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Molecular interactions between hepatitis B virus and delta virus

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Abstract

As a deficient virus due to the lack of envelope proteins, hepatitis D virus (HDV) causes chronic or fulminant "delta hepatitis" only in people with simultaneous hepatitis B virus (HBV) infection. HBV encodes three types of surface proteins known as small (S), medium (M) and large (L) envelope proteins. All three types of HBV surface antigens (HBsAg) are present on HDV virions. The envelopment process of HDV occurs through interactions between the HDV ribonucleoprotein (RNP) complex and

HBV HBsAg. While HBsAg is the only protein required by HDV, the exact interaction sites between the S protein and pre-mature HDV are not well defined yet. In fact, these sites are distributed along the S protein with some hot spots for the envelopment process. Moreover, in most clinically studied samples, HDV infection is associated with a dramatically reduced HBV viral load, temporarily or permanently, while HBsAg resources are available for HDV packaging. Thus, beyond interacting with HBV envelope proteins, controlling mechanisms exist by which HDV inhibits HBV-DNA replication while allowing a selective transcription of HBV proteins. Here we discuss the molecular interaction sites between HBsAg and the HDV-RNP complex and address the proposed indirect mechanisms, which are employed by HBV and HDV to facilitate or inhibit each other's viral replication. Understanding molecular interactions between HBV and HDV may help to design novel therapeutic strategies for delta hepatitis.

Key words: Viral hepatitis; Hepatitis B virus; Hepatitis D virus; Hepatitis B virus surface antigens; Hepatitis D virus antigen; Ag loop; Liver cirrhosis

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Core tip: Hepatitis D virus (HDV) causes accelerated liver disease in form of fulminant or chronic hepatitis in patients with hepatitis B virus (HBV) infection. HBV supports HDV replication by sharing its surface proteins. Even without overt HBV-DNA replication, transcription of HBV surface proteins (HBsAg) remains stable in HDV infected cells, which is essential for assembly of HDV virions containing HBsAg proteins. HDV replication is oftentimes associated with a suppression of HBV-DNA levels, and several mechanisms have been suggested how HBV or HDV may influence each other's replication. Understanding molecular interactions between HBV and HDV may help to design novel therapeutic strategies.

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INTRODUCTION

Globally, about 350 million people are chronically infected with hepatitis B virus (HBV), of which 15 million are positive for hepatitis D virus (HDV) antibodies^[1]. HDV causes chronic or fulminant "delta hepatitis", in form of co- or super-infection in HBV infected patients^[2]. Delta virus is considered as a very deleterious pathogen since its infection commonly leads to progression of hepatic fibrosis, cirrhosis and increased risk of hepatocellular carcinoma^[2,3]. There are eight known genotypes of HDV (from 1 to 8), of which genotype 1 has a worldwide distribution and genotype 3 has been associated with the most severe outcome of liver disease^[4,5]. With a virion size of 36 nm and a 1.7 Kb genomic circular RNA, HDV is the smallest known human virus. Its genome encodes only two structural proteins termed small- and large-HD-antigens (S- and L-HDAg). The proteins are transcribed from the same open reading frame (ORF) and are identical except for a 19 amino acid extension in C-terminal domain of L-HDAg^[2].

HDV requires the function of a helper virus as an envelope source for virion envelopment and propagation. This function can be provided through HBV (all genotypes from A to H) or other *Orthohepadnaviridae* members, such as Woodchuck hepatitis virus (WHV), by sharing the surface proteins^[2]. The 19 amino acid extension of L-HDAg, which is called the "packaging signal", is responsible for this interaction^[6]. While HBV thereby provides an essential basis for HDV viremia and infectivity, most clinical studies reported that HBV replication is diminished in HBV-HDV-infected patients and that HDV co-infection is associated with lower HBV viremia than HBV mono-infection^[7]. However, HBV-DNA, HDV-RNA and HBsAg apparently fluctuate in longitudinally studied patients indicating ongoing and dynamic interactions between HBV and HDV in infected cells^[8].

Although the direct contact between HBsAg and HDAg for HDV virion envelopment can be considered the main interaction, other less well understood mechanisms may also interfere with the replication of both viruses in infected cells^[9]. Here we describe possible mechanisms for HBV/HDV interactions and their probable molecular cross-talks in infected cells. These mechanisms include HBsAg-HDAg interactions and HDV-trans-controlling of HBV genome replication/transcription, cellular transcriptional pathways and RNA polymerase activity in dually infected hepatocytes.

HBsAg-HDAg INTERACTIONS

HBV encodes three surface proteins with different initiation-of-replication sites from one ORF. These proteins are large, medium and small HBsAg (L-, M- and S-HBsAg)^[10]. As an integral protein, S-HBsAg (226 amino acids) is anchored in the lipid bilayer of the endoplasmic reticulum (ER) through its N-terminal (residues 4-28 and 80-100) and C-terminal (residues 165-226) transmembrane domains (TMDs). It also includes an antigenic loop (Ag loop, residues 101-164) with immunodominant epitopes, facing the ER lumen. The rest of residues located between TMDs face the cytoplasm and are called cytosolic loops (CYLs). These are expected to be residues 29 to 79 (CYL- I) and 194 to 201 (CYL- II)^[11]. The M-HBsAg (281 amino acids), contains the whole S-HBsAg plus an N-terminal preS2 region facing the ER lumen. The L-HBsAg (389-400 amino acids), contains preS1, preS2 (preS) and S domains^[12]. This protein has two conformations based on the positioning of preS in ER membrane towards the cytoplasm (for virion formation) or ER lumen (for receptor binding)^[11]. All three types of HBsAg are found on the surface of mature HDV particles^[2]. The schematic features of HBsAg proteins and their localization in ER membrane are shown in Figure 1.

Both HDV small and large proteins form connections to one another as well as to HDV RNA through RNA binding domains to assemble the HDV ribonucleoprotein (RNP) complex^[6]. The L-HDAg is responsible for RNP localization in the ER membrane through a CXXX farnesylation signal (C stands for cytosine, and X for any amino acid) and also interactions with HBV surface proteins through its packaging signal (Figure 1)^[2]. The packaging signal is very genotype specific in HDV (74% divergence between genotypes 1 and 2) and plays an important role in the envelopment. Although an association between HDV-1/HBV-A and -D and HDV-3/HBV-F and -A has been observed, independent investigations suggest that the co-infections are mainly representative of common genotypes of each of the viruses in certain geographical areas and not specific for distinct HBV or HDV genotypes^[13-15]. Studies indicate that HDV genotype 2 is associated with a less aggressive disease compared to genotype 1 which has been attributed to the higher packaging efficiency of genotype 1 than that of genotype 2^[16,17]. Moreover, a variation in the packaging efficiency has been observed among different isolates of the same genotype, which reflects the critical role of this length in HDAg interactions with HBsAg. It has been reported that the hydrophobic nature of the L-HDAg C-terminal domain provided by C211-farnesylation as well as the number of hydrophobic residues of the packaging signal (which differs among HDV genotypes) enhance HDV interactions with surface proteins of HBV and therefore the packaging efficiency^[17].

While HBV requires both S- and L-HBsAg for viral

Mutational studies revealed that in addition to the receptor binding site on the pre-S1 domain of L-HBsAg, the Ag loop is also responsible for HDV virion infectivity^[27]. On the other hand, *in vitro* experiments using mutant HBsAg with deletions in Ag loop resulted in the lack of subviral particles as well as HDV virion secretion^[27]. In more detailed studies it was shown that N-glycosylation of S-HBsAg, which is mediated by the C-terminal domain of S protein and occurs partially on Asn-146, affects HDV envelopment and secretion^[12]. Based on these studies, HDV secretion is delayed or reduced (about ten folds) in the presence of non-glycosylated HBsAg, while HBV and HBsAg formation is not affected^[21]. Although a weakened interaction between HBsAg and other components of HDV-RNP complex (rather than L-HDAg) has been suggested for this reduction, based on the luminal positioning of Ag loop in the ER membrane, a direct interaction of this domain and HDV components is unlikely^[21,27]. Different mechanisms have been suggested to explain the effects of the antigenic domain, especially in its non-glycosylated form, on HDV packaging and secretion. One is a modified maturation and trafficking process for non-glycosylated HBsAg, which in turn will affect the rate of interactions with HDV^[21]. The association of HBsAg with calnexin (a molecular chaperon in

ER membrane) is also affected by the glycosylation process^[28]. Therefore, a non-glycosylated HBsAg is more prone to misfolding and late maturation^[29]. Furthermore, it has been suggested that a non-glycosylated Ag loop faces the cytoplasm, which possibly masks the cytosolic interaction sites with HDV or hinders appropriate connections between HBsAg and HDV (Figure 1)^[11]. Due to different propagation responses of HBV and HDV to non-glycosylated Ag loop, it is possible that these viruses apply different mechanisms to interact with HBsAg^[21]. Likewise, the lateral S-S interactions between S protein carbohydrates, which play a critical role in virion stability, are suggested to occur differently for HBV and HDV due to their particle sizes^[12].

INDIRECT INTERACTIONS BETWEEN HBV AND HDV AFFECTING VIRAL REPLICATION

There are several indications of low HBV replication levels in patients co-infected with HDV^[7,9,30]. On the other hand, longitudinal analyses of HBV/HDV co-infected individuals demonstrated a fluctuating pattern of HBV and HDV replication over time^[8]. In case that HDV is temporarily or permanently the dominant virus during dual infection with HBV, there should be a molecular scenario for these viruses to control each other's replication. Most of the studies so far, indicate a controlling role of HDV over HBV replication or its protein expression in infected cells^[7,9,31].

Previous investigations showed that HBV DNA in the host cell genome can produce enough surface antigen molecules for HDV virion assembly even in the absence of precore and pregenomic RNAs and regardless of an active HBV replication^[32,33]. These cells, which still produce some of the viral products, may be selected through immune responses, appear as a result of a resolved infection or just due to the support of the infected cells for parts of the viral proteins such as envelope antigens but not the complete replication of the virus^[33-35]. Nonetheless, regarding the role of HBV as an envelope provider, HDV nucleoproteins can be considered as competitors with HBV for HBsAg. Therefore, they may induce a selective suppression on HBV replication associated with an increase in PreS/S RNAs and HBsAg levels in co-infected patients^[9]. Investigations on the effects of S- and L-HDAgs on HBV replication have shown that these proteins inhibit HBV replication through a strong suppression of HBV enhancers (EnhI and II) and also trans-activation of the IFN- α -inducible MxA gene^[31]. The inhibitory effects of L-HDAg on RNA polymerase II, which is involved in replication of both HBV and HDV viruses, might be another reason for reduced HBV replication in the presence of co-infection with HDV^[36].

Another indication of HBV controlled replication/gene expression in HDV infected cells is the presence of basal core promoter (BCP) and precore (PC)

mutations in the HBV genome of patients co-infected with HDV. Occurrence of HBV BCP and PC mutations is associated with lower levels of HBV DNA in both serum and liver without affecting HDV replication and clinical manifestations in patients^[9,37]. In contrast, PC/BCP point mutations with HBeAg negative phenotype can significantly increase HBV viremia and replication of polymerase mutated strains in HDV-negative patients^[38].

Other instances of indirect effects of HBV and HDV on each other's replication include the synergistic activation of serum response element (SRE)-dependent pathways by HBxAg and L-HDAg, thus affecting factors which are involved in transcription regulation mediated by SRE^[39]. From the cross-talks between HBV and HDV we can also refer to the NF- κ B activation, which results from ER stress (induced by HBsAg) or TNF α secretion from immune cells (in response to HBV infection) and correlates with L-HDAg nuclear export and HDV secretion^[40].

CONCLUSION

The clinical observation of aggravated liver disease in patients with HBV/HDV co- or super-infection has prompted intense research on molecular interactions between both viruses. A major interaction between HBV and HDV is that they share a surface protein supply; this fact is currently being translated into novel therapeutic approaches using entry inhibitors in clinical trials for delta hepatitis^[41]. However, in spite of the direct interaction sites between HBsAg and HDV-RNP, it seems that the key interference between HBV and HDV cannot be devoted to a certain domain or residue of the S protein but connection spots are rather distributed along the HBsAg. Moreover, besides the main reason for HBV/HDV interactions to share a surface protein supply, this is not the only interface between the two viruses in infected cells. Further investigations are required to unravel yet unknown molecular interactions that are employed by HBV or HDV to dominate in dual infections.

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New advances on glial activation in health and disease

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and neurodegenerative disease are now aimed at targeting astrocyte responses to such insults including astrocyte activation, astrogliosis and other morphological changes, and innate and adaptive immune responses.

Key words: Astrocyte; Microglia; Neuroinflammation; Aging; Alzheimer's; Neurodegeneration

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Core tip: Over the past decade, research has begun to elucidate the role of astrocyte activation and changes in astrocyte morphology in the progression of neural pathologies, which has led to glial-specific interventions for drug development. This review addresses astrocyte response to central nervous system (CNS) injury and disease in relation to astrocyte activation, immune response, and changes in morphology. Further discussion addresses potential therapeutics targeting astrocytes, which consider these heterogeneous responses to CNS insults.

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Abstract

In addition to being the support cells of the central nervous system (CNS), astrocytes are now recognized as active players in the regulation of synaptic function, neural repair, and CNS immunity. Astrocytes are among the most structurally complex cells in the brain, and activation of these cells has been shown in a wide spectrum of CNS injuries and diseases. Over the past decade, research has begun to elucidate the role of astrocyte activation and changes in astrocyte morphology in the progression of neural pathologies, which has led to glial-specific interventions for drug development. Future therapies for CNS infection, injury,

INTRODUCTION

In addition to being the support cells of the central nervous system (CNS), glial cells, specifically astrocytes, are now recognized as active players in the regulation of synaptic function, neural repair, and CNS immunity^[1,2]. Astrocytes are among the most structurally complex cells in the brain, and activation of these cells has been shown in a wide spectrum of CNS injuries and diseases. Over the past decade, research has begun to elucidate the role of astrocyte activation and changes in astrocyte morphology in the progression of neural pathologies,

which has led to glial-specific interventions for drug development. Future therapies should look at targeting astrocyte responses to CNS insults including astrocyte activation, astrogliosis and other morphological changes, and innate and adaptive immune responses.

Astrocytes are the most numerous cells in the mammalian brain, yet much remains to be learned about their functional and morphological characteristics. Astrocytes have well-characterized roles in regulating cerebral blood flow, water transport, and extracellular concentrations of ions, metabolites, and neurotransmitters^[3]. Their processes comprise an important component of the blood-brain barrier (BBB), directly contacting endothelial cells with vascular endfeet and contributing to the structural and functional integrity of the BBB. Importantly, astrocytes contribute to the CNS's response to injury and infection^[4,5]. Recent studies have demonstrated the importance of astrocytes in innate and adaptive immune responses in the CNS and the roles that astrocyte morphology plays in these functions.

Astrocyte heterogeneity: Differences between protoplasmic and fibrous astrocytes

Although several types of astrocytes have been identified, pathological studies tend to classify them as protoplasmic or fibrous based on their morphology and localization in the CNS^[6,7]. Protoplasmic astrocytes are found in gray matter and are generally spongiform in nature. The processes of protoplasmic astrocytes spread radially from the cell body and have extensive fine branching that is distributed uniformly around the cell. The dense and complex ramifications of these fine processes extend from the primary processes reaching out to synaptic connections and contributing to metabolic, homeostatic, and BBB functions^[8].

Fibrous astrocytes, on the other hand, are present in white matter and have fewer, but longer, processes that extend along axon bundles providing structural support for axonal tracts^[9]. Studies indicate that both fibrous and protoplasmic astrocytes make contacts with blood vessels^[10]; however, fibrous astrocytes also send processes that contact axons at the nodes of Ranvier^[11] while protoplasmic astrocytic foot processes ensheath neuronal synapses^[12]. Additionally, protoplasmic astrocytes occupy their own domains in relatively independent structural units^[13], defining the micro-architecture of the parenchyma by "tiling" the gray matter. These domains are most clearly defined in areas of high synaptic density, such as the hippocampus, which suggests that domain organization may be important for modulation of synaptic transmission^[14]. Disruption of protoplasmic astrocytic domains is observed during glial scar formation in CNS trauma and infection as well as in the epileptic brain^[15,16]. Fibrous astrocytes, on the other hand, show extensive intersection of their processes, and therefore, do not appear to have the same organization as protoplasmic astrocytes^[17].

Astrocyte functions in the CNS

Gray and white matter astrocytes provide extensive metabolic support to the CNS as well as regulate water homeostasis and energy metabolism^[18]. Through gap junction communication, astrocytes can relay information from neurons to blood vessels in order to coordinate oxygen and glucose delivery with the energy demands of the tissue^[19]. Astrocytes also control extracellular ion concentrations; for instance, clearing extracellular potassium through inward rectifying channels^[20] and gap junction coupling^[21]. Furthermore, glutathione release by astrocytes provides antioxidant support^[22] protecting other neural cell types against the toxicity of various compounds by supplying glutathione precursors to neighboring cells^[23].

Astrocytes greatly outnumber neurons in the brain and play many roles essential for modulating synaptic formation and normal neurotransmission^[24]. Astrocytes have the potential to release their own chemical signals, or "gliotransmitters," such as glutamate, ATP, gamma-aminobutyric acid (GABA), and D-serine through Ca²⁺ mediated exocytosis, diffusion through pore channels, or the cysteine-glutamate antiporter system^[25]. Furthermore, studies have shown that astrocyte-neuron lactate shuttles couple synaptic plasticity and glucose metabolism in order to facilitate learning and memory^[26]. By forming connections to neuronal synapses as well as to each other through gap junctions, astrocytes can modulate neuronal activity and metabolic function.

The tripartite synapse, which includes astrocytic processes at the synaptic cleft, has thus replaced the traditional concept of a synapse as a contact between two neurons^[27]. Recently, Bernardinelli and colleagues demonstrated a bidirectional interaction between synapses and astrocytes^[28]. Synaptic activity, specifically long-term potentiation (LTP), was shown to regulate plasticity of astrocytic processes. In turn, coverage and motility of astrocytic endfeet in hippocampal synapses have been shown to predict synapse stability^[29]. For example, LTP increases the surface area of the astrocyte process enwrapping a synapse and the number of synapses receiving astrocyte coverage^[30]. Dynamic changes in astrocyte morphology were also found in electron microscopy studies of the visual cortex of rats raised in a complex environment^[31,32]. Astrocytes display a structural response to glutamate by increasing the number of astrocytic processes and surface filopodia contacting neuronal synapses^[12]. These actin-based cytoskeletal arrangements are closely linked to transformations in neighboring neuronal and vascular elements and appear as motile as dendritic processes in neurons^[33].

Research increasingly shows that astrocytes also serve important roles as an integral player in the brain's defense system^[6]. In the adaptive immune system, astrocytes have phagocytic and antigen presentation capabilities^[34,35], and summarized in Table 1. Astrocytes are able to express major histocompatibility complex (MHC) class

Table 1 Immunological molecules expressed in astrocytes and associated conditions

Immunological molecules	Effects	Conditions
Class II MHC	Autoimmune reactions	MS
ICAM-1, VCAM-1	Increased expression of pro-inflammatory cytokines	MS, AD
B7 (B7-1, B7-2)	T cell activation and differentiation	EAE
CD40	Promotes production of cytokines, chemokines, and neurotoxins	MS
CD1 (CD1b)	Antigen presentation to specialized T-cells	MS

MHC: Major immunohistocompatibility complex; ICAM: Intercellular adhesion molecule; VCAM: Vascular cell adhesion molecule; CD: Cluster of differentiation; MS: Multiple sclerosis; AD: Alzheimer's disease; EAE: Experimental autoimmune encephalitis.

I and II antigens and co-stimulatory molecules when stimulated by IFN- γ *in vitro*, which are important in T-cell activation and antigen presentation^[4]. The expression of MHC class II antigens in astrocytes *in vivo*, however, is controversial. Examination of post-mortem samples from multiple sclerosis (MS) patients showed evidence of MHC class II expression in astrocytes located in active MS lesions. Additionally, in MS lesions, reactive astrocytes express CD1 molecules (particularly CD1b), which then present lipid antigens to specialized T-cell subsets^[36], suggesting that astrocytes can participate in the presentation of non-peptide antigens to T cells. When stimulated, astrocytes also produce a wide array of cytokines and chemokines, which serve as immunological mediators in innate immune function^[1]. Glial cells may also perpetuate the progression and severity of brain pathologies associated with chronic inflammation, such as diabetes^[37] and Alzheimer's disease (AD)^[38]. Since astrocytes may serve as potential therapeutic targets, it is important to understand their functional and immunological roles in the CNS.

ASTROCYTE ACTIVATION: OVERVIEW

Astrocytes respond to CNS trauma and infection through a heterogeneous process that occurs on a continuum of molecular and cellular events. Generally, astrocytes react to CNS disturbances with increases in intermediate filament expression, progressive cellular hypertrophy and proliferation^[39,40]. Reactive astrocytes also respond with a diverse combination of intracellular and extracellular events including activation of ERK^[41] and c-Fos^[42] signaling pathways, increased production of cytokines and chemokines, and the recruitment of monocytes/microglia to the injured area^[4]. Recent research suggests that reactive astrocytes are key players in a number of neurological diseases, such as Alexander's disease, amyotrophic lateral sclerosis (ALS), and AD, underscoring the need for a better understanding of reactive astrocytes^[43-45].

Accumulating evidence indicates that reactive astrogliosis is not a simple all or none response. Instead, astrocyte activation is variable in regards to changes in cell morphology, proliferation, and molecular expression, all of which can be modified in a context-specific manner to different CNS insults^[8,16,39,46-48]. Additionally, these molecular and cellular changes are graded in a

manner that coincides with the level of injury to the CNS^[49]. Recent studies monitoring the progression of reactive gliosis show that a wide range of morphological changes occur in astrocytes and that their response varied depending on astrocyte subtype, type of injury and the location relative to the lesion site^[15,50,51]. For instance, gray and white matter astrocytes show different responses in reactive gliosis, with more dramatic morphological changes often observed in the gray matter^[15]. The signals that drive the reactive phenotype also differ with respect to the type and extent of injury sustained^[10,39]. For example, studies indicate that CNS injuries, such as ischemia and stab wounds, produce reactive astrocytes with neural stem cell potential, while astrocytes in neurodegenerative models lack such capabilities^[52,53].

Reactive astrocytes: Beneficial or harmful?

Astrocyte activation has often been classified into two categories: the first of which is beneficial and occurs soon after the CNS insult, and the second, which occurs later, inhibits neuronal regeneration, and contributes to sustained inflammation in the CNS^[54,55]. Perhaps the most well studied astroglial reaction is the formation of the glial scar from proliferative reactive astrocytes. Following an insult resulting in neuronal damage, astrocytes surround and isolate dying neurons. This is thought to prevent contact between dying and healthy neurons, preventing the progression of tissue damage, but may ultimately impede any functional recovery^[56]. Studies examining selectively ablated dividing astrocytes after spinal cord injury found that depletion of reactive astrocytes results in greatly expanded invasion of inflammatory cells beyond the lesion center resulting in a larger lesion volume and more extensive motor deficits^[57]. This suggests that the glial scar prevents inflammatory processes from spreading to healthy tissue. The glial scar reaction also produces a wide range of molecules, including tenascin-C, chondroitin sulfate proteoglycan, and matrix metalloproteinases (MMP), which inhibit axonal regeneration^[58,59].

Alternatively, further evidence shows that cytokine-activated astrocytes produce energy substrates and trophic factors for neurons and oligodendrocytes, aid in antioxidant support, promote revascularization, and restore CNS homeostasis^[60]. For instance, TGF- β signaling in astrocytes limits immune cell migration and decreases pro-inflammatory cytokine/chemokine

production, limiting neuronal injury in *Toxoplasma gondii* infection^[61]. Astrocytes also defend against oxidative stress, containing high concentrations of antioxidants^[23], and neuroprotection by reactive astrocytes is, thus, thought to occur through upregulation of glutathione following oxidative stress^[62,63].

Intermediate filaments, such as glial fibrillary acidic protein (GFAP) and vimentin, are upregulated in reactive astrocytes. While this increase aids in CNS protection and axonal regeneration, it has proved to be a double-edge sword. Intermediate filaments are thought to assist with synaptic elimination after lesion, guidance of axonal regrowth, formation of neuromuscular contacts, and timing of recovery^[64]. Conditional ablation of proliferating astrocytes leads to increased inflammation and increased neuronal death in spinal cord injury models and in experimental autoimmune encephalitis^[10]. However, studies in *GFAP^{-/-}Vim^{-/-}* aged mice demonstrated increased cell survival/proliferation in the hippocampus compared to control mice^[65]. Astrocytes of null mice exhibit fewer morphologic changes and less glial scarring after CNS insult than mice devoid of intermediate filament deficiencies^[66], indicating that chronically reactive astrocytes may restrict neurogenesis with increasing age. Furthermore, the absence of intermediate filament proteins has also been shown to decrease reactive gliosis, and subsequently, photoreceptor degeneration that results from retinal injury^[67].

Astrogliosis can be classified as anisomorphic, where astrocytes surround a lesion forming a glial scar, or isomorphic, whereby astrocytes remain distal to the site of injury and promote neurite outgrowth and facilitate synaptogenesis^[68]. Activation of astrocytes and other glial cells influence the rate and intensity of regeneration of peripheral nerves in the peripheral nervous system after injury^[64]. Experimentally, prevention of reactive gliosis improved the integration of neural progenitor cells grafted into the rodent hippocampus^[69], indicating that the survival and generation of new neurons may benefit from astroglial modifications. Overall, activation of astrocytes may be both beneficial and harmful in the setting of CNS trauma and/or disease. More research is needed to clarify therapeutic potential in astroglial responses.

Functional consequences of astrocyte activation

In the healthy CNS, astrocytes play an important role in maintaining homeostatic balance, directing the development of synapses, uptake and clearance of neurotransmitters, and modulation of cerebral blood flow^[2,19]. However, the degree to which reactive astrocytes maintain these functions, or gain new ones, remains to be elucidated. Recent studies in a transgenic mouse model of AD observed aberrant GABA production in reactive astrocytes surrounding amyloid plaques in the hippocampus^[45]. GABA, an inhibitory gliotransmitter, binds to neuronal GABAergic receptors inhibiting neuronal

synaptic release and impairing synaptic plasticity and memory function. Furthermore, studies in genetic null animal models can examine both benefits and detriments associated with gain or loss of reactive astrocytes^[70]. As mentioned above, loss intermediate filament expression attenuated reactive astrocytosis resulting, in some cases, progression of neuronal death and inflammation, and in others, increased neuronal survival. Further research will clarify the timing and situational consequence of activated astrocytes.

As such, therapeutics targeting astrocyte activation, like a recently developed TrkA agonist, has shown promise by reducing reactive gliosis and subsequent neural sequelae of neuroinflammation^[71]. Additionally, *in vitro* studies have shown that reactive astrogliosis can be suppressed by up-regulation of mitofusin 2 (Mfn2), a key protein in mitochondrial networks^[72]. Increasing Mfn2 expression in cells attenuated injury-induced astrocytic hyperplasia, activation-relevant protein synthesis, and cellular proliferation. Based on the impact of reactive astrogliosis in neurodegenerative pathologies, novel drugs targeting gliosis may be suitable for therapeutic applications in a wide number of neurological conditions.

CHANGES IN ASTROCYTE MORPHOLOGY

It is well established that astrocytes carry the potential to change their morphology in reaction to CNS injury^[73] as well as in interactions with CNS vasculature^[74] and neurons^[12]. In the same way that neuronal dendrites are adaptable and respond to changes in CNS activity by altering their structure, astrocytic processes dynamically alter their morphology and interact with synapses in response to their environment^[75]. Morphological changes in astrocytes have been documented in chronic stress^[76], traumatic brain injury^[77], neurodegenerative disease^[78], CNS viral and bacterial infections^[79,80], and behavioral and mood disorders^[81,82]. Experimentally, changes in astrocyte morphology have been reported after ethanol administration^[83], dietary-induced obesity^[84], and physical exercise^[85]. These structural changes can be detected not only at the level of their cell body and proximal processes, but more importantly, through their fine, lamellate distal processes that surround synapses and ensheath axonal nodes^[86]. Effective regulation of the perisynaptic space is attributed, in part, to astrocyte morphology^[87], and perturbations in fine morphology of these glial cells can ultimately contribute to synaptic dysfunction and disrupted neurotransmission^[88].

Astrocyte hypertrophy

Astrocyte hypertrophy is postulated to serve many functions in neuronal protection and recovery and repair. After traumatic injury, stroke, infection, or other severe CNS insult, areas of focal tissue damage become

filled with inflammatory, fibrotic, and other cells that derive from the perivascular cells, endothelia, bone marrow, and meninges. These tissue lesions become surrounded by reactive astrocytes forming glial scars that serve to separate necrotic from healthy tissue^[10,89]. Astrocytes and other glial cells surround infected or necrotic tissue providing a physical barrier between the CNS insult and healthy tissue. Longer and more complex processes would allow the astrocytes to envelop synaptic terminals and influence synaptic transmission through gliotransmitter release and neurotransmitter clearance^[90,91]. In experimental entorhinal lesions in the rat, hypertrophic astrocytes line the denervated outer molecular layer of the dentate gyrus, potentially providing trophic support for the sprouting process^[92]. Furthermore, astrocytes with more complex morphologies could come about as a compensatory mechanism for neuronal and synaptic degeneration^[93,94]. Studies have shown a significant increase in GFAP-positive hypertrophic astrocytes in the hippocampus in AD patients^[95].

The hypertrophic response in astrocytes may depend on the type and extent of CNS injury. It is hypothesized that glial scars are formed in two ways: one, through newly proliferated, elongated astrocytes that extensively overlap to form scar borders and secondly, through hypertrophic stellate reactive astrocytes that are derived from local populations of mature astrocytes^[51]. In contrast to microglia, which proliferate at a high frequency, reactive astrocytes proliferate very little in chronic disease^[53,78]. In a chronic disease model, low degrees of astrocyte proliferation were observed in the presence of pronounced astrocyte hypertrophy^[53]. Hypertrophy, but not proliferation, of GFAP-positive astrocytes also occurs alongside increased expression of proteins expressed in neural stem cells^[96,97]. Clarifying the roles that subsets of astrocytes have in injury response will have important implications for future therapeutics.

Astrocyte atrophy

While astrocyte hypertrophy/astrogliosis serves to contain brain damage and assist in neuronal survival^[39], the converse can be said about astroglial degeneration and atrophy. Atrophy of astrocyte processes has been detected in normal aging^[98] and chronic stress^[76] as well as in the early stages of various neurodegenerative diseases including AD^[99] and ALS^[44]. Atrophic astrocytes result in reduced support for neuronal networks, which may ultimately decrease neuronal connectivity and plasticity. We have recently shown that, in the setting of simian immunodeficiency virus (SIV) infection and SIV-induced encephalitis, gray and white matter astrocytes retract their processes resulting in an overall decreased arbor irrespective of encephalitic status^[79]. It is hypothesized that reduced numbers of astrocytes is directly linked to disruptions in cognitive behavior and that astrocyte loss may be a primary driver of

pathology^[100,101]. Furthermore, Tynan *et al.*^[76] observed decreases in astrocyte morphology without concomitant reductions in astrocyte number in rodents exposed to chronic stress. We observed similar effects in macaques that exhibited self-injurious behavior, a classic behavior following social stress^[81]. This suggests that atrophy and decreased GFAP expression, rather than reductions in astrocyte number, are related to neuropathological changes in stress and mood disorders^[76].

Conversely, global CNS insults, such as ischemia/hypoxia, induce changes in astrocyte morphology that are distinctly different from focal insults. Studies examining hypoxia/ischemia in the neonatal pig model showed significant decreases in astrocytic processes (length and number) with hypertrophy of the cell body post-insult^[102]. These changes were observed in both white and gray matter astrocytes and were evident as soon as eight hours after the insult and were concurrent with dysfunction in glutamate clearance^[102].

Furthermore, increasing or decreasing the numbers and sizes of astrocytes impacts the volume and alters the composition of the space between astrocytes^[103]. As a consequence of this, there would be neuronal dysfunction through excitotoxicity^[104], homeostatic imbalances^[105,106], damage to synapses^[107,108]. For instance, post-mortem examinations of human brains following TBI show enlarged perivascular spaces, which potentially reflect astrocyte retraction^[109]. The uncoupling of astrocytes and microvascular endothelium can interfere with homeostasis and metabolic support – ultimately resulting in an imbalanced energy supply to the brain^[110].

Factors controlling astrocyte morphology

There are two distinct mechanisms whereby astrocytes can be activated in the absence of infectious agents. In the first, gap junction proteins are down regulated^[111] restricting the overall syncytia of astrocytes. This would also alter the morphology of the astrocytes including the number of synapses they can form with neurons and the BBB. Alternatively, changes in astrocyte morphology can occur as a consequence of immune regulation and inflammation^[112].

Several genes are implicated in morphological alterations in astrocytes. GFAP, an intermediate filament protein highly expressed in white matter astrocytes and a subset of gray matter astrocytes, is thought to modulate astrocyte motility and shape, providing structural stability to processes^[113]. Studies in GFAP-null mice have shown that GFAP as well as vimentin, an intermediate filament necessary to stabilize GFAP, are required for proper glial scar formation in the injured CNS^[66]. Additionally, fibroblast growth factor (FGF) signaling has been shown to be responsible for alterations in astrocyte morphology during glial activation^[114]. The blockade of FGF signaling at the site of reactive gliosis reduced astrocyte branch formation and minimized hypertrophic responses during reactive gliosis. Selective deletion of transcription factor, signal

Table 2 Immune function of astrocytes

Pattern recognition receptors (expressed in astrocytes)	Effects	Conditions
TLRs (TLR2, TLR3, TLR4, TLR5, TLR9)	Upregulation cytokine/chemokine expression, induction of costimulatory molecules	Viral and bacterial infection, DAMPs
NOD receptors (NOD1, NOD2)	Upregulation of pro-inflammatory cytokines through NF- κ B	Bacterial CNS infections
Scavenger receptors (SR-BI, SR-MARCO, RAGE, SRCL)	Mediates adhesion/uptake of A-beta in the CNS	AD
Mannose receptors (expressed)	Receptor-mediated endocytosis, CD4 independent HIV-1 entry	HIV
Complement factors (C1q, C4, C2, C3, C3d, C5, C5b-9, C6, C8)	CNS inflammation, cell activation and astrogliosis	TBI, synaptic plasticity, Pick's disease, MS
Complement receptors (CR1, CR2, C3aR, C5aR)	CNS inflammation, cell activation and astrogliosis	TBI, synaptic plasticity

TLR: Toll-like receptor; NOD: Nucleotide-oligomerization domain; SR: Scavenger receptor; RAGE: Receptor for advanced glycation end products; DAMP: Damage-associated molecular pattern; TBI: Traumatic brain injury; CNS: Central nervous system; SR-BI: Scavenger receptor class B type I; SR-MARCO: Scavenger receptor - macrophage receptor with collagenous structure; SRCL: Scavenger receptor C-type lectin; CR1: Complement receptor type 1; CR2: Complement receptor type 2; C3aR: Complement component 3a receptor; C5aR: Complement component 5a receptor; NF- κ B: Nuclear factor kappa-light-chain enhancer of activated B cells; AD: Alzheimer's disease; HIV: Human immunodeficiency virus; MS: Multiple sclerosis.

transducer and activator of transcription 3, from astrocytes disrupted glial scar borders, which allowed the spread of inflammatory cells from the site of injury and increased neuronal loss^[51]. Furthermore, studies have shown that aquaporin-4 (AQP4) is important for sustaining astrocyte morphology, indicating a functional role of AQP4 in astrocyte plasticity. Knockdown of AQP4 in primary cultures resulted in a drastic reduction in membrane water permeability, impaired cell growth, and altered cell morphology^[115] as well as the down-regulation of three genes (glucose transporter 1, hexokinase, and metallothionein-1) involved in brain edema.

