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Systematic Review

Possible role of tocopherols in the modulation of host microRNA with potential antiviral activity in patients with hepatitis B virus-related persistent infection: a systematic review

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Abstract

Hepatitis B virus (HBV) infection represents a serious global health problem and persistent HBV infection is associated with an increased risk of cirrhosis, hepatocellular carcinoma and liver failure. Recently, the study of the role of microRNA (miRNA) in the pathogenesis of HBV has gained considerable interest as well as new treatments against this pathogen have been approved. A few studies have investigated the antiviral activity of vitamin E (VE) in chronic HBV carriers. Herein, we review the possible role of tocopherols in the modulation of host miRNA with potential anti-HBV activity. A systematic research of the scientific literature was performed by searching the MEDLINE, Cochrane Library and EMBASE databases. The keywords used were 'HBV therapy', 'HBV treatment', 'VE antiviral effects', 'tocopherol antiviral activity', 'miRNA antiviral activity' and 'VE microRNA'. Reports describing the role of miRNA in the regulation of HBV life cycle, *in vitro* and *in vivo* available studies reporting the effects of VE on miRNA expression profiles and epigenetic networks, and clinical trials reporting the use of VE in patients with HBV-related chronic hepatitis were identified and examined. Based on the clinical results obtained in VE-treated chronic HBV carriers, we provide a reliable hypothesis for the possible role of this vitamin in the modulation of host miRNA profiles perturbed by this viral pathogen and in the regulation of some cellular miRNA with a suggested potential anti-HBV activity. This approach may contribute to the improvement of our understanding of pathogenetic mechanisms involved in HBV infection and increase the possibility of its management and treatment.

Key words: Vitamin E: Hepatitis B virus: MicroRNA: Viral infections: Immune responses

Chronic hepatitis B virus (HBV) infection is a serious public health problem. Although remarkable differences are detectable across the world depending on geographical regions and ethnicity, it is estimated that approximately 350–400 million people are persistently infected with HBV[1]. A significant percentage of chronic HBV carriers develop a necroinflammatory liver disease with different patterns of severity and course. Long-lasting liver damage represents a high-risk condition for developing cirrhosis and hepatocellular carcinoma (HCC)[2]. In the last few years, an increasing number of studies have been conducted to investigate mechanisms involved in the regulation of HBV

Abbreviations: COX-2, cyclo-oxygenase-2; *DNMT*, DNA (cytosine-5-)-methyltransferase; EnhI, enhancer I; EnhII, enhancer II; HBV, hepatitis B virus; HBeAg, hepatitis B e-antigen; HBeAb, antibody to hepatitis B e-antigen; HBx, hepatitis B x protein; HCC, hepatocellular carcinoma; IFN-γ, interferon-γ; miRNA, microRNA; NASH, non-alcoholic steatohepatitis; Th1, T helper-1; UTR, untranslated regions; VE, vitamin E.

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transcription and replication, including epigenetic networks and microRNA (miRNA) profiles. There is a pressing need for a better understanding of virus–host interactions to improve our knowledge of HBV pathogenesis and to develop new therapeutic strategies. To date, approved therapeutic regimens for patients with persistent HBV infection have been interferons and nucleotide/nucleoside analogues. These therapeutic regimens generally decrease viral replication and substantially contribute to the attenuation of liver damage, but cannot definitively clear this pathogen in the majority of treated patients. Since 2001, some trials have been investigating the efficacy of vitamin E (VE) (α-tocopherol) as a treatment option for chronic HBV carriers, among both adults and children. Of the three published trials, two have reported that VE administration may promote serum hepatitis B e-antigen (HBeAg) clearance and antibody to hepatitis B e-antigen (HBeAb) development as well as concomitant HBV-DNA loss and alanine aminotransferase normalisation in a higher proportion of treated patients. The possible mechanisms that explain these clinical results have been reported in our previous review[3]. VE is a well-known essential lipid-soluble compound with both antioxidant effects, contributing to the protection of hepatocytes from oxidative stress, and immune-modulating properties, stimulating the functions of T-helper-1 (Th1) and natural killer cells[3]. Furthermore, VE has gene-regulatory activities at the transcriptional and post-transcriptional levels[4]. However, despite the significant improvement in the knowledge of the molecular mechanisms of VE activity, the understanding of tocopherol-mediated effects in supplemented subjects with persistent HBV infection still remains poor. Additional investigations are required to explain some of the clinical evidence observed in these patients. Herein, we discuss the potential role of tocopherols in the modulation of miRNA with potential antiviral activity in chronic HBV carriers treated with VE and briefly describe the current knowledge of HBV genome organisation and life cycle. We carried out a systematic research by focusing on the following: (1) reports describing the role of miRNA in the regulation of HBV life cycle; (2) *in vitro* and *in vivo* studies reporting the effects of VE on cellular miRNA expression profiles and epigenetic networks; (3) clinical studies reporting the use of VE in patients with HBV-related chronic hepatitis.

According to the studies available in the literature, we tried to correlate different miRNA, modulated by this fat-soluble compound, with biochemical and/or virological effects observed in clinical trials enrolling individuals with persistent HBV infection and treated with VE.

Hepatitis B virus genome organisation

HBV is a small, non-cytopathic, partially double-stranded DNA virus, belonging to the family of Hepadnaviridae[5,6]. The preferential site of HBV replication is the hepatocyte. The virus consists of an envelope and an icosahedral nucleocapsid core (HBcAg (hepatitis B core antigen)) containing a circular partially double-stranded DNA genome (3·2 kb in length) and a virus-encoded DNA polymerase. The viral genome has four overlapping open reading frames, which are as follows[7]: (1) S (nucleotides 834–2586), for surface or envelope genes; it is defined as pre-surface1 or pre-S1, pre-surface2 or pre-S2, and surface or S encoding the large, middle and small surface proteins, respectively; (2) C, for the core (nucleotides 1903–2458)/pre-core (nucleotides 1816–1902) genes encoding both core protein (HBcAg) and pre-core protein, which undergoes post-translational modification to become HBeAg; (3) P (nucleotides 1622–2309), for the polymerase RT, a multifunctional protein with a crucial role in viral replication; (4) X (nucleotides 1376–1837), for the hepatitis B x protein (HBx), with cellular and viral gene-transactivator properties[8].

The HBV genome also comprises four distinct promoters and two master regulators, defined as enhancer I (EnhI) and enhancer II (EnhII)[9], that control viral gene transcription and replication. EnhI (nucleotides 970–1240) can be detected between X and S genes, upstream of the X promoter[10]. Functional elements in EnhI have been defined as modulatory, core enhancer and basal promoter X-ORF (open reading frame) regions[11] or as E, EP, GB and R-S domains according to different studies[12] (Fig. 1). These domains contain a lot of binding sites for host liver-specific and ubiquitous transcription factors[13,14]. EnhI modulates X promoter activity and gene expression, and it may be transactivated by the HBx protein itself. EnhII (nucleotides 1627–1774) overlaps with the X protein gene and can be detected upstream of the core promoter[15–18]. It also contains important binding sites for several host nuclear factors. These sequences of HBV genome represent specific targets for cellular proteins that possess regulatory activities and are normally localised in an inactive form either in cell nucleus[19–23] or in cytoplasm[24–26]. Among these ubiquitous transcription factors, nuclear factor-1, activator protein-1, liver-enriched transcription factors (such as hepatocyte nuclear factor-3 and -4 and CAAT/enhancer-binding protein (C/EBP)) and p53 can bind to HBV enhancers. In addition, although both enhancers modulate the functions of all the four viral gene promoters and viral replication, a predominance of EnhI over EnhII in the regulation of HBV gene expression has been reported[27]. Following the entry of HBV virions into the cell, via receptor-mediated endocytosis, the viral capsid is carried along microtubules towards the nucleus. Within the nucleus, the relaxed circular form of the viral genome is converted to a covalently closed circular DNA. The covalently closed circular DNA is transcribed by cellular polymerase II and generates four major mRNA that are 0·7, 2·1, 2·4 and 3·5 kb in length[28]. The largest 3·5 kb RNA serves as a template not only for the e-antigen, core and polymerase protein translation, but also for HBV replication via reverse transcription[7]. The organisation of the HBV genome is shown in Fig. 1 and Fig. 2.

