA	IgG	[48]
Ehteram	2012	Central province of Iran	530	76	ELISA	IgG	[6]
Raofi	2012	Western Iran	400	31	ELISA	Not specified	[49]
Alavian	2012	Isfahan	274	78	ELISA	IgG	[50]
Rostamzadeh Khameneh	2013	Urmia	136	5	ELISA	IgG	[51]
Tabaraie	2013	Gorgan	150	6	ELISA	IgG and IgM	[52]
Joulaei	2013	Shiraz	158	26	ELISA	IgG and IgM	[53]
Naeimi	2013	Bushehr	628	105	ELISA	IgG	[48]
Sotoodeh Jahromi	2013	Jahrom	110	11	ELISA	IgG and IgM	[54]
Farshadpour	2014	Ahvaz	510	235	ELISA	IgG and IgM	[55]
Keshvari	2014	Tehran	559	45	ELISA	IgG	[56]

atic reviews of other types of research; STROB particularly evaluated the prevalence for any outcome. Items related to study type, sample size, research objectives, population and inclusion/exclusion criteria for primary research, methods of analysis and appropriate presentation of results were determined and a score was assigned to each item. We scored topic critical appraisal 0–2, a score was assigned to each sec-

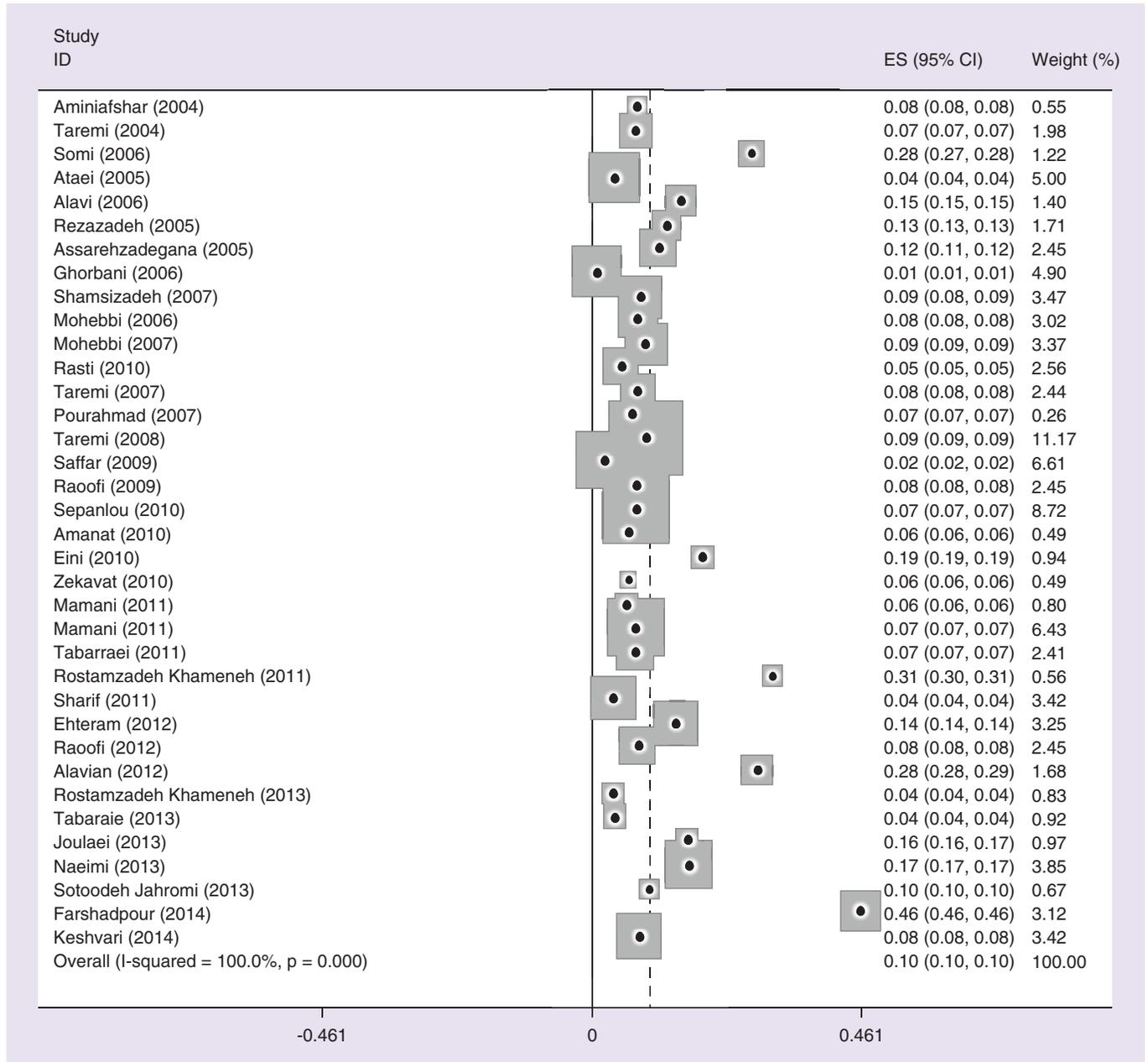
tion (e.g., title, abstract, methodology, result, discussion and so on), then articles scored between 0–15, and the studies achieved at least eight quality scores were considered eligible for final meta-analysis.