Furthermore, changes in astrocyte morphology may not necessarily be permanent and can change with amelioration of CNS insult^[116] and/or the administration of therapeutic medication (Lee *et al.*, under review). Recovery in changes in astrocyte morphology, such as decreases in process hypertrophy and an increase in primary processes, has been observed two weeks after optic nerve injury^[117]. We showed that changes in astrocyte morphology associated with self-injury in rhesus macaques were reversed with opioid antagonist treatment. Furthermore, valproate has been shown to reduce the overlap between adjacent astrocytic domains seen in epilepsy^[16]. Valproate was also used to treat a transgenic mouse model of AD. The investigators found that APP/PS1 mice had markedly improved symptoms as well as decreased astrogliosis and microgliosis after valproate treatment^[118].

ASTROCYTE ACTIVATION AND INFECTIOUS DISEASE

Immune function of astrocytes

The CNS is considered an immune-privileged system with the presence of the BBB, low levels of MHC molecules, and the absence of lymphatic irrigation^[119]. Increasing evidence shows that astrocytes participate in local innate immune responses triggered by a variety of insults.

Astrocytes are an important source of cytokines and have the capacity to respond to a wide variety of cytokines themselves^[60]. In the resting state, glial cells express a wide variety of receptors for inflammatory cytokines, chemokines, pathogen-associated molecular patterns (PAMPs), and damage-associated molecular patterns (DAMPs)^[120,121]. Once activated, glial cells have the capacity to induce numerous other receptors and inflammatory mediators following stimulation from other CNS cells, infiltrating leukocytes, and/or invading pathogens^[1]. Additionally, both microglia and astrocytes display an array of receptors involved in innate immunity and damage detection, including Toll-like receptors (TLRs), nucleotide-binding oligomerization domains, double-stranded RNA-dependent protein kinases, scavenger receptors, and mannose receptors^[122,123], and summarized in Table 2.

These pattern-recognition receptors detect infectious particles and damage-associated molecules associated with CNS trauma and neurodegeneration^[124]. TLRs, type I transmembrane receptors most commonly found in innate immune cells, are highly expressed in microglia and have also been observed in astrocytes^[125]. Under resting physiological conditions, astrocytes express TLR3^[126] as well as low levels of TLR2, TLR4, TLR5, and TLR9^[127,128]. Binding of PAMPs to TLRs on astrocytes alters cytokine secretion, cytoskeletal protein expression, and adhesion^[126].

Viral infection of astrocytes

Astrocytes can be targeted, as well as directly infected, by several pathogens and possess the ability to recognize structures belonging to various types of pathogens. For example, astrocytes display functional CXCR4 and CCR5 co-receptors, which render them permissive to HIV-1 infection^[129,130]. Direct infection of astrocytes has also been demonstrated in SIV^[131], group B streptococcal bacteria^[132], Borna virus^[133], and herpes simplex virus^[134]. Furthermore, TLRs may also increase or decrease susceptibility to viral infection in astrocytes, depending on the viral agent studied. For example, in rodent models,

Table 3 Mediators of astroglial function

Mediators	Examples	Effects on astroglial function
Cytokines	IL-6, IFN β , TNF- α , TGF- β , GM-CSF, BAFF, IL-1 β , MCP-1, RANTES	Increase BBB permeability, astrocyte activation, endothelial cell activation, microglial and monocyte activation, differentiation and proliferation, immunosuppression, release of neuroprotective mediators
Chemokines	CCL2, CCL5, CCL20, CXCL10, CXCL1, CXCL1, CXCL2, CX3CL1	Recruitment of monocytes and macrophages, dendritic T cells, T and B lymphocytes, and neutrophils/regulation of myelination and microglial activity, astrocyte proliferation and survival, migration of microglia and neural progenitors
Trophic Factors	EGF, FGF, NGF, BDNF, VEGF, IGF1	Astrocyte activation and morphological modification, neuronal/astrocytic survival, differentiation, function, and regeneration, oligodendrocyte survival, remyelination, neurogenesis
Endothelins	Et1, Et3	Inhibit gap junction coupling, disrupts direct intercellular communication in astrocytes, intracellular and extracellular ion homeostasis, metabolic trafficking, cellular swelling

IL: Interleukin; IFN: Interferon; TNF: Tumor necrosis factor; TGF: Transforming growth factor; GM-CSF: Granulocyte-macrophage colony-stimulating factor; BAFF: B-cell activating factor; MCP: Monocyte chemoattractant protein; CCL: Chemokine ligand; CXCL: CXC motif ligand; EGF: Epidermal growth factor; FGF: Fibroblast growth factor; NGF: Nerve growth factor; BDNF: Bone derived neurotrophic factor; VEGF: Vascular endothelial growth factor; IGF: Insulin-like growth factor; Et: Endothelin.

TLR3 in astrocytes protect against herpes simplex virus type-2 infection^[134], but has been reported to mediate entry of West Nile Virus into the CNS, causing encephalitis^[135].

Furthermore, recent evidence indicates that TLRs are also capable of sensing endogenous ligands produced during stress or injury called DAMPs, linking TLRs with the host response to CNS damage^[136]. Astrocytes can express receptors for DAMPs^[137]. Endogenous DAMP molecules released from damaged neurons can bind to TLR2 on nearby glia, and in turn, activate glial cells during CNS trauma and infection^[138]. As such, astrocyte and microglial activation was decreased in TLR2-null mice^[138]. Interestingly, studies in an intracerebral hemorrhage stroke model utilizing TLR2-null mice found no differences in microglial activation, indicating that inflammation and neurotoxicity were mediated by TLR2 on astrocytes^[124]. Since TLRs have been implicated in both infectious and noninfectious diseases of the CNS^[122], understanding their potential to influence the course of neuroinflammation is important in developing new therapeutic interventions aimed at minimizing tissue damage during neuroinflammatory disorders.

Following infection and/or activation, astrocytes secrete cytokines and chemokines, such as CXC motif ligand 10 (CXCL10), Chemokine ligand 2 (CCL2), interleukin-6 (IL-6), and BAFF, which influence both innate and adaptive immune responses^[4]. These responses are important in eliciting local CNS immune responses through inflammatory mediators and recruiting additional immune effector cells from the peripheral circulation. Increased CCL2 secretion in astrocytes initiates the recruitment of immune cells and activation of glial cells in the CNS during chronic neuroinflammatory disease and autoimmune inflammation^[139]. Experimentally, astrocytes activated by heat-killed bacteria or lipoproteins react by secreting chemokines, proliferate, or enter apoptosis^[140]. For instance, astrocyte infection by *Brucella* has been shown to induce MMPs, which are known to induce tissue remodeling^[80,141]. In cultured

astrocytes, viral mimic poly(I:C) induces the expression of several cytokines (TNF- α , IL-6, IFN β , granulocyte-macrophage colony-stimulating factor and transforming growth factor) and chemokines (CCL2, CCL5, CCL20, CXCL8 and CXCL10)^[142]. Astrocytes can also express receptors for and respond to a wide variety of other growth factors and cytokines, including, but by no means limited to, TNF- α , EGF, FGF, endothelins and interleukins (for review, see^[143]). Such factors can induce the expression of molecules associated with reactive astrogliosis, such as GFAP, and have also been implicated in astrocyte proliferation^[144]. The downstream effects are summarized in Table 3.

Astrocyte contributions to sustained inflammation

Evidence has demonstrated that astrocytes contribute to sustained inflammation in the CNS after trauma or infection^[145,146] and growing research implicates sustained glial inflammation in neurodegenerative disorders^[147]. Chronically activated microglia and astrocytes can release reactive oxygen intermediates, nitric oxide, inflammatory cytokines, which are toxic to neurons. In AD, amyloid β -peptide (A β) peptides activate astrocytes, which increase production of inflammatory mediators^[148]. Furthermore, astrocytes are able to remove and degrade A β , and chronically activated astrocytes may eventually lose their neuroprotective functions^[149]. Furthermore, in a rodent model of multiple sclerosis, investigators found that the enzyme, *LacCer*, which promotes astrocyte activation and controls the transcription of genes related to neuroinflammation and neurodegeneration, is upregulated in astrocytes^[150].

One mechanism by which astrocytes may contribute to sustained inflammation in the CNS is through upregulation of inflammatory pathways modulated by TLR expression. A single injection of LPS in aged rats, which mimics systemic infection in the elderly, resulted in sustained astrocyte activation and prolonged increases in cytokine expression^[151]. Increases in astrocytic TLR2 have been implicated in sustained inflammation

by increasing the likelihood of the cells to respond to subsequent inflammatory insults^[152]. HIV infection increases TLR2 expression in astrocytes, which can increase susceptibility to additional insults, either from a secondary/opportunistic infection or from a second round of virus entering the brain^[79,153]. Additionally, enhanced TLR expression would upregulate the secretion of proinflammatory cytokines by astrocytes^[154,155], triggering a self-sustaining inflammatory loop and long-term glial activation.

Astrocytes can release both pro- and anti-inflammatory factors, contributing crucially to inflammatory processes in the CNS. In addition, the astrocytes that are part of the BBB are among the first cells to encounter blood-derived leukocytes entering the brain during certain types of neuroinflammatory insult^[156]. Increased leukocyte migration also occurs in neurological conditions such as stroke or multiple sclerosis. As such, astrocytes are strategically located to influence direct interactions with leukocytes or interaction with endothelial cells of the BBB^[157]. Under inflammatory conditions, the integrity and function of the BBB is modified and enables greater leukocyte passage into the CNS^[158]. Recent studies examining human T-lymphocytic virus type-1 infection in the CNS show that astrocytes contribute to positive feedback loop that promotes chronic inflammation. Infected T cells produce INF- γ , which causes astrocytes to secrete CXCL10 and recruit more infected T cells, creating an immunological positive feedback loop^[159]. Another study by Owens and colleagues demonstrate that astrocyte ablation results in enhanced inflammatory monocyte cell migration into the CNS^[160,161]. Furthermore, astrocytes mediate microglial activation through RANTES-dependent mechanism in Borna disease virus infection^[162], indicating that activated astrocytes produce soluble factors that activate microglia.

Therapies targeting astrocyte contributions to chronic inflammation

Chronic activation of the innate immune system can indirectly contribute to neuropathology and neuronal death. Sustained neuroinflammation is implicated in HIV-associated neurocognitive disorder^[163], neurodegenerative disease^[150], and chronic pain^[164], and compromises CNS function causing progressive neurodegeneration and BBB compromise^[165]. In the clinical setting, pharmacological antagonists and immunosuppressive agents can be used to prevent chronic CNS inflammation responses. Such therapies can be appropriated from existing medications or can be the result of new developments in glial-activated neuroinflammation research^[166]. The development of novel therapeutic interventions targeted at glial activation pathways and glia-mediated inflammation appears to be promising and may lead to more effective prevention and treatment of neuroinflammation and resulting pathologies. For example, riluzole, the only FDA-approved treatment for

amyotrophic lateral sclerosis (ALS), enhances astrocytic glutamate uptake through increased GLT-1 activity reducing the activation of neurons by glutamate^[167]. Riluzole also stimulates astrocytic synthesis of NGF, BDNF and GDNF in culture^[168] as well as increase levels of BDNF and TGF- β in patients with Huntington's disease^[169]. Further research into novel methods for targeting inflammation by reducing the activity of glutamatergic system activation are thus necessary^[112].

Generally, astrocytic function in neuroprotection is greatly compromised during chronic neuroinflammation. New perspectives for therapeutic approaches include the replacement of dysfunctional astrocytes or pharmacological treatments that specifically target detrimental signaling pathways while preserving their neuroprotective functions. Signaling pathways, such as JNK and p38 MAPK, were found to be relevant to reactive gliosis in response to a variety of cytokines and pathogenetic stimuli; and as such, several MAPK inhibitors have been characterized *in vitro* and in animal models as potential therapeutic interventions targeting reactive astrocytes^[170,171].

Chronically proinflammatory astrocyte and microglia phenotypes, showing a reduction in genes involved in neuronal support and neuronal signaling, may contribute to neuronal dysfunction and cognitive decline in AD^[172]. Astrocytes contribute to the clearance of amyloid β -peptide^[173]. In sporadic AD, impaired removal of A β contributes to elevated extracellular levels that drive amyloid plaque pathogenesis. Enhancing lysosomal function in astrocytes with transcription factor EB, a master regulator of lysosome biogenesis, could promote A β uptake and catabolism and attenuate plaque pathogenesis^[174]. Furthermore, reactive astrocytes have recently been shown to produce and release the inhibitory gliotransmitter, GABA, which impaired synaptic plasticity in a rodent model of AD^[45]. Increased GABA synthesis and/or release may become a therapeutic target for treating memory impairment in neurodegenerative disease.

DIRECTIONS AND THERAPEUTICS

Research into the morphological changes in astrocytes will provide insight into the pathophysiology of the disease. In the future, disease models will consider "gliopathies" as a part of disease etiology. Further research on acute changes in astrocyte morphology would help elucidate the dynamics of astrocyte morphology. For example, analysis through the xCELLigence system provides data output in real time and is thought to measure cell adhesion^[175]. Studies using the xCELLigence system have shown that astrocytes exposed to cytokine treatment show loss of cellular adhesion^[176] and cell death^[177]. These changes occurred 24-48 h prior to astrocyte cell loss, demonstrating the ability of xCELLigence to detect changes in astrocyte composition long before cell death.

Furthermore, targets of intervention would seek to limit the inflammatory process where inflammatory environment is cytotoxic to the surrounding cells, or where glial cell damage would impact the ability of the CNS to repair itself. Reactive astrocytes have already emerged as an attractive target for improved recovery after stroke^[178]. Regardless of the type of ischemic injury, reactive astrocytes express hyperpolarization-activated cyclic nucleotide-gate channels, which have potential as a therapeutic target in post-stroke therapy^[179]. Post-traumatic axonal regeneration can be enhanced by inhibition of chondroitin sulfate proteoglycans produced by reactive astrocytes^[180].

Potential therapeutics targeting astrocytes should consider the heterogeneous responses to CNS insults including astrocyte activation, astrogliosis and other morphological changes, in addition to innate and adaptive immune responses. A key role in establishing a therapeutic intervention for astrocytes in CNS insults would be to clarify of the role of glial activation and the formation of the glial scar. A hallmark of CNS injury of any origin is the formation of scar tissue composed of activated or reactive astrocytes and microglia surrounding a distinctly inflammatory response. The cost-benefit analysis of the formation of this scar is debated as it restricts axonal growth within the lesion. However, several studies indicate that this process may have potential neuroprotective functions. Reactive astrocytes can also serve as potential sources of new neurons in the brain, replenishing the neurons damaged by neurodegenerative disease. Guo and colleagues have demonstrated the reprogramming of reactive astrocytes generated by brain injury or in a mouse AD model into functional glutamatergic neurons *in vivo*^[181]. Astrocytes represent an important therapeutic target in a number of neurological conditions, specifically where astrocyte activation exacerbates brain injury or where astrocyte loss may reduce BBB integrity or neuronal support. While CNS research in the past decade has dramatically shifted its focus to include astrocytes and other glial cells, more research to further clarify the roles of these cells in CNS injury and damage is needed to produce effective therapeutic interventions.

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Impact of antiretroviral therapy on lipid metabolism of human immunodeficiency virus-infected patients: Old and new drugs

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Abstract

For human immunodeficiency virus (HIV)-infected patients, the 1990s were marked by the introduction of highly active antiretroviral therapy (HAART) representing a new perspective of life for these patients. The use of HAART was shown to effectively suppress the replication of HIV-1 and dramatically reduce mortality and morbidity, which led to a better and longer quality of life for HIV-1-infected patients. Apart from the substantial benefits that result from the use of various HAART regimens, laboratory and clinical experience has shown that HAART can induce severe and considerable adverse effects related to metabolic complications of lipid metabolism, characterized by signs of lipodystrophy, insulin resistance, central adiposity, dyslipidemia, increased risk of cardiovascular disease and even an increased risk of atherosclerosis. New drugs are being studied, new therapeutic strategies are being implemented, and the use of statins, fibrates, and inhibitors of intestinal cholesterol absorption have been effective alternatives. Changes in diet and lifestyle have also shown satisfactory results.

Key words: Human immunodeficiency virus-1 infection; Highly active antiretroviral therapy; Protease inhibitors; Dyslipidemia; Atherosclerosis; Lipodystrophy; Statins; Fibrates; Diet; Lifestyle

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Core tip: Antiretroviral therapy inhibits human immunodeficiency virus (HIV)-1 replication, reduces mortality and increases survival. On the other hand, HIV-1 infection and antiretroviral therapy affect lipid metabolism. In fact, lipodystrophy is a well-documented

side effect of highly active antiretroviral therapy (HAART). Switching to a less metabolically active drug improve HAART-associated dyslipidemia. Other therapies may include statins, fibrates, inhibitors cholesterol absorption, fish oils, niacin. Moreover, changes in diet and lifestyle are needed to revert the dyslipidemia.

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INTRODUCTION

The introduction of highly active antiretroviral therapy (HAART) for human immunodeficiency virus (HIV)-infected patients in the early nineties (1990) represented a new perspective on life for these patients^[1]. The use of HAART was shown to effectively suppress the replication of HIV-1 and dramatically reduce mortality and morbidity, which has led to a better and longer quality of life for HIV-1 patients^[2]. The different HAART regimens, all composed of at least three different antiretroviral drugs, are effective in reducing viral load (HIV-1-RNA) to undetectable levels after its inception^[3]. HAART regimens inhibit viral replication by acting at different stages with their different combinations of drugs^[4]. This allows them to reach the viral cycle and/or viral enzymes and causes them to be classified in different therapeutic groups according to their mechanisms of action: nucleoside reverse transcriptase inhibitors (NRTIs)^[5], non-nucleoside reverse transcriptase inhibitors (NNRTIs)^[6], protease inhibitors (PIs)^[7], fusion inhibitors^[8], entry inhibitors [CC chemokine receptor-5 antagonists]^[9] and integrase strand transfer inhibitors (InSTIs)^[10] (Table 1). Apart from the substantial benefits that result from the use of various HAART regimens, laboratory and clinical experience has shown that HAART can induce severe and considerable adverse effects on metabolic complications of lipid metabolism, characterized by signs of lipodystrophy, insulin resistance, central adiposity, dyslipidemia, increased risk of cardiovascular disease and even an increased risk of atherosclerosis^[11-14]. However, other factors, such as virological, genetic, and individual immunological features, may be involved in the metabolic and lipid alterations observed because not all of the patients exposed to the same HAART regimens are similarly affected^[15-17]. All of these changes in the aspects of lipid metabolism during HIV-1 infection, specifically changes in high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL), very low-density lipoprotein cholesterol (VLDL), triglycerides (TG), lipid peroxidation, and their relationship with atherosclerosis in HIV-1 patients, are a

result of the critical role of cholesterol in the mechanism of HIV-1 replication^[11,12,18,19]. HIV-1 decreases plasma HDL by impairing the cholesterol-dependent efflux transporter ATP-binding cassette protein A1 in human macrophages, which is a condition that has a high atherogenic risk^[20,21]. The use of PI-based HAART currently constitutes a more potent option against HIV-1 infection, preventing the maturation of viral particles and effectively controlling the infection of new cells by HIV-1. However, observed changes in lipid metabolism in HIV-1 patients have been associated with this class of antiretroviral drugs^[13,14,22,23]. There is significant support in the literature showing that the PIs are associated with increased hepatic triglycerides-synthesis, VLDL, and to a lesser extent, total cholesterol (TC)^[11-14]. Moreover, it was observed that these drugs impair the hydrolysis of triglyceride-rich lipoproteins by lipase, which reduces the storage of free fatty acids and interferes with the normal postprandial metabolism of free fatty acids^[23,24]. The PIs are analogous substrates of the aspartyl protease enzyme of HIV-1 that are involved in the cleavage process of viral proteins and form smaller functional viral particles with infective capacity. After the cleavage process, the newly formed infectious viral particles are released from infected cells in a mature form^[7,25,26]. Once the PIs bind to the active site of the protease enzyme, and this process of cleavage is blocked, there is interference in the enzyme activity and inhibition in the process of viral maturation and the formation of infectious viral particles^[25,26]. The different mechanisms by which PIs promote these changes remain unknown. However, the main effect of PIs seems to be suppressing the breakdown of the nuclear form of sterol-regulatory element binding protein-1 in the liver and adipose tissue. This regulator is a key element in the proteolytic pathway responsible for regulating cellular and plasma levels of fat and cholesterol^[27]. Finally, other classes of antiretroviral drugs are available, including those with excellent activity against viral replication without having any apparent effect on lipid metabolism^[12,23,28]. However, it is clear that the use and recommendation of PIs occurs in situations where other drugs and/or regimens have not achieved the desired effect, either by non-adherence to treatment, viral resistance or lack of an immune response^[29,30]. Moreover, once the therapy with PIs is initiated, a change to a more conservative therapy without their use is not recommended nor used in clinical practice^[31,32]. Thus, a continuous search that considers the individual characteristics of each PI available as a current therapy is needed to achieve alternative HAART regimens that can maintain a suppression of viremia with minor effects on the lipid metabolism of HIV-1 patients^[32,33].

HIV-ASSOCIATED LIPID DISORDERS

Lipid disorders during the course of HIV-1 infection and acquired immunodeficiency syndrome (AIDS) were observed long before the advent of antiretroviral

Table 1 Antiretroviral drugs class

Antiretroviral class	Generic name drug	Trade name/manufacturer/approval (yr)
Nucleos(t)ide reverse transcriptase inhibitors	Abacavir (ABC)	Ziagen® ViiV Healthcare (1998)
	Didanosine (ddI)	Videx® Bristol-Myers Squibb Co. (1991)
	Emtricitabine (FTC)	Emtriva® Gilead Sci. (2003)
	Lamivudine (3TC)	Epivir® GlaxoSmithKline (1995)
	Stavudine (d4T)	Zerit® Bristol-Myers Squibb Co. (1994)
	Tenofovir (TDF)	Viread® Gilead Sci. (2001)
	Zidovudine (AZT)	Retrovir® ViiV Healthcare (1987)
	Zalcitabine (ddC)	Hivid® Roche (1992)
Non-nucleoside reverse transcriptase inhibitors	Delavirdine (DLV)	Rescriptor® Pfizer (1997)
	Efavirenz (EFV)	Sustiva® Bristol-Myers Squibb Co. (1998)
		Stocrin® Merck Sharp, Dohme (1998)
	Nevirapine (NVP)	Viramune® Boehringer Ingelheim (1996)
	Etravirine (ETR)	Intelence® Janssen-Cilag (2008)
	Rilpivirine (RPV)	Edurant® Janssen-Cilag (2011)
Protease inhibitors	Amprenavir	Agenerase® GlaxoSmithKline (1999)
	Atazanavir	Reyataz® Bristol-Myers Squibb Co. (2003)
	Darunavir	Prezista® Janssen-Cilag (2006)
	Fosamprenavir	Lexiva® ViiV Healthcare (2003)
	Indinavir	Crixivan® Merck and Co. (1996)
	Lopinavir	Kaletra® Abbott (2000)
	Nelfinavir	Viracept® ViiV Healthcare (1997)
	Ritonavir	Norvir® AbbVie Inc. (1996)
	Saquinavir	Invirase® Roche (1995)
	Tipranavir	Aptivus® Boehringer Ingelheim (2005)
	Enfuvirtide, T-20	Fuzeon® Hoffmann La Roche (2003)
Fusion inhibitors	Dolutegravir (DTG)	Tivicay® GlaxoSmithKline (2013)
Integrase strand transfer inhibitors	Elvitegravir (EVG)	Stribild® Gilead Sci. (2012)
	Raltegravir (RAL)	Isentress® Merck and Co. (2007)
Entry inhibitors (CC chemokine receptor 5 antagonists)	Selzentry	Maraviroc® Pfizer (2007)

regimens^[34,35]. In the early phase of acute HIV-1 infection, patients display several varied clinical signs of immunosuppression such as fever, intestinal infections, weight loss and depletion of protein reserves^[35,36]. The possibility of HIV-1 infection, by itself, causing changes in lipid metabolism was already postulated, because it is evident that plasma viremia may promote a decrease in the plasma concentrations of TC, HDL and LDL and, in later stages of infection, an elevation in the concentration of TG^[35,36]. Specifically, the reduction of HDL likely occurs as a result of an activation of the immune system in early HIV-1 infection, which promotes an increase in lipid peroxidation, inflammatory cytokine production, and alterations in the reverse cholesterol transport. This process promotes an imbalance in the antioxidant system, a decrease in the production of anti-inflammatory cytokines and an elevation of pro-inflammatory cytokines, which increases the chance of developing atherosclerotic diseases^[31-39]. As a result of the inflammatory process initiated by viral infection, the stimulation of endothelial lipase and phospholipase A2 occurs, which in turn can reduce HDL concentration^[38-40]. The inflammatory process may also be characterized by an elevation of interferon- γ levels (IFN γ) originating from lymphocytes and macrophages. IFN γ levels are elevated at early stages of infection and are also correlated with the presence of hypertriglyceridemia^[41,42]. Tumor necrosis factor- α (TNF α) is another potent pro-inflammatory mediator whose concentrations increase in

HIV-1 infected ART-naïve patients. TNF α promotes lipid peroxidation and disturbances in the metabolism of free fatty acids and also acts on the suppression of lipolysis mediated by hormones^[43] (Figure 1).

HAART-ASSOCIATED LIPID DISORDERS

HAART-associated dyslipidemia is complex and involves immunological, hormonal, and genetic predisposition aspects, as well as effects induced by various antiretroviral drugs^[13,44]. The observed dyslipidemia is characterized by hypertriglyceridemia, hypercholesterolemia, and decreased serum levels of HDL, either accompanied or not by increased levels of LDL (Table 2)^[44,45]. Other metabolic and/or clinical common disorders include insulin resistance with hyperinsulinemia, increased C-peptide levels, diabetes mellitus and lipodystrophy syndrome^[44-48]. HAART also affects the hydrolysis of triglyceride-rich lipoproteins and tissue lipase, disrupts normal post-prandial free fatty acid and lipoprotein catabolism and interferes with peripheral fatty acid trapping; all of these effects could be due to the interaction of these fatty acids with the master transcriptional regulator sterol regulatory element binding protein 1^[47-51]. Nevertheless, the presence of dyslipidemia in individuals who use HAART therapy is not necessarily accompanied by lipodystrophy and/or an evident insulin resistance, which suggests that the mechanisms involved in these disorders are independent^[44,46,51,52]. The NNRTI-based

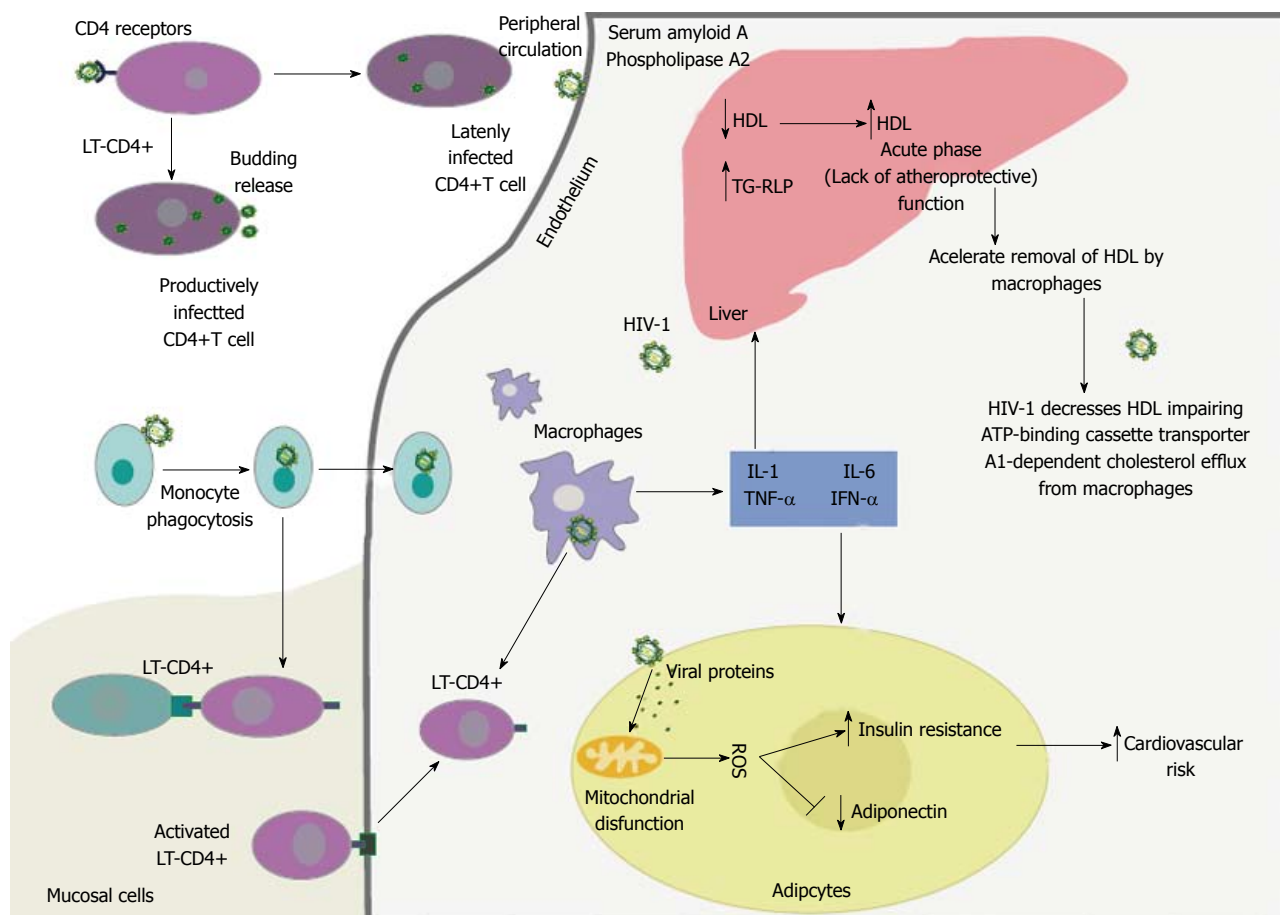


Figure 1 The human immunodeficiency virus type 1, upon entering peripheral circulation, will infect lymphocytes and macrophages. The viral proteins gp120 and gp41 of HIV-1 bind to the CD4+ receptor and coreceptors, C-C chemokine receptor type 5 and C-X-C chemokine receptor type 4, on the surface of these cells. The lymphocytes T-CD4 that are infected with HIV-1 produce viral particles and may remain in a latent form within circulation. Infected monocytes can directly present antigen to lymphocytes T-CD4, or transform into tissue macrophages. This process stimulates the host inflammatory response and amplifies the production of proinflammatory cytokines and promotes increased cellular oxidative stress. The production of proinflammatory cytokines by macrophages and lymphocytes promotes a decrease in plasma high-density lipoprotein cholesterol by impairing the cholesterol dependent efflux transporter ATP-binding cassette protein A1 in human macrophages. Additionally, viral proteins and proinflammatory cytokines including interleukin-1, interleukin-6, tumor necrosis factor α and interferon gamma stimulate endothelial lipase enzyme and different acute phase proteins, such as serum amyloid A. Viral proteins also exert effects on adipocytes resulting in mitochondrial dysfunction, production of reactive oxygen species, increased insulin resistance, decreased adiponectin, and change the clearance of triglyceride-rich lipoproteins and insulin resistance. Finally, all of the different cellular mechanisms involved and affected by HIV-1 infection promote an increased risk of cardiovascular disease. Source: de Almeida *et al.*^[21]. Gp120: Glycoprotein 120; gp41: Glycoprotein 41; CCR5: C-C chemokine receptor type 5; CXCR4: C-X-C chemokine receptor type 4; LT-CD4: Lymphocytes T-CD4; HDL: High-density lipoprotein; ABCA1: ATP-binding cassette protein A1; IL-1: Interleukin-1; IL-6: Interleukin-6; TNF α : Tumor necrosis factor α ; IFN- γ : Interferon gamma; TG-RLP: Triglyceride-rich lipoproteins; ROS: Reactive oxygen species; HIV-1: Human immunodeficiency virus type 1.

HAART, zidovudine, stavudine or lamivudine, have become associated with the occurrence of dyslipidemia; however, lipid metabolism disorders are most evident in individuals who make use of PI-based therapy^[44,45,52,53]. The mechanisms involved in PI-associated dyslipidemia are not fully understood; however, the prevailing hypothesis is based on the structural similarity between the catalytic region of the HIV-1 protease and two homologous human proteins involved in the metabolism of lipids, called cytoplasmic retinoic acid-binding protein type 1 (CRABP-1) and low-density lipoprotein-receptor-related protein type 1 (LRP1) (Figure 2).