Systematic review

Materials and methods

Search strategy. A systematic computer-based search for published articles, according to the PRISMA (Preferred Reporting Items for Systematic reviews and Meta-Analysis)

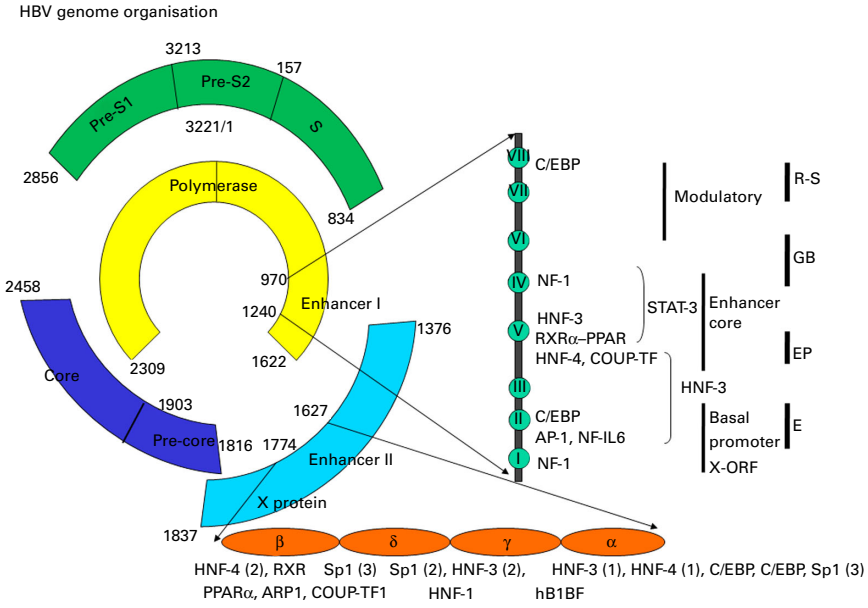

Fig. 1. Schematic representation of hepatitis B virus (HBV) genome organisation. The most important binding sites for nuclear factors in enhancers I and II, such as CAAT/enhancer-binding protein (C/EBP), hepatocyte nuclear factor (HNF)-3, HNF-4, activator protein-1 (AP-1), retinoid X receptor-α–PPAR (RXRα–PPAR), nuclear factor-IL-6 (NF-IL-6), specificity protein-1 (Sp1), chicken ovalbumin upstream promoter transcription factor 1 (COUP-TF1) and signal transducer and activator of transcription 3 (STAT-3), are shown[7–13]. The functional elements in enhancer 1 have been described as modulatory, enhancer core and basal promoter X-ORF (open reading frame) regions or as E, EP, GB and R-S domains. Enhancer core domain plays a key role in the functions of enhancer I. Pre-S1, pre-surface 1; Pre-S2, pre-surface 2; NF-1, nuclear factor-1; ARP1, nuclear receptor subfamily 2, group F, member 2; hB1BF, human B1 binding factor. A colour version of this figure can be found online at http://www.journals.cambridge.org/bjn

Statement[29] through OVID interface, was performed to conduct this review. Relevant articles investigating the role of miRNA in the regulation of HBV life cycle and the possible role of VE/tocopherols/tocotrienols in the modulation of cellular miRNA in patients with persistent HBV infection were identified. The following databases were used: MEDLINE (1950 to 31 January 2014); the Cochrane Library (until the third quarter of 2013); EMBASE (1980 to 31 January 2014) for all potentially relevant articles. The search terms were developed with the support of a professional research librarian. The search key words were identified by means of controlled vocabulary, such as the National Library of Medicine's MESH (Medical Subject Headings). The following MESH terms were used: 'Anti-viral Agents'; 'Hepatitis B'; 'Therapeutics'; 'Epigenetics'; 'Vitamin E'; 'Tocopherols'; 'Tocotrienols'; 'microRNA/miRNAs'; 'molecular mechanisms'. The following key words were identified: 'HBV'; 'Treatment'; 'Therapy'; 'Chronic Infection'; 'Antiviral Activity'. If a study was considered potentially eligible, its full text was further evaluated and its assessment was carried out in accordance with eligibility criteria developed to systematically include studies in this review. The selected studies were considered eligible if one or more of the following criteria were met: (1) the research was designed to evaluate either a single miRNA or distinct panels of miRNA in the serum and/or in the hepatic tissue and/or immune cells of patients with HBV-related chronic hepatitis or HCC as well as in transgenic mice expressing individual viral genes or in HBV-producing cultured cells; (2) the research was designed to assess the modulatory effects of VE supplementation on miRNA expression and functions in humans, animals or cell lines; (3) the research was a controlled or uncontrolled clinical trial, a pilot study or a case series designed to assess VE use in adult and paediatric patients with HBV-related chronic hepatitis. Moreover, the research had to be reported in English, as peer-reviewed, full-text publication. Articles not published as full reports, such as conference abstracts, case reports and editorials, were excluded.

Study selection and data extraction. Literature review was performed independently and in parallel by two authors (M. M. and S. S.), followed by identification and screening of relevant articles on the basis of titles and abstracts. If a study was considered potentially eligible by either of the two reviewers, the full text of the article was retrieved for additional assessment. All relevant data from the identified studies were independently extracted and tabulated by another two authors (A. F. and D. d. B.) by means of a standardised flow path, according to an adapted schedule, obtained from the Cochrane handbook section 7.3a checklist of domains[30].

The following information was extracted from each research: first author's name, study design, year of publication, country of origin, methods used for HBV, miRNA and VE/tocopherol/tocotrienol detection, as well as quality control for viral, miRNA and VE/tocopherol/tocotrienol testing methods, and ethnicity of enrolled subjects, matching criteria and number of cases and controls for studies carried out in human subjects. A third reviewer (A. C.) checked the accuracy of data collection, and disagreements concerning the results were settled by consensus among all authors.

The key words were associated according to the following scheme: 'HBV therapy' (12 846 citations); 'HBV treatment' (14 655 citations); 'VE and HBV' (fifteen citations); 'VE and

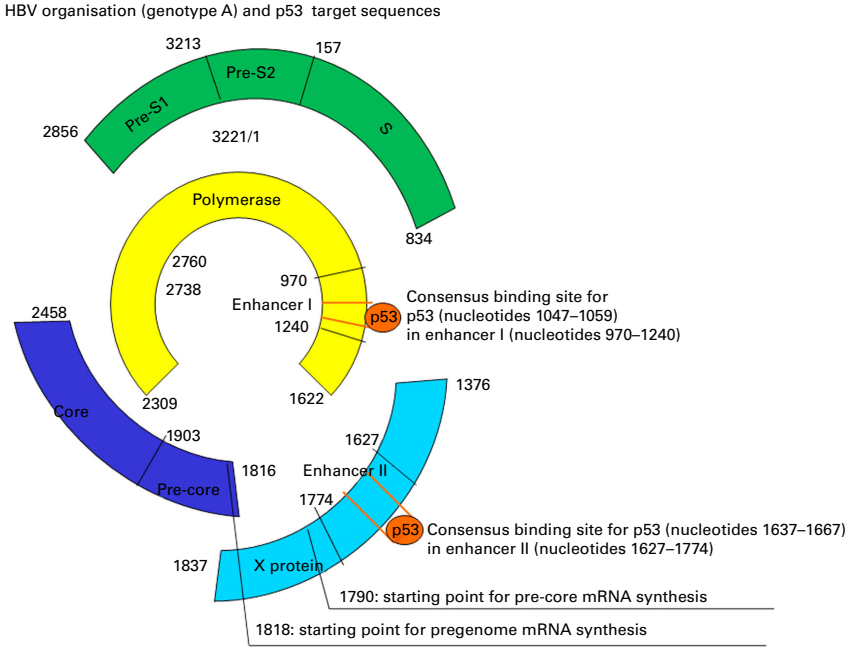

Fig. 2. Schematic representation of the binding of p53 to its target consensus sequences localised within hepatitis B virus (HBV). Enhancer I (nucleotides 1047–1059) and enhancer II (nucleotides 1637–1667). Numbers indicate relative positions at the 5′-terminus of HBV genome. This interaction inhibits the function of both viral enhancers and causes a decrease in the gene expression of HBV[13–18]. Pre-S1, pre-surface 1; Pre-S2, pre-surface 2. A colour version of this figure can be found online at http://www.journals.cambridge.org/bjn

chronic HBV infection' (six citations); 'VE and antiviral activity' (twenty-one citations); 'VE and microRNA/miRNAs' (nine citations); 'HBV and microRNA/miRNAs' (148 citations); 'tocopherols and HBV' (0 citations); 'tocopherols and microRNA/miRNAs' (one citation); 'tocotrienols and HBV' (one citation); 'tocotrienols and microRNA/miRNAs' (one citation); 'epigenetic and HBV' (eighty-seven citations); 'epigenetic mechanisms and HBV' (thirty-one citations); 'VE and epigenetics' (twenty-one citations); 'epigenetics and VE and HBV' (0 citations); 'tocopherols and epigenetics' (five citations); 'tocotrienols and epigenetics' (three citations). The reference list of the retrieved articles was also checked to identify additional pertinent studies. A total of 27 501 citations were identified. Among these, 27 227 were excluded after a preliminary review of the titles and/or abstracts. The full text of the remaining 274 articles was considered for a more detailed assessment. The eligibility criteria were not met by eighty-eight reports. Overall, 186 potentially relevant articles were identified and considered for this review. A selection of these papers is reported in the reference list. Because of the characteristics and heterogeneity of the identified reports as well as the difference in study designs both in trials enrolling patients and in studies carried out in cell cultures, sensitivity and subgroup analyses were considered inappropriate. Therefore, no quantitative assessment of these studies was performed.

Results and discussion

Taking advantage of the data reported in the selected articles, results and conclusions from the reports are summarised and organised into three sections and used to propose a systematic hypothesis, describing how VE may exert an antiviral effect by modulating cellular miRNA profiles.