• **Statistical analysis**

The numbers of total participants with rotavirus were used to estimate the RF which was con-

verted to log RF and its standard error for the meta-analysis [26]. We used the random effect model, when heterogeneity existed ( $I^2 \geq 50\%$ ). The pooled prevalence was derived from a random effect model that takes between-study variation into account [27]. Statistical heterogeneity of the statistics between studies was evaluated by using Cochran's Q test and quantified with I-squared [26]. In order to identify sources of heterogeneity, which the pooled prevalence

might depend on, sensitivity analysis was used for a particular study or a group of publications (if any) that had the highest impact on the heterogeneity test. Publication bias and asymmetry was checked by Funnel plots, Begg's adjusted rank-correlation test and Egger's regression [28]. All statistical analyses were performed using STATA 11.0 (STATA Corp., TX, USA) and p-values less than 0.05 were considered statistically significant.



**Figure 2. Results of meta-analysis of pooled prevalence with 95% CI and its forest plot.**  
ES: Estimation of prevalence

**Results**

A total of 9246 articles were retrieved by the database search. Summary of the literature search and study selection is shown in the **Figure 1**. In a primary screening process, 3484 of the publications were excluded due to duplication. In the secondary screening process, 5129 of the publications were excluded based on title and abstract evaluation, and 633 articles were retained for detailed full-text evaluation. After full-text evaluation, 36 articles describing the prevalence of Hepatitis E infection in Iran, with average of quality scores was 11.75 (SD = 2), were selected for the present study which are listed in **Table 1**. In all studies, the hepatitis E infectious samples are taken from the general population. As it is clear; most of the studies were conducted in central and west of Iran followed by the south. **Figure 2** shows the forest plot and results of meta-analysis for the pooled estimation of the prevalence with 95% CI.

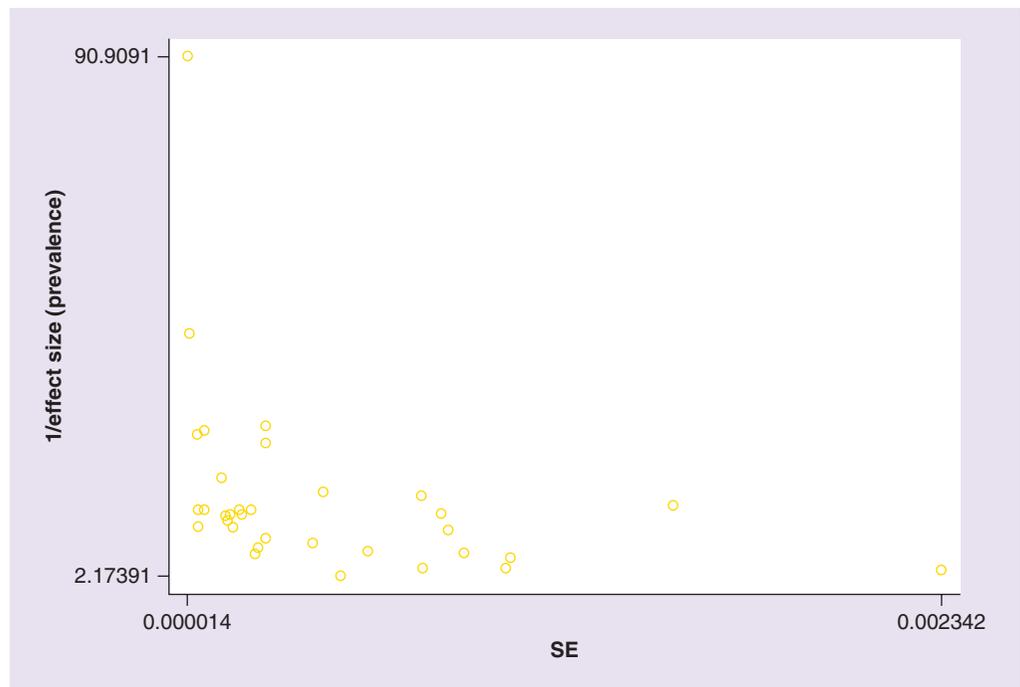
The status of HEV in Iran is represented in **Table 1**. The pooled prevalence of hepatitis E infection calculated using the random-effects model was found to be 9.7% (95% CI: 9.5–9.9%) (**Figure 2**). However, the evident heterogeneity of HEV-RF was observed among studies (Cochran Q test, p-value = 0.001; I<sup>2</sup> = 90%). Although

a slight asymmetry was seen in the funnel plot (more detail was shown in the **Figure 3**), this was not confirmed by statistical tests (p-value = 0.075 for Begg rank correlation analysis; p-value = 0.052 for Egger weighted regression analysis).