CRABP-1

CRABP-1 exhibits 58% homology in its amino acid sequence of the C-terminal region of the catalytic

region of the HIV-1 protease. CRABP-1 usually binds intracellular retinoic acid and presents it to cytochrome P450 (CYP450) or 3A (CYP3A) enzymes, which convert retinoic acid to cis-9-retinoic acid and bind to the retinoid X receptor-peroxisome proliferator-activated receptor γ (RXR-PPAR γ) heterodimer, stimulating adipocyte differentiation and inhibiting apoptosis^[20,45,54]. The PIs likely bind to CRABP-1, which is homologous to the viral protease and erroneously inhibits the formation of cis-9-retinoic acid, leading to reduced RXR-PPAR γ activity, increased apoptosis and diminished proliferation of peripheral adipocytes. Such events cause peripheral lipoatrophy syndrome and hyperlipidemia due to adipocyte loss, decreased lipid storage and lipid release into the bloodstream. The inhibition of CYP3A by ritonavir is another possible mechanism involved in lipid

Table 2 Antiretroviral drugs: Impact on lipid and glucose metabolism

Antiretroviral class	Drug	Effects on lipids	Effects on glucose
NRTIs	Abacavir (ABC)	↑ Dyslipidemia	No effect
	Didanosine (ddI)	↑↑ Dyslipidemia	Insulin resistance
	Emtricitabine (FTC)	↑ Dyslipidemia	No effect
	Lamivudine (3TC)	↑ Dyslipidemia	No effect
	Stavudine (d4T)	↑↑ Dyslipidemia	Insulin resistance
	Tenofovir (TDF)	↑ Dyslipidemia	No effect
	Zidovudine (AZT)	↑↑ Dyslipidemia	Insulin resistance
NNRTIs	Efavirenz (EFV)	↑↑ HDL, ↑ Dyslipidemia	No effect
	Etravirine (ETR)	Neutral effects	No effect
	Nevirapine (NVP)	↑↑ HDL, ↑LDL	
	Rilpivirine (RPV)	Neutral effect	
PIs	Amprenavir/ritonavir	↑↑↑ Dyslipidemia	Insulin resistance
	Atazanavir/ritonavir	↑ Dyslipidemia	Insulin resistance
	Darunavir/ritonavir	↑ Dyslipidemia	Insulin resistance
	Fosamprenavir/ritonavir	↑↑↑ Dyslipidemia	Insulin resistance
	Indinavir	↑↑ Dyslipidemia	Insulin resistance
	Lopinavir/ritonavir	↑↑↑ Dyslipidemia	Insulin resistance
	Nelfinavir	↑↑ Dyslipidemia	Insulin resistance
	Saquinavir	↑ Dyslipidemia	Insulin resistance
	Tipranavir/ritonavir	↑↑↑ Dyslipidemia	Insulin resistance
	Enfuvirtide, T-20	Neutral effect	No effect
Fusion inhibitors			
InSTIs	Dolutegravir (DTG)	Neutral effect	No effect
	Elvitegravir (EVG)	Neutral effect	No effect
	Raltegravir (RAL)	Neutral effect	No effect
Entry inhibitors	Selzentry	Neutral effect	No effect

NRTIs: Nucleos(t)ide reverse transcriptase inhibitors; NNRTIs: Non-nucleoside reverse transcriptase inhibitors; PIs: Protease inhibitors; InSTIs: Integrase strand transfer inhibitors.

abnormalities in HIV-1-infected patients and associated PI-based therapy and would promote a reduction in the formation of cis-9-retinoic acid and reduced enzymatic activity of RXR-PPAR γ . The decrease in RXR-PPAR γ activity results in apoptosis within peripheral adipose stores, decreased adiponectin, and insulin resistance. However, central and visceral adipose stores are spared and expand with weight gain, contributing to insulin resistance^[20,45,54].

LRP

LRP shares 63% homology with the catalytic region of HIV-1 protease. LRP binds to lipoprotein lipase (LPL) on the capillary endothelium, and the formation of this LRP-LPL complex promotes cleavage of fatty acids from TG, thereby promoting free fatty acid accumulation in peripheral adipocytes. A possible hypothesis is that the binding of PIs to LRP may inhibit the complex normal function of LRP-LPL and interfere with fatty acid storage, leading to hyperlipidemia. This hyperlipidemia is characterized by elevations in cholesterol levels, principally in the LDL and VLDL cholesterol fractions, because fatty acids released into the bloodstream subsequently reach the liver and promote a secondary hepatic synthesis of TG and VLDL^[4,55].

Mitochondrial alterations

Another proposed mechanism for HAART-associated dyslipidemia is the mitochondrial alterations induced by HAART, especially with PI-based therapy. The hypothesis

is that the HAART regimen will cause mitochondrial disturbances by inhibiting the mitochondrial DNA (mtDNA)-polymerase γ , leading to mitochondrial DNA depletion, respiratory chain dysfunction and reduced energy production by cells^[56,57]. This disturbance in the mitochondrial respiratory chain may promote metabolic disorders in adipocytes, promote lipodystrophy syndrome and increase plasma lipid levels. Moreover, interference between PIs and cellular proteases could also trigger the development of metabolic alterations because some proteases are essential for mitochondrial biogenesis and metabolic function. Furthermore, functional changes of mitochondria in skeletal tissue promote insulin resistance and consequent dyslipidemia^[56-58].

Genetic factors

HAART-associated lipodystrophy and dyslipidemia may be related to genetic predisposition, and studies with HIV-1 patients with hypertriglyceridemia and low HDL subjects were associated with different polymorphisms in the *APOCIII* gene. Promoter polymorphisms -455T > C and -482C > T in the *APOCIII* gene are both associated with increased levels of TG containing lipoproteins (VLDL) and low HDL values. Carriers of the -455T > C genetic variant had 30% lower levels of HDL cholesterol compared to those without this polymorphism, and plasma lipid concentrations increase according to the number of these variant alleles. Another variant nucleoside, the -1131T > C promoter

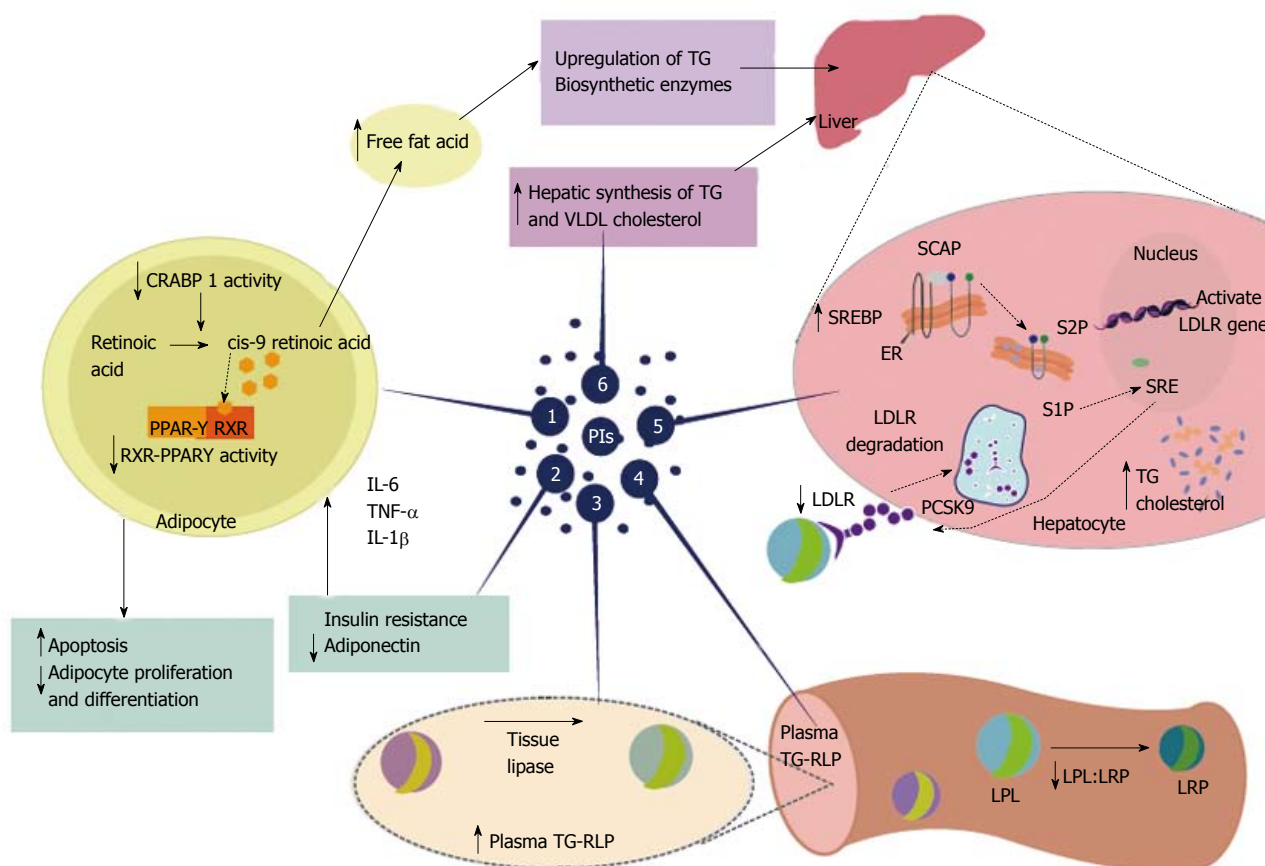


Figure 2 Highly active antiretroviral therapy-associated dyslipidemia is especially evident with the use of protease inhibitors. Protease inhibitors (PIs) promote a decrease in plasma high-density lipoprotein cholesterol and increased overall cholesterol, triglycerides (TG), and low-density lipoprotein cholesterol. These changes, induced by PIs, promote an increased risk of cardiovascular disease. Proposed mechanisms for PI-based dyslipidemia include the following: (1) There is structural similarity with the amino acid sequence of the C-terminal region of cytoplasmic retinoic acid-binding protein type 1 (CRABP1); thus, the PIs likely bind to CRABP-1, increasing apoptosis and diminishing the proliferation of peripheral adipocytes; (2) PI-mediated increases in the expression and secretion of proinflammatory cytokines, such as tumor necrosis factor alpha, interleukin 1 β and interleukin-6 are involved in altered adipocyte functions and decreased adiponectin; (3-4) PI-induced dyslipidemia is based on the structural similarity between the catalytic region of HIV-1 protease and the LDL-receptor-related protein that interferes with lipoprotein lipase complex formation (LRP-LPL). As a result the adipose storage capacity is reduced and plasma TG-rich lipoproteins are increased; (5) PI suppresses proteasome-mediated degradation of the sterol regulatory element binding proteins (SREBP) in the liver and adipocytes, which are transcription factors responsible for fatty acid and triglyceride synthesis in the liver and adipose tissue and control several steps of cholesterol synthesis. The suppression promotes nSREBP accumulation in the liver and an increase in the biosynthesis of total cholesterol and triglycerides, and adipose tissue, promoting increased insulin resistance, reduced expression of leptin and lipodystrophy; (6) PI-based therapy increases the hepatic synthesis of triglycerides, and to a lesser extent, very-low density lipoprotein cholesterol. Source: de Almeida *et al.*^[21]. PIs: Protease inhibitors; HDL: High-density lipoprotein; TG: Triglycerides; LDL: Low-density lipoprotein; CRABP1: C-terminal region of cytoplasmic retinoic acid-binding protein type 1; TNF- α : Tumor necrosis factor alpha; IL-1 β : Interleukin 1 β ; IL-6: Interleukin-6; LRP: LDL-receptor-related protein; LPL: Lipoprotein lipase; TG-RLP: Triglyceride-rich lipoproteins are increased; SREBP: Sterol regulatory element binding proteins; VLDL: Very-low density lipoprotein; RXR-PPAR γ : Retinoid X receptor-peroxisome proliferator-activated receptor γ ; LDL-R: Low-density lipoprotein-receptor; PCSK9: Proprotein convertase subtilisin-kexin type 9; SCAP: Sterol regulatory element binding protein cleavage activating protein; S1P: Site 1 protease; S2P: Site 2 protease.

polymorphism in the *APOA5* gene, was associated with hypertriglyceridemia in PI-based patients^[59-62].

Paraoxonases

Changes in antioxidant enzymes, such as the family of paraoxonases (PONs), may partially explain some of the mechanisms involved in HAART-associated dyslipidemia and consequently characterize a higher risk for cardiovascular diseases and atherosclerosis^[63]. The hypothesis that the PIs can promote reductions in the activity of PONs and an increased risk for atherosclerotic disease in HIV-1 patients has been shown through previous evidence. PON1 is an antioxidant enzyme

present in serum that is strongly associated with apolipoprotein-A1 (apoA1) from HDL and protects LDL against oxidative modifications^[63,64]. The action of serum PON1 most likely occurs through the involvement of the enzyme in reverse cholesterol transport, a well-established anti-atherogenic propriety of HDL^[65]. PON1 has the ability to inhibit LDL oxidation (oxLDL) and significantly reduce the lipid peroxidase enzyme, which decreases the accumulation of cholesterol in peripheral tissues^[66]. The oxidative modification of LDL in the arterial wall plays a central role in the pathogenesis of atherosclerosis, which is characterized by the deposition of lipids and the formation of atherosclerotic plaques

Table 3 Clinical diagnosis and treatment to human immunodeficiency virus-associated lipodystrophy syndrome

Clinical diagnosis	Treatment options
Lipoatrophy Sunken eyes, sunken cheeks, prominent zygomatic arch, prominent veins, skinny or muscular appearance, loose skin folds loss of contour	Switching antiviral therapies: stavudine or zidovudine to abacavir or tenofovir, other switch, and/or reconstructive procedures
Lipohypertrophy Increased abdominal girth with visceral fat accumulation, dorsocervical or supraclavicular fat pad	Diet, exercise, liposuction
Related findings Hypertriglyceridemia, usually with depressed HDL, hypercholesterolemia, insulin resistance, glucose intolerance	Statins, fibrates, inhibits intestinal cholesterol absorption, fish oils, diet, exercise, drugs (metformin, acarbose, sulfonyleureas, glinides or leptin)

that cause narrowing of the blood vessels^[67]. The inhibition of LDL oxidation by HDL is attributed to the high antioxidant content of this lipoprotein due to the antioxidant properties of apoA1 and by the presence of other different antioxidant enzymes, such as glutathione peroxidase and PON itself, which prevent the formation of or degrade bioactive products of LDL oxidation^[68]. Some studies have shown that the activity of PON1 may be affected and/or inactivated by oxidative stress, which could explain its reduced activity during HIV-1 infection^[63-65]. In HIV-1 patients and those who undergo HAART, there is a significant increase in oxidative stress. In turn, in asymptomatic individuals infected with HIV-1 and/or with AIDS, there is an increase in oxidative stress characterized by increased plasma metabolites of lipid peroxidation and/or a quantitative decrease in antioxidants compared to seronegative controls that are considered to be in a healthy condition. Therefore, possible reductions in the activity of PON1 and HDL concentrations may characterize an increased cardiovascular risk in individuals infected with HIV-1^[64,65,69]. The PON1 activity that was reduced in ART-naïve patients, and restored in patients treated with HAART, suggested that the activity of PON1 is associated with the immune status in HIV-1 patients. However, in individuals treated with lopinavir/ritonavir, even with low plasma viremia, PON1 activity was reduced and a higher atherogenic risk was shown by the high TC:HDL ratio, suggesting that a PI-based regimen affects the mechanisms involved in the oxidation of LDL, thereby promoting greater atherogenic risk^[63-68].

LDL oxidation

Oxidation is a common feature in lipid metabolism^[70-72]. Oxidative modifications to LDL, which are considered the initial event in the pathogenesis of atherosclerosis, are attributed to oxidative stress mechanisms initiated by agents such as superoxide, nitric oxide and hydrogen peroxide that transform LDL into oxLDL^[73,74]. The deposition of oxLDL in the arterial intimal layer promotes a cytotoxic effect on the vascular endothelium, followed by inflammation and modification of monocytes into macrophages that phagocytose oxLDL particles to form the foam cells that accumulate in the intima and lead to the development of atheromatous plaques^[75]. The

oxLDL particles are immunogenic, and serum levels of anti-oxLDL antibodies (Abs) can be used as indicators of oxidative stress^[73-75]. The IgG anti-oxLDL Abs are pro-atherogenic and can predict the progression of coronary and carotid atherosclerosis, whereas IgM anti-oxLDL Abs appear to be associated with a possible protective role against the development of atheromatous plaques^[76]. During the process of infection by HIV-1, the increase in atherogenic risk results from changes in lipid metabolism associated with the severity, duration, and stages of infection. Different degrees of lipodystrophy occur in patients along with a decrease in LDL receptor expression, which could lead to increased oxidation of LDL particles and the consequent development of atherosclerosis^[77]. HIV-1 patients treated with lopinavir/ritonavir have shown higher levels of IgG anti-oxLDL Abs compared to patients treated with efavirenz or nevirapine regimens, and these levels were associated with an increase in the atherogenic indices^[75-77].

HAART-ASSOCIATED LIPODYSTROPHY

Lipodystrophy is a syndrome that includes peripheral fat wasting and central obesity and is a well-documented side effect of HAART (Table 3)^[16,48,78]. In addition to the decrease in the expression of LDL receptors, and a consequent increase in serum concentrations of LDL, the most obvious mechanisms involved in HAART-associated lipodystrophy and dyslipidemia are the mitochondrial changes induced by HAART^[13,56-58]. The inhibition of mtDNA-polymerase γ , which leads to mitochondrial DNA depletion in respiratory chain dysfunction and a reduced energy production in cells, may promote metabolic disorders in adipocytes and promote increased lipodystrophy syndrome and plasma lipid levels^[56-58,79,80]. Both therapies, PIs- and NRTIs-based, are associated with the inhibition of mtDNA-polymerase γ ^[79-81]. The abnormalities observed in lipodystrophy syndrome include lipoatrophy, lipohypertrophy, and metabolic disturbances. Lipoatrophy is associated with the loss of subcutaneous fat, usually in the lower limbs, face and buttocks. The observation of lipoatrophy in HIV patients has been demonstrated in therapy with both PIs- and NRTIs-based therapies. Several studies initially suggested that lipoatrophy in HIV-1 patients is primarily associated with the use of PI-based therapies; however,

more recent reports show that the incidence of lipotrophy was significantly higher in the efavirenz plus two NRTIs group than in the lopinavir or efavirenz plus two NRTIs plus lopinavir groups^[82-84]. The association of lipotrophy with efavirenz use was mainly in combination with either stavudine or zidovudine but not with tenofovir/lamivudine.

Lipohypertrophy is the result of a metabolic disorder in which there is excess fat accumulation in the adipose tissue, resulting in a central obesity process. The most affected regions are the intra-abdominal, trunk and/or breast, anterior neck, and dorsocervical region (*i.e.*, buffalo hump)^[14,85]. There may be co-existing fat deposition in the liver, muscle, myocardium, and epicardium^[86,87]. The most accepted hypothesis regarding the development of lipohypertrophy suggests that a defect in peripheral adipocytes promotes increased availability of fatty acids in the general circulation. The available fatty acids are then selectively deposited in visceral adipose tissue owing to the higher rate of lipid turnover and uptake in visceral adipocytes^[88]. This disruption in the metabolism of fatty acids characterized by increased uptake in the visceral adipose tissue could be related to the effects of HIV itself *via* the HIV-1 accessory protein Vpr or to the effects of HAART^[89]. In patients infected with HIV and treated with HAART, especially with PIs, there seems to be an association between HIV treatment and the development of lipohypertrophy^[84,90,91]. However, various longitudinal studies have failed to demonstrate that HAART is the main cause of lipohypertrophy in HIV-1 patients^[92-95]. The contribution of PIs to lipohypertrophy is based on several hypothetical mechanisms. PIs impair adipocyte differentiation through interactions with adipocyte proteasomal gene expression systems, down-regulation of cellular retinoic acid binding protein (CRABP), sterol regulatory binding protein levels with resultant dysregulation of gene expression stimulated by cortisol, activation of the adipocyte renin-angiotensin system and adipokine effects (including adiponectin and leptin), and decrease in peroxisomal proliferator-activator receptors α and γ ^[96-98]. This metabolic disorder results in the hypertrophy of adipose tissue, particularly in visceral tissues, resulting in increased TG levels, lowered HDL cholesterol levels, hypertension, increased propensity for type 2 diabetes, and increased insulin resistance^[98-100]. This metabolic disorder results in hypertrophy of adipose tissue, particularly in visceral tissues, with the consequent increase of TG and reduced HDL cholesterol, and hypertension, increased propensity for type 2 diabetes, and an increased insulin resistance in adipocytes^[98-100]. Insulin resistance is a common metabolic disorder that can accompany lipodystrophy (*i.e.*, lipohypertrophy) and is associated with an increased cardiovascular risk, especially among HIV-infected individuals with lipodystrophy^[101]. As described in the literature, HIV-infected individuals exhibit a higher prevalence of dyslipidemia, including both

abnormal distribution of fatty acids and altered glucose homeostasis, compared to HIV seronegative individuals after adjustment for age and body mass index^[102]. The disturbance in glucose metabolism appears to be closely linked to abnormal fat distribution, particularly visceral adiposity and lower extremity lipotrophy. Lipodystrophy promotes accumulation of intramuscular lipids, which is associated with a reduction of insulin action in this tissue^[103]. Importantly, in addition to the lipohypertrophy observed in HIV-infected individuals taking HAART, there appears to be an increase in fat distribution and deposition in places such as the liver and muscles regardless of the use of HAART^[86,104]. The mechanisms involved in HIV-associated lipodystrophy are diverse, but it is suggested that HAART plays an important role^[105], as well as the endothelial dysfunction associated with the HIV infection itself^[106], vascular endothelial injury^[107], and inflammation with elevated serum levels of C-reactive protein^[108], TNF- α , IL-6, and adiponectin^[102,109-111].

SWITCHING ANTIVIRAL THERAPIES

The search for different therapeutic strategies to reverse HAART-associated dyslipidemia has led to the use of less metabolically active antiretroviral drugs without compromising antiretroviral efficacy. Ritonavir is the most representative drug in HAART-associated dyslipidemia, and in combination with lopinavir confers higher risks for cardiovascular disease in HIV-1 patients. Amprenavir and nelfinavir promote lower impacts compared to the therapy with lopinavir/ritonavir^[29,45,64,77,112]. In turn, the use of indinavir and saquinavir shows even less of an effect on lipid metabolism in HIV-1 patients receiving HAART. Currently, atazanavir has the least impact on lipid metabolism^[113,114]. In contrast, nelfinavir promotes the elevation of TC, TG and LDL levels, and its replacement by atazanavir permits the reduction of the concentrations of these parameters without affecting antiretroviral activity^[115]. A more recent alternative is tipranavir, a non-peptide PI prescribed for patients with multidrug resistance. However, it has shown deleterious effects that promote atherogenic risk by increasing the levels of TC and TG^[116]. Another strategy to control dyslipidemia has been the discontinuation of the PI-based regimens and a switch to a NRTI- or NNRTI-based protocol. For ART-naïve patients, HAART regimens that include at least one NNRTI, or abacavir and two NRTIs, might be as efficient as PI-based therapy, although they are not the standard choice. This exchange of HAART in patients with viral suppression did not reduce antiretroviral efficacy during long-term use^[116,117]. A strategy that must be better evaluated is the long-term use of the NRTI/NNRTI class of drugs before the use of PI-based therapy. The use of NRTI-associated nevirapine reduces levels of TC and TG promotes an increase in HDL and a decrease in atherogenic risk. The use of NNRTIs may also alter the lipid profile due

Table 4 Statins to highly active antiretroviral therapy-associated dyslipidemia

Drug	Metabolism and interactions
Simvastatin	Considerable CYP3A4 metabolism. ↑ simvastatin levels with PIs and ↓↓ levels with efavirenz. Not recommended with atazanavir, atazanavir/ritonavir, fosamprenavir/ritonavir, saquinavir/ritonavir, tipranavir/ritonavir, lopinavir/ritonavir, indinavir/ritonavir, darunavir/ritonavir and nelfinavir. Doses of 80 mg/d with NNRTIs, raltegravir and selzentry
Lovastatin	Not recommended with atazanavir, atazanavir/ritonavir, fosamprenavir/ritonavir, saquinavir/ritonavir, tipranavir/ritonavir, lopinavir/ritonavir, indinavir/ritonavir, darunavir/ritonavir and nelfinavir. Doses of 80 mg/d with NNRTIs, raltegravir and selzentry
Atorvastatin	Somewhat CYP3A4 metabolism, ↑ levels with PIs darunavir, lopinavir, saquinavir/ritonavir, fosamprenavir. ↓ levels with efavirenz. Doses of 20 mg/d with PIs, 80 mg/d with NNRTIs, raltegravir and selzentry
Pravastatin	Reduced interaction with CYP450 metabolism, primarily renal excretion but 50% ↓ with lopinavir/ritonavir, 45% ↓ with nelfinavir, 80% ↑ with darunavir/ritonavir, and 40% ↓ with efavirenz. Doses of 80 mg/d with PIs, NNRTIs, raltegravir and selzentry
Fluvastatin	Metabolized by CYP2C9, and occasional interactions with nelfinavir and efavirenz. Doses of 80 mg/d with PIs, NNRTIs, raltegravir and selzentry
Rosuvastatin	Not CYP3A4 metabolized but 5 × ↑ levels with lopinavir/ritonavir and darunavir/ritonavir (uncertain). Low starting doses (5-10 mg) recommended with PIs. Doses of 20 mg/d with PIs, 40 mg/d with NNRTIs, raltegravir and selzentry

NNRTIs: Non-nucleoside reverse transcriptase inhibitors.

mostly to the use of efavirenz. Using this medication, TG levels were higher in comparison to the use of nevirapine. However, in studies with a large number of HIV-1 patients, accompanied at intervals of ninety days and with undetectable HIV-1-RNA, the levels of TC, LDL and TG were kept within the desirable limit in the groups treated with nevirapine and efavirenz, including HDL levels within the reference values^[116-118]. Only the HIV-1 patients treated with a PI-based regimen showed lipid abnormalities and increased risks for cardiovascular disease^[13,22,117]. In addition, possible alterations in lipid metabolism resulting from the use of NNRTI-based therapy are easier and faster to reverse with the use of statins, fibrates, diet and lifestyle. Although the individual effects of NRTIs remain unclear, stavudine was associated with TC and TG elevations greater than zidovudine and tenofovir. The addition of fusion inhibitors to the existing therapies, such as enfuvirtide/T-20, had little effect on plasma lipids. The possibility of different HAART strategies eliminating or reducing the dyslipidemia in HIV-1 patients must be evaluated, and the risk of development of variants of the virus with multi-drug resistance must be taken into account^[119]. In HIV-1 patients with favorable historical responses to HAART and accompanied by a physician experienced in HIV-infection, the transition from a PI-based to a therapy with nevirapine, abacavir, or even atazanavir may be preferable to the use of a hypolipidemic. In practice, many patients will show pre-existing resistance to the drugs, limiting options for the exchange of the treatment^[77,113-115]. Experts must assess the risks of toxicity of the new treatment and the possibility of virologic relapse when switching HAART regimens.

OTHER THERAPIES FOR HAART-ASSOCIATED DYSLIPIDEMIA

The use of hypolipidemic drug therapy becomes necessary when HAART-associated dyslipidemia occurs or persists for a long period and when alterations in diet, exercise and other HAART strategies are

ineffective. Difficulties in the treatment of dyslipidemia in HIV-1 patients involve potential interaction between drugs, toxicity, intolerance, and low patient adherence to multiple drug regimens. Several alternatives are available, which, when adequately monitored, may be beneficial in reducing HAART-associated dyslipidemia.

Statins

Statins are a group of drugs that inhibit the enzyme HMG-CoA reductase (3-hydroxy-3-methylglutaryl coenzyme A reductase) and are considered the primary drugs for the treatment of primary hypercholesterolemia^[120] in addition to others effects^[121,122]. In clinical practice, the use of statins has achieved excellent results in reducing TC and LDL, leading to a decreased risk of coronary artery events and in the primary and secondary prevention of heart diseases^[123,124]. Statins inhibit the key rate-controlling enzyme in the de novo synthesis of cholesterol, which is responsible for production of > 50% of total body cholesterol. Inhibition of HMG-CoA reductase also promotes an increase in the synthesis of hepatic LDL receptors and reduced VLDL production^[123-125]. The most important drugs of this class are simvastatin, fluvastatin, atorvastatin, lovastatin, pravastatin and rosuvastatin. All of these drugs reduce LDL concentrations, although the use of simvastatin and atorvastatin has shown superior effects in HIV-1 seronegative patients^[123-125]. In HIV-1-infected patients affected with dyslipidemia, the use of simvastatin, pravastatin, fluvastatin and rosuvastatin promotes reduction of dyslipidemia, but not is complete remission once other factors and elements are associated with the dyslipidemia in these patients^[123-126]. The different drugs that compose HAART have metabolizing effects similar to statin (Table 4). Most of these compounds are metabolized by CYP3A4 and may cause clinically relevant interactions with other agents that are changed by this enzymatic complex, such as cyclosporine, erythromycin, itaconazole, ketoconazole, oral anticoagulants, PIs and NNRTIs^[126-128]. An additional complicating feature is that individual statins are metabolized at differing degrees,

in some cases producing active metabolites. They are also substrates for P-glycoprotein, a drug transporter present in the small intestine, which may influence their oral bioavailability^[127-129]. The presence of elevated statin levels in plasma increases the risk of liver toxicity, promoting elevations of serum transaminases and possible toxic hepatitis as well as skeletal muscle toxicity and myalgia with elevations of serum creatine kinase elevations, especially in the case simvastatin and atorvastatin^[127-131]. Fluvastatin is metabolized by CYP2C9 enzyme; pravastatin and rosuvastatin are not significantly metabolized by the CYP450 system and have a very low risk of drug interactions. Reductions in the levels of TC and TG were observed in patients with dyslipidemia associated with HIV-1 and treated with a PI and the use of rosuvastatin. Simvastatin, lovastatin and atorvastatin should be avoided because they present a high risk of pharmacological interactions with PIs. Moreover, in a recent study, pravastatin had the lowest binding to plasma proteins of the statin agents and dietary advice associated with this statin compound significantly reduced total cholesterol levels in HIV patients treated with HAART, without significant adverse events^[126-130]. It is reasonable to recommend the use of pravastatin and/or rosuvastatin as a first-line treatment for hypercholesterolemia in PI-treated patients and the use of fluvastatin, characterized by a slightly lower efficacy, as a second-line regimen. Additional benefits are obtained in patients treated with indinavir or pravastatin and fluvastatin, which significantly reduces the levels of TC and LDL, while maintaining good tolerability. Different associations between statins and antiretrovirals present considerable tolerability but always require monitoring of serum transaminases and creatine kinase. Different clinical studies and the routine use of fluvastatin, pravastatin, or rosuvastatin have shown that they are most suitable and safe to reduce LDL cholesterol levels in HIV patients^[126-132].

Fibrates

Fibrates represent the cornerstone of drug therapy for hypertriglyceridemia and mixed hyperlipidemia. These compounds are characterized by an extended activity on the hepatic synthesis of both TC and TG, LPL and acetyl-CoA-carboxylase, and the favorable effects on peripheral lipolysis inhibition and glycemic control^[133]. Fibrates are also metabolized by CYP450 system, but they appear to affect only CYP4A enzymes and do not show clinically relevant interactions with PIs. However, concomitant use of both fibrates and statins can increase the risk of skeletal muscle toxicity and should be avoided^[134-136]. In HIV-1 seronegative individuals, the use of a fibrate and a statin in a monotherapy regimen exhibits moderate lipid-lowering effects and good tolerability^[136-138]. In HIV-1 patients, fibrates do not have the same efficacy of statins in preventing cardiovascular disease. Studies with HIV-1 patients treated with PI-based therapy and fibrates, including

gemfibrozil, bezafibrate or fenofibrate, showed a significant reduction in the concentration of TC, TG and hypertriglyceridemia^[135,137,138]. Fibrates appear as a suitable alternative for the treatment of dyslipidemia associated with HIV, especially in the presence of hypertriglyceridemia. Periodic monitoring of serum creatinine, creatine kinase, and transaminases should be performed for the use of fibrates^[137-139]. The association between fibrates and statins has been used with relative safety and demonstrated in different studies with large numbers of HIV-1 seronegative volunteers, except for the use of the combination of statins and gemfibrozil, which is not recommended^[138-140]. The use of statins, fibrates, or associates has shown positive results in HIV-associated dyslipidemia, and the pravastatin/fenofibrate combination has promoted an improvement in lipid parameters and is safe and efficacious^[141,142]. However, as already described, there is a need for clinical and laboratory monitoring, with careful evaluations of possible clinical symptoms, such as myalgia, and laboratory symptoms such as serum transaminases, creatine kinase and creatinine.

Inhibitors of intestinal cholesterol absorption

Ezetimibe is effective at lowering lipid levels because it has the ability to inhibit the intestinal cholesterol absorption, and it shows good tolerability because it does not interact with the metabolism of CYP4A enzymes^[143,144]. In non-HIV-1-infected patients who have dyslipidemia, the monotherapy with ezetimibe or when combined with statins or fenofibrate has shown considerable efficacy and safety^[145,146]. In HIV-1 patients with high serum levels of LDL, the use of ezetimibe has also been considered an effective alternative^[144]. Monotherapy using 10 mg/d of ezetimibe has promoted reductions of more than 20% of serum LDL and, in addition, reduces the concentrations of TC and TG and increases HDL concentrations^[143-146]. Studies have shown that in individuals with HIV that is beyond effective treatments, ezetimibe has no interaction with HAART, and those receiving a PI-based association of fenofibrate/ezetimibe showed greater efficacy compared with pravastatin in monotherapy resolution of dyslipidemia^[147-149].

Fish oil

The ability of fish oil, commonly known as omega-3 fatty acids [namely, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)], to reduce elevated TG concentrations has been observed in different studies^[150,151]. HIV-1 patients using both HAART and fish oil showed an effective reduction in the concentration of TG^[152]. This ability to reduce TG levels promotes a direct benefit in risk reduction of atherogenic cardiovascular disease through a combination of anti-inflammatory and anti-platelet actions^[152-154]. For HIV-1 patients, the use of fish oil associated with fenofibrate showed additive effects in reducing TG. Given these considerable results, the American Heart Association's (AHA) dietary

guidelines, recommends that healthy adults have a minimum of two portions of fish per week, and those who have elevated TG should consume 2-4 g of EPA and DHA daily as a dietary supplement^[152-155].