Role of microRNA in the regulation of hepatitis B virus life cycle

In the last few years, an increasing body of evidence has shown miRNA to play a key role in the modulation of the expression of critical cellular genes at the post-transcriptional level[31]. These endogenous non-coding RNA (19–25 nucleotides in length) target specific sites in the 3′-untranslated regions (UTR) of cellular mRNA, negatively regulating their stability and translation processes[32]. miRNA function in a concerted manner and in association with other control systems to maintain cellular homeostasis. In particular, these molecules exert combined effects, as they influence multiple and crucial interconnected signalling pathways that take part in distinct types of negative or positive intracellular feedback regulatory loops. miRNA modulate several cellular activities and biological events, including proliferation, differentiation, apoptosis, inflammation and immune responses[33,34]. miRNA may regulate more than 50 % of cellular mRNA, and a single miRNA may target more than 100 mRNA[35]. Alterations in their expression or activities characterise several diseases, such as cancers[36,37] and viral infections[38–40]. miRNA have a key regulatory role in the complex virus–host interplay developing in the course of an acute or chronic viral infection[41]. Both DNA and RNA viruses have evolved fine strategies to promote a favourable environment for their replication and survival. These pathogens modulate the expression patterns of cellular miRNA and some of them may encode their own miRNA. HBV has also developed mechanisms to

persistently colonise its hosts, escaping their defence mechanisms. To date, no HBV-encoded miRNA has been demonstrated, although the existence of one putative candidate has been suggested[40]. HBV may directly or indirectly manipulate cellular miRNA profiles, thereby causing an increase or a decrease in the expression of these molecules. As a consequence, the transcription and replication of HBV are enhanced or reduced. However, it is not yet well known how this pathogen reprogrammes host miRNA machinery[41]. HBV may directly elicit the binding of cellular miRNA to specific viral transcripts, such as the mRNA for surface antigen and for X protein and DNA polymerase, with the subsequent change in the quantitative expression of these proteins. Furthermore, the production of some miRNA, induced by HBV infection as a direct host-specific antiviral response, may also be exploited by this pathogen to indirectly modulate its transcription and replication. These miRNA generally control the activity of critical cellular mRNA encoding key regulatory proteins with important functions in cells. These proteins (i.e. liver-enriched transcription factors, nuclear receptors and enzymes such as methyltransferase) are critical to the life cycle of HBV and to its survival. In the last few years, significant efforts have been made using *in vitro* or *in vivo* models, with the aim of identifying qualitative and/or quantitative modifications in miRNA profiles induced by HBV during persistent or acute infection. The results of these studies are not univocal and even substantial differences exist in their conclusions. There are various reasons for these discrepancies. Heterogeneity among these studies in terms of aims and methods represents one of the most important factors. In particular, some studies have focused on HBV-infected patients in different stages of persistent liver diseases, ranging from chronic hepatitis to cirrhosis and to HCC, as well as have assessed miRNA patterns either in the serum or in the hepatic tissue[42,43]. On the other hand, additional studies have been carried out *in vitro*. However, taking into account these potential limitations and according to available evidence, some miRNA with critical roles in cell physiology and with aberrant expression in patients with chronic HBV-related liver diseases have been described. Several studies have analysed distinct panels of miRNA in cultured human as well as animal cell lines and have identified some of the endogenous molecules that may modulate viral genome expression[41–44]. In a model of HBV-producing HepG2.2.15 cells, the expression of miRNA-199a3p and miRNA-210 has been found to be up-regulated due to the direct binding of these molecules to viral RNA with the consequent decrease in HBV replication and reduction of hepatitis B surface antigen (HBsAg) expression[44]. Binding sites for miRNA-199a3p and miRNA-210 have been detected in the HBsAg-coding region as well as in the pre-S1 region, respectively[44]. Furthermore, miRNA-125a-5p and miRNA-151-5p have been reported to modulate viral genome expression in HepG2 cell lines, and an increase in miRNA-125a-5p levels seems to be associated with suppressed HBV replication[45,46]. miRNA-155 inhibits *in vitro* HBV protein expression and replication, promoting innate antiviral immune responses in hepatoma cells[47]. Studies carried out in cultured cell lines have demonstrated that HBV mediates, via some viral transcripts such as *HBx*, the down-regulation of some cellular miRNA with tumour-suppressor activities, including miRNA-15a, miRNA-16, miRNA-199a-3p and Let-7. This event may contribute to the promotion of HCC[48]. In addition, the HBx protein up-regulates the expression of miRNA-29a[49] and miRNA-143[50] and down-regulates that of miRNA-101, miRNA-122, miRNA-132, miRNA-148a and miRNA-152[51–58]. Differential profiles of circulating miRNA have been described in individuals with chronic HBV-related infection in comparison with healthy controls as well as the number and type of these aberrantly expressed endogenous molecules vary, depending on disease severity. In particular, among the host miRNA that are most frequently perturbed by viral products, miRNA-19b, miRNA-20a, miRNA-22, miRNA-92a, miRNA-99a, miRNA-106a, miRNA-125a, miRNA-125b, miRNA-146a, miRNA-194 and miRNA-223 levels have been found to be up-regulated in the serum of HBV-infected patients[41,59,60]. According to some reports, the serum levels of miRNA-122, a specific and highly expressed molecule in normal liver with crucial functions in hepatocytes, are higher in HBV carriers[61]. However, other investigators have reported opposite results and have shown an inverse linear correlation between miRNA-122 levels and viral loads detectable in peripheral blood mononuclear cells of HBV-positive individuals[62]. The expression of miRNA-122 is significantly down-regulated in the hepatic tissue of patients with chronic HBV infection and its levels are negatively associated with intrahepatic viral loads and inflammation[62]. Therefore, HBV might decrease the levels of miRNA-122 and prevent this molecule from binding to viral mRNA, causing the suppression of its replication. During persistent infection, HBV induces infected hepatocytes to produce and release not only complete virions but also subviral elements. These circulating HBsAg particles contain selective pools of miRNA with specific functions in the liver, including miRNA-27a, miRNA-30b, miRNA-122, miRNA-126 and miRNA-145, and with immune-regulatory activities, such as miRNA-106b and miRNA-223[63]. HBV might have developed this strategy to sequester and to expel from hepatocytes cellular molecules that interfere with its life cycle and exert antiviral and immune-regulatory effects to maintain a chronic infection[59].

Further studies have focused on the differential expression profiles of miRNA in the hepatic tissue of individuals with HBV-related liver diseases of different severity. In a research enrolling HBsAg/HBeAb-positive carriers, miRNA-125a has been detected in the liver of all the included patients and its levels correlated with serum and hepatic HBV-DNA levels as well as with histological activity index, fibrosis score and more severe disease progression[64]. According to the results of available studies carried out *in vitro* and *in vivo*, miRNA-125a may be involved in a regulatory negative feedback loop, limiting HBV replication[64]. The role of miRNA in HBV-related hepatic carcinogenesis has also been investigated and several miRNA involved in this complex process have been identified, because they function as tumour suppressors or oncogenes. Specific profiles of these endogenous molecules have been reported to be associated with the clinical and pathological features of HCC. Some miRNA have been

considered to be the predictors of HCC prognosis or early recurrence. In particular, miRNA-22, miRNA-29c and miRNA-101 inhibit the gene expression of HBV and are down-regulated in HCC patients[54,65,66]. In addition, hepatic overexpression of miRNA-29a-5p and miRNA-221 has been reported to be associated with an early recurrence or with a poor prognosis and with a higher risk of multifocal tumour development, respectively, in individuals with HBV-related HCC[67,68]. A recent study has profiled 667 miRNA in the cancerous and adjacent non-tumour tissues of HBV-positive individuals with HCC and reported ten up-regulated miRNA (miRNA-217, miRNA-512-3p, miRNA-517c, miRNA-518a-3p, miRNA-518b, miRNA-518e, miRNA-519a, miRNA-520, miRNA-522 and miRNA-525-3p) and eleven down-regulated miRNA (miRNA-138, miRNA-199a-5p, miRNA-214, miR-214*, miRNA-433, miRNA-483-3p, miRNA-483-5p, miRNA-511, miRNA-592, miRNA-708 and miRNA-1275). Most of these miRNA, regulating several critical physiological processes and cellular pathways, have been proven for the first time to be involved in the development of HCC. In particular, miRNA profiles associated with the progressive stages of liver diseases ranging from chronic hepatitis to cirrhosis and to HCC after HBV infection have been reviewed[69]. Viral replication causes methylation of both host DNA and HBV-DNA, through the up-regulation of DNA (cytosine-5-)-methyltransferase (DNMT) genes, such as *DNMT-1*, *DNMT-2* and *DNMT-3*. This event induces decreased viral gene expression and replication, but it may also constitute a mechanism for liver carcinogenesis[70,71]. In a recent research carried out in a group of patients with HCC on HBV-related cirrhosis, the expression of miRNA-152 has been found to be down-regulated. The reduced expression of miRNA-152 is responsible for aberrant DNA hypermethylation via *DNMT-1* repression and may contribute to the development of cancer[52,72].