Sensitivity analysis was performed by sequential omission of individual studies. The combined RFs of HEV prevalence from sequential omission was 11.1% and 95% CI: 9.1–13.2% (CI consists of the pooled prevalence of HEV). Therefore, the pooled prevalence of HEV not altered after omission, indicating that our results were statistically robust (**Figure 4**).

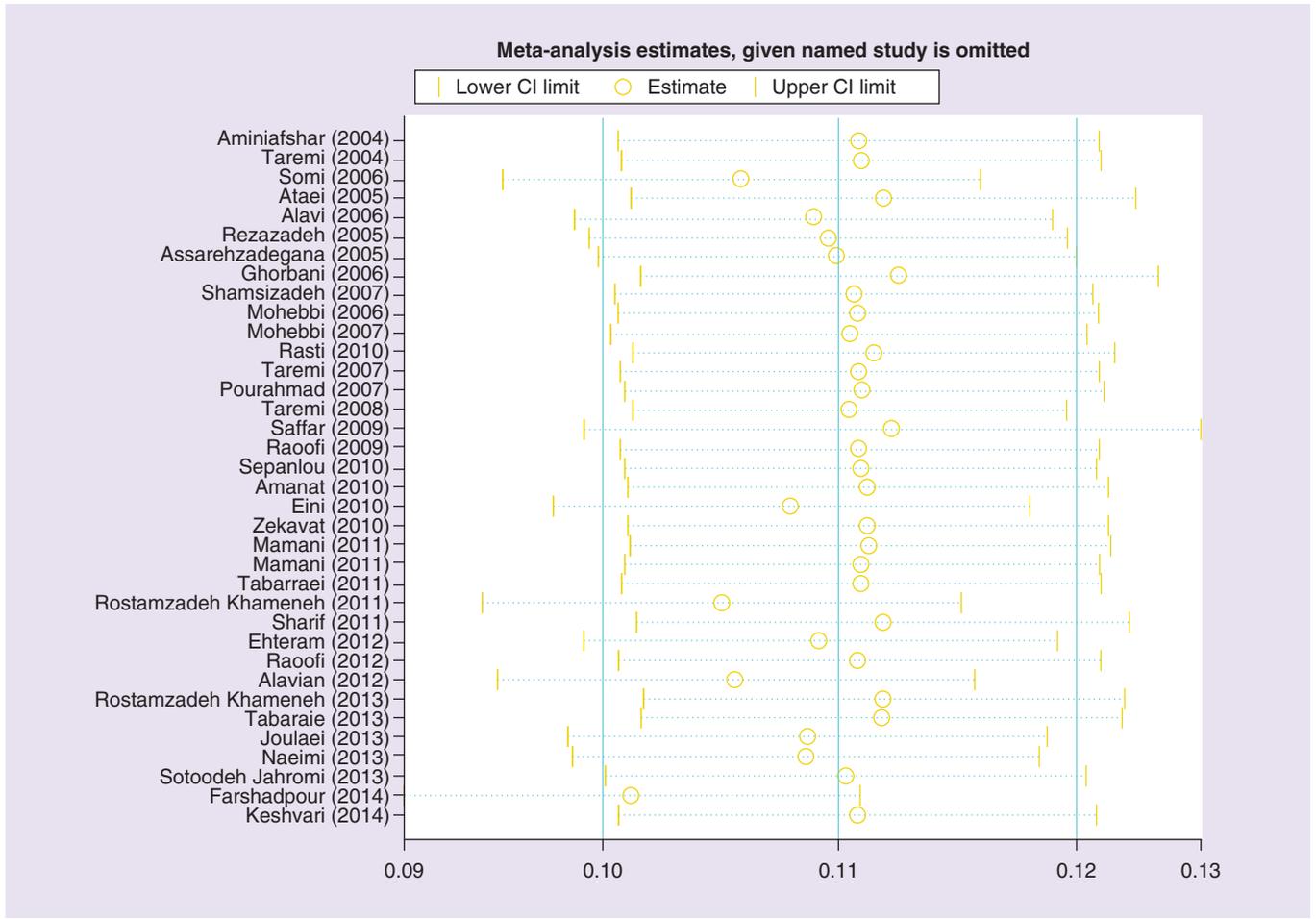
**Discussion**

Iran with few suspected outbreaks of HEV is expected to have a high rate of hepatitis E prevalence. Although, there have been few reports of HEV suspected outbreaks in this geographic region [22]. The first documented report of Iran came from a pregnant woman in Kermanshah province in 1991 [57]. In recent years, Iranian researchers investigated HEV seroprevalence and reported more heterogenic data about local prevalence of HEV infection [22]. This study aimed to determine the RF of HEV from different parts of Iran. In order to do so, the related published



**Figure 3. Funnel plot of the relative frequencies in the studies that evaluated the seroprevalence of hepatitis E virus in Iran.**

RF: Relative frequencies.