Niacin

Niacin (water-soluble vitamin B3), or nicotinic acid, is a powerful reducing agent of serum lipids when administered at pharmacological doses. Its ability to reduce the levels of lipoproteins and apolipoprotein-B-containing lipoproteins and to raise HDL levels has been shown, characterizing it as an atheroprotective drug^[156,157]. Niacin has beneficial effects on other cardiovascular risk factors, including lipoprotein (a), C-reactive protein, platelet-activating factor acetylhydrolase, plasminogen activator inhibitor 1 and fibrinogen^[158,159]. The molecular mechanisms involving the action of niacin are not fully understood, but its effect on hypertriglyceridemia in uninfected individuals is recognized^[157-159]. In HIV-1 patients, the use of niacin in an extended release formulation significantly reduced the levels of TC, TG and HDL. However, the use of niacin in HIV-1 patients with dyslipidemia need to be carefully monitored because the presence of adverse events have been commonly shown, including headache, flushing, pruritus, rash, hyperuricemia, and exacerbation of insulin resistance^[160,161].

OTHER AGENTS

Other agents may contribute to HIV-associated dyslipidemia. The use of recombinant methionyl human leptin was associated with reduced insulin resistance and increased HDL levels^[162]. Tetradecylthio acetic acid, an agent whose mechanism is still unknown, promotes a reduction in levels of plasma lipoproteins^[163]. Additionally, Acipimox, a drug with sustained action and a structure similar to niacin, has been associated with decreased insulin resistance and significantly reduced levels of TG in HIV-1 adults^[164]. In a double-blind study, the use of cholestin was able to reduce the levels of TC and LDL cholesterol without modifying HDL and TG, and without showing adverse effects^[165]. The use of L-carnitine (3 g/d) resulted in a significant reduction in serum triglycerides in patients with HIV-associated dyslipidemia^[166]. These and other drugs studied aimed to revert the HIV-associated dyslipidemia but require more control to be considered appropriate for the treatment of dyslipidemia.

NEW DRUGS TO TREATMENT HIV INFECTION

Since the introduction of zidovudine (1987) for the treatment of HIV-1 infection, followed by the emergence of fusion inhibitors, such as enfuvirtide/T-20 (2003), and more recently the approval by the Food and Drug Administration (FDA) of raltegravir (2007) and

dolutegravir (2013), both InSTIs drugs, HIV-1 treatments have been adapting to new challenges. Once the inability of different HAART regimens to cure infection was recognized, new drugs, strategies and therapeutic regimens were developed with the goal of greater efficiency associated with safety and fewer adverse effects. The common adverse effects observed by the use of the first class of drugs such as zidovudine, and the dyslipidemia caused by the use of PIs, are obstacles that are being minimized in newer experimental drugs. Currently, more than 30 drugs are approved and available in various forms (the different classes of antiretroviral drugs), and many others are in experimental stages.

NRTIs

Festonavir (BMS986001) is a thymidine analogue drug, derived from stavudine, but with less potential toxicity^[167]. It has been used in cases where there is HIV-1 resistance to abacavir and tenofovir and is an oral drug recommended for HIV-1 patients with multi-drug resistance. The compound has a 50% effective concentration (EC₅₀) for the inhibition of mtDNA-polymerase γ and is 100 times less toxic to the mtDNA-polymerase γ in renal proximal tubular cells, muscle cells, and adipocytes and on the cellular levels of adenosine triphosphate and/or lactate production (ATP) than stavudine. The mitochondrial toxic effects of stavudine are the main cause of the adverse effects associated with lipodystrophy and peripheral neuropathy, which has led to the decline in its use and indicated that festonavir has a minor impact on lipid metabolism^[167-169]. Apricitabine (AVX754, formerly SPD754) is a drug for oral administration and is currently in the experimental phase (Phase IIB clinical trial). It is structurally related to lamivudine and emtricitabine and, as such, is an analog of cytidine^[170]. This drug is well tolerated, and its most common side effects include headache, nausea, muscle aches and diarrhea. The use of apricitabine in HIV-1 infected patients had no effect on bone marrow, liver or kidney toxicity, and lipase. However, its use caused changes in lipid metabolism, most noticeably elevated serum TG, indicating that its use should be evaluated in patients who initiated therapy with apricitabine or who already have a dyslipidemic profile^[170-172]. GS-7340 is a prodrug of tenofovir called tenofovir disoproxil fumarate. Unlike tenofovir, GS-7340 is stable in plasma and is then converted to tenofovir inside of cells by the cellular enzyme cathepsin, which is highly expressed in lymphoid tissue^[173]. Within the cell, the drug is transformed into the active metabolite tenofovir diphosphate, an inhibitor of HIV-1 reverse transcriptase. Phase III studies are underway to better define the safety profile and efficacy, and initially, the drug does not show effects on lipid metabolism. However, formulations containing 300 mg of the drug promoted adverse effects on the kidneys and bone marrow toxicity^[173-175]. Other drugs of the NRTI class are in the experimental phase, such as racivir (an enantiomer of emtricitabine), elvucitabine (Phase

II clinical trial), and amdoxovir (AMDX or DAPD). For these drugs, current data about the adverse effects are insufficient to characterize their impact on lipid metabolism^[174-178].

NNRTIs

Etravirine (ETR, Intelence®) is a drug from the second generation of NNRTIs and shows efficacy, safety and good tolerability in HIV-1 patients^[179]. One of the primary advantages of etravirine is as a replacement for other NNRTIs to which the HIV-1 virus is resistant, mainly due to the presence of the K103N and Y181C mutations in the case of efavirenz and nevirapine, respectively. The FDA approved the drug in 2008 for use in patients with multiple drug resistance. However, the drug is a substrate and an inhibitor of different CYP3A4 enzymes, which in turn are contraindicated in the use with antimicrobial and anticonvulsant drugs metabolized by the CYP450 system. In patients receiving HAART who have alterations in lipid metabolism, the switch to a therapy containing etravirine has shown satisfactory results and a reversal of dyslipidemia^[179-182]. Rilpivirine (Edurant®) is a second-generation NNRTI class drug. It is more potent than diarylpyrimidine (DAPY), and adverse effects are considerably reduced compared to older NNRTIs such as efavirenz. After clinical trials, rilpivirine was approved by the FDA in 2011, and its use is often combined with emtricitabine and tenofovir. Rilpivirine produces few changes in TC, LDL, HDL and TG in HIV-1 patients. In comparison to treatment with efavirenz, this drug promotes an increase in lipids and in the TC:HDL ratio, which is characterized by an increased risk of cardiovascular diseases in these patients^[183,184]. MK-1439 is a new and effective drug against a variety of HIV-1 mutants that are resistant to NNRTIs^[185]. Preclinical studies (Phase I clinical trial) that are currently in progress show that this drug has a good pharmacokinetic profile with the possibility of a low concentration daily dose needed to obtain an optimal effect. Additionally, it has good absorption, low potential for toxicity and the ability to be used with other antiretroviral agents. MK-1439 showed good results in cases where the K103N mutation of HIV-1 led to resistance against nevirapine and efavirenz, as well as in the presence of the Y1818C mutation, which leads to a lower susceptibility in treatment with nevirapine, rilpivirine and etravirine. *In vitro* data suggest that MK-1439 has beneficial properties that warrant additional development as a new antiviral drug; however, no data are available about its potential impact on lipid metabolism^[185-187]. New drugs of the third generation of NNRTIs are in various experimental stages such as BILR 355 BS (Phase IIa), (+)-Calanolide A (Phase I), GSK 2248761 (Phase IIb), MK-4965 (Phase I), MK-6186 (Phase I), RDEA806 (Phase IIa), and UK-453061 (Phase IIb). These new drugs have not been approved by the FDA and still require different clinical trials prior to their release as drugs available for the treatment of HIV-1 infection, and they currently have

no scientific information regarding their possible effects on lipid metabolism.

Fusion/entry inhibitors

The HIV-1 envelope glycoprotein (Env) complex, which is composed of three receptor-binding gp120 subunits and three fusion protein gp41 subunits, mediates virus entry by fusing viral and cellular membranes and offers an attractive target for developing antiviral agents^[188]. In succession with enfuvirtide/T20, a number of design strategies have been applied to develop new peptide-based fusion inhibitors with improved stability, bioavailability and potency^[188,189]. There are several drug classes that are in two experimental phases. Albuviride (FB006M), T649, T2634, T2544, T1249, SC34EK, and SC29EK are in the class of fusion inhibitors. BMS 663068, BMS 626529, vicriviroc (SCH 417690), and cenicriviroc (TAK-652, TBR-652) are in the class of entry inhibitors. These and other drugs are in experimental stages or have been suspended, and there are no initial and/or conclusive data about their potential toxic effects and the impact on lipid metabolism.

InSTIs

Cobicistat (GS-9350) is a new InSTIs drug recently approved by the FDA (2012). This drug, similar to ritonavir, has the ability to inhibit hepatic enzymes that metabolize other drugs used to treat HIV-1 infection, such as raltegravir^[190]. Cobicistat has become increasingly important, and its use has been associated with elvitegravir, permitting it to have higher blood concentrations with the use of smaller doses, which theoretically allows for greater suppression of viral replication when used with elvitegravir, and with fewer adverse effects. Cobicistat has been employed in combination with elvitegravir/emtricitabine/tenofovir (Stribild®)^[190,191]. Cobicistat is a potent inhibitor of CYP3A enzymes that concurrently affect administered medications metabolized by this pathway. It also inhibits intestinal transport proteins, increasing the overall absorption of several drugs including atazanavir, darunavir, and tenofovir alafenamide fumarate. Phase III trials of the cobicistat-containing combination antiretroviral therapy regimens in ART-naïve patients have shown a small elevation of serum fasting lipid, with a relative increase in the levels of TC and TG, in addition to bilirubin elevations, jaundice, nausea and diarrhea^[190-192]. Other drugs of the InSTI class are experimental, such as MK2048. MK-2048 represents a prototype second-generation InSTIs developed with the goal of retaining activity against viruses containing mutations associated with resistance to first-generation InSTIs (raltegravir and elvitegravir)^[193]. It is a drug that acts by inhibiting the integrase enzyme four times longer and shows superior efficacy to raltegravir. Additionally, it is being investigated for use as part of a pre-exposure prophylaxis^[193,194]. BI 224436 is the first non-catalytic site integrase inhibitor. It inhibits HIV

replication *via* binding to a conserved allosteric pocket of the HIV integrase enzyme. This makes the drug distinct in its mechanism of action compared to raltegravir and elvitegravir, which bind at the catalytic site^[195,196]. Another experimental drug is GSK744 (S/GSK1265744, Cabotegravir®), which has a structure similar to that of carbamoyl pyridone and dolutegravir. In investigational studies, the therapeutic agent has been packaged into nanoparticles (GSK744LAP), which confer an exceptionally long half-life of 21–50 d following a single dose. In theory, this would make suppression of HIV possible when dosing as infrequently as once every three months. These drugs do not have sufficient data on their toxicity profile and/or on lipid metabolism; however, they have been previously considered to have low metabolic toxicity^[197,198].

DIET AND LIFESTYLE

Changes in diet and lifestyle, and the adequacy of a hypocaloric diet, are recommendations that seek to reduce the concentrations of TC and its fractions, especially LDL^[199-201]. These changes bring benefits over short periods of time and reduce the risk for cardiovascular and atherosclerotic diseases. These recommendations are addressed to the entire population, as well as HIV-1 infected patients, and are measures that should be applied to delay the need for lipid-lowering drugs, even before the treatment of dyslipidemia is needed^[199-202]. Changes in diet can directly alter the levels of circulating LDL including saturated fats, cholesterol, and trans-unsaturated fats. The biggest impact comes from saturated fats, which are generally those that have a solid state at room temperature or under refrigeration. The major sources of saturated fats are meat and meat products (poultry, pork, beef, lard, and sausages), dairy (milk and cheeses), and vegetable oils (derived from palm or coconut). For an adequate daily diet, the recommended consumption is equal or < 7% of saturated fats, for the total daily caloric intake. Dietary cholesterol is exclusively found in animal products such as meats (particularly organ meats and tissues such as brain, kidney, and liver), egg yolks, and dairy products^[203,204]. It is recommended to keep dietary cholesterol consumption to < 200 mg/d. Trans fats and unsaturated fats are found in breads and cookies, doughnuts, stick margarine, and fried foods. This type of fat is added to foods to enhance the substance or texture of the product, to replace some of the animal fats, and even to increase the shelf life of certain products. The recommendation is to keep the consumption of trans fats as low as possible. The amount of trans fat is not included in the < 7% of calories/day allowed from saturated fats^[203-205]. The consumption of unsaturated fats is preferred; sources include fish such as salmon, mackerel, tuna, and vegetables such as avocado, olives and olive oil and vegetable oils^[206]. Other foods that are recommended for their maintenance and/or lipid-lowering effects are the omega-3 fats, which are

polyunsaturated fats that can lower TG levels. Omega-3 fats are frequently referred to as fish oils because the most common sources are fatty fishes such as salmon, tuna, mackerel, and halibut. However, they are also found in krill and flax seed oil. The current recommendation is that 25%-35% of daily calories can come from fat sources, including saturated fats, which should be < 7%^[206]. In addition, physical activity improves cardiorespiratory function, promotes the reduction of LDL and TG, and decreases insulin resistance (in both uninfected and HIV-1 patients)^[207,208]. Physical exercise is effective in reducing TC and TG, reducing total fat mass and increasing muscle mass in HIV-1 infected patients with hypertriglyceridemia^[45,119,208]. Additionally, physical exercise is associated with greater cardiovascular fitness, improved muscle strength and endurance, and the reduction of depression and anxiety. In addition, exercise lessens problems resulting from lipodystrophy (dyslipidemia, insulin resistance, and osteoporosis) and cardiovascular disease^[208-210]. However, there are several factors that can directly influence the reduction of metabolic disorders observed in seropositive patients. The common observation of gastrointestinal diseases in patients in advanced stages of infection may offset the positive effects of a balanced dietary regimen^[209,210].

PERSPECTIVES

The advances in antiretroviral therapy are clear, and practical results are observed in clinical practice where HIV-1 infected patients enjoy a better quality of life and a higher rate of survival, something unthinkable upon the discovery of HIV/AIDS in the early 1980s. New challenges for curing HIV-1 infection continue, and different approaches are the focus of several studies. The development of vaccines, the use of cell therapy, and the continuous development of new drugs that are more effective and have fewer side effects are obstacles that persist. Recently, approaches that target the intracellular trafficking of viral proteins and post-translational modifications of viral proteins have been considered as promising new treatments. Knowledge of the intracellular trafficking of viral proteins and the role of the polyprotein Gag of HIV-1 suggests that this process, once locked, would change the viral replication cycle by preventing formation of mature forms of the virus. Therefore, inhibitors could block viral maturation by interrupting the final stage of processing the Gag protein or by inhibiting intermolecular bond to the capsid protein immature.

This immature form, when connected to a new host cell, would suffer a disruption of the protein structure by the action of potent intracellular factors that restrict the subsequent phase of viral replication^[211-213]. Additionally, viral PIs for HIV-1 that block viral maturation have become a therapeutic target. In addition to the maturation inhibitors that inhibit the formation of viral capsids, another issue of interest is that the cells themselves have intrinsic antiviral factors that may

inhibit or restrict viral replication. One of the major families of cell restriction factors is tripartite motif 5 (TRIM5) composed of proteins that block retroviral infection, represented by two distinct forms of TRIM: TRIM5, which is expressed in most primates^[214,215], and TRIM-Cyp, which is expressed in monkeys^[216]. Both recognize the viral capsid but by different routes. The TRIM5 proteins are trimeric structures bind to one or two sites on the surface of the viral capsid and prevent the accumulation of reverse transcriptase. However, in late stages of viral replication, blocking is observed under some conditions of viral restriction^[217]. The TRIM5α is associated with an accelerated degree of dissociation of the viral capsid, suggesting that this protein and its cofactors destabilize the structure of the capsid^[218,219].

Other therapeutic factors that restrict the cellular antiviral protein APOBEC (apolipoprotein B mRNA-editing catalytic polypeptide) are the group of cytidine deaminases, which include APOBEC1 (A1), AID, APOBEC2 (A2), a subgroup of APOBEC3 (A3) proteins in humans and recently a protein, APOBEC4 (A4), expressed in some humans. These proteins have been presented as intracellular antiviral factors capable of blocking viral replication^[220,221]. The function of the A3 gene remains unknown, but it has been reported that human A3G has the ability to block viral replication^[222]. Similarly, A3G, A3B and A3F are also able to inhibit viral replication of HIV-1 and of other viruses, such as simian immunodeficiency virus and Hepatitis B virus^[221-223]. Additionally, the tetherin protein, originally described as BST-2 (CD137/HM1.24), was identified as a new surface marker of malignant B cells and characterized as an antiviral intrinsic factor with the ability to restrict the exit of viral capsids from the membrane surface^[224,225]. The same protein was also recognized as a target of the Vpu protein of HIV-1, a potential antagonist against tetherin^[226,227]. Further studies on TRIM5, tetherin and APOBEC proteins, as well as potential inhibitors of the viral capsid maturation acting on the Gag polyprotein are necessary; however, the information obtained so far allow us to suggest that understanding the intracellular trafficking of viral proteins and mechanisms for post-translational modification of viral proteins could turn out to elucidate the complex replication cycle of HIV-1 from HIV-1 fusion in the host cell until the final stage of release of mature, infectious viral particles.

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Therapeutic and prevention strategies against human enterovirus 71 infection

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Abstract

Human enterovirus 71 (HEV71) is the cause of hand, foot and mouth disease and associated neurological complications in children under five years of age. There has been an increase in HEV71 epidemic activity throughout the Asia-Pacific region in the past decade, and it is predicted to replace poliovirus as the extant neurotropic enterovirus of highest global public health significance. To date there is no effective antiviral treatment and no vaccine is available to prevent HEV71 infection. The increase in prevalence, virulence and

geographic spread of HEV71 infection over the past decade provides increasing incentive for the development of new therapeutic and prevention strategies against this emerging viral infection. The current review focuses on the potential, advantages and disadvantages of these strategies. Since the explosion of outbreaks leading to large epidemics in China, research in natural therapeutic products has identified several groups of compounds with anti-HEV71 activities. Concurrently, the search for effective synthetic antivirals has produced promising results. Other therapeutic strategies including immunotherapy and the use of oligonucleotides have also been explored. A sound prevention strategy is crucial in order to control the spread of HEV71. To this end the ultimate goal is the rapid development, regulatory approval and widespread implementation of a safe and effective vaccine. The various forms of HEV71 vaccine designs are highlighted in this review. Given the rapid progress of research in this area, eradication of the virus is likely to be achieved.

Key words: Human enterovirus 71; Infection; Therapy; Prevention; Drugs; Vaccine

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Core tip: This review focuses on therapeutic and prevention strategies for the control of human enterovirus 71 infection. Therapeutic strategies highlighted include natural products, synthetic antivirals, immunotherapy, and the use of oligonucleotides. Prevention strategies such as surveillance, physical prevention, and vaccine development form the second part of the review.

Kok CC. Therapeutic and prevention strategies against human enterovirus 71 infection. *World J Virol* 2015; 4(2): 78-95 Available from: URL: <http://www.wjgnet.com/2220-3249/full/v4/i2/78.htm> DOI: <http://dx.doi.org/10.5501/wjv.v4.i2.78>

INTRODUCTION

Human enterovirus 71 (HEV71) is a member of the human enterovirus A species within the genus *Enterovirus* of the family *Picornaviridae*. It is a positive-stranded RNA virus of approximately 7500 nucleotides. The viral genome contains an open reading frame (ORF) encoding a polyprotein of 2194 amino acids. The ORF is divided into three regions: P1 encodes four structural proteins (VP1-VP4); P2 (2A-2C) and P3 (3A-3D) encode seven non-structural proteins. The ORF is flanked by 5' and 3' untranslated regions. A poly-A tail of variable length is covalently attached to the 3' terminus of the genome^[1].

Since its discovery in 1969, HEV71 has been identified as the cause of epidemics of hand-foot-and-mouth disease (HFMD) associated with severe neurological complications, including aseptic meningitis, brainstem encephalitis, acute flaccid paralysis and neurogenic pulmonary oedema, in children under five years of age^[1]. There has been a large increase in HEV71 epidemic activity throughout the Asia-Pacific region since 1997. A large epidemic occurred in Taiwan in 1998, with 1.3×10^5 cases of HFMD, 405 cases of severe neurological disease and 78 fatalities attributed to HEV71 infection^[2-4]. In 1999, a large HFMD outbreak occurred in Perth, Western Australia, with approximately 6×10^3 cases reported and 29 cases of severe neurological disease identified^[5]. From 2008 to 2011, circulating HFMD outbreaks occurred throughout mainland China, increasing the annual number of HFMD cases from 488955 (126 deaths) to 1619706 (509 deaths)^[6]. In 2010, the largest recorded outbreak of HEV71-associated HFMD occurred in the country, comprising more than 1.7 million cases, including 27000 patients who exhibited severe neurological complications, and 905 deaths^[7]. Smaller epidemics have been detected in the United States and European countries, such as Austria, Germany, France, Norway, United Kingdom, Hungary and Greece^[8-14].

The reasons for the emergence of HEV71 as a cause of large epidemics of HFMD and acute neurological disease in the Asia-Pacific region remain elusive. Upon successful completion of the WHO-sponsored eradication of poliomyelitis, HEV71 will become the extant neurotropic enterovirus of highest global public health significance. However there are currently no effective clinical therapies or vaccine for HEV71 associated HFMD. Symptoms such as fever, encephalitis and meningitis are eased by supportive medication. In some cases viral infections are treated with broad-spectrum antiviral drugs, including Ribavirin, Ganciclovir, and Acyclovir^[15]. These common remedies only partially alleviate the symptoms instead of controlling the infections, and usually come with high cytotoxicity. Although ribavirin has been reported to inhibit virus production *in vivo*, a very high dose is used for treatment, which may raise safety concerns. Other than symptomatic treatment, intravenous immunoglobulin (IVIG) is clinically used to neutralise the virus and to non-specifically suppress

inflammation. Considering the morbidity and mortality caused by the disease, it is important to develop new specialised drugs and ultimately a safe and effective vaccine for the control of HEV71 infection. This review focuses on the efforts and progress towards development of effective therapeutic and prevention strategies.

THERAPEUTIC STRATEGIES

In recent years, significant amount of effort has been made to develop antiviral drugs for the treatment of HEV71 associated HFMD. Promising candidates have been identified through the screening of natural therapeutic products, repositioning of existing antiviral drugs, as well as the development of new synthetic compounds. Many of these drugs show anti-HEV71 activity *in vitro*, and some have been evaluated in animal models. However, clinical application of these drugs is not yet available.

Natural therapeutic products

Natural therapeutic products have been used in many countries in Asia for centuries, and have gradually been adopted by Western medical treatment and health care^[16,17]. The WHO estimates that approximately 80% of the global population still relies on traditional medicine for primary health care^[18]. As such, the search for new bioactive molecules in plants is still an active part of pharmaceutical research in many key therapeutic areas, including immunosuppression and infectious disease^[19]. Antiviral activities have been identified in several hundred natural compounds worldwide. Compared to synthetic pharmaceutical drugs, an advantage of natural molecules is the exclusion of extra chemical synthesis. This may reduce the cost of production, which is particularly attractive to affected patient population from low income countries.

Most natural therapeutic products work as a mixture, and thus it is difficult to characterise the detailed antiviral mechanisms and to further develop into effective clinical drugs. Up till recently, no single compound has been identified to potently inhibit HEV71. However, during the HFMD outbreaks in China, traditional Chinese medicines have demonstrated therapeutic efficacy by ameliorating the symptoms of the disease and/or shortening the course of the disease^[20]. Most of the herbs with reported therapeutic effectiveness have been used traditionally or folklorically for inflammatory and/or infectious diseases. As disease outbreaks become more common in China, a significant increase of research in this area followed. Table 1 compares natural therapeutic products that have been well studied.

Hydrolysable ellagitannins: The most widely published natural molecules in association with HEV71 infection are ellagitannins, from the family of hydrolysable tannins. Ellagitannins are characterised by the presence of one or more hexahydroxydiphenoyl

Table 1 Natural therapeutic products tested for anti-human enterovirus 71 activity

Natural product (Group)	Tested	Possible mechanism	Advantages	Disadvantages	Ref.
Hydrolysable Ellagitannins	<i>in vitro/in vivo</i>	Inhibit viral absorption/penetration	No obvious side effects	Weak oral activity	[21-30]
Flavonoids	<i>in vitro</i>	Inhibit viral RNA/protein synthesis	Low escape mutants	Mechanism not clear	[18,31-35]
Alkaloids	<i>in vitro/in vivo</i>	Inhibit protein synthesis	No obvious side effects	Mechanism not clear	[36-38]
Deferoxamine	<i>in vitro/in vivo</i>	Upregulation of B cells	Previous US FDA approval for treatment of iron overload	N/A	[39,40]

N/A: Not available; US FDA : United States Food and Drug Administration.

(HHDP) unit(s) on a glucopyranose core. The HHDP group is biosynthetically formed through intramolecular, oxidative C-C bond formation between neighboring galloyl groups in galloylglucoses^[21]. They are easily hydrolysed, either enzymatically or with acid, to liberate a stable ellagic acid as the dilactone form of hexahydroxydiphenic acid. Hydrolysable ellagitannins have previously shown medicinal values and antiviral effects^[22-25].

Treatment with hydrolysable ellagitannins such as corilagin^[26], geraniin^[27], punicalagin^[25] and chebulagic acid^[28] enhanced the survival of HEV71-infected cells *in vitro* with low cytotoxicity. Further, geraniin, punicalagin and chebulagic acid was shown to greatly prolong the survival time and reduce mortality of HEV71-infected mice. Virus replication in the muscle of treated mice was shown to be significantly inhibited. In general, treatment did not cause any obvious side effects in the mice and full recovery was observed after two weeks. The antiviral mechanism of chebulagic acid against herpes simplex virus-1 (HSV-1) was previously published^[22]. It was found to block interactions between cell surface glycosaminoglycans and HSV-1 glycoproteins, and could prevent binding, entry, and cell-to-cell spread, as well as secondary infection. Based on these observations, it is possible that chebulagic acid activity against HEV71 is related to the inhibition of viral absorption and/or entry. Further studies are required to elucidate the anti-HEV71 mechanism of hydrolysable ellagitannins, but results thus far suggest that they constitute a potential source for antiviral discovery, particularly in the field of HEV71 infection. Interestingly another hydrolysable tannin, punicalin, did not demonstrate obvious antiviral efficacy. This prompted the suggestion of key structural requirements for anti-HEV71 activity^[28]. Although the *in vitro* antiviral activity of corilagin seemed promising, oral administration of corilagin was not shown to induce significant biological activity^[29,30]. On the contrary, intraperitoneally administered geraniin, punicalagin and chebulagic acid demonstrated good inhibitory effects on HEV71^[25,27,28]. This may have been due to the difficulty in the absorption and metabolism of corilagin by intestinal microflora. The incubation of tannins with anaerobic microflora in faeces of animal led to the hydrolysis of the compound into metabolites including gallic acid and ellagic acid^[30]. To circumvent this problem, *in vivo* studies using intravenous or

intraperitoneal administration may be required.

Flavonoids: Another group of compounds commonly tested for anti-HEV71 activity are the flavonoids. Flavonoids are a broad class of low molecular weight secondary metabolites that are present in all vascular plants. The flavonoid structure is usually characterised by a C6-C3-C6 carbon skeleton^[31]. These phenolic compounds are known to be responsible for the bioactivities of plant crude extracts to confer protection against UV radiation, pathogens, and herbivores^[32]. Their relatively low toxicity and strong bioactive potential to increase human health prompted many studies in the field of pharmaceutical drug development.

Chrysosplenetin and penduletin^[33], 7-hydroxyisoflavone^[34], chrysin and its phosphate ester^[18], epigenin and its analog luteoline^[35], are flavonoids that have all been shown to exhibit *in vitro* anti-HEV71 activity. Experimental evidence indicated that these compounds could inhibit viral RNA and protein synthesis. To understand the mechanism of action, Zhu *et al*^[33] attempted to select chrysosplenetin- and penduletin-resistant HEV71 through continuous passage in the presence of the compounds. However, after 13 passages, HEV71 remained sensitive to the compounds. Although the mechanism of action is still unclear, time-of-addition studies suggested that flavonoids function in post virus-attachment, during the early stages of virus infection^[33-35].

Alkaloids: Alkaloids have also been shown to possess anti-HEV71 activities. Liu *et al*^[36] found that lycorine, one of the most abundant alkaloids of Amaryllidaceae, inhibited HEV71 replication in cultured cells, and lycorine treatment significantly enhanced the survival rate of HEV71-infected mice. Further investigation suggested that the drug inhibits the elongation of viral polyprotein during protein synthesis, and may lead to imbalanced synthesis of viral proteins and interrupted packaging of the virus. Matrine, a quinolizidine alkaloid, is one of the main active components of the root of Chinese *Sophora* herb plants^[37]. It proved effective in reducing the mortality rate of HEV71-infected mice^[38]. Treatment with matrine delayed the appearance of paralysis, reduced the clinical scores and prevented other symptoms of the infected mice compared with that of the placebo. Virus replication in mouse muscle tissues was significantly decreased and no obvious side effects

Table 2 Synthetic antiviral compounds tested for anti- human enterovirus 71 activity

Synthetic antivirals	Tested	Mechanism	Advantages	Disadvantages	Ref.
Pre-infection					
Pleconaril	<i>In vivo</i>	Prevents attachment by binding to viral capsid	High oral availability	Varied capacity of inhibition	[47-49]
BPROZ	<i>In vitro</i>	Prevents attachment by binding to viral capsid	High oral availability	Resistant mutants	[49-54]
Soluble and anti-SCARB2/PSGL-1	<i>In vitro</i>	Prevents attachment	N/A	N/A	[55-57]
Lactoferrin	<i>In vitro / in vivo</i>	Prevents entry by binding to VP1/ cellular receptor	No obvious side effects (animal)	Mechanism not clear	[58-62]
Suramin	<i>In vitro</i>	Prevents attachment	May inhibit other multiple stages of HEV71 life cycle	Mechanism not clear	[63]
Peptides (SP40)	<i>In vitro</i>	Prevents attachment by binding to glycosaminoglycans	Small size, high activity/specificity, low toxicity	Low bioavailability	[64-66]
Post-infection					
Rupintrivir	<i>In vitro / in vivo</i>	Inhibits viral 3C protein	Low quantity, low toxicity, high barrier for drug resistance	Lack efficacy in natural infection	[67,68]
DTrip-22	<i>In vitro</i>	Inhibits viral 3D polymerase activity	Broad spectrum activity	N/A	[69]
Aurintricarboxylic acid	<i>In vitro</i>	Inhibits viral 3D polymerase activity	N/A	N/A	[76-81]
NITD008	<i>In vitro / in vivo</i>	Inhibits viral 3D polymerase activity	More potent than ribavirin in vivo	May have toxicity issue, resistant mutants	[82,83]
Sorafenib	<i>In vitro</i>	Block virus induced activation of ERK/p38 signalling pathways	Licensed for cancer treatment	N/A	[84,85]

N/A: Not available.

were observed.

Deferoxamine: Besides plants, marine microorganisms are also a major source for natural products^[39]. Deferoxamine (DFO), a marine natural product derived from *Streptomyces pilosus*, was found to compensate for the decreased levels of B cells caused by HEV71 infection in mice, and to improve the levels of the neutralising antibodies against the virus^[40]. The clinical symptoms, muscle damage and mortality were ameliorated by DFO treatment. Interestingly DFO did not significantly inhibit viral replication in Rhabdomyosarcoma (RD) cells. In contrast, viral replication in the muscle tissues of DFO-treated mice was slightly inhibited. These results suggested that the possible mechanism of DFO activity against HEV71 in infected mice was through the upregulation of B cells, and not the direct inhibition of HEV71.

Other natural products: Other natural products shown to exhibit antiviral activity against HEV71 include *Glycyrrhiza* spp. and its active component glycyrrhizic acid^[20,41], *Fructus gardenia* and its primary component geniposide^[42], chlorogenic acid^[43], the *Ganoderma lucidum* triterpenoids, Lanosta-7,9(11),24-trien-3-one,15;26-dihydroxy and Ganoderic acid Y^[15], and hederasaponin B^[44]. Whilst the *in vitro* results of these compounds looked promising, *in vivo* studies were not performed.

Synthetic antiviral compounds

A growing body of literature on synthetic anti-HEV71

drug development has been published in recent years, but most of these drugs are still in the early phase of development and need further optimisation of their pharmacokinetics and absorption, distribution, metabolism, excretion, and toxicity profiles. Ribavirin, a wide spectrum synthetic antiviral, was reported to reduce mortality caused by HEV71 in Institute for Cancer Research (ICR) mice^[45]. However, the dosage used was much higher than the clinical recommended dosage prescribed to adults with Hepatitis C Virus (HCV) infection. Given that most HEV71 infections affect children younger than 5 years old, high dose of ribavirin may raise serious safety concerns.

The life cycle of HEV71 generally involves virus attachment, uncoating and entry, polyprotein translation and cleavage, viral RNA replication, and virus assembly. These critical steps are currently considered targets for synthetic antiviral development. Lead compounds that inhibit virus attachment, uncoating and entry are being actively pursued and may be used as potential prophylactic against HEV71, whereas inhibitors of post-infection stages may be suitable for treatment. Both pre- and post-infection inhibitors of HEV71 are discussed in detail below and further summarised in Table 2.

Pre-infection inhibitors: The most widely studied chemical structures amongst capsid binding molecules as antiviral agents for HEV71 are the series of "WIN" compounds^[46]. Pleconaril (WIN 61893) was the first of a new generation of metabolically stable capsid

function inhibitors. In a mouse model of infection following intracranial inoculation of enteroviruses, pleconaril reduced viral titres in all affected organs and prevented death in animals. Furthermore, there was high oral bioavailability in humans and other animals^[47,48]. However, the HEV71 inhibition capacity of pleconaril could vary for different isolates of the virus. It was nearly ineffective in neutralising HEV71 isolates from the outbreak in Taiwan^[49]. Using pleconaril as a template for computational drug design, a Taiwanese group succeeded in discovering a new class of pyridyl imidazolidinones with anti-HEV71 activity. A series of imidazolidinone derivatives, designated BPROZ (e.g., BPROZ-194, BPROZ-103 and BPROZ-074), demonstrated effectiveness against HEV71 infection^[49-54]. Their therapeutic potential is still under active investigation.