Modulatory effects of vitamin E on host microRNA expression profiles: in vitro *and* in vivo *studies*

To date, the potential regulatory effects of VE on the expression profiles of cellular miRNA remain poorly understood and only a small number of studies have been carried out to explain the possible modulatory effects of this compound on miRNA machinery function. In 2008, the role of VE in the expression of miRNA in rats was studied. The rats were randomised to receive either a VE-sufficient or a VE-deficient diet for 6 months. The hepatic levels of miRNA-122a and miRNA-125b were assessed[73]. These molecules have previously been reported to be involved in several processes, such as lipid metabolism and inflammation[74]. Decreased hepatic miRNA-122a and miRNA-125b levels were detected in rats fed the VE-deficient diet for 6 months. In 2013, a study evaluated the effects of DL-α-tocopherol acetate on the expression of eight stress-associated miRNA, including miRNA-16, miRNA-21, miRNA-122, miRNA-125b, miRNA-146a, miRNA-155, miRNA-181a and miRNA-223, in the hepatic tissue of Nile tilapia (*Oreochromis niloticus*)[75]. In this study, juvenile Nile tilapia fish were randomised to receive three doses of VE supplementation, including α-tocopherol acetate-deficient (9·02 mg/kg), -containing (59·4 mg/kg) and -excessive (2735·95 mg/kg) diets, for 8 weeks. Hepatic miRNA-16, miRNA-122, miRNA-146a and miRNA-223 levels as well as superoxide dismutase activity were decreased in fish fed the VE-deficient diet. On the other hand, fish fed the diet containing excessive amounts of α-tocopherol acetate exhibited an enhanced expression of all the eight miRNA and a reduced superoxide dismutase activity. For the first time, these studies have analysed the role of VE in the modulation of the production of some miRNA that control and influence critical cellular functions. The possible role of VE in the regulation of cellular epigenetic machinery activity and miRNA network functions was also assessed[76]. However, the molecular mechanisms involved in these complex processes are still unclear and have to be investigated further.

Use of vitamin E in clinical studies as a therapeutic agent for patients with hepatitis B virus-related chronic hepatitis

Only three studies have assessed the effects of VE (α-tocopherol) administration in patients with persistent HBV infection, with promising results (Table 1). In 2001, a small randomised controlled pilot trial evaluated the efficacy of VE administration in chronic HBV carriers in inducing HBV-DNA clearance and alanine aminotransferase normalisation. In total, thirty-two adults (twelve HBeAg-positive/HBeAb-negative and twenty HBeAg-negative/HBeAb-positive) were given VE at a dose of 300 mg twice a day for 3 months or were not treated. After 12 months of follow-up, endpoints were achieved in seven patients (47 %) in the VE-supplemented group in comparison with no subject in the control arm[77]. In 2007, a trial was carried out in fifty-eight HBeAg-positive children with high viral loads and normal alanine aminotransferase levels. These immune-tolerant patients were randomly assigned to receive either VE at a dose of 100 mg/day for 3 months or no therapy. Children were followed up for 6 months. At the end of the study, none of the scheduled endpoints, including HBeAg loss and HBV-DNA clearance, was achieved[78]. Different conclusions have been drawn by another randomised research that enrolled ninety-two children at a 3:1 ratio to receive either *RRR*-α-tocopheryl acetate supplementation, at a dose ranging between 200 and 600 IU (5 and 15 mg) on the basis of body weight, or placebo for 6 months with 12 months of follow-up[79]. HBeAg loss was observed in sixteen (23·2 %) of the sixty-nine treated children in comparison with two (8·7 %) of the twenty-three children in the placebo group. Although the seroconversion rate did not reach the statistical significance between the two groups, the authors concluded that VE therapy might promote HBeAg clearance and HBeAb development. Discrepancies in the results of these studies may be only apparent and determined by differences in research designs, such as a lower dose of α-tocopheryl acetate and a shorter period of therapy and follow-up in Turkish trials in comparison with Italian and German trials. Interesting findings, emerging from both European studies, are represented by the progressive, substantial and persistent decrease in serum HBV viral loads with the subsequent HBeAb seroconversion in patients who responded efficaciously to VE therapy. Further

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Table 1. Summary of clinical studies assessing the effects of vitamin E administration in patients with chronic hepatitis B virus (HBV) infection

Author and publication year	Country of origin	Study design	Sample size	Therapy	Study duration	Endpoints	Results
Andreone[77] (2001)	Italy	RCT	Thirty-two adults: twelve HBeAg+ and twenty HBeAg−	Vitamin E 300 mg/twice a day *v.* no treatment	Treatment: 3 months; FU: 12 months	HBV-DNA clearance and ALT normalisation	Seven (47 %) of the fifteen patients in the vitamin E-supplemented group *v.* none of the seventeen subjects in the control arm achieved both endpoints (P= 0·0019)
Dikici[78] (2007)	Turkey	RCT	Fifty-eight children: HBeAg+ in immune-tolerant phase	Vitamin E 100 mg/d *v.* no treatment	Treatment: 3 months; FU: 6 months	HBV-DNA clearance and HBeAg clearance	No endpoint achieved
Gerner[79] (2008)	Germany	RCT at a 3:1 ratio	Ninety-two children: HBeAg+	Vitamin E dose ranging from 5 to 15 mg/d, depending on body weight, *v.* placebo	Treatment: 6 months; FU: 12 months	HBV-DNA clearance and HBeAg loss/ HBeAb seroconversion	Sixteen (23 %) of the sixty-nine patients in the vitamin E-supplemented group *v.* two (9 %) of the twenty-three patients in the placebo arm achieved HbeAg loss (P= 0·13)

RCT, randomised controlled trial; HBeAg, hepatitis B e-antigen; FU, follow-up; ALT, alanine aminotransferase; HBeAb, antibody to HBeAg.

studies have to be carried out to confirm or to reject these apparently promising results and to improve our knowledge concerning the possible regulatory effects of VE supplementation on miRNA expression in patients with chronic HBV infection.

Recognised roles of distinct tocopherol-modulated microRNA in living organisms and their possible effects on hepatitis B virus expression and replication

A total of eight miRNA (i.e. miRNA-16, miRNA-21, miRNA-122, miRNA-125b, miRNA-146a, miRNA-155, miRNA-181a and miRNA-223), the hepatic expression of which has been shown to be influenced by VE supplementation in the 'Nile tilapia' fish, play key regulatory roles in living organisms[73,75]. In particular, three groups of these fish were fed the same semi-purified diet, but with three different doses of this fat-soluble compound, and subdivided into VE-deficient, VE-containing and VE-excessive groups, respectively. The VE-deficient diet significantly reduced the expression of miRNA-16, miRNA-122, miRNA-146a and miRNA-223, but did not affect that of miRNA-21, miRNA-125b, miRNA-155 and miRNA181a. On the other hand, the VE-excessive diet induced an increase in the expression of miRNA-16, miRNA-21, miRNA-122, miRNA-125b, miRNA-146a, miRNA-155, miRNA181a and miRNA-223. In this section, the mechanisms that up to now have been recognised to regulate the complex interactions between HBV and host are described, in particular, the possible or recognised roles of these molecules in the modulation of the activity of the host immune system against HBV expression and replication.

MicroRNA-16. miRNA-16 acts as a tumour-suppressor molecule, modulating multiple cell-cycle genes, including cyclin D1, cyclin D3 and cyclin E1[80–82]. Only few studies have investigated the functional interplay between HBV and miRNA-16. In an *in vitro* model of HBx-overproducing HepG2 cells, this viral protein has been found to be able to induce indirect down-regulation of miRNA-16 via c-Myc induction, causing anchorage-independent cell growth and cellular apoptosis inhibition[56]. miRNA-16 is transcribed with either miRNA-15a (miRNA-15a/miRNA-16-1 cluster) or miRNA-15b (miRNA-15b/miRNA-16-2 cluster). A recent study in HBx-expressing human malignant cells has demonstrated not only that *HBx* viral RNA can directly promote miRNA-15a/miRNA-16-1 repression, but also that miRNA-15a/miRNA-16-1 can inhibit HBV replication by targeting HBV-RNA sequences[48]. In this research, miRNA-15/mRNA-16 have been found to bind to specific sequences in viral transcripts, including three sites in the HBV polymerase RNA and one site in the X protein RNA (Fig. 3). The miRNA machinery represents one of the antiviral defence systems that contribute to the modulation of cell–pathogen interactions. The miRNA network contributes to the protection of the host by preventing the spread of invading pathogens. miRNA-15a/miRNA-16 are crucial components in the miRNA machinery as they contribute to the modulation of the intricate balance between cellular antiviral defence mechanisms and strategies that this pathogen develops to support its survival