**Figure 4. Influence or sensitivity plot.**

and unpublished data from the last 10 years were collected. The finding of this study revealed the mean prevalence of HEV infection moderately was at a high level and quantitatively was equal to 9.7%. Fifty percent of Iranian cities were afflicted by the infection. We tried to compare current study with similar studies performed in different parts of the world. Based on different studies, Hepatitis E globally has impacted human health and has infected almost a third of the world population [16,58,59]. The infection rates vary in the different geographical regions and high prevalence of HEV infection has been reported in India (up to 50%), Africa (25%) and Eastern-Oriental countries (15–20%), respectively [15]. In Iraq as parts of the Middle East and an endemic region, Nasrawi *et al.* reported that the prevalence of HEV infection was 38.1% [60]. The reported prevalence rate of HEV infection was 14% for Pakistan [61] and 6% for Egypt [62]. In industrialized countries, the circumstances of the infection

is different in Far East Asia and North America. In these regions, HEV infection is much less frequent and only several sporadic cases have been reported [58,59]. Also, a recent study by Yapa *et al.* described first HEV outbreak in Australia [63,64]. In addition, recent studies carried out on population of European countries revealed an increase in seroprevalence of anti-HEV IgG. In southwest England, HEV IgG seroprevalence was determined in 500 blood donors and the study revealed that 16% were IgG positive [65]. In Eastern Germany (2012), seroprevalence of anti-HEV IgG detected 18% among blood donors and 11% among forestry workers [66]. Petrovic *et al.* reported 15% anti-HEV IgG among 200 volunteer blood donors. Also, it was found that HEV seroprevalence is enhanced by age [67]. Maral *et al.* indicated that seroprevalence of anti-HEV IgG among primary school students was 1.7% and 2.1% in November 2003 and in January 2005 in Turkey, respectively [68]. Seroprevalence of anti-

HEV IgG in Iran varied from 1.1% in Tehran [2] to 30.76% in Urmia [47] and 46.1% in Ahvaz [55]. These widely varying rates of HEV infection in different geographic areas could be related to several factors such as different socioeconomic and hygienic states, variability in the circulation of types of virus and tropical climates. Also, in developed countries, occupational exposure and consumption of some foods such as pork products [69] are considered as important factors in distribution of the infection. ELISA, as a sensitive and specific diagnostic method [70], has been used in most of the studies that have been applied in the review for HEV detection. The result revealed that ELISA assay could reduce bias in the virus detection. The findings showed that HEV infection is globally distributed evenly in developed countries and healthcare systems may face difficulties in controlling the infection worldwide. There were several limitations that should be considered in our meta-analysis, including: due to the language barrier, non-English literature could not be considered in the study; missed studies, due to limited access to the press articles, however, the funnel plot suggests that publication bias was not obvious; in some part of Iran, no study was not performed on prevalence of HEV infection, so these results cannot fully be extensible to some

parts of Iran; there was heterogeneity between the included studies.

### Conclusion

In conclusion, the findings of meta-analysis revealed that HEV infection is endemic in Iran. Therefore, sanitation programs, public health education, careful monitoring and early detection of HEV infection through sensitive and specific laboratory methods are recommended for prevention and control of the infection in Iranian population. Furthermore, studies on the role of HEV, especially in cases of clinical hepatitis and asymptomatic are recommended.

### Future perspective

Iran has the highest prevalence rate of hepatitis E and thus more surveys in this region are needed.

### Financial & competing interests disclosure

*The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending or royalties.*

*No writing assistance was utilized in the production of this manuscript.*

## EXECUTIVE SUMMARY

### Aim

- The aim of this study is to evaluate the hepatitis E virus (HEV) prevalence in Iran and provide an overall relative frequency for Iran.

### Methods

- We searched all the related databases.
- Studies were selected using inclusion and exclusion criteria.
- The data were analyzed by software and the prevalence of HEV was reported.

### Results & conclusion

- Thirty two studies were selected among 9246 studies.
- Pooled estimate prevalence of HEV positive was 9.7%.
- Relative frequency values varied from 1.1% in Tehran to 46.1% in Ahvaz.
- HEV is common in Iran, although the prevalence is lower than some neighboring countries.

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