The soluble form of HEV71 receptors, SCARB2 and PSGL-1, has been shown to block virus-host interaction^[55,56]. It was proposed that these soluble receptors could act as molecular decoys of cell-associated receptors^[57]. Antibodies against these receptors have also been shown to inhibit *in vitro* virus infection^[55,56]. However, further studies are required to determine the potential of these molecules as therapeutic antivirals. In their study, Weng *et al.*^[58] demonstrated that lactoferrin (LF) inhibited HEV71 infection *in vitro* and *in vivo* by binding to the VP1 protein of HEV71, as well as to host cells. The anti-HEV71 mechanism of LF is unclear, but may relate to the prevention of viral entry by blocking cellular receptors and/or by direct binding to the virus particles, as suggested by the above finding. Binding of LF to several different cell ligands such as heparan sulfate, chondroitin sulphate and nucleolin has been reported^[59-61]. However, antiviral activity of LF analogues is only partly related to their affinity for heparin sulfate^[62]. Although lactoferrin has not been approved for therapeutic purposes, it could be considered an agent for preventing virus entry. Another group of researchers screened a library of compounds and identified suramin as having the ability to inhibit HEV71 proliferation by blocking the attachment of HEV71 to host cells, as well as affect other steps of the HEV71 life cycle^[63].

Peptides have also been used as therapeutic agents to block viral attachment or entry into host cells. A major advantage is their small size and their high activity and specificity when compared to antibodies and other larger molecules. Peptides accumulate in lesser quantity in tissues, and have very low cell toxicity when compared to synthetic molecules^[64]. A 15-mer peptide spanning from position 118 to 132 in the VP1 capsid region, SP40, exhibited antiviral activity in all three genotypes of HEV71 (genotypes A, B and C), coxsackievirus A16 (CVA16) and poliovirus Mahoney (PV1)^[65]. It also reduced viral induced CPE and viral RNA synthesis in Vero, HeLa and HT-29 cell lines in a dose-dependent manner. Data from further research suggested that the SP40 peptide could have interacted with cell surface glycosaminoglycans and prevented

HEV71 attachment. A major disadvantage of peptides is their low bioavailability due to their rapid degradation in the gastrointestinal system. To circumvent this issue, new formulations such as the D-isomer peptide^[64], addition of N-terminal pyroglutamate and C-terminal homoserine lactone to the peptide, are being developed to improve the resistance to peptidase^[66].

Attachment and entry inhibitors stop the virus from entering cells, and therefore may be useful as prophylactic agents. However, a major obstacle of this approach is for it to be cost-effective for resource-limited countries where large outbreaks frequently occur. Furthermore, the effectiveness of the drug itself would be highly dependent on the timing of the treatment provided. It is a challenge to deliver a sufficient amount of the inhibitor to the targeted site early enough to prevent disease progression, or to prevent the spread of infection to others.

Post-infection inhibitors: Various synthetic antiviral compounds were designed to target post-entry stages of the HEV71 life-cycle. The anti-HEV71 activity of rupintrivir, an irreversible peptidomimetic inhibitor of viral 3C protein, has been evaluated in a mouse model^[67]. Complete protection against HEV71-induced cell death was observed at low nanomolar concentrations, with very little cell toxicity. Consistent with the symptoms, a significant decline in viral RNA was witnessed in intestine, lung, muscle, brain stem, and cardiac muscle when rupintrivir was administered *in vivo*. Rupintrivir also significantly improved the integrity of limb muscle structure and suppressed the expression of VP1 in infected mouse muscle. Another potential clinical advantage is the high barrier for emergence of drug resistance, as tested by the researchers^[67]. However, it is worth noting that a previous clinical trial for rupintrivir for the treatment of human rhinovirus infection was halted due to a lack of efficacy in natural infection studies^[68].

Several compounds were found to inhibit the 3D polymerase. DTriP-22, a piperazine-containing pyrazolo [3,4-d] pyrimidine derivative, was shown to inhibit HEV71 RNA accumulation during virus infection, but not IRES-driven translation^[69]. It may interfere with 3D activity by obstructing the nucleoside triphosphate entry cavity of 3D polymerase but not by incorporation into the growing RNA chains. This compound is considered novel because most other polymerase inhibitors that exhibit anti-enterovirus activity are nucleoside analogues^[70-75]. DTriP-22 has a broad spectrum activity against RNA viruses, including different genotypes of HEV71, coxsackieviruses A and B, and echovirus 9^[69]. Aurintricarboxylic acid (ATA), a polyanionic compound originally reported to be an inhibitor for the replication of HIV, HCV and SARS-CoV^[76-80], also exhibited the ability to inhibit HEV71 3D polymerase^[81]. Results showed that ATA slows down viral RNA synthesis at early stages after a single round of viral replication in HEV71-infected cells. However, ATA did not inhibit the activity of HEV71 viral

2A/3C protease activity. A nucleoside analog, NITD008, has been reported to selectively inhibit viruses within the family *Flaviviridae*^[82]. Although NITD008 showed efficacy in a dengue mouse model, it was not further developed due to the adverse findings observed in a preclinical toxicity study^[83]. Deng *et al*^[83] reported that NITD008 potently inhibits HEV71 in cell culture and in a mouse model, and demonstrated the feasibility that this compound could potentially be developed for HEV71 therapy, if the toxicity issue is resolved. Their data further showed that mutations in viral 3A and 3D polymerase regions could confer resistance against NITD008, suggesting an intimate crosstalk between 3A and 3D during viral replication.

Sorafenib, previously known as BAY 43-9006 and marketed commercially as Nexavar, is a multi-target tyrosine and serine-threonine kinase inhibitor currently used in cancer therapy^[84]. A significant reduction of infectious HEV71 titres and viral RNA was observed in infected cells when sorafenib was added 1 and 3 h post-infection. However, no difference was seen compared to non-treated cells when sorafenib was added 2 h pre-infection and during virus adsorption. Experimental data indicated that sorafenib treatment was able to block the HEV71 mediated CPE through blocking of virus induced activation of the ERK and p38 signaling pathways. A previous study has shown that HEV71 infection induced cyclooxygenase-2 (COX-2)/prostaglandins (PG) E₂ expression *via* mitogen-activated protein kinases (MAPKs) including ERK and p38, and further that inhibition of HEV71-induced COX-2/PGE₂ expression may reduce CNS inflammation^[85]. Thus it was proposed that sorafenib treatment may alleviate HEV71-induced inflammatory responses^[84]. Further *in vivo* studies are required to validate the effectiveness of the drug.

Other therapeutic strategies

Immunoglobulin: A number of animal studies have shown that neutralising antibodies stimulated by immunisation with inactivated virus, virus-like proteins, or VP1 subunit vaccines, are cross-protective against heterologous strains of HEV71 and can passively protect mice and monkeys (see section on vaccine development). Further, studies on patients have indicated that HEV71 infection is cleared by humoral immunity, and clinical trials have shown the presence of neutralising antibodies in the serum of immunised healthy adults and children^[86-88]. The significant involvement of neutralising antibody responses in the control of HEV71 infection in humans would render IVIG treatment an ideal complimentary therapeutic agent. In fact since the year 2000, IVIG has been used in China as the last resort for treatment of severe cases of HEV71 infection, with some measure of success^[89].

However, treatment of patients with IVIG has its disadvantages. Besides the risk of transmitting human pathogens using pooled human sera, necessitating screening and treatment, it also requires donor availability. Other disadvantages include batch to batch

variability, and the presence in the serum of virus specific but non-neutralising antibodies^[90]. A phenomenon termed antibody-dependent enhancement (ADE) was recently confirmed in experimental and clinical settings^[91,92], in which sub-neutralising concentration of antibodies was evidenced to enhance HEV71 infection in Fc receptor-bearing human monocytes and contributed to exacerbation of HEV71 infection in mice. The wide existence of cross reactivity between enterovirus antibodies may also become the underlying risk for HEV71 ADE infections.

A solution would be to exploit future passive immunotherapy based on monoclonal antibodies (mAb) produced in cell culture. They offer a selective advantage over pooled human sera that are more commonly used in IVIG treatment by reducing the risks mentioned above. Based on the success of a United States Food and Drug Administration (FDA) approved humanised mAb for respiratory syncytial virus infection of the lower respiratory tract^[93], a similar approach was taken to develop neutralising anti-HEV71 mAb for the treatment of severe HFMD caused by HEV71^[89]. Using previously identified peptides containing amino acids of the VP1 region known to be potent in eliciting neutralising antibody^[94,95], a mAb (clone 22A12) with strong neutralising activity against HEV71 in an *in vitro* neutralisation assay was successfully generated. Because clone 22A12 is a murine antibody, further work for the chimerisation and/or humanisation of the antibody is currently underway to reduce human anti-mouse antibody response for therapeutic application. Another group of researchers generated and characterised several mAbs by immunising mice with purified HEV71 virus, strain Henan2^[96]. They identified a mAb, clone 4E8, with strong neutralising activity against HEV71 and that specifically reacted with synthetic peptides containing amino acids 240-250 and 250-260 of VP1 by Enzyme-Linked Immunosorbent Assay (ELISA) assay. Clone 4E8 partially protected mice against the lethal challenge of HEV71 strain Henan2. Kiener *et al*^[90] succeeded in isolating a novel mAb against HEV71 that targets a conformational neutralisation epitope outside of VP1. The mAb 10D3 targets the highly conserved "knob" region of VP3. The protective efficacy of mAb 10D3 was evaluated and verified by an animal challenge experiment using a lethal dose of HEV71. All mice prophylactically treated with mAb 10D3 survived the lethal challenge without showing any disease symptoms.

Several factors have to be considered when using mAbs instead of polyclonal serum. First, due to the antigenic variability of circulating strains, the mAb must cross-neutralise all existing subtypes to be useful. Second, there is a risk of escape mutations, which may be circumvented by administering two or more antiviral mAbs against non-overlapping epitopes. A combination of synergistic mAbs may also reduce the required dosage^[97,98].

The use of non-human immunoglobulins in the

treatment of HEV71 infection has also been investigated. Immunoglobulin Y (IgY) antibodies are the predominant serum immunoglobulin in birds, reptiles, and amphibians, and are transferred from serum to egg yolk in the females to confer passive immunity to their embryos and neonates^[99]. The potential of orally administered IgY for the prevention and treatment of many pathogens has been widely reported^[100-103]. It was found that chicken as bio-factory can produce a higher yield of IgY antibodies compared to the production of IgG in mammals. In HEV71-infected ICR mice, a survival rate of 98.3% was achieved when the challenged mice were given intraperitoneal injection 1 to 3 d post-infection for 3 consecutive days with a purified IgY antibody at neutralisation titre of 128 or more^[104]. Oral administration at a higher dose also conferred protection to infected mice. The study suggested that IgY in the form of an egg-yolk-added drink, yolk powder tablet, or capsule, can potentially be used to prevent the early infection of HEV71.

Adoptive transfer of macrophage: The adoptive transfer or activation of macrophages has been used in the immunotherapy of cancer, liver ischemia, reperfusion injury and pneumonia^[105-108]. Liu *et al*^[109] showed that the adoptive transfer of macrophage cells from adult mice can partly protect young mice from lethal HEV71 infection. The macrophages displayed anti-HEV71 activity *in vitro* and could alleviate the pathology of infected mice, possibly by engulfing the virus directly through phagocytosis. The application of macrophages in antiviral therapy *via* adoptive transfer is a novel proposal. Unlike human macrophage, murine macrophage can be easily obtained either from the peritoneal cavity or grown from bone marrow precursor cells. Technology for the isolation or growth of large scale human macrophage is still unavailable. Future studies using activated macrophage derived from peripheral blood monocytes of adults were proposed.

Interferons: The effectiveness of interferons (IFNs) in the treatment of HEV71 infection has been studied with contradictory findings. Liu *et al*^[110] demonstrated that early treatment of HEV71-infected newborn mice with a recombinant murine IFN- α resulted in an increased survival rate. However another study demonstrated that HEV71 2A^{pro} could be an IFN antagonist, because it reduces the expression level of the type I IFN receptor^[111], making it questionable whether type I IFN will be active against HEV71 infection. There are about 20 different human type I IFNs identified to date^[112]. Although they are highly homologous in amino acid sequence and share the same receptors, the biological effect of each IFN is apparently different. It has been shown that the anti-HEV71 activities of various IFN subtypes differ from each other^[113]. Based on their antiviral activities, they can be divided into three subgroups: IFNs with high anti-HEV71 activities at low

concentrations, IFNs with moderate anti-HEV71 activity at high concentrations, and IFNs with nearly no antiviral activities. Hung *et al*^[114] showed that the 3C^{pro} of HEV71 was able to cleave IRF9, a host protein involved in the signaling cascade triggered by type I IFN. They found that HEV71 could be effectively inhibited by a combination of IFN- α and a 3C^{pro} inhibitor such as rupintrivir.

All-trans-retinoic-acid: Most HEV71-infected children present with vitamin A (VA) deficiency, which is associated with decreased immunity and more severe pathogenic conditions^[115]. It was shown that serum IFN- α levels were markedly reduced and positively related to the lack of VA in HEV71-infected children. The active VA metabolite, all-trans-retinoic acid (ATRA), is the natural ligand for the retinoic acid receptors (RAR). In various *in vitro* systems, ATRA has been shown to regulate the expression of a number of IFN-stimulated genes, including retinoid-induced gene I (RIG-I), a pattern recognition receptor involved in the innate immune response of the host^[116-118]. It was proposed that the inhibition of RIG-I-mediated type I IFN responses may contribute to the pathogenesis of HEV71 infection^[119]. Chen *et al*^[120] demonstrated that ATRA is a potent IFN inducer that effectively inhibits HEV71 and significantly regulates the RIG-I signalling pathway in the human monocytic cell line. They proposed that the antiviral effect of ATRA occurred through a RAR- α pathway, and further suggested that ATRA may directly contribute to anti-HEV71 infection by reinforcing innate immunity.

Oligonucleotides: Previous reports have described the antiviral effects of RNA-based therapeutics, such as siRNA, shRNA and miRNA, targeting the VP1, 3D, 2C genes, or the 3' UTR of the HEV71 genome, resulting in antiviral activity^[121-128]. However, whilst plasmid-derived shRNAs are widely used for laboratory studies, they are not suitable for antiviral therapy. Further, the limitations of RNAs are short half-life and the requirement of a delivery agent that may be toxic to the host. There is currently no approved marketed siRNA drug. On the contrary, the use of antisense oligodeoxynucleotide (ASODN) technology to inhibit pathogen replication has shown promising results. Since the United States FDA approved the first antisense drug, Fomivirsen, for the treatment of cytomegalovirus (CMV) retinitis in 1998, more than 30 types of ASODNs have been evaluated in clinical trials^[129].

Unmodified oligonucleotides are highly unstable *in vivo* due to rapid nuclease digestion. In order to circumvent this problem, a number of chemically modified oligonucleotides such as classic phosphorothioate oligonucleotides, phosphorodiamidate morpholino oligomers, locked nucleic acids, and gene-silencing oligonucleotides have been developed^[130].

Liu *et al*^[131] designed and tested 5 antisense

phosphorothioate oligonucleotides targeting the 5'-terminal conserved sequence found in HEV71 RNA. One of the oligonucleotides, EV5, effectively inhibited HEV71 amplification both *in vitro* and *in vivo* in a sequence-specific and dose-dependent manner. It was also capable of providing effective protection to HEV71-infected mice and inhibited virus replication in the lungs, intestines, muscle, but not brain, of infected mice. Tan *et al*^[132] tested 3 octoguanidium dendrimer conjugated-morpholino oligomers (vivo-MOs) that are complementary to the HEV71 IRES (vivo-MO-1 and -2) and 3D polymerase (vivo-MO-3). Vivo-MO-1 and -2 showed significantly reduced plaque numbers, viral RNA copies, and viral capsid expression in RD cells in a dose-dependent manner. In contrast, vivo-MO-3 exhibited less antiviral activity. Both vivo-MO-1 and 2 remained active when administered within 4 h before or 6 h after HEV71 infection. Resistant mutants arose after serial passages in the presence of vivo-MO-1, but not vivo-MO-2. Thus vivo-MO-2 was proposed to be a favourable candidate for further development as an antiviral agent.

PREVENTION STRATEGIES

HEV71 is highly contagious and can be isolated from throat swabs, rectal swabs, and stool specimens of sick children. Virus shedding can persist for nearly 4-5 wk in the respiratory tract and through faeces^[133,134]. As a result, HEV71 transmission may occur not only through direct contact with infected people, but also contact with respiratory secretions or faeces of an infected person. The virus can subsequently spread from one person to another through the faecal-oral route by contaminated hands or objects^[135], rapidly causing outbreaks. Due to the long periods of viral shedding in children, HEV71 is frequently transmitted in families, kindergartens, and schools^[136]. Therefore to successfully control the devastating outcome of HEV71 epidemics, prevention of infection remains the top priority.

Surveillance

Until a vaccine becomes available, the best way to prevent HEV71 infection is through infection control practices such as hand-washing, disinfection and social distancing during epidemics^[137]. Early intervention can lessen the spread of the virus. For these actions to be effective, adequate clinical and laboratory surveillance of HEV71 activity and identity in the community is essential to provide early warning of impending epidemics. As such many countries in the Asia-Pacific region, including Japan, Malaysia, Singapore, Taiwan, Vietnam and China, have implemented heightened surveillance for HEV71^[138-142]. HFMD has now become a notifiable disease in many countries in the region. However, since other enteroviruses such as CVA8, CVA10, and CVA16 can also cause HFMD, concurrent virological surveillance may provide invaluable molecular epidemiological data to help track the spread of the virus across the

region^[143]. In some instances surveillance programs have provided information that resulted in early control of HEV71 epidemics and reduced the total number of cases of acute neurological disease^[144].

Physical prevention

Transmission of the viruses responsible for HFMD, including HEV71 and CVA16, is mainly through the faecal-oral route. Therefore the first line of defense is to contain the disease causing agent. Infected children are quarantined and non-infected children are also kept from crowds. During the 2000 outbreak in Singapore, spread of viruses was prevalent in child-care centres. One of the measures taken to break the chain of transmission was a 2-wk nationwide closure of preschool centres^[145]. However, it was suggested that even though such controls may decrease the peak incidence of disease, the outbreak may be prolonged, and therefore the overall number of cases may not be lowered^[143].

Health education plays an important role to inform and educate parents about the virus infection and prevention strategies. It should focus on observance of good personal hygiene, and cleaning and disinfection of premises and articles. Alcohols are widely used as active ingredients in many hand disinfectants. However, their effectiveness is largely dependent on the type and concentration used. A recent study showed that 95% ethanol instead of 70%-95% isopropanol has the most virucidal activity against HEV71, but did not result in complete inactivation of HEV71^[146]. Further, high concentration of ethanol may cause skin irritation and a decrease in antibacterial activity. New formulations are needed for routine use to prevent the spread of enteroviruses.

Vaccine development

Similarities between HEV71 and poliovirus in many virological and clinical aspects have strongly suggested that a vaccine strategy, similar to that against poliovirus infection, could be effectively adopted to control HEV71 infection. Because it mainly threatens the children in developing countries, an ideal HEV71 vaccine would have to be inexpensive, safe, convenient to administer, and acceptable to parents. In addition, a successful vaccine strain would also provide cross-protection to different HEV71 genotypes.

Live-attenuated vaccine: Based on the similarities between PV and HEV71, Arita *et al*^[147] developed a HEV71 attenuated strain carrying mutations in the 5'- and 3'-untranslated regions and 3D polymerase, based on the temperature-sensitive determinants of poliovirus Sabin 1 vaccine strain. The EV71 (S1-3') strain, which belongs to HEV71 genotype A, was characterised by attenuated neurovirulence and limited spread of virus. In a subsequent study, cynomolgus monkeys inoculated with EV71 (S1-3') *via* the intravenous

route had a mild neurological symptom in the form of tremor, but survived lethal challenge by virulent HEV71 (BrCr-TR) without exacerbation of the symptom^[148]. The immunised monkey sera demonstrated a broad spectrum of cross-genotype neutralising activity, including genotypes A, B1, B4, C2, and C4. Although EV71 (S1-3') demonstrated promise as a live attenuated vaccine against HEV71, the vaccine itself was not completely attenuated, as evidenced by mild neurological symptoms and isolation of virus from the spinal cord.

Due to the lack of proof-reading activity by enteroviral 3D polymerase, a high incidence of error leading to random mutations occur during replication. This phenomenon makes it easier for the reversion of mutants to wild-type virus. To overcome this issue, researchers have explored the possibility of replacement or deletion of bigger fragments. Replacement of the PV internal ribosome entry site (IRES), with that of a non-neurotropic human rhinovirus (HRV), was found to stably attenuate PV in animal models^[149,150]. In HEV71, it was shown that deletion of stem-loop domain Z within the 3'-untranslated region attenuates the growth of a HEV71-HRV2-IRES chimera in neuroblastoma cells^[151]. Another strategy employed to generate stably attenuated vaccine strains is to increase the replication fidelity of the 3D polymerase. Mutations at amino acid positions G64R and S264L in the HEV71 3D polymerase have recently been shown to increase replication fidelity and the genetic stability of the HEV71 genome by greater than ten-fold during growth in cell culture^[152]. Further, the HEV71 3D-G64R and 3D-S264L mutant virus populations were attenuated in a mouse model of HEV71 infection^[153].

Inactivated vaccine: In response to the Bulgarian outbreak in 1975, a formalin-inactivated HEV71 vaccine was developed, but was not used to control the epidemic^[154]. However since then, the value of inactivated vaccine for the effective control of HEV71 has been shown by various researchers. Suckling mice immunised with the adjuvant-carrying formaldehyde-inactivated mouse-adapted HEV71 vaccine were effectively protected from lethal virus challenge and disease^[155]. Another experimentally inactivated vaccine produced using the FY-23K-B strain of HEV71 was capable of inducing an immune response and offered protection to rhesus monkeys against future virus attacks^[156]. Additionally, passive transfer of serum from formalin-inactivated and heat-inactivated virus vaccine immunised adult mice, could provide protection against HEV71 challenge in neonatal mice^[157,158]. The efficacy of this model of maternal vaccination-neonatal challenge is consistent with the results of other similar studies using maternal vaccination to protect offspring from infectious disease^[159-162]. Bek *et al*^[159] provided the first demonstration of cross-genotype protective efficacy of a candidate HEV71 vaccine which

suggested that inactivated vaccines may confer broad protection against HEV71 infection. On the other hand, another study showed that HEV71 type specificity of neutralisation was unidirectional^[163]. The antisera used against newly emerging subgenogroups could cross-neutralise their ancestor subgenogroups, but not vice versa. Chen *et al*^[164] demonstrated that co-immunisation of a formaldehyde-inactivated HEV71 vaccine with a commercial pentavalent vaccine that contained inactivated polio vaccine, did not interfere in antibody production nor protective efficacy of the HEV71 vaccine. This indicates that the two vaccines are compatible after co-immunisation, and that formaldehyde-inactivated HEV71 vaccine may be used in designing multivalent vaccines.

Due to their inability to replicate, inactivated HEV71 vaccines are favoured over the live attenuated vaccines for safety reasons. However, the manufacturing costs of inactivated vaccines and potential supply problems cause substantial difficulties in practical implementation, particularly in developing countries. Further, viruses are sensitive to chemical treatment and neutralising epitopes could be destroyed during inactivation, as it is reported in formalin inactivated C4D HEV71 vaccine strain^[165]. Nevertheless, research and development of HEV71 inactivated vaccines have progressed further than the other types of HEV71 vaccines, with some currently in phase III clinical trial^[166].

Subunit vaccine: Like all enterovirus the antigenic diversity of HEV71 is caused by variations within capsid proteins VP1, VP2 and VP3, but the VP1 protein displays a number of important neutralising epitopes^[157,167,168]. Key neutralising antibody determinants have been found in the N-terminal half of VP1 when tested with high titre human neutralising antibodies^[169,170]. The potential safety advantage of subunit vaccines over conventional whole virus vaccines has prompted researchers to query whether the VP1 subunit of HEV71 is sufficient to provoke adequate protective immunity against viral infection. Different delivery systems have been tested for their suitability in expressing the VP1 and to stimulate immune response. They include recombinant VP1 protein expressed in *Escherichia coli* BL21^[157], recombinant Newcastle disease virus capsid displaying VP1^[171], and VP1 expressed in yeast *Pichia pastoris*^[172]. All induced high levels of neutralising antibodies.

The mucosal immune system serves as the first line of defense against HEV71 as it initiates disease following implantation in the gut mucosa^[173]. Thus an oral vaccine for immunisation against HEV71 has its advantages over injected vaccines. Oral subunit vaccines stimulate production of mucosal antibodies more effectively than is the usual case with injected vaccines^[174]. Oral administration is also widely accepted in children who need a HEV71 vaccine. The use of attenuated *Salmonella* as a vector for the VP1 subunit demonstrated the advantages of oral vaccine vectors^[175].

Yu *et al*^[176] showed that VP1-expressing *Bifidobacterium longum*, a gastrointestinal probiotic, can confer protection from the mother to neonatal mice, suggesting the potential of this recombinant *B. longum* as an oral vaccine against HEV71 infection. Transgenic plants and animals are possible alternatives to prokaryotic and eukaryotic vectors. They offer a palatable oral delivery system that can elicit a good mucosal immune response as well as systemic humoral and cellular immune responses, making it particularly suitable for protecting against infectious agents intruding *via* the mucosal surface^[177,178]. In one study, transgenic tomato fruit expressing the VP1 subunit was developed as a free-feeding oral vaccine^[179]. Serum from immunised mice was able to neutralise the infection of HEV71 in RD cells. In another study, the bovine α -lactalbumin promoter and α S1-casein signal peptide sequence were fused with the VP1 cDNA to generate transgenic mice with mammary gland-specific VP1 expression^[180]. Expression of the HEV71 VP1 capsid protein was shown to be highly specific to the mammary gland and was secreted in the milk of transgenic mice, reaching satisfactory expression level for oral vaccine development and is much higher than that achieved in bacterial or transgenic plant system^[157,158,179,181,182].

Gastric acid and enzymatic digestion are major concerns for oral vaccines because they may interfere with vaccine conformation and absorption. Moreover, it is difficult to determine the precise dose of antigens for immunisation, since competition with food and microbial antigens interferes with the absorption rate of vaccine components. Many strategies have been employed to improve oral vaccine delivery, including the use of tissue-specific promoters, mucosal immune adjuvant, liposomes, and N-trimethyl chitosan nanoparticles^[173,174]. New strategies are necessary to achieve a high level of expression of VP1 protein in the correct antigen conformation. If these prototypes can be refined to yield similar immunogenicity levels as inactivated vaccines, they could become strong preventive options.

Synthetic peptide: Epitope-based vaccination using synthetic peptides is another area under intense investigation for the delivery of precise vaccine components to the immune system. A series of overlapping synthetic peptides spanning the VP1 capsid protein of HEV71 was used to immunise BALB/c mice in order to identify neutralising linear epitopes^[94]. Peptides containing amino acids 163–177 and 208–222 of the VP1 were capable of eliciting neutralising antibodies against HEV71. Additionally, mouse antisera raised against the peptide 208–222, designated SP70, demonstrated *in vivo* passive protective efficacy in BALB/c mice^[183]. Hydrophobic profile assays showed that this highly conserved sequence is located within the major hydrophilic regions and is expected to be exposed at the surface of the protein, hence making it a promising and attractive candidate for

synthetic peptide-based HEV71 vaccine^[94]. Further, the amino acid sequence represented by SP70 was totally conserved amongst 25 HEV71 strains from subgenogroups A, B1–B5 and C1–C4, which suggested possible cross-protection against infectivity of all HEV71 strains. A different delivery approach for the synthetic peptide was explored using adenovirus (Ad) vectors^[184]. Compared to the recombinant GST-fused SP70 protein, immunisation with the Ads containing SP70 elicited higher SP70-specific IgG titres, higher neutralisation titres, and conferred more effective protection to neonatal mice.

Nevertheless, mouse antisera raised against HEV71 whole virions provide higher *in vivo* passive protection to suckling mice against lethal HEV71 challenge when compared with the anti-SP70 antisera, possibly due to higher titres of neutralising antibodies elicited by several neutralising epitopes located on the virus other than that represented by the synthetic peptide SP70 alone. Further, the short epitopes can easily change to avoid antibody mediated neutralisation. To circumvent this issue, 6 peptides without cross-reactivity were selected and combined into three vaccine candidates and applied in further evaluation in neonatal mice^[185]. The Vac6 comprising the peptides of P70–159, P140–249, P324–443 and P746–876 of the structural proteins could provide effective protection on pups against virus infection.

Virus-like particles: Another method of vaccine development is the construction of virus-like particles (VLPs). The baculovirus expression system is the most widely used platform for generating VLPs. To assemble the HEV71 VLPs, the P1 polyprotein needs to be cleaved by viral protease 3CD into individual structural proteins. Hu *et al*^[186] developed VLPs by co-expressing the P1 and 3CD regions of HEV71 in the pFastBac™ Dual vector, which contains two strong baculovirus promoters, polyhedron (PPH) and p10 (Pp10). The P1 region was controlled by a strong baculovirus promoter, PPH, whilst the protease 3CD was controlled by weak promoters such as CMV promoter or baculovirus IE1 promoter. The expressed 3CD successfully cleaved P1 *in vitro* and *in vivo*. Also, the co-infection in insect cells resulted in crystalline virus-like particle structures morphologically resembling the authentic HEV71 aggregates. A patent for these recombinant baculoviruses has been applied for in Taiwan, the United States and mainland China^[166].

In a study using monkeys, Lin *et al*^[187] found that VLPs and formalin-inactivated vaccines generated comparable amount of HEV71-binding antibodies measured by ELISA, and induced memory T and B cell responses. However, monkeys immunised with inactivated HEV71 virus showed relatively greater neutralisation titre, proliferation, and cytokine production than those immunised with VLPs. This may be partially due to the conformation difference between VLPs and viral particles, which was not detected under the

Table 3 Comparison of human enterovirus 71 vaccine strategies

Vaccines	Tested	Advantages	Disadvantages	Ref.
Live-attenuated vaccine	<i>In vitro / in vivo</i>	Broad spectrum, low cost	Incomplete attenuation	[143-149]
Inactivated vaccine	<i>In vitro / in vivo / clinical trial</i>	Inability to replicate	High cost	[150-162]
Subunit vaccine	<i>In vitro / in vivo</i>	Safe to use	Low immunogenicity	[153,154,163-178]
Synthetic peptides	<i>In vitro / in vivo</i>	Small and safe to use	Low immunogenicity, escape mutants	[94,179-181]
Virus-like particles	<i>In vitro / in vivo</i>	Safe to use	Unstable, need purification, high cost	[162,182-185]
DNA vaccine	<i>In vitro / in vivo</i>	Most resemble native virus, fast production, low cost, can be manipulated	Low neutralising effect	[153,186-190]

assays performed. Even though immunisation with VLPs has less of a response than inactivated vaccine, nevertheless they provide a safer method for preventing viral infection with regards to clinical treatment.

The main problem associated with VLPs is their stability, purification and cost. At present, the VLPs are mostly developed using insect cells and the strict culture conditions limit the required large scale of vaccine production. Thus, transgenic plants or yeast that can produce VLPs to be delivered by either oral administration or injection may prove to be promising platforms. Recently Li *et al*^[188] coexpressed the P1 and 3CD regions in *Saccharomyces cerevisiae* to yield VLPs. The *S. cerevisiae* system is a low cost platform and it is easy to scale-up production. As a eukaryotic expression system, it benefits from the processes of protein expression, folding, and modification, which are lacking in prokaryotic expression systems. Compared to the insect cell expression systems, the use of stable yeast transformants avoid the generation of initial large quantities of recombinant baculoviruses. In laboratory conditions, however, the yield of *S. cerevisiae*-derived VLPs was not sufficient for clinical use. However the use of fermentation engineering and automation control, which have been used for the production of other types of VLPs^[189], may overcome this issue. The patent for *S. cerevisiae* production of VLPs has been applied for in China^[166].

DNA vaccine: DNA immunisation offers many advantages over the traditional forms of vaccination. It is able to induce the expression of antigens that resemble native viral epitopes more closely than standard vaccines do, since live attenuated and inactivated vaccines are often altered in their protein structure and antigenicity. Plasmid vectors can be constructed and produced quickly, at relatively lower cost, and the coding sequence can be manipulated in many ways. Further, DNA vaccines encoding several antigens or proteins can be delivered to the host in a single dose at low quantity to induce immune responses. They are also very temperature stable making storage and transport much easier.

Tung *et al*^[190] developed a HEV71 DNA vaccine by inserting the VP1 gene into a eukaryotic expression vector and evaluated the immune response in mice.

They showed that whilst anti-VP1 IgG level was increased in immunised mice, the level declined after boosting immunisation. Further, although the anti-VP1 IgG exhibited neutralising activity against HEV71, the neutralising effect of the sera of mice immunised with the VP1 DNA vaccine was much lower than that of HEV71-infected human serum. Another DNA vaccine was developed by inserting the entire VP1 gene into plasmid pcDNA3^[157]. Intramuscular administration elicited a high and stable level of neutralisation titre in both ICR and BALB/c mice, which could be detected post-immunisation. However, it induced a weaker immune stimulation compared to whole virus particles.

Various strategies to increase the immune stimulation ability of DNA vaccines have been explored. Amongst these are the incorporation of immunostimulatory sequences in the backbone of the plasmid, co-expression of stimulatory molecules, use of localisation/secretory signals, and an appropriate delivery system, as well as adjuvants and optimisation of transgene expression^[191-194]. While therapeutic and prophylactic DNA vaccine clinical trials are underway for a variety of infectious diseases and cancers, the scientific basis of DNA vaccines has yet to be clearly defined. If DNA vaccines pass all scientific and regulatory scrutiny, they promise to be products of the next generation. A comparison of DNA vaccine with other vaccine strategies is shown in Table 3.

CONCLUSION

During the past decade, HFMD and associated neurological complications caused by HEV71 infection have resulted in the loss of many paediatric lives in the Asia-Pacific region. Whilst a significant amount of research have been published in the field of HEV71 antivirals and vaccine development lately, an effective therapeutic and/or prevention strategy is still elusive. Various groups of natural compounds have demonstrated anti-HEV71 activities. However more work is needed to characterise the detailed antiviral mechanisms and to further develop into effective clinical drugs. The use of synthetic antiviral compounds in clinical setting has been hampered by potential adverse effects to the host and emergence of drug resistance mutants. New strategies such as computer-aided drug

design, screening of licensed drugs against HEV71 infection, and combination therapy targeting different replication steps of HEV71, may play an important role in antiviral drug development.