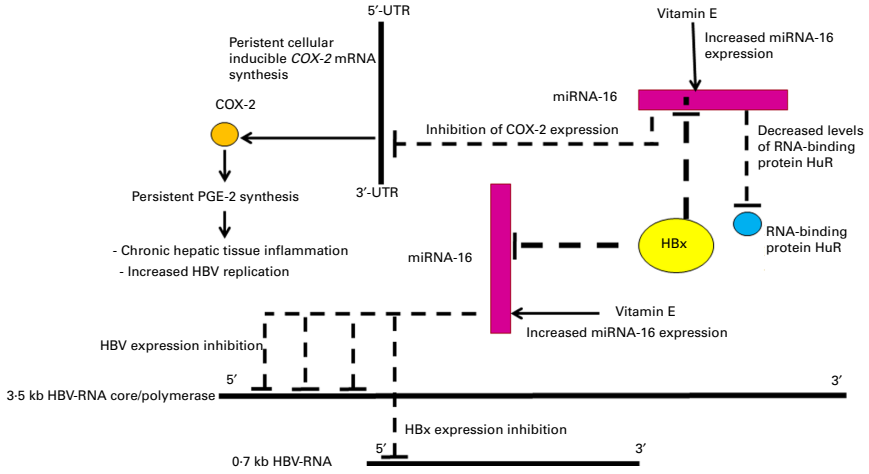

Fig. 3. Schematic representation of microRNA (miRNA)-16-mediated repression of hepatitis B virus (HBV) replication. miRNA-16 promotes the silencing of cellular inducible cyclo-oxygenase-2 (COX-2) expression by (1) directly binding to the miRNA response element motif in the 3′-untranslated region (UTR) of *COX-2* gene and (2) decreasing the levels of the RNA-binding protein human antigen R (HuR). Inhibition of COX-2 and PGE-2 promotes the inhibition of inflammatory processes and down-regulation of HBV expression. In addition, miRNA-16 targets specific binding sites in viral transcripts, including three sites in polymerase and one site in X protein, and down-regulates viral genome expression. On the other hand, HBV may induce the down-regulation of miRNA-16 expression, via hepatitis B x (HBx) protein synthesis[48,83,84]. A colour version of this figure can be found online at http://www.journals.cambridge.org/bjn

in infected individuals. In particular, some studies have shown that some viruses, including HBV, may benefit from the induction of a strong inflammatory response[83]. One of the mechanisms by which this pathogen promotes its persistence is represented by its ability to up-regulate the expression of cyclo-oxygenase-2 (COX-2)[84,85]. This enzyme catalyses the first step of prostanoid production and it is involved in PGE-2 synthesis[86]. The induction of COX-2 and release of PGE-2 represent critical events for an efficient viral replication in infected hosts. The inhibition of COX-2 decreases viral progeny levels in virus-expressing cultured cell models[87]. In addition, PGE-2 exerts an immune-suppressive effect, modulating both the innate and adaptive components of the immune system[88]. The immune-inhibitory effects of PGE-2 have also been observed in patients with persistent liver diseases. The down-regulation of COX-2 is associated with an increased production of interferon-γ (IFN-γ) by peripheral blood mononuclear cells and with an enhanced Th1 response[89,90]. The suppression of PGE-2, via COX-2 down-regulation, induces an improvement in the antiviral activity of the immune system[91,92]. Recently, miRNA-16 has been shown to down-regulate COX-2 synthesis at the post-transcriptional level, through two mechanisms: (1) direct binding to the *COX-2* RNA messengers in the 3′-untranslated miRNA response element motif and (2) reduction of the levels of the RNA-binding protein human antigen R[93]. These events induce the inhibition of *COX-2* mRNA translation. Studies carried out *in vitro* and *in vivo* in aged mice have proved that VE reduces the production of free radicals and PGE-2 by activated macrophages through a decrease in COX and lipoxygenase activities[94–96]. Furthermore, VE increases Th1 cytokine production and improves immune responses in elderly and diseased individuals[97,98]. High-dose, short-term supplementation with VE has been found to be safe and well tolerated in healthy old adults[99]. It can be hypothesised that the intake of VE influences the production and activity of COX-2 as well as PGE-2, exerting a post-transcriptional control on their biosynthesis via the up-regulation of miRNA-16 expression. These events may improve host anti-HBV activity, through two mechanisms: the direct inhibition of viral replication and the stimulation of innate and adaptive immune responses against this pathogen (Fig. 3).

MicroRNA-21. miRNA-21 is involved in several biological processes, such as development, immunity and epithelial-to-mesenchymal transition.

This endogenous molecule is a well-known onco-miRNA, and it is overexpressed in several malignancies, such as lung, stomach, breast, prostate, colon and pancreatic cancers[100,101], by targeting tumour-suppressor genes, including programmed cell death protein 4 (*PDCD4*)[102] and phosphatase and tensin homolog (*PTEN*)[103]. Furthermore, miRNA-21 is overexpressed in HCC cells, and it protects cultured human cells against apoptosis and increases their proliferation rate and migration capabilities[104]. This molecule is rapidly induced after the stimulation of transforming growth factor-β[105], and it is involved in organ fibrosis, as has been observed in some animal models[106,107]. miRNA-21 is also involved in the regulation of the immune system. This miRNA exhibits a low expression in naïve T cells, but its production increases progressively in effector and memory T cells. Therefore, this molecule plays a pivotal role in the maintenance of the functions of these cell subsets[108]. In animal models, miRNA-21 has been found to target critical regulatory mechanisms of adaptive immune responses via the mRNA repression of IL-12p53, a cytokine that contributes to the polarisation of Th-cell population towards Th1-cell subsets[109], as well as via the elevation of forkhead protein box 3 (FOXP3) levels in T-regulatory cells[110]. VE supplementation, via the modulation of miRNA-21 expression, might have a beneficial impact on the host anti-HBV responses, by decreasing too vigorous and potentially dangerous

necroinflammatory processes as well as by modulating the functions of distinct T-lymphocyte subsets. Therefore, this fatsoluble compound might contribute to the rebalancing of the whole activity of the immune system. On the other hand, it is not understood whether VE influences the known pro-carcinogenic activities of miRNA-21.

MicroRNA-122. miRNA-122 is a liver-specific molecule, accounting for about 70% of the miRNA in the hepatic tissue of healthy individuals[111]. miRNA-122 plays a crucial role in the differentiation of hepatocytes and development of hepatic cells, and its expression increases during embryogenesis[112]. In addition, miRNA-122 contributes to the regulation of distinct metabolic processes, including cholesterol and fatty acid synthesis, as well as to the inhibition of fatty acid oxidation[72], via modulation of the functions of distinct genes[113]. miRNA-122 levels are increased in the serum, but are decreased in the hepatic tissue of HBV-positive patients with chronic hepatitis or with HCC. Recent studies have shown that miRNA-122 in the hepatic tissue can affect the life cycle of HBV, negatively regulating its gene expression and replication via direct and indirect mechanisms. In particular, miRNA-122 binds to a highly conserved region in the 3′-UTR of the viral mRNA, coding for the core protein, as well as to specific sequences of the mRNA for the polymerase (at nucleotides 2738–2760), modulating their stability and translation. As a consequence of these interactions, the expression of these viral genes is down-regulated at the post-transcriptional level. According to a study carried out in a cultured cell model, HBV replication is decreased when miRNA-122 levels are enhanced, whereas the levels of this endogenous molecule are reduced when HBV loads increase[59]. On the basis of these results, the authors of this research have suggested that the inhibition of HBV replication, via miRNA-122 induction, may contribute to the promotion of viral persistence in the host as well as that the induction/generation of this endogenous molecule might serve as an effective therapeutic strategy to suppress HBV infection and replication. Complex mechanisms are involved in HBV down-regulation, mediated by miRNA-122. Several cellular genes have been shown to be modulated by this miRNA, including cyclin G1[114]. In this intricate process, both p53 and cyclin G1 play a crucial role. In particular, p53 can target a specific region (defined as R-S) in HBV EnhI at its 5′-terminus (nucleotides 1043–1115) and also specific sequences in EnhII at its 5′-terminus (nucleotides 1637–1667). The presence of p53 seems to cause interference between this protein and liver-enriched transcription factors[115]. Therefore, this interaction prevents the binding of the liver-specific proteins to their target sequences in both HBV enhancers and block cooperative activities of these viral regulatory elements. This event induces as the final effect a transcriptional repression of the HBV pregenome/core promoter as well as of the surface, pre-S and X promoters with a consequent reduced viral replication/translation[115–117]. However, it is also known that cyclin G1, interacting with p53, forms a complex with it and prevents its association with HBV enhancers, contributing to the modulation of their activity and viral genome expression. The binding of miRNA-122 to specific sequences in the 3′-UTR of cyclin G1 mRNA down-regulates the expression of this cyclin[118]. Therefore, the miRNA-122/cyclin G1/p53/HBV-enhancer pathway constitutes a system with critical regulatory roles in HBV life cycle. In particular, low miRNA-122 levels are associated with an enhanced expression of cyclin G1 and with a consequent attenuation of p53 function, promoting an increased HBV replication. On the other hand, the up-regulation of miRNA-122 expression has inverse effects[62]. Therefore, VE supplementation may potentially play a critical role in the above-mentioned miRNA-122/cyclin G1/p53/HBV-enhancer pathway. VE, by enhancing miRNA-122 levels, may contribute to the reduction of viral replication and loads, as has been observed in clinical trials in patients responding to a course of VE treatment (Fig. 4).