The recent identification of HEV71 receptors SCARB2 and PSGL-1 will enable the development of humanised transgenic mice for testing of antivirals and vaccines. Vaccine candidates in the form of inactivated HEV71 have progressed into clinical trials and look most promising. However, the unit cost of inactivated HEV71 vaccines is likely to be high, restricting their usefulness in resource-limited countries in Southeast Asia. By contrast, self-propagating live attenuated vaccines can be produced at much lower unit cost and are thus likely to be more cost-effective for use in vaccine prevention programs in developing countries and in regional and global control strategies. However, in order for this potential to be realised, it will be necessary to design a HEV71 vaccine in which attenuation is fully defined and which possesses a demonstrably higher stability and safety profile than the oral polio vaccine. Together with a good surveillance program, these strategies will hopefully lead to the containment and eradication of HEV71.

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Viral hepatitis and human immunodeficiency virus co-infections in Asia

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and human immunodeficiency virus (HIV) affect many people in Asian countries, although there are geographic differences. Both HBV and HIV (HBV/HIV) and HCV/HIV co-infections are highly prevalent in Asia. Hetero- and homosexual, injection drug use, and geographic area are strong predictors of HBV, HCV, and HIV serostatus. In HBV endemic regions, the prevalence and genotype distribution of HBV/HIV co-infection is almost comparable with that in the general population. In Japan, where HBV has low endemicity, the prevalence of HBV/HIV co-infection is approximately 10-fold higher than that in the general population, and HBV Ae is the most common subgenotype among HIV infected individuals. Highly active antiretroviral therapy (HAART) is an effective treatment for HIV/Acquired Immune Deficiency Syndrome. Lamivudine, a component of HAART, is an effective treatment for HBV, HIV, and HBV/HIV co-infection; however, cost, emerging drug resistance, antiretroviral-associated liver toxicity and liver-related morbidity due to HCV progression are particular concerns. HCV/HIV co-infection may accelerate the clinical progression of both HCV and HIV. The high prevalence of HBV/HIV and HCV/HIV co-infections in Asia underscores the need to improve prevention and control measures, as fewer evidence-based prevention strategies are available (compared with Western countries). In this review, the most recent publications on the prevalence of HBV/HIV and HCV/HIV co-infections and related issues, such as therapy and problems in Asia, are updated and summarized.

Key words: Hepatitis B virus; Hepatitis C virus; Co-infection; Human immunodeficiency virus; Prevalence; Asia; Pathogenicity; Natural history; Problems; Drug resistance

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Abstract

Hepatitis B virus (HBV), hepatitis C virus (HCV),

Core tip: Hepatitis B virus (HBV) and hepatitis C virus (HCV), infections are common among human

immunodeficiency virus (HIV) positive individuals due to similar blood-borne transmission routes. Highly active antiretroviral therapy is an effective treatment for HIV/acquired immune deficiency syndrome; however, emerging drug-resistant viruses and drug-induced hepatotoxicity are particular concerns. The high prevalence of HBV/HIV and HCV/HIV co-infections in Asia highlights the need to improve prevention and control measures because, unlike in Western countries, few evidence-based prevention strategies are available. Here, we review the epidemiologically and clinically important aspects of HBV/HIV and HCV/HIV co-infections in Asian countries.

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INTRODUCTION

An estimated 240 million people worldwide are chronically infected with hepatitis B virus (HBV), and 170 and 34 million people are infected with HCV and human immunodeficiency virus (HIV), respectively^[1]. Although the prevalence of these viral infections varies according to geographic region, the majority of cases occur in developing Asian and African countries^[2,3]. Co-infection with HBV or HCV is common among HIV-positive patients due to their similar blood-borne transmission routes (e.g., sexual and perinatal for HBV^[4], and percutaneous for HCV).

HIV co-infection has a deleterious effect on the outcome of patients with chronic viral hepatitis and greatly complicates their management. HIV-positive individuals with chronic hepatitis (caused by either HBV or HCV) have greater liver mortality than those infected with HIV alone. Moreover, the highest reported mortality is among those with multiple hepatitis infections^[5]. Although the burden of HBV and HCV infection is greatest in Asia, the biological characteristics associated with either HBV or HCV infection among HIV-infected individuals are unclear. YMDD motif mutants in HBV and occult hepatitis virus infection were observed in Asia^[6] and these were found to be related to severe liver diseases and resistance to treatment and prevention^[7]. This article provides an overview of the epidemiology of hepatitis B and C virus infections among HIV-infected individuals in Asian countries.

HIV EPIDEMIOLOGY IN ASIA

In the early-to-mid 1980s, much of the world was dealing with serious HIV and acquired immune deficiency syndrome (AIDS) epidemics, although Asia remained relatively unaffected. However, by the early 1990s, AIDS epidemics had emerged in several Asian

countries and, by the end of the decade, had spread rapidly across the continent. Today, almost 5 million people in South, East, and Southeast Asia are infected with HIV. The epidemiology of the disease in different Asian countries is unique. In fact, the epidemiology can be different among areas/districts/provinces within the same country. By 2010, between 3.0 and 3.9 million people in Southeast Asia were living with HIV/AIDS, which was up from 3.3 million in 2009. Women account for 37% of those infected, the majority of whom were infected by a partner. However, the HIV epidemic in Southeast Asia is now declining: the number of new infections has fallen by 34% (from 320000 in 2001 to 210000 in 2010). According to the Joint United Nations Program on the global HIV/AIDS epidemic, 2011, Southeast Asia recorded above-average declines in the number of new HIV infections^[8]. The WHO Southeast Asia Region (SEAR) comprises 11 countries: Bangladesh, Bhutan, South Korea, India, Indonesia, the Maldives, Myanmar, Nepal, Sri Lanka, Thailand, and Timor-Leste. The region has a combined population of over 1.8 billion. Five countries (India, Indonesia, Myanmar, Nepal and Thailand) account for the majority (99%) of HIV cases. India has the second highest HIV burden of any country in the world. There are few reports of HIV cases in Korea, Bangladesh, Bhutan, the Maldives, Sri Lanka, or Timor-Leste, which together represent less than 1% of all HIV infections recorded in the SEAR. The annual number of new infections reported in four of the five countries with a high HIV burden (namely India, Myanmar, Nepal, and Thailand) is declining. However, the number of cases in Indonesia continues to rise, making the epidemic in this country one of the fastest growing in Asia^[9,10].

HBV/HIV CO-INFECTION IN ASIA

Epidemiology and risk factors

Approximately 240 million people are chronically infected with HBV, approximately 600000 of whom die each year of HBV-related diseases or hepatocellular carcinoma (HCC)^[11]. Although the global prevalence of HBV varies from region to region, approximately 5%-10% of HIV-infected individuals are chronically infected, which is defined as persistent detection of hepatitis B surface antigen (HBsAg) for more than 6 mo. In areas with a low HBV prevalence (< 2% of individuals are HBsAg-positive), such as Western countries and Japan, chronic HBV infection among HIV-positive individuals is approximately 10-fold higher than that in the general population^[12]. On the other hand, the prevalence of HBV infection in some parts of Africa and Asia is approximately 10%-15%, regardless of HIV co-infection^[13].

The major route of HBV infection, especially in HBV endemic regions, is mother-to-child transmission. In HBV endemic regions, the prevalence and genotype distribution of HBV in HIV-infected patients is comparable

Table 1 Prevalence, risk factors, and the main vital genotypes identified in hepatitis B virus/human immunodeficiency virus co-infected individuals in Asia

Country	Prevalence (%)	Risk factors	Main genotype	Ref.
Southeast Asia				
Indonesia	3.2	Sexual	B3	Anggorowati <i>et al</i> ^[15] , 2012
	15.3	IDU	B3	Utsumi <i>et al</i> ^[6] , 2013
	7.0	Men with IDU	B3, D1, B2, C1	Fibriani <i>et al</i> ^[16] , 2014
Myanmar	8.7	Homosexual men		Zaw SK <i>et al</i> ^[17] , 2013
Thailand	13.0	HB vaccination women		Aurpibul <i>et al</i> ^[18] , 2012
	3.3			Peters <i>et al</i> ^[19] , 2013
	11.9			Tsuchiya <i>et al</i> ^[20] , 2013
Vietnam	28.0	IDU	B4, B2, C1,	Dunford <i>et al</i> ^[21] , 2012
	15.2	CSW	C5	
	10.3			Sereno <i>et al</i> ^[22] , 2012
East Asia				
China	4.9	Older children ethnicity		Zhou <i>et al</i> ^[23] , 2010
	6.3	Sexual		He <i>et al</i> ^[24] , 2011
	7.2	Sexual		Maimaiti ^[25] , 2012
	6.1			Chen <i>et al</i> ^[26] , 2013
Japan	8.8	Homosexual men		Gatanaga <i>et al</i> ^[27] , 2007
	6.4	Homosexual men		Koike <i>et al</i> ^[28] , 2008
	7.9	Homosexual men	Ae	Fujisaki <i>et al</i> ^[29] , 2011
	6.0	Homosexual men		Yanagimoto <i>et al</i> ^[30] , 2012
South Asia				
India	9.0	Hetero sexual		Saravanan <i>et al</i> ^[31] , 2007
	11.3	Hetero sexual men		Saha <i>et al</i> ^[32] , 2013
	1.5		D > A > C	Saravanan <i>et al</i> ^[33] , 2014

IDU: Injecting drug user; CSW: Commercial sex worker.

with that in the general population^[6,14]. Table 1 shows the prevalence of HBsAg and lists the risk factors and major HBV genotypes identified in HIV-infected individuals in Asia. Japan is the only country with low endemicity of HBV infection in Asia^[7]; however, it is 10 times more prevalent among HIV-infected individuals. A higher prevalence of HBV/HIV co-infection compared with the prevalence of HBV infection alone is observed in Indonesia, Vietnam, and India. Interestingly, Ae (HBV/Ae), which originated in Europe and the United States, is the most common HBV genotype in HBV/HIV co-infected patients in Japan, even though HBV/B and HBV/C are indigenous^[34]. An individual infected with HBV/Ae (as opposed to other HBV genotypes) is at a higher risk of co-infection with HIV. Furthermore, HBV/Ae (which tends to be chronic)^[29] is detected almost exclusively in homosexual men^[28-30]. Indeed, in Myanmar, homosexual men carry the greatest risk of being co-infected with HBV and HIV^[17], and an increased prevalence of HBV/HIV infection is also observed in men with a history of IDU^[26]. A substantial number of HBV-infected individuals in Indonesia, Vietnam, Thailand, and India are also infected with HIV (15.3%, 28.0%, 13.0% and 11.3%, respectively)^[6,15,16,19,21,32,35]. Indonesia is currently experiencing an increasing HIV incidence and a high HBV burden^[6,15,17]; however, no HBV/HIV co-infection cases have been identified in commercial sex workers (CSW)^[35]. In Thailand, HBV/HIV co-infection is more common in HIV-infected adolescents who are negative for anti-HBV antibodies^[18]. HBV/HIV co-infection is also common in China, where sexual transmission is

an independent risk factor, followed by ethnicity and occupation^[24]. Additionally, the incidence of co-infection among HIV-infected children receiving antiretroviral therapy (ART) is high. Significant levels of co-infection by blood-borne viruses are observed among IDUs and CSWs in Vietnam^[21]. The main risk factor for HBV/HIV co-infection in India is heterosexual sex, whereas it is homosexual sex and IDU in other Asian countries.

Treatment and drug resistance

Several of the antiretroviral drugs used to treat HIV infection can also be used to treat HBV infection; therefore, they can be used to treat some co-infected patients^[36]. On the other hand, HBV/HIV co-infection may complicate the delivery of ART by increasing the risk of drug-related hepatotoxicity and impacting the selection of specific agents, such as drugs that are effective against both HBV and HIV^[37].

Lamivudine, a component of highly active antiretroviral therapy (HAART), is used widely because it is easy to obtain, relatively cheap, and its clinical efficacy has been shown in long-term follow-up studies^[36,38,39]. However, lamivudine often induces mutations within the RT domain of the viral polymerase region, resulting in multidrug-resistance and a poor prognosis for some HBV/HIV co-infected individuals. Table 2 shows the effects of ART on HBV/HIV co-infection and drug resistance in Asian countries. Because lamivudine is associated with the emergence of drug-resistant strains, the current recommended treatment for co-infected individuals is a tenofovir (TDF)-based regimen^[22]. Drug-

Table 2 Antiretroviral therapies for hepatitis B virus/human immunodeficiency virus co-infection, and drug-resistant mutations in Asian countries

	Indonesia	Japan	Thailand	Vietnam	China	India
Recommendation for ART on HBV/HIV co-infection	Initiate with WHO clinical stage IV and/or a CD4 count less than 200/mm ³ ^[40]	TDF + 3TC/FTC-based regimen	Screening for HBV. Two antiretroviral regimen with anti-HBV/anti-HIV activity (<i>e.g.</i> , TDF, 3TC)	TDF-based regimen	Combination of TDF with FTC or 3TC	HBsAg positivity is indicator to initiate ART with combination of two dually-active drugs
Currently used therapy for HBV/HIV co-infection	Telbivudine (LDT) Lamivudine (3TC) Zidovudine (ZDV) Nevirapine (NVP) Efavirenz (EFV) Stavudine (d4T) Tenofovir (TDF)	3TC Entecavir (ETV) TDF Adefovir dipivoxil (ADV) Emtricitabine (FTC)	3TC Nevirapine (NVP) EFV Stavudine (d4T)	Stavudine (d4T) 3TC NVP	d4T ZDV 3TC NVP EFV ^[41]	ZDV 3TC NVP EFV d4T ^[42]
Drug-resistant mutation	Lamivudine M204I M204I + L180M	Lamivudine ¹ V173L + L180M + M204V Lamivudine L180M, M204V, L217R M184V, I195M	Lamivudine M204V/I	Lamivudine L180M M204V		Lamivudine ¹ M204V L180M

¹Lamivudine naïve case. WHO: World Health Organization; HBV: Hepatitis B virus; HIV: Human immunodeficiency virus; FTC: Emtricitabine; TDF: Tenofovir; LDT: Telbivudine; ETV: Entecavir; ZDV: Zidovudine; ADV: Adefovir dipivoxil; NVP: Nevirapine.

resistant mutations, particularly multidrug-resistant mutations, are the major concern for patients receiving long-term therapy with nucleoside analogues, such as lamivudine^[7]. A high prevalence of lamivudine-resistant mutation has been reported in adolescents from Thailand (M204V/I)^[18], Vietnam (L180M and M204V)^[13], and Indonesia (M204I and M204I + L180M)^[6]. However, V173L + L180M + M204V was identified in HBV/HIV co-infected patients who were not treated with lamivudine^[29]; L217R, M184V, and I195M were identified in lamivudine-treated patients^[43] in Japan; and L180M and M204V were identified in ART-naïve patients in India^[32]. One study suggests that all patients should be screened for HBV prior to ART initiation, as this would enable the most appropriate regimen to be selected^[18]. Combination treatment with TDF plus either emtricitabine or lamivudine is recommended for patients who are co-infected with HBV/HIV^[25]. However, many countries/regions are experiencing difficulty in accessing effective drugs (even the drugs recommended in their own guidelines) due to the lack of availability and/or high cost. Moreover, some developing Asian countries/regions lack experienced hepatologists and HIV specialists who can manage HBV, HIV, and HBV/HIV co-infection effectively.

Antiretroviral-associated liver toxicity (due to use of nevirapine use) was reported in both Thailand and Indonesia, and in some cases, ART with lamivudine did not suppress HBV^[6,19]. In Vietnam, the precore mutation, G1896A, which is highly associated with HCC, is more common in HBV/HIV co-infected individuals harboring the HBV genotype B (HBV/B) than in those harboring HBV/C^[32]. HBV replication can occur in the absence of HBsAg [known as occult HBV (OHBV) infection]. OHBV has been reported in HIV-infected individuals, although the underlying mechanisms are unclear^[6,44]. The

majority of perinatally infected HIV-positive adolescents in Thailand do not produce HBV protective antibodies, even though hepatitis B vaccination coverage is high. Thus, the HBV seroprotection level is low despite childhood vaccination, suggesting that the populations at risk for HBV infection require a booster dose of an HBV vaccine^[18]. The current recommendation in Japan is that all newborn babies are vaccinated against hepatitis B.

HCV/HIV CO-INFECTION IN ASIA

Epidemiology

HCV and HIV are among the top ten leading causes of death due to infectious disease worldwide. HCV accounts for an estimated 170 million chronic infections and HIV accounts for approximately 34 million infections^[1]. These viruses, together with HBV, share transmission routes, although their prevalence differs according to the transmission efficiency and geographic region^[4].

The overall prevalence of HCV/HIV co-infection ranges from 1.2% to 98.5% in South and Southeast Asia^[45]. These estimates are influenced by several factors, including geographic differences in the prevalence of chronic infection in different age groups, transmission efficiency *via* certain routes, and the number of individuals at high risk for infection^[4]. The HCV transmission risk is significantly higher among patients who acquired HIV infection *via* the parenteral route rather than the sexual route. Although the sexual route is a common mode of HIV transmission, it is less common for HCV^[46]. Today approximately one-quarter of HIV-infected individuals in Europe and the USA are co-infected with HCV^[47]. HCV infection outbreaks have been reported in HIV-positive men who have sex with men (MSM) in North America, Europe, and Asia.

Transmission is believed to be the result of exposure to blood during sexual contact^[48]. HCV-RNA was detected in 38.2% of anti-HCV-negative samples in Indonesia^[10]. Sexual contact is still the main HIV transmission route in Indonesia, whereas HCV is less easily transmitted via the sexual route. Thus, among the heterosexual transmission history, the HCV-seropositive rate (26.9%) was significantly higher compared with the HCV infection rate alone, which may be due to the HCV transmission mechanisms between sexual partners, particularly those who engage in practices that are associated with a high virus transmission risk^[10]. Sulkovski *et al.*^[49] and others also reported HCV infection with sexual transmission between HIV-infected MSM and other individuals with abnormal sexual activities^[49]. The European AIDS Clinical Society guidelines on co-infection suggest that patients who are beginning HIV therapy should be serologically tested for HCV. It also suggests that HCV RNA-negative and anti-HCV antibody-negative patients exhibiting an unexplained increase in alanine transaminase levels, and those at high risk for HCV infection (e.g., IDU and those likely to suffer mucosal trauma during intercourse) should be tested annually thereafter^[46].

A study regarding HIV and HCV infection among those receiving methadone maintenance treatment (MMT) in clinics in Yunnan, China, showed that the prevalence of HIV seropositivity in rural and urban areas was 27.7% and 10.0%, respectively, and the prevalence of HCV seropositivity was 75.6% and 46%, respectively; however, the prevalence of HIV/HCV co-infection was 20% and 7%, respectively. Over three-quarters (76.2%) of the HIV-infected participants in this study were also infected with HCV^[50]. The majority of heroin is smuggled into Yunnan province from Myanmar. It then moves along drug trafficking routes to other areas of the country. Thus, high levels of illicit drug use and HIV and HCV epidemics are common in Yunnan^[51-53]. Urban and rural MMT patients know little about HIV and HCV; therefore, education programs in MMT clinics must be improved. Studies performed in Beijing, Henan, Guangxi, Kunming, Sichuan, Hunan, Xinjiang, and Shanxi reveal that the prevalence of HCV co-infection among HIV-infected individuals ranges from 11.6% to 85.0%, depending on the area surveyed. The primary transmission route favored by each of these viruses may explain these differences. The co-infection rates in IDUs (58.2%-91.6%) and commercial blood donors (15.8%-71.6%) are significantly higher than those in individuals who become co-infected *via* sex (5.3%-20.0%)^[54]. Another study from Central China shows that only 62.4% of HIV-infected individuals have anti-HCV antibodies^[26]. In Vietnam, 89.8%-98.5% of HIV-positive IDUs are infected with HCV. A study in southern India found that 18 (15%) and 10 (8.3%) out of 120 HIV-infected patients were also positive for HBsAg and anti-HCV antibodies, respectively. The study, which was carried out in a tertiary care center, also found that the most common transmission routes

were sexual promiscuity (79%), followed by sex with a positive spouse (15%), and a blood transfusion history (6%)^[55]. A cross-sectional study performed in Mazandaran province, Iran, from 2008 to 2010 showed that of 80 HIV-positive patients, only 33.8% were co-infected with HCV, whereas 25% were co-infected with both HBV and HCV. Thus, 58.8% of HIV-positive patients were also infected with HCV^[56]. A study of 33255 blood samples from in Kathmandu, Nepal, reported that the HIV seroprevalence was 0.19% and that 10.8% of the donors were co-infected with HCV^[57]. Additionally, the study found similar HIV seroprevalence rates between first-time and repeat donors, and between volunteer and replacement donors, indicating the need for more effective donor recruitment, education, and counseling strategies.

Natural history

HCV and HIV co-infection enhances liver damage and increases the risk of developing end-stage liver disease and HCC. From a clinical perspective, HCV/HIV co-infection is the most common cause of liver cirrhosis in these patients; therefore, monitoring and treatment of these infections must be prioritized, even though this is more difficult to achieve than in HCV-monoinfected individuals^[58,59]. A meta-analysis that examined the impact of HIV infection on HCV-infected individuals revealed that HCV/HIV co-infection was associated with a 6.14-fold increase in the relative risk for end-stage liver disease and a 2.07-fold increase in the relative risk for cirrhosis compared with HCV monoinfection^[60,61]. On the other hand, HCV/HIV co-infected patients respond less well to antiviral therapy with peginterferon + ribavirin (pegIFN + RBV), resulting in a lower sustained virological response (SVR) after antiviral therapy. HIV/HCV co-infected individuals develop end-stage liver disease more quickly than either HIV- or HCV-monoinfected patients^[26,55], particularly those receiving long-term immunosuppressive regimens. Thus, early HCV/HIV co-infection diagnosis may allow for prompt co-morbidity recognition of and prevent future complications.

Problems

Unsafe therapeutic injections performed by both professionals and non-professionals appear to be the predominant HCV transmission mode in countries/regions with moderate-to-high prevalence; indeed, such cases account for up to 40% of all HCV infections worldwide. The predominant transmission mode in most low prevalence areas is IDU^[4,62-64]. Although transfusion- and transplant-associated HCV infections are minimized by routine testing of donors, and preventing a new generation of young injectors from becoming infected with HIV or HCV is paramount^[65].

The introduction of HAART has meant that HCV infection is now considered the principal cause of morbidity and mortality among HIV-infected indi-

viduals^[10,49,66,67]. Liver-related morbidity occurs due to the acceleration of HCV-related disease, drug-induced hepatotoxicity, and, possibly, direct damage caused by HIV itself. Chronic viral hepatitis accounts for > 80% of liver-related deaths. End-stage liver disease and HCC are common complications in HIV-infected patients. Co-infection may accelerate clinical progression in both of these diseases, which are caused by HCV and HIV, and successful treatment for one disease is undermined if the other is neglected^[45]. The infectious diseases physicians that care for HIV-infected patients with advanced HCV-related liver disease need to know how to assess a patient for advanced fibrosis, when to refer a patient for endoscopic screening for varices, and when/how to enroll patients in an HCC screening program^[67]. Data from The TREAT Asia HIV Observational Database, a multi-center cohort of HIV patients in the Asia-Pacific region, showed that the impact of hepatitis co-infection on immunological and virological responses to ART, and on AIDS progression, are similar among Asian and Western populations^[68]. That said, the high prevalence of HIV/HCV co-infection in Asian countries underscores the need to implement improved prevention and control measures because, compared with Western nations, fewer evidence-based prevention strategies are available.

Medical management

Hepatitis C has a limited impact on HIV disease progression. However, HIV does affect HCV with regard to several important areas^[69]. The hepatitis C treatment goal is to eradicate HCV infection. Only then can complications associated with HCV-related liver disease, including HCC, be prevented. The therapy endpoint is the achievement of SVR, defined as undetectable HCV RNA levels in the serum 24 wk after the completion of antiviral therapy. SVR is associated with an improved outcome in terms of liver fibrosis and reduced liver-related morbidity and mortality^[59]. Similar results have been described in HIV/HCV co-infected patients^[59].

The availability of Direct Acting Antivirals (DAAs) for the treatment of patients infected with HCV genotype 1 has markedly improved SVR^[70]. The Asian Pacific Association for the Study of the Liver^[69] and other studies^[49,71] suggest that DAAs, either with or without pegIFN + RBV, show much higher HCV eradication rates in HCV/HIV co-infected individuals than conventional pegIFN + RBV, with manageable toxicity and pharmacologic interactions. However, the promise of new oral DAAs comes with a substantial up-front financial cost, particularly for poorer Asian countries. Indeed, the majority of HCV-infected patients in low- or middle-income countries remain untreated. The global rollout of ART for HIV shows us that it is possible to make these agents both widely available and affordable^[72]. Robust efforts to ensure equitable access to these advanced drugs for co-infected patients are imperative.

In the past, HIV-individuals were not considered candidates for solid organ transplantation due to concerns about a heightened risk of opportunistic infection and malignancy. However, recent single- and multi-center studies show that liver transplantation can be performed in HIV-infected patients who satisfy commonly accepted eligibility criteria, including an undetectable HIV RNA load in the plasma, current treatment with a stable HAART regimen or the ability to tolerate ART after transplantation, a minimum CD4+ T-cell count of 100-200 cells/mm³, and an absence of opportunistic infections^[66].

HBV/HCV/HIV TRIPLE INFECTION IN ASIA

Few reports are available regarding the prevalence of HIV/HBV/HCV triple infection in Asia. The prevalence of triple infection in Chinese IDUs (19.1%) is a little bit higher than that in Burmese IDUs (10.4%) in the China-Myanmar border region, which is an important transfer station for drug trafficking from the "Golden Triangle"^[53]. As we have seen in mainland Myanmar, triple infection is markedly low (0.35%)^[17]. The other studies in mainland China show triple infection were 3.3% in a cohort study, that was carried out between 2010-2012^[73] and 12.2% in central China^[26]. Therefore, the endemicity of triple infection greatly varies even within the (China) country. Triple infection was not detected in North India^[74], but it was manifested in 2.5% of subjects in South India^[56], and in 4.8% in Indonesia^[15].

HBV/HCV/HIV triple infection raised the chance of death, virological failure, and dropping out of care programs^[73]. The incidence of hepatic decompensation was higher in patients with triple infection than in those with HIV/HCV co-infection^[75].

CONCLUSION

Both HIV/HBV and HIV/HCV co-infection are highly prevalent in Asia. IDU, MSM, and geographic area are strong predictors for HBV, HCV, and HIV serostatus. Differences in the HBV and HCV co-infection rates among Asian countries may be due to the epidemiology of these viruses in specific countries/areas. Differences in the HBV/HIV co-infection rates among countries are more pronounced than the differences in the HCV/HIV co-infection rates. The success of HBV vaccination programs and the HBV endemicity in a particular country may also play a role. Unlike HBV infection, the prevalence of which has been reduced by vaccination, HCV (which is mostly transmitted through the blood) cannot be tackled effectively in developing countries unless people are educated about the transmission routes and the dangers of certain practices. A detailed analysis of the progression and activity of liver disease in HIV co-infected patients is needed, along with the urgent implementation of comprehensive prevention

strategies, such as community education and control programs, for both HBV/HIV and HIV/HCV co-infected individuals.

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Debunking the myths perpetuating low implementation of isoniazid preventive therapy amongst human immunodeficiency virus-infected persons

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Abstract

Isoniazid preventive therapy (IPT) is the administration of isoniazid (INH) to people with latent tuberculosis (TB) infection (LTBI) to prevent progression to active TB disease. Despite being life-saving for human immunodeficiency virus (HIV)-infected persons who do not have active TB, IPT is poorly implemented globally due to misconceptions shared by healthcare providers and policy makers. However, amongst HIV-infected patients especially those living in resource-limited settings with a high burden of TB, available evidence speaks for IPT: Among HIV-infected persons, active TB- the major contraindication to IPT, can be excluded with symptom screening; chest X-ray and tuberculin skin testing are unreliable and often lead to logistic delays resulting in increased numbers of people with LTBI progressing to active TB; the use of IPT has not been found to increase the risk of the development of INH mono-resistance; IPT is cost-effective and cheaper than the cost of treating cases of active TB that would develop without IPT; ART and IPT have an additive effect on the prevention of TB, and both are safe and beneficial even in children. In order to sustain the recorded gains from ART scale-up and to further reduce TB-related morbidity and mortality, more efforts are needed to scale-up IPT implementation globally.

Key words: Human immunodeficiency virus; Isoniazid preventive therapy; Tuberculosis; Chemoprophylaxis

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Core tip: To better inform healthcare providers, policy makers and human immunodeficiency virus-infected persons about isoniazid preventive therapy (IPT), this article summarizes the existing evidence in support

of IPT including recommendations for scale-up of implementation globally.

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INTRODUCTION

The human immunodeficiency virus (HIV) infection and tuberculosis (TB) have both remained significant global health challenges claiming millions of lives every year. Despite improved access to antiretroviral therapy (ART), the burden of TB among HIV-infected individuals has remained high. In 2012, the World Health Organization (WHO), reported an estimated 8.6 million TB cases and 1.3 million deaths from the disease (including 320000 deaths among HIV-positive people)^[1]. Majority of these deaths are preventable with the use of available evidence-based strategies.

Active TB disease can be prevented among HIV-infected individuals either by protecting them from being exposed to *Mycobacterium tuberculosis* (*M. tuberculosis*), the organism responsible for the disease or by preventing those already infected from progressing from latent infection to active disease. This is important because HIV-infected persons who are co-infected with latent TB are not likely to transmit TB to others nor develop drug resistant TB. Therefore, treatment of latent TB infection (LTBI) has the added benefit of reducing the incidence of resistant TB and thus contributing to the control of multi-drug resistant TB and extensively drug resistant TB.

A Cochrane review that included 12 trials with a total of 8578 randomized participants showed that preventive therapy with any anti-TB drug vs placebo was associated with a 32% lower incidence of active TB [Risk ratio (RR) 0.68, 95%CI: 0.54 to 0.85], although this benefit was found to be more pronounced in individuals who were tuberculin skin test (TST) positive (RR 0.38, 95%CI: 0.25 to 0.57) than in those who had a negative test (RR 0.89, 95%CI: 0.64 to 1.24) and efficacy was similar for all regimens (regardless of drug type, frequency or duration of treatment)^[2]. However, among the available regimens for treatment of LTBI, isoniazid (INH) preventive therapy (IPT) is the one commonly recommended and has been shown to be very effective and safe among people living with HIV^[2]. IPT is the administration of INH to people with latent tuberculosis (TB) infection (LTBI) to prevent progression to active TB disease. Its use is a component of the TB/HIV collaborative activities recommended by the WHO

to decrease the burden of TB in people living with HIV^[3].

The use of IPT for at least six months has been recommended by the WHO for HIV-infected children and adults without active TB including pregnant women, those receiving ART, and those who have successfully completed TB treatment^[4]. Furthermore, the guidelines also emphasize that a TST is no longer required for the initiation of IPT in people living with HIV. However, despite available evidence regarding the efficacy of IPT, and the recommendation from WHO that IPT be included in the minimum care package for people living with HIV, this life saving and cost-effective intervention is still not being widely implemented.

In 2008, WHO reported that the provision of IPT remains at very low levels, with the number of people who received IPT reaching only 27056 in 2006 – equivalent to less than 0.1% of the 33.0 million people estimated to be infected with HIV globally and Botswana alone accounted for 70% of the total number of people reported globally^[5]. In a cross-sectional survey conducted via email by the WHO amongst HIV programme officers in 69 selected countries having a high burden of HIV and HIV/TB co-infection, 21 of 41 countries (51.0%) that responded had a national policy but only 6 (28.0%) had achieved nationwide IPT implementation^[6]. This picture seems to have improved but is still far below what is generally expected. According to the WHO, an estimated 50.0% of those newly enrolled in HIV care globally meet the eligibility criteria for IPT^[4]. However, of the reported 1.6 million people newly enrolled in HIV care in 2012, only 0.5 million (31.0%) were provided with IPT with South Africa accounting for 71.0% of the global total with 370000 people^[1]. In contrast, of the 0.14 million HIV-infected people screened for TB in Nigeria in 2012, only 2300 (1.6%) of them received IPT while of the 69000 HIV-infected persons screened for TB in Swaziland in 2012, only 1900 (2.8%) of them were prescribed IPT^[1].

The low level of implementation can be attributed to several reasons given by healthcare providers and policy makers. Most of these excuses or challenges can be termed myths because there is enough evidence in support of full scale implementation of IPT globally. These myths include the following: (1) it is difficult to exclude active TB among people living with HIV; (2) chest X-ray is necessary before initiating IPT; (3) use of IPT will increase the risk of the development of INH mono-resistance; (4) ART alone is sufficient for preventing TB among people living with HIV; (5) it is difficult for those on ART to adhere to treatment with IPT; (6) the use of IPT is associated with increased side effects of INH and therefore not safe; (7) use of IPT is not cost effective; (8) IPT cannot be used in children; and (9) TST is needed before prescribing IPT.

We hereby discuss the range of evidence available in support of IPT implementation even in the face of the above challenges or myths.

AVAILABLE EVIDENCE

Active TB can be excluded using symptom screening

Excluding active TB disease before the initiation of preventive therapy is required to minimize the risk of drug resistance as a result of inadvertent treatment of active TB with an inadequate regimen^[7]. Within all HIV care and treatment centers, TB screening should be considered as one of the first services to be offered to all patients irrespective of their treatment status. Among asymptomatic HIV-positive patients, it is possible to use symptom screening to exclude active TB. Interestingly, reports from Botswana and Zambia suggest that the rate of TB is very low among asymptomatic HIV-positive individuals^[8,9]. In a study conducted in South Africa, it was reported that symptoms alone were adequate to exclude TB in 129 Cape Town patients, all of whom were in WHO stage 3 or 4^[10].

A good screening rule was developed using results from a meta-analysis of 12 observational studies that involved over 8000 HIV-infected persons^[11]. The analysis showed that individuals exhibiting none of 4 symptoms namely current cough, night sweats, fever or weight loss have a very low probability of having TB disease (negative predictive value of 97.7% at 5.0% TB prevalence among people living with HIV). Therefore, those who do not have current cough, fever, weight loss or night sweats are unlikely to have active TB and should be offered IPT^[4] while those with symptoms should have further work-up for TB and those found positive should be offered a full treatment course for TB. There is the need to avoid a situation where HIV-infected persons are not placed on anti-TB medications and are also not offered the benefits of IPT. Therefore, the algorithm for TB screening in adults and adolescents living with HIV in HIV-prevalent and resource-constrained settings developed by the WHO should be adequately followed^[4].