MicroRNA-125b. miRNA-125b, a member of the miRNA-125 family that includes three homologues (miRNA-125a, miRNA-125b-1 and miRNA-125b-2), plays a critical role in several physiological processes and in different types of diseases. In particular, this molecule affects myelopoiesis, influencing the fate of some crucial cells. It contributes to the regulation of both the innate and adaptive arms of the immune system in a coordinated manner. In particular, miRNA-125b modulates the differentiation of granulocytes[119] and diversification of B lymphocytes in germinal centres[120] as well as affects the expression of critical factors such as type I IFN, TNF-α and IFN-γ, contributing to the activation of macrophages[121]. Enhanced miRNA-125b levels increase the ability of macrophages to present Ag to T lymphocytes and to mediate their activation, stimulating also IFN-γ secretion. Therefore, miRNA-125b plays a critical role, orchestrating an effective immune response against pathogens, by linking the innate and adaptive arms of the immune system[122,123]. In addition, reduced expression of miRNA-125b may contribute to the limitation of the strength of inflammatory processes. Therefore, this molecule, regulating different target genes at the post-transcriptional level, modulates both antibacterial and antiviral immunological host defence mechanisms. Furthermore, miRNA-125b is deregulated in different types of human cancer cells, although the results are not univocal. This molecule may act either as a tumour suppressor or as a cancer promoter. In particular, miRNA-125b is underexpressed in prostate, oral, lung, breast, and bladder carcinoma cells and in HCC cells, whereas its expression is up-regulated in urothelial and gastric cancer cells as well as in leukaemic cells[124,125]. The reasons for these different behaviours are unknown, but several oncogenes or regulatory proteins involved in cell-cycle control and in the process of carcinogenesis are targeted by miRNA-125b, such as B-cell lymphoma 3 (BCL3), E2F transcription factor 3 (E2F3), lin28 homolog B (LIN28B), v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2 and 3 (ERBB2 and ERBB3), v-ets avian erythroblastosis virus E26 oncogene homolog 1 (ETS1), caudal type homeobox 2 (CDX2) and BCL2-antagonist/killer 1 (BAK1)[126]. Furthermore, miRNA-125b can negatively regulate p53, by directly binding to the 3′-UTR of its mRNA and decreasing its expression in human neuroblastoma cells as well as in lung fibroblast cells[127].

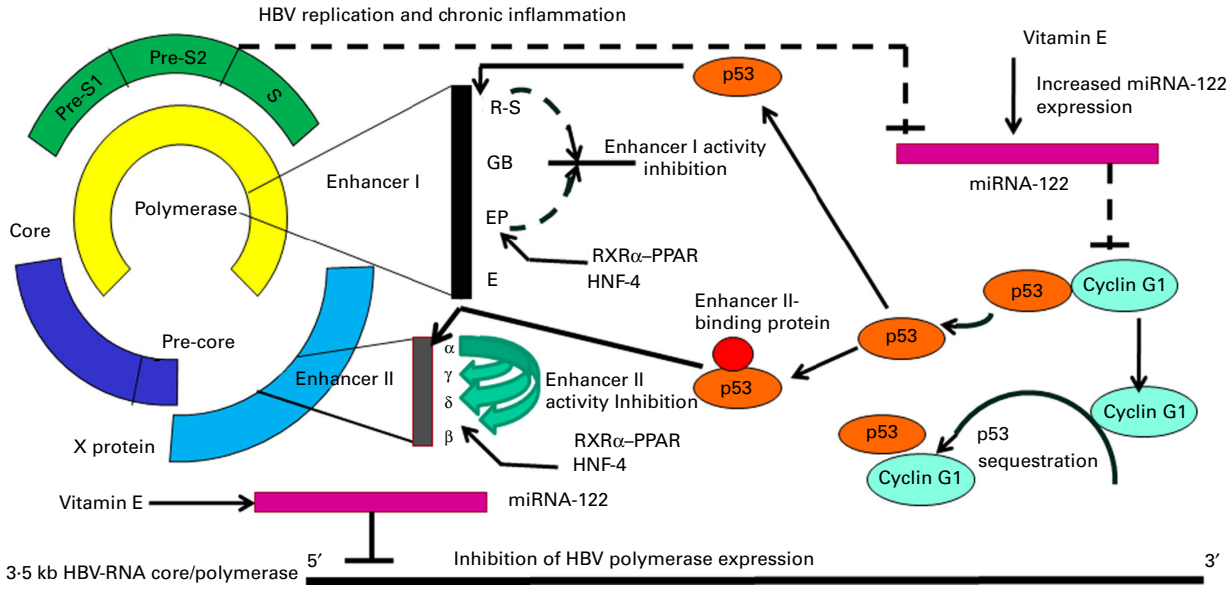

Fig. 4. Schematic representation explaining (1) the role of p53 in the repression of hepatitis B virus (HBV) enhancers I and II, leading to a decreased viral replication, (2) the effects of microRNA (miRNA)-122 on HBV transcription, and (3) the possible role of vitamin E in the regulation of miRNA-122 production and in the modulation of viral genome expression[115–117]. (1) p53 targets the R-S domain in enhancer I, preventing the coordinated cooperation among EP, GB, and E elements and cell transcription factors as well as forms a complex with an enhancer-binding protein, interacting with the box-α in enhancer II. These events block the activity of enhancers and viral replication. (2) In liver of chronic HBV carriers, miRNA-122 levels are decreased and this down-regulation leads to the following: (a) an increase in the levels of cyclin G1 protein, which sequestrates p53, leading to a p53–cyclin G1 complex, and (b) a decreased binding of miRNA-122-specific sequences at the 5′-untranslated regions (UTR) of the viral mRNA coding for HBV core protein and polymerase (nucleotides 2738–2760). These events promote viral core and polymerase transcription and consequently HBV replication. (3) Enhanced miRNA-122 levels, potentially induced by vitamin E, may restore these two inhibitory pathways of viral genome expression (also see Figs. 1 and 2)[62]. Pre-S1, pre-surface 1; Pre-S2, pre-surface 2. A colour version of this figure can be found online at http://www.journals.cambridge.org/bjn

A study carried out in human HCC cells has suggested that p53 levels are not altered by this miRNA[128] and that it mediates its tumour-suppressor effects, modulating different intracellular pathways. The expression of miRNA-125b is correlated with the post-operative survival time in patients with HCC, with lower levels of this molecule being associated with a poorer prognosis[128]. Further studies are required to adequately assess miRNA-125b-regulated expression of p53 in normal hepatocytes, as an increase in the levels of this regulatory protein might affect the miRNA-122/cyclin G1/p53/HBV-enhancer pathway and stimulate HBV replication. It has been demonstrated that miRNA-125a may target specific sequences in hepatitis B surface antigen mRNA (nucleotides 3037–3065), down-regulating its translation[4]. Unpublished observations seem to suggest that miRNA-125b may exert a positive effect on HBV replication[129]. Therefore, VE-induced increase of miRNA-125b levels might exert a stimulatory effect on antiviral innate and adaptive immune responses, by potentiating the functions of macrophages and by decreasing the strength of inflammatory processes, but it might promote viral genome expression. Further studies will have to be carried to explain this crucial point in the life cycle of HBV (Figs. 4 and 5).

MicroRNA-146a. miRNA-146a, along with miRNA-155, miRNA-181a and miRNA-223, acts as a critical player in the modulation of both innate[130] and adaptive[131] immune systems and it is involved in distinct pathological processes. According to the results of available studies, miRNA-146a may play an extensive role in innate immunity, as a regulator of pro-inflammatory signals, via a tightly regulated negative feedback loop. In addition, this molecule plays a crucial role in the orchestration of efficacious and efficient T-cell-mediated adaptive responses. miRNA-146a controls different steps of T-lymphocyte activation. In particular, different expression patterns of this molecule have been observed, depending on the T-cell subtypes considered. miRNA-146a levels are higher in human memory T cells than in naïve T lymphocytes[132], and this molecule is expressed at lower levels in murine Th2 cells and naïve T cells in comparison with Th1 lymphocytes[132]. In addition, miRNA-146a overexpression has been reported to be associated with decreased IL-2 release and reduced activator protein-1 function, following T cell receptor engagement. Therefore, according to these results, miRNA-146a functions as a modulator of IL-2 production, preventing T lymphocytes from undergoing Fas-mediated apoptosis. Some pro-inflammatory molecules, including IL-1 and TNF, modulate its expression. In animal models, deficient miRNA-146a expression has been shown to be associated with the development of autoimmune disorders and cancers. A recent research has assessed the possible role of miRNA-146a in the modulatory activity of T lymphocytes in patients with persistent HBV infection[133]. This pathological condition is associated with an impaired overall immune response and, in particular, with a worsening of T-cell functions. According to the results of this study, miRNA-146a is significantly overexpressed in $CD4^+$ and