Chest X-ray is not mandatory before prescribing IPT

The data on the utility of chest X-ray on IPT programmes is still not very clear^[8,9]. Though chest X-ray is helpful in the diagnosis of active TB, it must be noted that HIV-infected patients with active TB may have normal chest X-rays. In one study, about 8.0% of HIV-infected patients with pulmonary TB had normal chest X-rays^[12] and chest X-rays were normal or not consistent with TB in 23.0% of patients in another study^[13]. One study that evaluated the impact of HIV co-infection on the chest radiographic pattern and extent of pulmonary TB in Ethiopian out-patients showed that HIV-infected patients had chest X-rays classified as normal or with minimal involvement compared with HIV-negative individuals^[14]. These findings may be partially due to the subjective components of reviewing X-rays which include correctly taking and interpreting the X-rays.

In view of the above, symptom screening alone is recommended currently for the exclusion of TB in resource limited settings^[4]. This recommendation is

based on the burden of evidence that currently exists. A study conducted in Cape Town, South Africa to validate screening instruments found that a combination of 2 or more of weight loss, cough, night sweats or fever had a sensitivity of 100.0% and specificity of 81.0% and had the best fit using logistic regression (Wald statistic 19.64, $P < 0.001$) and also that including Mantoux Testing and Chest X-ray did not improve the performance of the screening instruments^[10]. This finding is in line with several other studies that have found that chest X-rays are not sensitive especially in patients with HIV.

A study by Samandari *et al*^[15] comparing 3 screening policies namely symptom screening alone, symptom screening with Chest X-ray and Symptom screening with Chest X-ray and tracking showed that though the inclusion of Chest X-ray reduced the number of new cases of INH resistance (because additional cases of active TB were recognized and therefore IPT was given to fewer people with active TB), the inclusion of chest X-rays actually increased the number of TB cases by 15.8% and the number of deaths from TB by 13.0% because there was attrition of patients during the Chest X-ray screening process and less people benefitted from the protective benefits of IPT^[15]. Thus according to the WHO, for IPT, chest X-ray can be done if available, but is not required to classify patients into TB and non-TB groups^[4].

IPT does not increase the risk of the development of INH mono-resistance

One of the major reasons given for poor utilization of IPT to prevent active TB is the belief that IPT can result in subsequent resistance to INH in patients who later develop active TB^[16,17]. Theoretically, if active TB is missed and the bacterial load is large enough, treatment with monotherapy or an inadequate regimen has the potential to generate drug resistance^[18]. Though the impact of widespread use of IPT on drug resistance is not well known, a systematic review of data from studies published in English, French and Spanish between 1951 and October, 2003 that assessed the effect of primary IPT on the risk of INH-resistant TB in populations without HIV reported that the risk of resistance in those given IPT was not statistically different from those that received placebo^[16]. The study authors support the expansion of IPT use in line with the recommendations from the HIV/TB working group of the Stop TB partnership^[3].

Van Halsema *et al*^[19] described a case series of miners derived from a cluster randomized trial in which clusters were randomized either to receive TB screening and IPT or routine TB control consisting of annual case finding by chest radiograph and targeted IPT offered to individuals with HIV or silicosis with results that do not suggest an increase in the proportion of INH resistance cases among those exposed to TB screening and IPT^[19]. Randomized controlled trials (RCT) of the effect

of IPT in HIV-infected patients in Botswana, India and South Africa also did not show an increased risk of INH resistance amongst patients given IPT^[20-22].

Furthermore, it has been reported that patients with INH-resistant TB respond to standard short course anti-TB therapy just as well as patients without INH-resistant TB, though those with INH-resistant TB do suffer a slightly increased risk of relapse^[23]. Therefore, even though there is a possibility of INH-resistant TB following the use of IPT in HIV-infected people, the benefits in terms of its effectiveness and efficacy must be balanced against this risk.

IPT is useful in combination with ART

To reduce the burden of TB among HIV-infected persons, the WHO recommends intensified case finding (ICF), IPT, infection control, and early initiation of ART^[4]. ART is the most potent and widely implemented TB preventive intervention among people living with HIV (PLHIV)^[4]; its use profoundly reduces the incidence of TB in PLHIV and with continued use, the risk of TB progressively declines. Although treatment with ART has been estimated to result in more than 80.0% reduction in the risk of TB^[24], some reports showed that even after ART initiation TB incidence remains very high^[24-26]. This suggests that, even among those with adequate response to ART, other interventions are needed to control the TB epidemic in PLHIV^[25].

Observational studies from Brazil and South Africa have shown that the combined effect of ART and IPT in preventing TB among PLHIV is significantly higher compared to ART alone^[27,28]. Two retrospective analyses on assessing the advantages of using IPT with ART concluded that the benefit of combining INH and ART was additive^[27,29]. In a study in Ethiopia it was found that using either IPT or ART alone among PLHIVs reduced the incidence of TB by 68.0% and 65.0% respectively while co administration of IPT and ART reduced the incidence by 80.0% to 82.0% when either initiated together or IPT was initiated before ART^[30]. Concomitant use of IPT and ART also improves adherence to IPT, as shown in a study in Brazil where being on ART was associated with higher completion of IPT^[31].

There is good treatment adherence with the use of IPT

Good treatment adherence with the use of IPT has been reported and has been found to be associated with several factors, including availability and access to quality health care, favorable economic, social and cultural environments^[32]. A study conducted in Thailand to determine the level of and reasons associated with adherence to TB preventive therapy among asymptomatic HIV-infected individuals recorded 74.3% completion of a nine-month IPT regimen^[33]. Swaminathan *et al.*^[22] in their RCT conducted in India to compare the efficacy of a 6 mo and 36 mo regimen for prevention of TB in HIV-infected patients also recorded high adherence, even with the 36-mo IPT regimen.

In addition to the existing evidence in support of good adherence to IPT are the results obtained from a cross-sectional study conducted in Ethiopia to assess adherence to IPT and associated factors among PLHIV^[34]. In this study, the level of self-reported adherence of IPT was found to be 89.5% (CI 86.1 to 92.3). Another important finding in this study was the fact that patients who were on ART were more likely to be adherent [95%CI, COR = 1.97 (1.01–3.84)] than patients who were on Pre-ART^[34].

Good adherence to IPT has also been recorded among children. In a cohort study conducted in Cape Town, South Africa to investigate the combined effect of IPT and ART on TB risk amongst HIV-infected children, INH was well tolerated with excellent adherence^[35]. Similar results were also obtained in another RCT conducted in the same city but compared daily to three times a week dosing of INH among HIV-infected children^[36]. The overall adherence to INH was excellent, with a mean adherence of 94.7%^[36]. From these studies, it is clear that good adherence with the use of IPT can be achieved even with concurrent treatment with ART.

IPT is safe and is not associated with increased risk of INH side-effects

Like most other medications, anti-TB medications are primarily metabolized by the liver and potentially can lead to drug-induced hepatitis and other adverse events (e.g., nausea, vomiting, gastritis, peripheral neuropathy, and rashes)^[22]. This understanding has retarded the implementation of INH as a prophylaxis for TB among many care givers despite WHO recommendations. The side effect of major concern with regards to IPT is hepato-toxicity, which has been found to occur in a very small proportion of individuals receiving treatment^[37,38]. The hepato-toxic effect of INH could be mild (subclinical) with good prognosis or fatal which is less common. Fatal INH-induced hepatitis occurs in 0.001% to 0.06% depending on several other factors such as increasing age (*i.e.*, over 35 years) and frequent alcohol ingestion^[39,40]. Clinical monitoring and good patient education can help in reducing the risk of toxicity^[41]. In a study in Seattle, without laboratory monitoring, only 11 cases of hepatitis were reported after about seven years of monitoring over 11000 patients on INH and only one case needed hospitalization^[41]. The authors concluded that the rate of INH hepato-toxicity during clinically monitored preventive therapy was lower than has been reported previously suggesting that clinicians should have greater confidence in the safety of IPT.

A report from a RCT conducted in Botswana showed no difference in adverse events between participants on placebo vs those on INH for 30 mo (1.0% vs 1.3% respectively; $P = 0.36$). A similar trial conducted in India reported only 3.0% (22/683) of participants in both study arms (*i.e.*, Ethambutol vs INH) with side effects related to study drugs^[22]. This risk of side effects was marginally higher in another trial reported by

Rangaka *et al.*^[42] with 1.5% of participants on placebo vs 2.9% of participants on INH developing side effects (*i.e.*, grade 3 or above raised alanine transaminase level; clinical hepatitis; grade 2 or above rash or peripheral neuropathy), but the use of INH by participants on ART had no additive toxic effect^[42]. The experience from Brazil indicated that expanded use of IPT in HIV-infected persons is achievable with high adherence and low adverse events^[31]. Therefore, the fear of INH side effects should not prevent the implementation of IPT.

IPT use is cost-effective

Cost-effectiveness analytical studies conducted in the United States and South Africa found that compared with no prophylaxis, short and long course IPT use amongst PLHIV saved an average of \$5 in medical care, for every \$1 spent on prophylaxis^[43,44]. In addition, the 6-mo regimen reduced the incidence of TB by an average of 23.0% to 47.5% and increased life expectancy by an average of 7.2 mo^[43,45]. The cost-utility analysis of an IPT program in Uganda showed that the provision of IPT for HIV-infected persons was cost-effective^[46].

In the pre-ART era, Bell *et al.*^[47] found that in sub-Saharan Africa, when IPT was given daily for 6 mo, there was a savings of \$24.16 per person on medical care, social costs and costs associated with treating secondary infections. In resource-limited settings, where ART was not always available, the savings made and demonstrated reduction of TB and HIV-associated morbidity and mortality using IPT is desirable^[47]. Even as recent as 2012, with expanded access to ART, an analysis in Southern India found that the ART-induced increases in CD4 counts attenuated the absolute IPT efficacy of reducing the risk of TB infection and related mortality, thus increasing the cost-effectiveness of IPT and making it good value for money^[48].

IPT is recommended for use in children

TB is a leading cause of death in adults and more so in children due to their increased vulnerability to infection^[49]. This vulnerability is even more pronounced in children living with HIV as TB is the leading cause of death among children with HIV in TB endemic areas^[50]. HIV-infected children are also more likely to have severe respiratory disease and extra pulmonary TB and acquire TB at all ages compared with HIV negative children who are more at risk only during infancy^[35,51]. Therefore, the need to protect HIV-infected children from acquiring TB cannot be over emphasized. Over the years the efficacy of INH as prophylaxis for TB in children has given rise to a lot of controversy due to inadequate data and trials revealing conflicting results^[35,49].

A study by Madhi *et al.*^[51] showed no significant effect when INH is used for prophylaxis in children with or without HIV. This study was included in a recent meta-analysis by Ayieko *et al.*^[50] and the authors explained that the reason for the null results could be due to the fact that TB was over-diagnosed in the study since few

cases were confirmed microbiologically and many of the TB cases met only minimal criteria^[49,50]. Another explanation given was that the initial study involved mostly infants (median age 96 d, range 90-120 d) while the studies with a positive effect included older children suggesting that age may be an effect modifier of TB development in children receiving IPT.

On the other hand more recent studies have shown promising results making the body of evidence available stronger. The safety of the use of INH in children has also been reported by several studies^[49,52]. In 2006 a randomized control trial found a 72.0% risk reduction in TB in children receiving INH compared to placebo. This study also found a 54.0% risk reduction in mortality^[53]. A cohort analysis of another RCT conducted by Frigati *et al.*^[35] found a reduction in TB incidence in HIV-infected children randomized to receive IPT compared with placebo^[35]. Further reduction in the risk of TB was found when comparing children receiving ART and IPT to those receiving ART and placebo^[35]. The meta-analysis by Ayieko *et al.*^[50] found a strong positive effect against TB in HIV negative children although, the results for the effect on HIV-infected children was inconclusive because the analysis included only 2 studies.

Based on this moderate quality of evidence, the WHO strongly recommends IPT for use amongst HIV-infected children above 12 mo of age who are unlikely to have active TB or have not had any contact with a person infected with TB and for those less than 12 mo of age, prophylaxis is strongly recommended for those who have had contact with an infected person and in whom active TB has been ruled out^[4].

TST is not necessarily required for the implementation of IPT

TST is the administration of purified protein derivative (PPD) in individuals with exposure risk to TB in order to identify those who may have acquired latent infection and for whom prevention would be beneficial^[54]. The previous WHO policy statement on preventive therapy against TB in people living with HIV recommended TST as a condition for IPT implementation in developing countries^[55]. Additionally, results of a meta-analysis of RCT showed that TB preventive therapy was more effective amongst HIV-infected person who are TST positive than those who are TST negative^[2]. However, while TST was successfully implemented as a screening tool in developed countries^[56,57], it has not been so well received in resource-poor nations where TB burden is greatest. Apart from limitations of low sensitivity and specificity (51.0% in HIV-infected persons vs 94.0% in HIV negative persons with active pulmonary TB)^[56,58], it requires a lot of resources including adequately trained manpower to administer and read the test, need for repeat visits by patients, difficult logistics of cold chain maintenance and cost of tuberculin procurement which may be prohibitive for a large scale prevention program^[4,54].

TST status of an individual is influenced largely by the degree of immunodeficiency^[59]. An RCT conducted in India assessed two cohorts of HIV-infected patients; one with active pulmonary TB and the other without evidence of active TB. The cohort without active TB was found to have a lower TST-positive rate of 27.6% at CD4 < 100 cells/ μ L against 42.0%-48.0% of those with CD4 > 100 cells/ μ L in the same group^[58]. The authors concluded that TST is a poor predictor of both latent and active TB in HIV-infected individuals in TB endemic countries and that programmes offering treatment for LTBI should consider including all HIV-infected individuals regardless of TST status, or use other indicators, such as CD4 count^[58]. Thus a negative TST in an HIV-infected person may be due to anergy leading to missed opportunity for those who should have been offered chemoprophylaxis^[4].

Botswana, one of the few African countries that have implemented a successful national IPT program since 2001 uses the WHO symptom checklist alone without the need for a TST or chest X-ray as this was found to increase loss to follow up^[21]. After its pilot in 2005, Brazil made a similar recommendation to WHO that TST not be used as a screening tool to reduce waiting time between diagnosis and those who are TST positive would likely benefit more from implementation of IPT in patients^[31].

In its 2011 revised guidelines, WHO makes a strong recommendation for the provision of IPT to all HIV-infected patients in TB-endemic countries (prevalence of latent TB > 30%) irrespective of TST status^[4]. However, since TST positive individuals derive greater benefits from treatment of LTBI, TST could still be requested where feasible^[4].

CONCLUSION

With the available evidence discussed above, the benefits of IPT are far more than the perceived risks. Therefore, to scale up implementation of IPT at both global and country levels, more efforts are needed in order to fully implement the recommendations contained in the WHO Policy Guidelines for IPT (2008)^[45]. HIV programs should own IPT services and provision of IPT must be fully included as part of the basic care package for all PLHIV. Perhaps, the use of IPT should be included in the range of palliative care services provided to all PLHIV, like Cotrimoxazole, nutritional supplements and anti-malarial medication during visits to most ART clinics. Additionally, patients should be properly educated in order to know the importance of IPT and thus be able to demand prescription of IPT from their providers. Others measures include the inclusion of IPT as part of ART scale-up, integration of HIV and TB services, full development of national policies for IPT, continued promotion of the concept of the Three I's, improved and stronger advocacy at all levels, improved monitoring and evaluation of IPT programmes, and pursuing the

possibility of co-formulation of Cotrimoxazole and INH to further aid treatment adherence and improve access.

Furthermore, implementation studies to further understand the best models for IPT implementation and scale-up at country level are needed. Since the fear of INH mono-resistance is one of the barriers to full scale IPT implementation, reports on the risks and benefits associated with the administration of INH in error to undiagnosed people with active TB are also needed^[4]. Although, a recent report showed that in HIV-infected persons, 36 mo IPT was more effective than the current 6 mo regimen^[21], additional studies are needed to clarify this. With all these efforts, the gains achieved through ART scale-up globally would be better consolidated with further reduction in TB incidence, improved survival and lower mortality among PLHIV. Conclusively, more needs to be done by the policy makers and the experts to ensure effective and strategic implementation of IPT especially in high HIV burden resource-constraint settings.

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Is transfusion-transmitted dengue fever a potential public health threat?

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enveloped ribonucleic acid viruses, named dengue viruses (DENV), that include four serotypes and are mainly transmitted *via* the bite of mosquitoes of the genus *Aedes* (*A. aegypti* and *A. albopictus*). The distribution of the disease was historically limited to intertropical areas; however, during the last thirty years, the perimeter of the disease extended considerably and temperate areas are now at risk of outbreaks. The present global burden of dengue is considerable: 2.5 billion people over more than 100 countries are concerned; 50 to 100 million infections occur every year, with a number of fatal cases of approximately 20000. Although frequently asymptomatic or limited to a mild fever, dengue is responsible for severe cases mainly consecutive to the occurrence of hemorrhagic complications that can lead to shock and death, notably in children from poor-resource settings. The place of DENV as a transfusion-transmitted pathogen has been recognized only in 2008. At the present time, only five cases of transfusion-transmitted dengue, including one case of dengue hemorrhagic fever, have been formerly documented. This review provides a general overview of dengue, its viruses and their vectors. It replaces the disease in the context of other viral diseases transmitted by arthropods. It discusses the threat of dengue on the supply of blood products in endemic and non endemic areas. Finally, it describes the specific and non specific measures available for improving the security of blood products with regards to this emerging risk. Interestingly, in 2009, the American Association of Blood Banks placed DENV in the highest category of emerging infectious agents for their potential impact on transfusion recipient safety for the next years in North America.

Key words: Dengue; Dengue viruses; *A. aegypti*; *A. albopictus*; Transfusion-transmitted virus; Blood safety

Abstract

Dengue is an arboviruses due to single-stranded

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Core tip: The place of dengue viruses as transfusion-transmitted pathogens has been recognized only in 2008. By now, only five cases of transfusion-transmitted dengue, including one case of dengue haemorrhagic fever, have been formerly documented. This review provides a general overview of dengue, its viruses and their vectors. It replaces the disease in the context of other viral diseases transmitted by arthropods. It discusses the threat of dengue on the supply of blood products in endemic and non-endemic areas. Finally, it describes the specific and non-specific measures available for improving the security of blood products concerning this emerging risk.

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INTRODUCTION

Dengue is an arboviruses mainly transmitted by mosquito bite that constitutes a major public health concern: two-fifths of the world's population, mainly located in the intertropical regions, are exposed to the risk of infection. According to the World Health Organization (WHO), an estimated 500000 people with severe dengue require hospitalization each year, a large proportion of whom are children; about 2.5% of those affected die^[1]. Despite the large distribution of this "old" infection and the fact that the virus can be present for about one week in the blood of infected patients, the risk of dengue as a transfusion-transmitted disease emerged very recently (first publications in 2008). An attempt to explain this paradox is proposed later in this review. After a few recalls concerning dengue, its viruses and their vectors, the disease is replaced in the larger context of arboviruses associated to a demonstrated or possible risk of transmission *via* blood products. The third part of the manuscript intends to answer the question formulated in the title of the paper: "Is transfusion-transmitted dengue fever a potential public health threat?" The last part of the study describes the measures available for reducing this risk.

RECALLS ON DENGUE, ITS VIRUSES AND THEIR VECTORS

Dengue viruses

Dengue viruses (DENV) are single-stranded ribonucleic acid (RNA) viruses, 40 to 60 nm in size, belonging to the *Flaviviridae* family (Table 1) and exhibiting an icosahedral capsid and a lipid envelope. The viral genome codes for ten viral proteins: three structural

(core, membrane-associated and envelope) and seven non structural ones. The envelope protein is responsible for the specific recognition of host cells and for the development of protective neutralizing antibodies. Non structural proteins have been associated with the pathogenesis of severe forms of the disease. Dengue viruses include four serotypes entitled DEN-1, DEN-2, DEN-3 and DEN-4. The infection by one serotype confers a strong protection against the corresponding serotype but only a partial immunity against the three other ones, which explains that an individual can be infected several times during life by DENV. It is worthwhile to note that a fifth dengue serotype has been identified on virus samples that were collected during an outbreak in Malaysia in 2007^[2]. More data are awaited about the epidemiological significance of this observation.

Vectors of DENV

The main vectors of DENV are mosquitoes of the *Aedes* genus (also called *Stegomyia*).

The most common vector of dengue viruses is *Aedes aegypti* whose distribution is very large in intertropical regions of the world (Figure 1). In the Americas, discontinuation of *Aedes aegypti* control efforts in the mid-20th century has led to a resurgence of dengue throughout South and Central America, resulting in hundreds of thousands of dengue cases in these areas. In October 2012, an outbreak of DEN-1 infection was documented for the first time in the Portuguese island of Madeira^[3]; the viral strain was shown to be very close to a virus strain originated from Venezuela^[4].

Aedes albopictus (the tiger mosquito) is also involved in dengue outbreaks or isolated cases, notably in temperate regions as Europe where the mosquito is able to survive in cooler environment and expended very quickly (Figure 1) from Asia following the international trade in used tyres and other goods such as lucky bamboo. In 2010, an autochthonous outbreak of dengue was documented in Croatia^[5] and two sporadic cases were identified in Nice city in the South-East of France^[6].

A third species, *Aedes polynesiensis*, has been involved in rare cases. *Aedes* mosquitoes are highly domesticated mosquitoes that are able to grow in urban environment, notably in human-made containers filled with stagnant water (*e.g.*, water storage tanks, subterranean pits, flowerpot trays). Interestingly, when both viruses are present in the same area, *Aedes albopictus* is able to displace *Aedes aegypti* from competing environment, which would facilitate the dissemination of DENV into temperate regions that are refractory to colonization by *Aedes aegypti*^[7].

Routes of transmission of DENV

Dengue is mainly a mosquito-borne infectious disease. Besides the sylvatic reservoir that involves not human primates with occasional contamination of humans, the human cases are mostly related to the urban or

Table 1 Main arboviruses exhibiting a potential or demonstrated transfusion-associated risk

	Dengue virus	West Nile virus	Saint-Louis encephalitis virus	Tick-borne encephalitis virus	Chikungunya virus	Colorado tick fever virus
Family	Flaviviridae	Flaviviridae	Flaviviridae	Flaviviridae	Togaviridae	Reoviridae
Virus characteristics						
Nucleic acid	ssRNA	ssRNA	ssRNA	ssRNA	ssRNA	dsRNA
Envelope	Yes	Yes	Yes	Yes	Yes	No
Vectors	Mosquitoes (<i>Aedes aegypti</i> and <i>Aedes albopictus</i>)	Mosquitoes (genus <i>Culex</i> but also <i>Aedes albopictus</i>)	Mosquitoes (genus <i>Culex</i>)	Ticks (genus <i>Ixodes</i>)	Mosquitoes (<i>Aedes aegypti</i> , <i>Aedes albopictus</i>)	Ticks (<i>Dermacentor andersoni</i>)
Usual vertebrate hosts	Humans	Birds	Birds	Rodents	Humans, primates	Humans
Geographical distribution	World (mainly intertropical regions)	Asia, Africa, Europe, Americas	Americas	Europe, Asia	Africa, Asia, West Pacific, Europe,	Western USA and Canada
Clinical features						
Incubation period in days	2-14	2-14	4-21	7-14	1-12	3-6
Asymptomatic forms	75%	80%	> 99%	80%	15%	low%
Clinical manifestations	DF-DHF-DSS	Fever- encephalitis	Fever- encephalitis	Fever- encephalitis	Fever- joint pains	Fever- encephalitis
Vaccine	Phase III trials	No	No	Yes	No	No
Demonstrated transfusion-transmitted cases	Yes	Yes (high number)	No	Yes	No	Yes

ssRNA: Single-stranded RNA; dsRNA: Double-stranded RNA; DF: Dengue fever; DHF: Dengue hemorrhagic fever; DSS: Dengue shock syndrome; CHIKV: Chikungunya virus.

peri-urban cycle where human beings are the main amplifying host for DENV (Figure 2). Female mosquitoes get infected by biting infected humans during their viremic phase; after 7 to 14 d of incubation, the mosquito is able to transmit the virus *via* blood feeding. Besides mosquito biting, DENV may be accidentally acquired after vertical transmission, especially in near-term pregnant women through the placenta^[8], *via* the organ transplantation process^[9,10], after needle-stick injury^[11] and, as evidenced below, after transfusion of blood products.

Clinical presentation

The infection occurs after an incubation period of 3-14 d (average 3-7 d). Approximately 75% of all DENV infections are asymptomatic, notably in adults. The common symptomatic infection, which appears as a mild febrile illness associated or not with more evocative symptoms, represent approximately 20% of DENV infections. In endemic areas, about 5% of all acute febrile illnesses can be related to DENV^[12]. Severe forms may represent up to 5% of symptomatic infections; they are more frequent at the two extremes of life (very young children and elderly) and in patients with diabetes mellitus, hypertension and renal insufficiency^[13]. As shown in Figure 3, the classification of dengue presentations evolved through time^[14]. According to the WHO classifications of 1975 and 1997, symptomatic dengue was divided in undifferentiated fever, dengue fever (DF) and dengue hemorrhagic fever (DHF) ranging from mild hemorrhagic symptoms (grade I) to dengue shock syndrome (DSS) (grades III and IV). In 2009, WHO proposed a new simplified classification in two presentations: dengue (without or with warning signs) and severe dengue (Figure 3). The latter classification

is more adapted to clinical evaluations in primary care or resource-limited settings; however, it does not differentiate hemorrhagic forms from other severe presentations. A trend to capillary fragility together with the risk of thrombocytopenia is common features of all dengue cases, even those without hemorrhagic complications. It can be searched for by the tourniquet test that consists in applying and inflating a blood pressure cuff to the midpoint between the systolic and diastolic blood pressures for five minutes. The test is positive if more than 10 to 20 petechiae per square inch develop.

Pathophysiology

From a pathophysiological point of view, many aspects of disease remain unsolved (for a review, see^[15]). The first targets of DENV after mosquito bite seems to be Langerhans cells, dermal cells and interstitial dendritic cells, but many other cells can replicate the virus, including hepatocytes, lymphocytes, endothelial cells, neuronal cells and muscle satellite cells^[16]. Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN)^[17] and the mannose receptor (CD206)^[18] have been described as potential host receptors for virus entry. As for other flaviviruses, both signal transducer and activator of transcription 1 and 2 possess the ability to independently limit the severity of DENV pathogenesis. When these signalling pathways are inactivated, notably within the hepatosplenic compartment, the deregulation of cell-mediated immunity may lead to the activation of CD4+ and CD8+ T cells, which results in a "cytokine/chemokine storm" that plays an important role in the vascular permeability leading to leakage of plasma into the extravascular compartment seen in DHF. The resulting

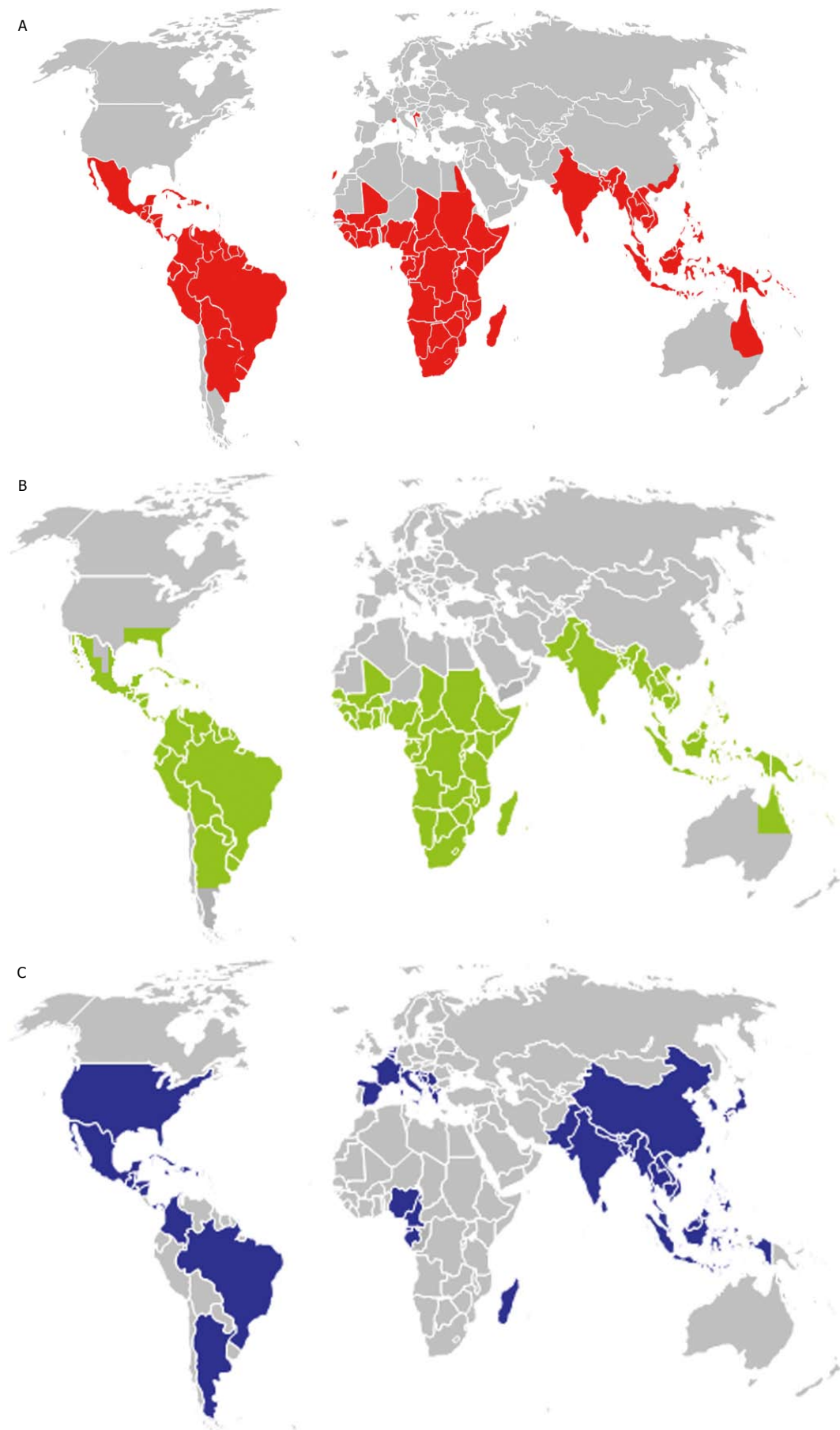


Figure 1 Overall distribution of dengue cases (endemic or epidemic) worldwide (A) and perimeter of expansion of the two main vectors of dengue viruses, *Aedes aegypti* (B) and *Aedes albopictus* (C).

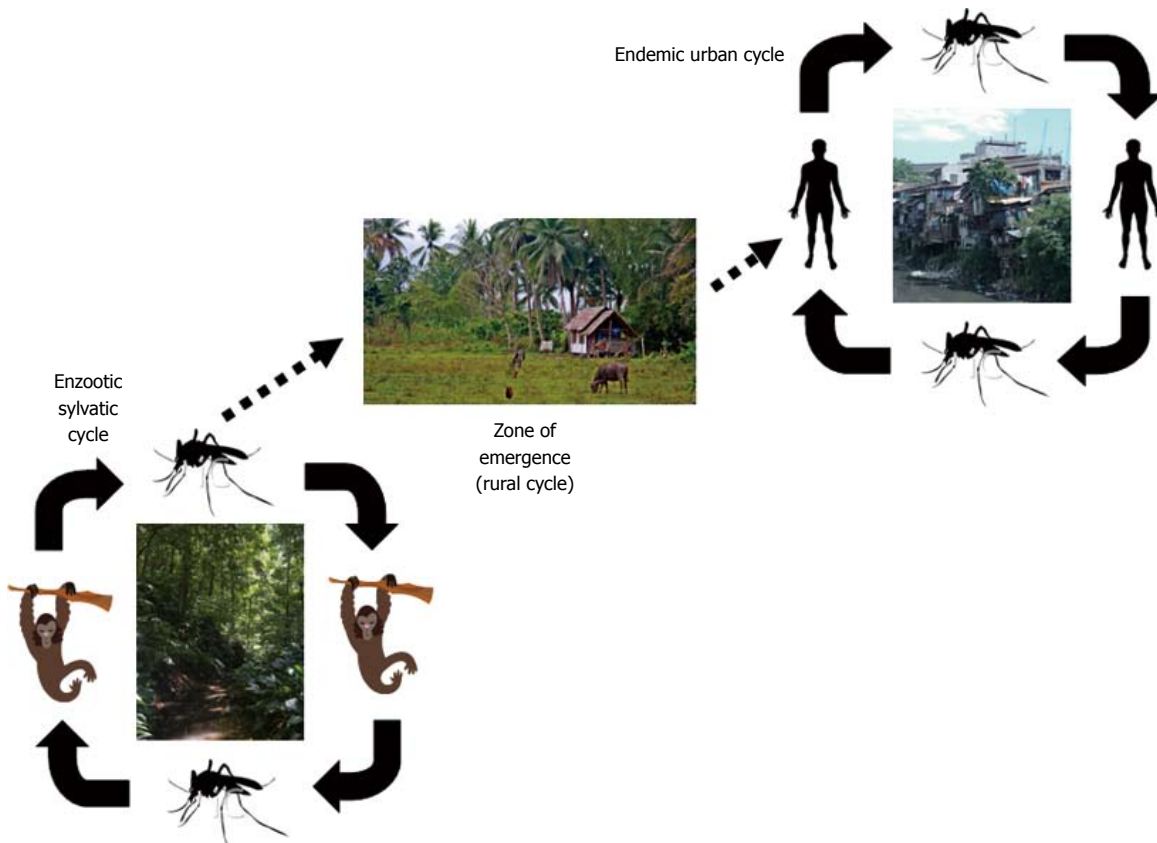


Figure 2 Simplified representation of the sylvatic and urban/peri-urban cycles of dengue that models the natural spread of dengue viruses through bites by infected mosquitoes.

hemoconcentration and decreased blood pressure may result in DSS.

More severe infections are known to occur after secondary infection than after primary infection. It has been suggested that facilitating antibodies against the envelope glycoprotein^[19] and the "original antibody sin" theory^[20] are involved in this observation.

Laboratory diagnosis

The virological diagnosis of dengue is required in case of severe infection and for confirming an outbreak. Different tools of direct diagnosis including cell culture, antigen detection and nucleic acid technologies (NAT), and of indirect diagnosis (serological tests) are available for documenting a recent infection.

Virus isolation from blood or tissues is possible by inoculation to mosquitoes or cell culture but these techniques are fastidious and limited to specialised laboratories.