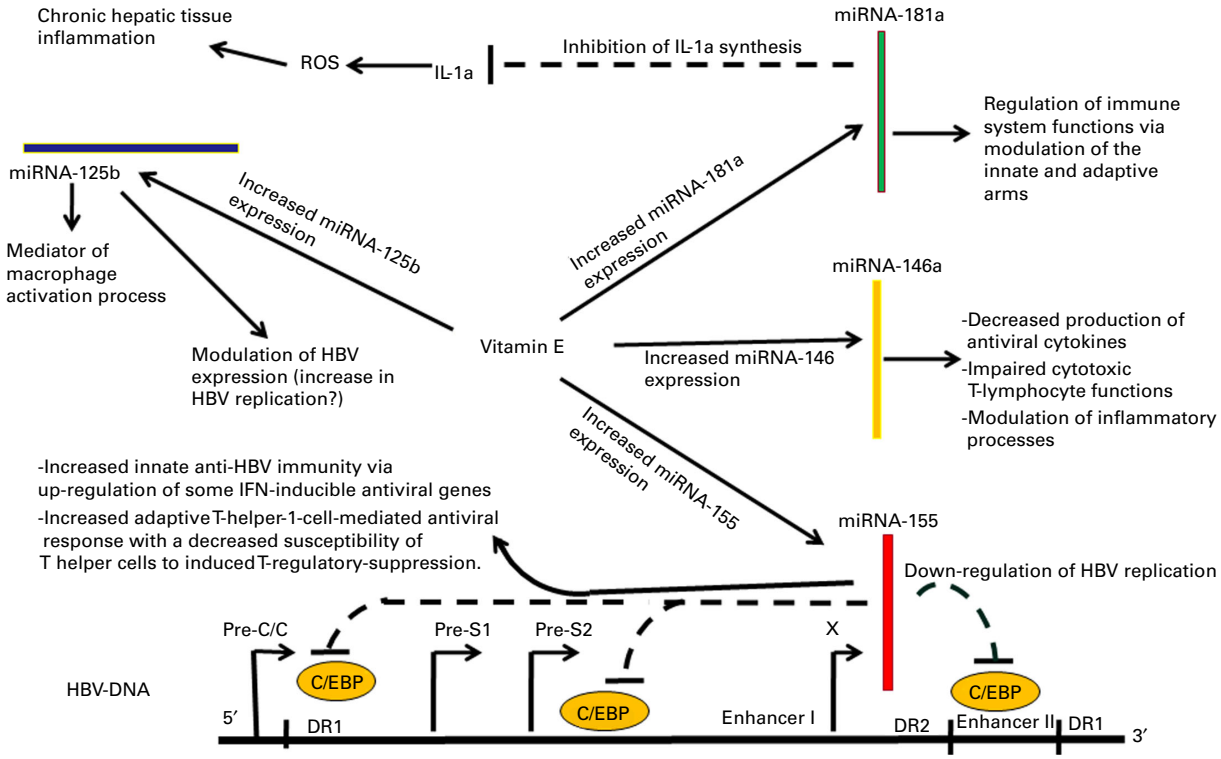

Fig. 5. Schematic representation of the immune-modulatory activities of microRNA (miRNA)-125b, miRNA-146, miRNA-155 and miRNA-181a, including the regulation of immune system functions and control of inflammatory response strength. These miRNA may stimulate anti-hepatitis B virus (HBV) innate and adaptive immune responses. Furthermore, miRNA-155 can target and negatively modulate CAAT/enhancer-binding protein (C/EBP-β). This event may down-regulate HBV transcription. On the other hand, increased levels of miRNA-125b could promote HBV replication. Angled arrows indicate the HBV-RNA start site for the most important viral transcripts (pre-core/core (pre-C/C), pre-surface1 (pre-S1), pre-S and X) and boxes schematically indicate viral enhancers I and II[72]. IFN, interferon; ROS, reactive oxygen species; DR1, direct repeat 1. A colour version of this figure can be found online at http://www.journals.cambridge.org/bjn

$CD8^+$ T cells detectable in subjects with chronic HBV-related hepatitis in comparison with healthy controls. The up-regulation of this molecule is associated with serum aminotransaminase levels as well as with necroinflammatory injury, and it induces not only a decreased production of antiviral cytokines, but also an impaired function of cytotoxic T cells by targeting signal transducer and activator of transcription 1 (STAT-1). Reduced miRNA-146a expression in specific T cells of patients with persistent HBV infection leads to an increase in their antiviral activity. In the intricate network developing between the host and HBV during persistent infection, the up-regulation of miRNA-146a expression may lead to beneficial consequences for the host, by preventing and counteracting the potentially dangerous effects of the hyperactivation of inflammatory responses. On the other hand, this event might contribute to the prevention of viral clearance and promotion of its persistence. The enhancement of miRNA-146a production, mediated by VE, might, therefore, impair T-cell activities against HBV and might induce a defective antiviral immune response. However, it should be taken into consideration that the negative impact on T-lymphocyte functions, through the VE-induced increase of miRNA-146a expression, represents only one among the distinct and potentially relevant effects of this lipid-soluble antioxidant in this context. On the whole, taking into account the activities of miRNA-146a alone or in cooperation with miRNA-155, miRNA-181a and miRNA-223, it may be hypothesised that this micronutrient promotes a progressive rebalancing of several immune system responses and prevents the development of severe liver injury and that it is generated by a deleterious overactivation of host immunity (Fig. 5).

MicroRNA-155. miRNA-155 plays a critical multifunctional role in the modulation of several key cellular activities, such as proliferation, growth, differentiation and survival. Therefore, this endogenous molecule is involved in physiological and pathological processes, including haematopoiesis and immune responses as well as inflammation, CVD, viral infections and different types of cancers[134,135]. A miRNA-155 deficit is associated with hypertension and numerous cardiac pathologies. Furthermore, this small RNA is overexpressed in different types of solid malignancies, such as breast, thyroid, cervical, colon, lung and pancreatic carcinomas as well as in B-cell lymphomas[136], and it is involved in inflammatory responses. The role of miRNA-155 in this process is not yet defined and deserves to be investigated further. It has been suggested that this molecule is induced by a wide range of inflammatory mediators and may have a crucial role in the regulation of host defence mechanisms against pathogens, linking inflammation with innate and adaptive immune responses. Its overall activities are complex and multiform;

miRNA-155 acts not only by stimulating immune responses, but also by controlling tissue damage caused by inflammation. This molecule affects both human and murine $CD4^+$ Th-cell components of the immune system. In particular, a recent research has shown that miRNA-155 has no significant detectable effects on the suppressive capacity of T-regulatory cells. Nevertheless, enhanced levels of this small non-protein-coding RNA are associated with a decreased susceptibility of Th cells to induced T-regulatory suppression, whereas reduced miRNA-155 levels in $CD4^+$ Th lymphocytes sensitise these cells to T-regulatory suppression[137]. In addition, miRNA-155-deficient T cells tend to differentiate into Th2 cells, via suppression of IL-12 and IFN-γ signalling. An analysis of putative miRNA-155 targets in $CD4^+$ T cells has revealed a panel of ninety-three genes with expression regulated by this endogenous molecule[138,139]. Enhanced production of this endogenous molecule might contribute to the stimulation of the antiviral activity of the innate and/or adaptive arms of the immune system. However, the results of available studies investigating the role of miRNA-155 during viral infections are still inconclusive and not univocal. Some viruses such as Epstein–Barr virus and Kaposi's sarcoma-associated herpes virus may, respectively, up-regulate and mimic the expression of host miRNA, including miRNA-155, to promote their survival, but mechanisms involved in these complex processes are poorly understood and conclusions remain unclear. However, a feedback regulatory loop is generated between this endogenous molecule and the invading viruses in the host, through the activation of the type I IFN signalling pathway, and it contributes to the lowering of their transcription and replication[140]. However, it is well known that HBV exerts very strong inhibitory effects on the IFN signalling pathway in infected cells and this is one of the most important mechanisms contributing to the persistence of HBV in individuals who are chronic carriers and who are unable to clear this virus efficaciously[141,142]. Overexpression of miRNA-155 results in increased innate immunity against this pathogen in human hepatoma cells, via up-regulation of some IFN-inducible antiviral genes with a consequent mild attenuation of HBV infection[47]. Enhanced levels of Mx (*MxA*) and interferon-stimulated gene-15 (*ISG15*) and decreased levels of the suppressor of cytokine signaling 1 (*SOCS1*) gene in miRNA-155-overexpressing HepG2 cells with the consequent prolonged activation of the Janus kinase–STAT-1 pathway have been reported to mediate the antiviral activity of miRNA-155. It can be suggested that increased levels of miRNA-155, induced by VE treatment, improve host innate and adaptive immune responses against HBV, stimulating intracellular IFN-inducible antiviral genes and promoting Th1 polarisation of T lymphocytes and attenuating their susceptibility to T-regulatory suppression and their tendency to differentiate into Th2 cells (Fig. 5).

MicroRNA-181a. miRNA-181a is a multifaceted molecule that it is involved in several cellular processes, including determination of cell fate, such as proliferation and promotion of migration and invasion as well as immune system functions and inflammatory responses. In particular, miRNA-181a modulates, along with miRNA-146a, miRNA-155 and miRNA-223, the differentiation of cells of haematopoietic lineage[143] and controls the development of B-, T- and natural killer-cell compartments[144]. miRNA-181a plays a key role in the innate and adaptive arms of the host immune system as it contributes to the development of a protective response against cancer cells and viruses. Furthermore, some studies have shown that miRNA-181a expression is up-regulated by inflammatory stimuli and that it is strongly correlated with the expression of IL-1β, IL-6 and TNF-α. miRNA-181a seems to be involved in homeostatic responses to inflammation *in vivo*, contributing to the modulation of damage in injured tissue[145]. In addition, this miRNA induces feedback regulation to TNF-α-mediated transcription of pro-inflammatory genes in hepatic epithelial cells, via suppression of p300/CREB-binding protein-associated factor[146]. Increased expression of this miRNA-181a might contribute to the stimulation of an efficacious activity of the innate and/or adaptive arms of the immune system against HBV. However, the real role of miRNA-181a in the host antiviral response in patients with persistent HBV-related hepatitis is not yet understood, and further studies are required to definitively improve our knowledge concerning this subject. Recently, it has been reported that miRNA-181a expression is altered in patients with cirrhosis/HCC and that the up-regulation of this molecule may mediate transforming growth factor-β-induced hepatocyte epithelial-to-mesenchymal transition[147] as well as may promote cell growth and carcinogenesis in patients with HBV-related HCC, by targeting the sequences in the 3′-UTR of the *E2F5* mRNA[148]. It may be hypothesised that the modulation of miRNA-181a levels, induced by VE treatment during persistent HBV infection, improves the activities of both the innate and adaptive arms of the host immune system against this virus, in particular, via stimulation of natural killer cell activity. In addition, the enhancement of miRNA-181a levels might contribute to the modulation of inflammatory response intensity and, therefore, protect the host from serious organ damage. In particular, miRNA-181a might prevent the development of hepatic tissue injury that may be caused by the hyperactivation of inflammatory responses during persistent viral infection (Fig. 5). On the other hand, it is still not understood whether VE influences the known carcinogenic promoter activities of miRNA-181a.

MicroRNA-223. miRNA-223 modulates several processes, including haematopoiesis, immune system functions and cell-cycle regulation[149]. This molecule plays a crucial role during myelopoiesis. The expression of miRNA-223 varies widely, depending on the cell lineages considered. The levels of this molecule either rise progressively in progenitor cells that differentiate into natural killer lymphocytes and into erythrocytes or decrease in precursor cells that are committed to a granulocytic lineage. In addition, miRNA-223 is involved in the modulation of normal macrophage development, via NF-κB signalling and in association with miRNA-15a and miRNA-16[150], as well as its expression is altered in distinct inflammatory processes. To date, several specific miRNA-223 targets have been detected, including C/EBP-β, nuclear factor-1-A, stathmin 1 and insulin growth factor 1 receptor. On the basis of its critical role in the control of

immune system functions, it is likely that miRNA-223 regulates different steps of infectious processes induced by viruses, bacteria or fungi. In particular, serum levels of this miRNA are increased in patients with HBV-related chronic hepatitis or HCC[58]. On the other hand, miRNA-223 expression is down-regulated in the liver of HBV-positive subjects with persistent liver injury or HCC. In particular, its expression is considerably repressed in cancerous *v.* non-malignant hepatic tissue. miRNA-223 interacts with the 3′-UTR of the mRNA for stathmin 1, a key microtubule-regulatory protein that modulates the function of microtubules, and controls its synthesis. Therefore, miRNA-223 influences cell proliferation and S-phase of the cell cycle[151]. Down-regulation of this miRNA promotes the increased synthesis of stathmin 1, and this event is associated with chromosomal instability and cytogenetic abnormalities[152]. This mechanism might also be involved in HCC development. VE supplementation might act by restoring immune system homeostasis and improving its anti-HBV activity as well as by affecting and decreasing inflammatory responses. VE might exert a protective role by preventing excessive hepatic tissue injury. In addition, VE might act as an active anti-cancer compound.

Vitamin E and the prevention of non-alcoholic steatohepatitis

Evidence for a potential promising role of VE in the treatment of non-alcoholic steatohepatitis (NASH) has emerged last year. Following some small open-label studies[153,154], two randomised controlled trials[155,156] carried out in adults with NASH have indicated that VE supplementation at a high dose (800 mg once a day) and for 96 weeks leads to a significant decrease in serum aminotransferase levels as well as an improvement in liver histology. Another study involving children and adolescents with non-alcoholic fatty liver disease[157], who received VE at a dose of 800 mg/d for 96 weeks, has confirmed a significant improvement in liver histology in the group of subjects with NASH. However, no significant decrease in serum aminotransferase levels was detected in these patients in the trial. To date, mechanisms potentially promoting the improvement of liver diseases in VE-supplemented patients have not been understood. According to the current view, the pathogenesis of NASH depends on altered liver lipid metabolism, inflammation and oxidative stress, and substantial changes in hepatic or serum miRNA expression patterns have been observed in patients with this disease. Some miRNA are known to modulate the pathogenesis of NASH[158–161]. In particular, in these patients, hepatic expression of miRNA-122 and miRNA-223 is down-regulated and that of miRNA-16, miRNA-21 and miRNA-125b is up-regulated[158,159]. On the other hand, serum miRNA-122 and miRNA-125b levels are increased in patients with NASH[160]. According to the available studies, the promising preventive effects of VE on NASH might be due to the modulatory effects of this compound on serum and/or hepatic miRNA expression patterns. Therefore, common mechanisms might be involved both in patients with chronic HBV infection and in patients with NASH. Some miRNA, such as miRNA-16, miRNA-155 and miRNA-223, might influence inflammatory responses as well as immune responses, modulating the synthesis of inflammatory molecules (such as COX-2 and PGE-2) or the type and quality of immune responses, contributing to the rebalancing of immune system function and to the reduction of inflammation severity[158–161].

Conclusions

To the best of our knowledge, this report represents the first attempt to describe in a structured and detailed way the possible role of tocopherols in the modulation of host miRNA with potential antiviral activity. In the past few years, several studies have demonstrated that VE supplementation at a high dosage can exert a strong immune-enhancing effect both in animals and in humans and in particular in elderly healthy individuals as well as in patients with persistent viral infections. In the past, the immune-stimulating effects of VE have been attributed to its well-known antioxidant and anti-inflammatory activities. In the last few years, the progressive improvement of our knowledge regarding the complex molecular mechanisms that are at work during virus–host interactions have allowed us to understand that VE exerts its potential modulatory effects in cells mainly at the post-transcriptional level. In particular, it is now well known that the clinical effects observed in VE-supplemented subjects or animals are mediated, at least in part, via the regulation of cellular miRNA expression profiles. In addition, several studies have reported changes in the serum and hepatic miRNA patterns of patients with chronic HBV infection. The results of our research suggest that miRNA-21, miRNA-146a, miRNA-155, miRNA-181a and miRNA-223 modulate the immune system by controlling the activities of its innate and adaptive arms, whereas miRNA-16, miRNA-146a and miRNA-181a influence the strength and size of inflammatory responses in host tissue. In addition, miRNA-122 and miRNA-125b have been demonstrated to influence cell differentiation and several cellular metabolic processes. Both these endogenous molecules have been demonstrated to modulate HBV protein synthesis and translation, influencing the function of distinct viral promoters and enhancers. To date, available results indicate that at least one of these miRNA (miRNA-122a) represses HBV replication, whereas miRNA-125b might up-regulate, almost in part, viral genome expression. Most of these miRNA exhibit tumour-promoter or -suppressor activities. Based on the results obtained in clinical studies, we hypothesised that VE-mediated regulation of miRNA synthesis, at the post-transcriptional level, might have a critical role in the modulation of HBV expression/replication, although, up to now, only a small number, among the hundreds identified in humans, of these endogenous molecules have been recognised to be affected by this fat-soluble compound. Therefore, it may be hypothesised that as further studies will be performed additional miRNA with possible pivotal direct antiviral functions or immune-stimulating activities will be identified and characterised and, as a consequence, our knowledge of these complex host–virus interactions will probably improve and a more detailed description of this complex and intricate pattern will become available. The importance

of this hypothesis has also been underlined by two recent studies[59,63] that have reported a new modality of intercellular communication, based on the transfer of distinct miRNA between cells and viruses. Therefore, this network might not only act as a host defence system against pathogens or tumour cell proliferation, but also be used by micro-organisms or cells to counteract immune responses, contributing to the establishment of a chronic infection or promotion of cellular malignant transformation. However, several questions still remain unresolved. Although results obtained in the above-mentioned studies investigating the potential effects of VE supplementation on clinical outcomes in patients with persistent HBV infection as well as its possible *in vitro* and *in vivo* positive effects on the modulation of critical cellular miRNA are promising, the low number of patients enrolled, the short follow-up period, the heterogeneity in design, and the small number of examined miRNA make the studies inconclusive and not univocal.

The results of some meta-analyses reporting potentially harmful effects in patients or healthy subjects supplemented with higher doses of VE[162] should be taken into account, although these studies are highly questionable for the inclusion criteria adopted. In the past, reports of several authors have been published against these conclusions[163–165]. Should the described potential and beneficial effects of VE be demonstrated, this fat-soluble compound might become part of a new approach that helps to develop useful therapeutic strategies, with the aim of promoting the coordinated activation of the innate and adaptive arms of the immune system as well as of regulating several crucial cellular roles via active modulation of miRNA profiles.

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None of the authors has any conflicts of interest to declare.

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