In contrast, serological tests are very useful because they are relatively simple to implement, even in the absence of a laboratory of virology. They consist in microplate immunoassays that can measure IgM-specific antibodies, positive as soon as 4-5 d after the beginning of symptoms and lasting for up to 6 mo^[21] (with a peak at week 2), and IgG-specific antibodies that become positive a few days after IgM and are

a long-lasting marker of past infection. In patients infected at any time by other flaviviruses, which is relatively common in endemic areas, cross-reactive antibodies may interact with dengue serology and lead to false-positive results. The measure of neutralizing antibodies on a late serum specimen, a technique that requires cell culture within a specialised laboratory, may be useful to distinguish specific from unspecific IgM response.

An antigen test detecting the DENV NS1 protein in blood by immunoassay is now available. It is positive during the first 5 d following the initial symptoms. The sensitivity of the test is optimal during primary infection^[22]. A negative test does not exclude the diagnosis in case of secondary infection^[23,24].

The detection of DENV genome in blood or tissues by NAT has become the gold standard for the diagnosis of recent infection. It is positive within the first 5 d of disease. NAT tests are very sensitive and specific. Different molecular technologies are used for the diagnosis of DENV infection, including realtime polymerase chain reaction (RT-PCR), transcription-mediated amplification (TMA) and other isothermal amplification assays. The choice of primers may apply either on highly conserved parts of RNA genome within the 4 serotypes or on a combination of sequences specific of each of the 4 serotypes.

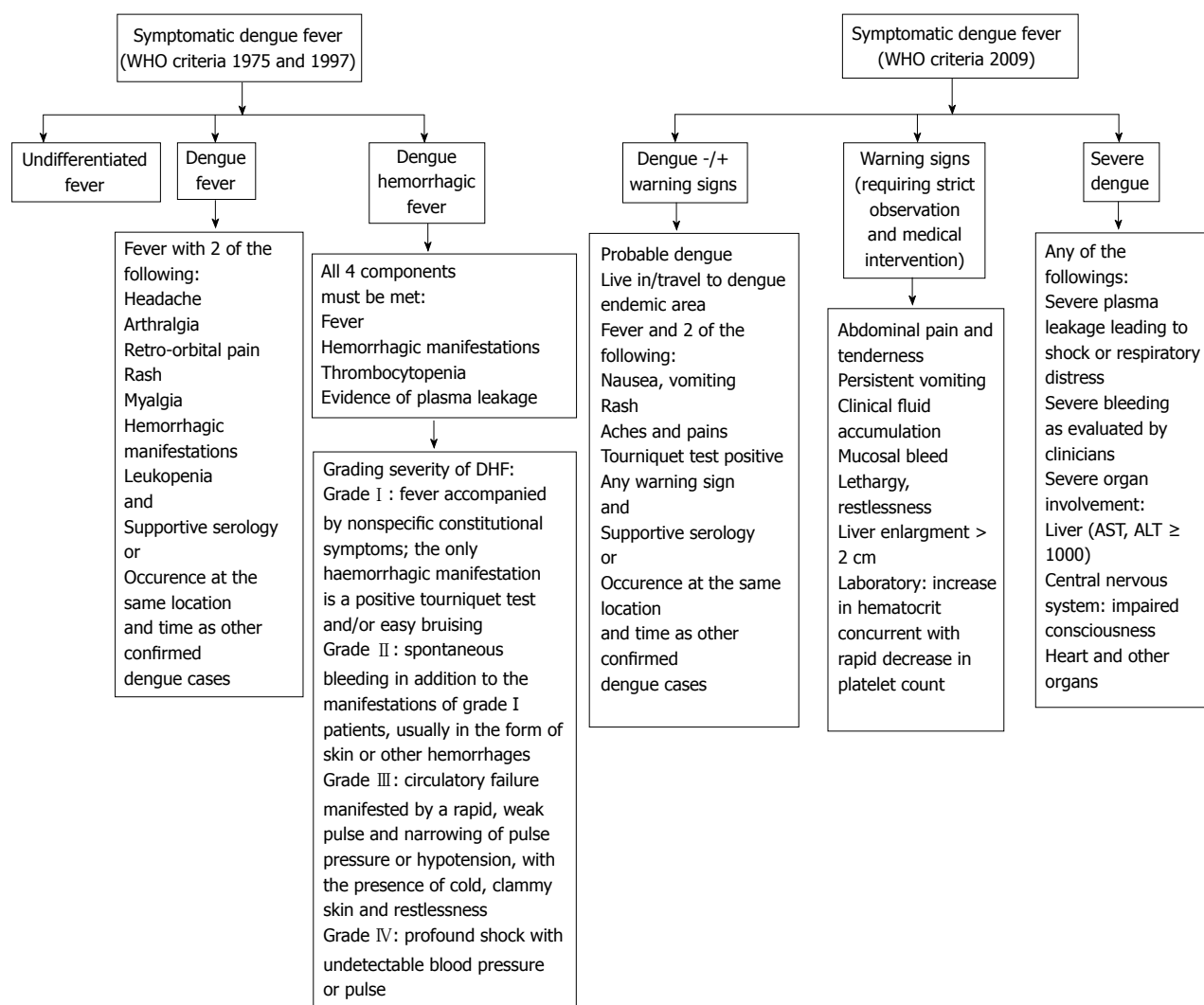


Figure 3 Successive classifications of dengue clinical presentations according to the World Health Organisation definitions. WHO: World Health Organisation. AST: Aspartate transaminase; ALT: Alanine transaminase.

Prevention

At the individual level, the vaccinal approach is certainly the more suitable way to control dengue durably. The existence of at least four serotypes that are sufficiently antigenically different necessitates the use of four monovalent vaccines. However, as mentioned above, there is safety concern about a possible increase of virus infectivity *via* antibody dependent enhancement when a vaccinated subject is exposed to a wild virus. Although no vaccine against dengue is presently available, several approaches have been proposed for controlling the spread of disease (for review, see^[25,26]). The most advanced solution is a live-attenuated tetravalent vaccine based on chimeric yellow fever dengue virus that is produced by Sanofi and could be commercially-available before the end of this year.

At the vector level, the eradication of susceptible mosquitoes is the more effective way to contain the epidemic. However, the large use of insecticides has shown its limits in terms of toxicity for the environment together with the rapid development of cross-

resistances. A vector control program has been launched by the WHO^[27]. It is based on actions combining the elimination of containers harbouring larval and adult mosquitoes (plastic cups, broken bottles, used tyres, flowerpots), the use of insect repellents, mosquito traps and mosquito net in the home. Future strategies are in progress to modify the vectors by biological interventions including transgenic mosquitoes or their infection by the intracellular bacterium *Wolbachia* that reduces the replication of arboviruses in susceptible vectors^[28,29].

Curative treatment

The curative treatment is mainly symptomatic. No antiviral drug has yet demonstrated any effect against DENV. DF resolves spontaneously within a few days; analgesics containing ibuprofen and aspirin must be avoided to prevent hemorrhagic complications. Cases of DHF must be hospitalised; with replacement of fluid leakage and intensive monitoring; the mortality can be reduced under 1% when adequate cares are given but may reach up to 20% in case of poor medical intake.

DSS and severe forms of dengue involving organ failure constitute a critical medical issue that needs urgent hospitalisation in an emergency unit.

DENGUE IN THE LARGER CONTEXT OF ARBOVIRAL DISEASES ASSOCIATED TO A DEMONSTRATED OR POSSIBLE RISK OF TRANSMISSION *VIA* BLOOD PRODUCTS

A total of approximately 130 arboviruses are known to cause disease in humans. Since they are transmitted *via* arthropod bite, these viruses are present in the bloodstream for a few days, which imply an at least theoretical risk of transmission *via* blood products if the patients are sampled during the viremic stage. The arboviruses known or suspected to be transmitted to recipients *via* blood products are presented in Table 1.

As reviewed by Petersen *et al.*^[30], the emergence of West Nile virus (WNV) in New York City in 1999 and its rapid dissemination through Northern America during the following years is a good illustration of the sudden recognition of the role of transfusions in the spread of the virus, a fact that had been completely occulted before, despite many decades of circulation of WNV in the Ancient world. At the early phase of the USA outbreak, it was relatively difficult to establish a relationship between WNV infection and blood products^[31,32], mainly due to the limits of contemporary diagnostic tools (IgM serology and NAT) that were insufficiently sensitive to identify infected donors, even retrospectively^[30]. Another lesson driven from the WNV outbreak in USA was the decreased sensitivity of NAT when tested on minipools, a measure intended to decrease the costs and delay of WNV screening in blood donors.

The very successful emergence of Chikungunya virus (CHIKV) in the Indian Ocean and notably in the French Reunion Island is another illustration of the recent recognition of a new transfusion-transmitted risk. Even if no positive case was documented by NAT, probably for the same reasons as those evoked just before, it was modelled that, given a mean duration of 7.5 d for viremia and an exposition rate to CHIKV of 38% in inhabitants of the island, the prevention measures taken (eviction of autochthonous donors for red blood cells and systematic treatment of platelets by the Intercept® technology) had prevented the use of approximately 40 infected gifts during the whole epidemic period^[33].

Concerning arboviral diseases in general, these examples illustrate that "planning efforts are hindered by the notoriously unpredictable nature of outbreaks and that importations of exotic arboviruses are random events with uncertain consequences"^[30]. The rapid extension of dengue suggests that the subsequent

transfusion transmission risk can be partly anticipated.

EVIDENCE FOR THE TRANSMISSION OF DENV BY BLOOD PRODUCTS AND ITS IMPACT ON PUBLIC HEALTH

As stated above, the global burden of dengue is considerable: according to the WHO, 2.5 billion people over more than 100 countries are concerned; 50 to 100 million infections occur every year, with a number of fatal cases of approximately 20000. A recent study^[34] estimated that these figures could be increased by a factor of 3 to 4 to reflect the real load of dengue.

Despite the fact that dengue is the leading arboviruses in the world, there are only three reported observations of DENV transmission *via* blood products in the literature. The first report concerned a 76 year-old woman who received a blood transfusion in 2002 in a Hong-Kong hospital following a severe anaemia; two days later, she developed low-grade fever that resolved spontaneously (she received antibiotics for a suspicion of urinary infection). The case was secondarily related to dengue because the donor presented a typical dengue infection documented by serology. Molecular testing performed on the donated blood product was positive for DEN-1. Two months after transfusion, the recipient exhibited IgM antibodies confirmed by seroneutralisation assay. The case was published only six years later^[35]. The second study, also published in 2008^[36], involved a cluster of three cases contaminated in Singapore by the same donor who developed fever and myalgia after blood donation. Two days after transfusion, 2 of the 3 recipients developed a symptomatic infection that resolved spontaneously. The 3 recipients demonstrated serological evidence of acute dengue infection. A PCR assay performed on blood specimens from the donor and the 2 symptomatic recipients was positive for DEN-2.

The third observation, published in 2012^[37] was documented from the outbreak of dengue that occurred in Puerto-Rico in 2007. Of 15350 donation samples tested retrospectively, 29 were found positive for DENV genome by TMA assay. Three of the recipients of these contaminated samples could be tested by NAT and one of them, who received red blood cells containing 10⁸ copies/mL DEN-2, was found positive. Three days after transfusion, he developed DHF. Both donor and recipient were shown to harbour viruses with the same envelope sequence. This is the first case of severe dengue infection transmitted by blood products.

One may wonder about the gap between the important role played by dengue in Public Health worldwide and the limited number of transfusion-transmitted documented cases reported so far. Different arguments can be advanced for explaining such a paradox: (1) in the absence of documented inquiry between donor and recipient, it is often difficult to differentiate infection

transmitted by mosquitoes and blood products; (2) the disease is frequently asymptomatic or mild in donor, recipient or both, with spontaneous resolution within a few days; (3) most of transfusion-transmitted cases are intended to occur in areas where dengue is endemic, which contributes to minimize the risk, especially in low-income countries where the virological documentation of dengue cases is not available easily, and, last but not least, and (4) most recipients of blood products have been already exposed to mosquito-transmitted DENV early in their life, which prevents them from being infected again via infected blood products.

In 2009, the American Association of Blood Banks stratified in four levels (red, orange, yellow and green) the emergent or re-emergent infectious agents that could represent a potential threat to transfusion in North America for the next years^[38]. Besides epidemiological considerations and subjective assessment of public perception, the following scientific criteria were taken into consideration: (1) the agent must be present in blood at least for a few hours or days; (2) this blood phase must be at least in part asymptomatic for allowing the blood donor to pass through the filter of clinical selection; (3) the infectious agent must be able to induce, at least in some cases, a severe disease; and (4) finally, the blood pathogen must resist to inactivation by the innate or adaptative immunity of the donor (*i.e.*, bacterial power of serum). According to these criteria, DENV was classified in the upper red level, together with *Babesia sp* and the human variant of Creutzfeldt-Jakob disease. These agents were considered as low to high scientific/epidemiologic evidence of risk regarding blood safety with the potential for severe clinical outcomes.

The arguments that pleaded for the upper-level classification of DENV with regard to blood safety in North America were as follows^[38]: (1) the viremia is frequently asymptomatic and usually lasts for 2 to 7 d; (2) the viral load may be relatively high (from 10^4 to 10^8 copies/mL by NAT) in blood with the four serotypes of DENV, as exemplified by retrospective studies conducted in blood donors from Honduras, Brazil^[39] and Puerto-Rico^[37], with recovery of live virus from PCR-positive products in a few cases; (3) the disease can occur as important outbreaks; (4) the competent mosquitoes have a large distribution in the considered area (here United States); (5) the viral infection has a high seroprevalence in populations boarding the considered area; and (6) infected blood products could be imported from epidemic or endemic areas. At the opposite, the prevalence of positive samples was relatively low in the retrospective studies cited above (0.07% in 16521 blood gifts from Puerto-Rico^[40], 0.30% in 2994 blood gifts from Honduras^[39] and 0.06% in 4858 blood gifts from Brazil^[39]).

The potential threat of dengue to transfusion safety is majored by the rapid spread of the disease worldwide whose incidence has increased 30-fold in the

past 50 years^[41]. Half of the planet is already exposed (Figure 1A) and the distribution of competent vectors (Figures 1B and C) is progressing very rapidly, notably with the climate changes^[42] and the development of transcontinental travels. Regions with temperate climate as Europe or North America^[43] can be the target of future outbreaks as illustrated by the recent cases observed in Croatia^[5], Nice^[6] or Florida^[44]. In non-dengue endemic areas, asymptomatic infection is primarily associated with travellers returning from dengue-endemic areas. A few years ago, the recovery of areas endemic for malaria and dengue favoured the selection of blood donors returning from these countries. By now, dengue, as well as other arbovirolos, constitutes a risk that needs to be taken into consideration specifically.

MEASURES AVAILABLE FOR REDUCING THE RISK OF TRANSFUSION-TRANSMITTED DENGUE

Until a vaccine is widely used for preventing the expansion of dengue through the world population, it will be necessary to implement measures able to reduce the risk of transfusion-transmitted dengue. These measures include (1) the clinical selection of donors; (2) the implementation of screening tests specific for dengue; and (3) the non-specific reduction or inactivation of pathogens by the use of physical or chemical treatments applied to blood products. Their indications may differ in endemic and non-endemic areas^[45].

Clinical selection of donors

In endemic areas, this measure would consist in excluding donors who may be at higher risk of infection. Given the fact that the exposition to mosquito bite is rather unpredictable, such a measure is not realistic. On the other hand, the presence of fever in donors of blood products is a general contra-indication of blood gift.

In non endemic areas, the clinical selection of donors consists in excluding travellers returning from endemic regions for a period of 4 wk. For instance, the latter measure was adapted in Europe towards tourists returning from Madeira during the recent 2012-2013 outbreak. The main limit of this strategy is the need for continuous adaptation of these exclusion measures to various epidemiological situations, which may lead to complicate the work of personnel in charge of this selection and to discourage donors from coming again for blood gift.

Screening tests specific for dengue

This strategy is useful in endemic areas or during an outbreak. Serology is not adapted for screening purpose because the viremia precedes of a few days the antibody answer. Only NAT could allow detecting the

presence of viral genome in blood from infected donors. Such a strategy was applied in the Puerto-Rico outbreak in 2005^[40] and 2007^[37]. During the Madeira outbreak, an in-house RT-PCR assay was implemented for screening blood products; 43 of 1948 donations tested positive for DENV genome (further identified as DEN-1) between 9 September 2012 and 11 March 2013^[46]. For large-scale screening purpose as in blood donors, Gen-Probe Inc. (San Diego, CA, United States) developed a prototype TMA assay using highly conserved primers; the analytical sensitivity of the test was of approximately 15 copies/mL for each serotype^[39]. The low levels of viremia in many donors with dengue justify the individual testing of blood products, which limit this strategy to countries with high-income economy. By contrast to West Nile virus, no automated molecular screening test is currently commercially available.

In the future, the development of fully automated multiplexing assays detecting simultaneously several blood-transmitted pathogens in microarray plates or using nanotechnology would be very useful for areas where multiple infectious agents at risk for blood safety may circulate at the same time (*i.e.*, in the Caribbean or in South-East Asia)^[47].

Non specific reduction or inactivation of pathogens

Many systems are now available for treating blood products in order to inactivate some pathogens (for reviews see^[38,48-50]). Most of these techniques are able to inactivate bacteria and lipid-enveloped viruses as DENV. Due to technical purposes, they can be applied to plasma, platelets or red blood cells. The main techniques that are efficient on DENV are briefly described thereafter.

Some techniques are exclusively dedicated to plasma. Solvent-detergent treatment is able to disrupt viral envelopes. Dyes containing phenothiazine like methylene blue, when activated by visible light, are responsible for an oxidation of guanine present in viral genomes. Nanofiltration is able to retain viral particles whose size is over that of the pores of the nanofilter.

Other techniques based on photoactivation by ultra-violet (UV) rays may be applied to both plasma and platelet concentrates. The Intercept® system from Cerus Corporation (Concord, CA, United States) uses a psoralen derivative, amotosalen, as active compound. The Mirasol® system from Terumo BCT (Lakewood, CO, United States) use riboflavin (vitamin B2) as active compound. The Theraflex UV® system from MacoPharma (Tourcoing, France), by combining an exposition to UV light and strong shaking, induces the formation of cyclobutyl rings. Using those different technologies, a small proportion of platelets may be lost but the properties of activation, adhesion and aggregation of the cells resisting to the treatment are sufficiently well conserved to warrant their clinical use.

For red blood concentrates, some processes are in experimentation, including riboflavin (Caridian),

Inactine® (PEN110 from the Vitex Company, Prestons, NSW, Australia) and an alkylating agent, Amustaline, from Cerus Corporation, whose activation occurs through exposition to acidic pH.

The main advantage of these strategies is the inactivation or reduction of a wide range of pathogens, including those that are still unidentified. However, the benefit-risk of each treatment needs a careful evaluation.

Economic considerations

The measures listed above regarding the prevention of transfusion-transmitted dengue represent an extra-cost for the Health system, especially those involving screening molecular tests specific for dengue that would be dedicated to the transmission of a single pathogen. No cost-effectiveness study has already been conducted to evaluate the economic burden of the implementation of a molecular screening targeting DENV neither in endemic or non endemic areas.

Lessons can be drawn from the experience acquired with the systematic screening of blood products for the presence of WNV in the United States during the epidemic period. Two studies were published on this topic in 2005^[51] and 2006^[52]. They demonstrated that the optimal cost-effectiveness strategy for WNV screening in blood products depends on different factors, including mainly the prevalence of the agent in the considered population, but also the ability to pool or not the samples before screening (*i.e.*, mean viral load), the seasonal period concerned by the screening and the consequences for the recipients. Globally, these studies demonstrated that targeted donor screening seems to be more cost-effective than mass donor screening.

It is too early to consider whether these conclusions regarding WNV in a developed country may be applied to DENV in endemic and non endemic area. In dengue non endemic countries that correspond mostly to places with high living standards, it is likely that the emergence of a dengue outbreak will conduct to the set-up of a molecular screening, as it was done in Madeira recently^[46]. In the epidemic of DENV that occurred in northern Queensland, Australia, in 2008-2009, the risk for a dengue-infectious blood donation was estimated as 1 in 7146^[53]. Although the temporary exclusion of potentially infected donors was chosen to limit transfusion-transmitted dengue during these outbreaks, the authors raised the question of the better cost-effectiveness of a strategy involving the use of a suitable screening test or of a pathogen reduction technology^[53].

In dengue-endemic areas, the risk may be higher, as shown during the 2005 outbreak in Singapore through a mathematical modelling, with an estimated risk for a dengue-infectious blood donation of 1 in 1667 to 6154^[54]. The implementation of a screening test would be probably cost-effective as compared to the exclusion of blood donors but it is likely that neither of these two strategies could be implemented in low income

countries where the disease is the more prevalent, at least in a near future.

CONCLUSION

Dengue provides an excellent model of transfusion-transmitted disease. Despite the large distribution of the disease worldwide, the risk with blood products from infected donors was only recognized recently. Except for one case of DHF^[37], the disease, when transmitted by blood, does not seem to be more severe than after mosquito bite. However, the area of dengue extended considerably during the last 50 years; after having been limited to intertropical regions for a long time, the disease is now reaching temperate areas because of the worldwide distribution of its two main vectors (Figure 1) and of the climate change^[42]. Considering these emerging risks, there is an urgent need for mathematical models able to predict the spread of DENV and its consequence on the supply of blood products. While waiting for an efficient prophylactic vaccine that could be able to reduce the burden of the disease, it is important to develop efficient measures for securing blood products in endemic and non endemic areas. The attention paid to DENV as a transfusion-transmitted pathogen could help to prevent the emergence of other more harmful known or unknown viruses.

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Key role of human leukocyte antigen in modulating human immunodeficiency virus progression: An overview of the possible applications

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locus have shown the peculiar capability to modulate both innate and adaptive immune responses. In particular, HLA class I molecules are recognized by CD8⁺ T-cells and natural killers (NK) cells towards the interaction with T cell receptor (TCR) and Killer Immunoglobulin Receptor (KIR) 3DL1 respectively. Polymorphisms within the different HLA alleles generate structural changes in HLA class I peptide-binding pockets. Amino acid changes in the peptide-binding pocket lead to the presentation of a different set of peptides to T and NK cells. This review summarizes the role of HLA in HIV progression toward acquired immunodeficiency disease syndrome and its receptors. Recently, many studies have been focused on determining the HLA binding-peptides. The novel use of immune-informatics tools, from the prediction of the HLA-bound peptides to the modification of the HLA-receptor complexes, is considered. A better knowledge of HLA peptide presentation and recognition are allowing new strategies for immune response manipulation to be applied against HIV virus.

Key words: Human immunodeficiency virus progression; Human leukocyte antigen; Epitope; Immunoinformatics; CD8⁺ T lymphocytes

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Core tip: Human immunodeficiency virus (HIV) disease progression depends on several host factors. Among them human leukocyte antigen (HLA) locus has a main role due to the peculiar capability to modulate both innate and adaptive immune response. In this review, the role of HLA molecules and its receptors in HIV progression toward acquired immunodeficiency disease syndrome is summarized. A better knowledge about HLA-peptide presentation and recognition by immune cells will open new applications in HIV vaccine and diagnostics design.

Abstract

Host and viral factors deeply influence the human immunodeficiency virus (HIV) disease progression. Among them human leukocyte antigen (HLA) locus plays a key role at different levels. In fact, genes of the HLA

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INTRODUCTION

Different host's genetic factors have been associated both with rapid and slow progression to acquired immunodeficiency disease syndrome (AIDS). This suggests that the efficient control of human immunodeficiency virus (HIV) -1 infection lays on different variants of immune response associated genes. In this context, genetic association studies have been strongly limited by different factors. HIV-1 is a quasi-species virus with large variability among the population even if small geographical areas are examined. In this review, the strong contribution of human leukocyte antigen (HLA) locus in HIV progression is highlighted. The use of immune-informatics is capable to efficiently predict the HLA binding peptides, adding important information in this context. Overall, this might lead to the design of preventive vaccine and immunotherapies capable to improve the HIV immune response.

HIV IMMUNE RESPONSE

HIV immune response depends on both innate and adaptive compartment of the immune system. The primary HIV infection typically occurs in the mucosa. At this level, resident memory CD4⁺ T cells are infected together with dendritic cells, granulocytes, natural killer (NK) cells and macrophages^[1]. Subsequently, infected cells and virus particles bounded by dendritic cells and B lymphocytes reach the lymph nodes. Within the lymph nodes, HIV-1 infects also the effector-memory and the activated CD4⁺ T cells.

These processes are responsible for the increase of viral spread, viremia and decrease in the number of CD4⁺ T cells^[1]. Early events, which occur directly after HIV infection, determine the course of HIV disease progression. The reduction of viral replication often occurs before the development of the adaptive immune response against HIV, suggesting that the innate immune system has an essential role in controlling the infection^[2,3].

Studies on primary HIV infection before the seroconversion show the presence of HIV-specific adaptive immune response exert by CD8⁺ T lymphocytes (CTL)^[4-6]. CTL immune responses play a central role in the control of viral replication as it has been observed in Long Term Non-Progressor (LTNP) patients. Different mechanisms for viral inhibition mediated by CTL immune response have been observed.

HIV infected cells are recognized by the TCR of HIV-

specific CTLs when viral peptides are presented at the cell surface in the context of HLA class I molecules. This recognition leads to CTL cytotoxic immune response^[7].

Humoral immune response has a secondary role in the control of HIV infection. Although neutralizing antibodies reduce the virus particles and therefore the viral spread. However, serum of the infected patients does not reduce the viral infectivity *in vitro* and the efficacy of gp120 neutralizing antibodies is reduced. This is due to the fact that gp120, HIV glycoprotein responsible for the viral entry, has a high mutation frequency which leads to conformational changes impairing the antibody binding^[7].

Due to the lack of capability of the immune response to eradicate HIV, the infection becomes chronic and the virus is integrated in a latent form in the human genome. Despite the return of circulating CD4⁺T cells to normal levels, massive immune activation and accelerated cell turnover takes place. The ultimate consequence of immune activation is the depletion of CD4⁺ T cells. In absence of T helper response the immune system is not able to control other infections, therefore opportunistic infections occur and lead to AIDS^[1].

In general, HIV protective immune response is associated with recognition and activation of the cytolytic function exerted mostly by NK and CD8⁺ T cells. Thus, the contribution of HLA molecules and its ligands play a key role in controlling HIV disease progression^[8].

HIV PROGRESSION

The progression of HIV infection has different phases. In the primary infection, HIV infects mainly macrophages and dendritic cells by using the co-receptor C-C chemokine receptor 5 (CCR5) together with the CD4 molecule. Virus replication in the lymph nodes leads to the viremic peak characteristic of acute infection^[4,5]. The viremia increases the viral spread in the other lymph nodes of the entire organism. The immune system mounts a response to control the viremia, which decreases towards a stationary phase named "set point". In most of the cases, the immune response is not capable to eradicate the infection. Therefore, an equilibrium between host and virus occurs and the viral DNA is integrated in a latent form that could not be detected by the immune system^[6].

In the late phase of the infection, the constant viral replication induces a tropism shift. The virus prefers C-X-C chemokine receptor type 4 co-receptor (CXCR4) and infects mainly the CD4⁺ T-cells. The CD4⁺ T-cells depletion (< 200 cell/mm³) and the increase of the viral load lead to an impairment of the entire immune system. Therefore, opportunistic infections occur, leading to AIDS and often to death^[7]. Clinical latency period has a large variability in the HIV-infected subjects with different disease progression rates in absence of antiretroviral therapy.

Most of the infected individuals (70%-80%) are defined as slow progressors (SP). SP are characterized by increasing of viral load and CD4⁺ T-cell count decline towards AIDS within 6-10 years of HIV infection. A smaller percentage of individuals (10%-15%), defined as fast progressors, have a fast CD4 count decline and develop AIDS within few years after infection. The LTNP represent about 5% of the infected cases and do not have significant changes in CD4 count, viral load or clinical symptoms for over 10 years^[7-9]. Among them, a subgroup named elite controllers (EC) is characterized by stable CD4⁺ T-cell count, undetectable viremia and no clinical symptoms overtime^[10].

Overall, the strong individual variability to HIV infection highlights the importance of the host factors in delaying HIV progression toward AIDS. Different host factors have been widely associated with HIV progression and can be classically divided into two different groups: one related to a reduction in the viral entry capability and the other one with the interference with the viral replication process.

Reduction of viral entry has been associated with different receptors, co-receptors and ligands. Among them the CCR5Δ32 in combination with higher C-C Chemokine ligand 3-like 1 (CCL3L1) copy number and RANTES or Stromal cell-derived factor 1 chemokine variants have been extensively studied^[9,11-14]. Regarding the viral replication processes, a pioneer work of Brass led to the identification of all the possible host endogenous proteins related with HIV infection^[15].

Among them Zinc Ribbon Domain-containing 1 (ZNRD1), HLA Complex P5, (HCP5) Apoipoprotein B mRNA editing enzyme catalytic polypeptide-like 3G (APOBEC3G) genes have been extensively studied in association with delayed HIV progression. However, further studies regarding other possible interacting proteins still need to be addressed^[16-18].

More recently, a contribution of micro-RNA has been described in HIV context leading towards interesting alternative approaches^[19].

In addition, other immune related mechanisms have been associated with HIV control by immune response. This is the case of TNF-α and Ig enhancer HS1, 2 last but not least in showing a role in controlling HIV progression. Although, these factors barely play a role in delaying HIV progression compared with other host factors^[20,21].

Beside the constant discovery of novel host variants, multiple issues such as population dependency might increase the difficulty to perform an association with HIV progression. For these reasons, HLA locus remains the unique factor clearly associated with HIV progression among the human population. However, different HLA alleles play a main role in HIV disease progression depending on the population considered.

Moreover, the HLA locus is the only one capable to modulate both innate and adaptive immune responses against viral infections respect to other immune related

genes. Therefore, HLA locus might be used not only for diagnostic purpose, but also for drug and vaccine design approaches.

HLA

The *HLA* gene products are highly polymorphic molecules, characterized by co-dominant expression and polygeny. The combination of polygenicity and polymorphism has two important consequences. First, it ensures that each individual will be able to present a broad range of peptides. Second, the population will be consisted of individuals presenting different peptide's repertoires^[22].

It is possible to distinguish the HLA molecules in two different classes: HLA class I and HLA class II^[23].

HLA class I is expressed on all nucleated cells and are recognized by CD8⁺ T-cells^[23]. The overall structure of HLA class I molecule is shown in Figure 1A. The β2-microglobulin is a monomorphic polypeptidic chain and its main role is to keep the tridimensional structure of HLA class I molecules. The α-chain is responsible for the peptide binding and interacts with TCR, CD8 and innate immune receptors. The binding of the peptide as well as the TCR interaction are mediated by α1 and α2 domains. Both of them present two main interaction pockets (B and F), which directly interact with the bound peptide (Figure 1B). HLA class I molecules bind peptides between 8–12 amino acids long which are derived from proteolysed endogenous protein fragments^[23].

HLA class II is expressed only on antigen presenting cells and are recognized by CD4⁺ T-cells^[23].

HLA class II molecule is composed by two polypeptidic chains (α and β) with a similar structure and belong to Ig superfamily. Both α and β chains participate in the peptide binding (Figure 2A). The two chains are bound in a non-covalent manner and can be further divided in two different domains. The first domain of each chain (α1 and β1) is responsible for the peptide binding and TCR interaction. The second domain of each chain (α2 and β2) has an important role in the HLA class II structure and in the interaction with CD4 molecules.

The two HLA molecules have a distinct pattern of expression and cellular interaction. HLA class I molecules are expressed by all nucleated cells and recognized by CTL. HLA class II molecules are selectively expressed on antigen presenting cells such as macrophages, monocytes, B lymphocytes and dendritic cells. HLA class II molecules are recognized by T helper lymphocytes^[24]. The main role of HLA molecules is to present the antigen to different immunological receptors. In first approximation, HLA class I molecule presents peptides derived from endogenous/cytosolic proteins, while HLA class II presents peptides derived from exogenous proteins^[25].

In addition, HLA class I molecules play an important role in the activation of the innate immune response. In fact, HLA class I molecules interact also with innate immune receptor expressed by NK cells^[26]. The wide inter- and intra-population diversity in HLA locus

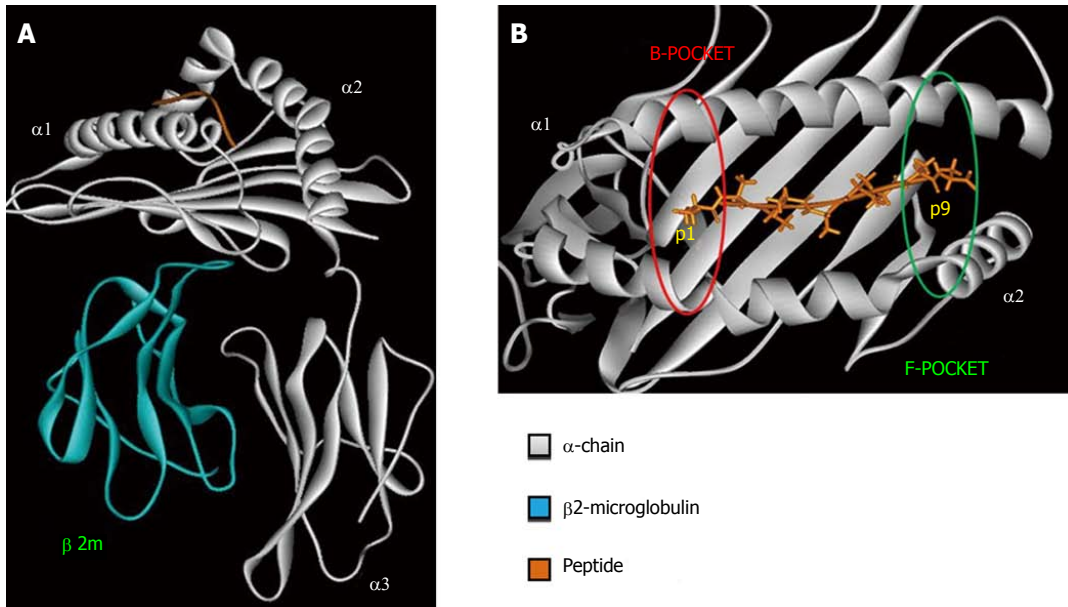


Figure 1 Human leukocyte antigen class I tridimensional structure. Crystal structure of the HLA-B*57:03 (PDB ID: 2YPK); HLA α -chain in gray, β 2-microglobulin in blue, the peptide in orange. A: HLA class I overall structure; B: HLA class I peptide binding pocket. In red is shown the HLA pocket B, in green HLA pocket F the most polymorphic regions in HLA peptide binding pocket. The figure has been made using WebLab Viewer Pro 3.7. HLA: Human leukocyte antigen.

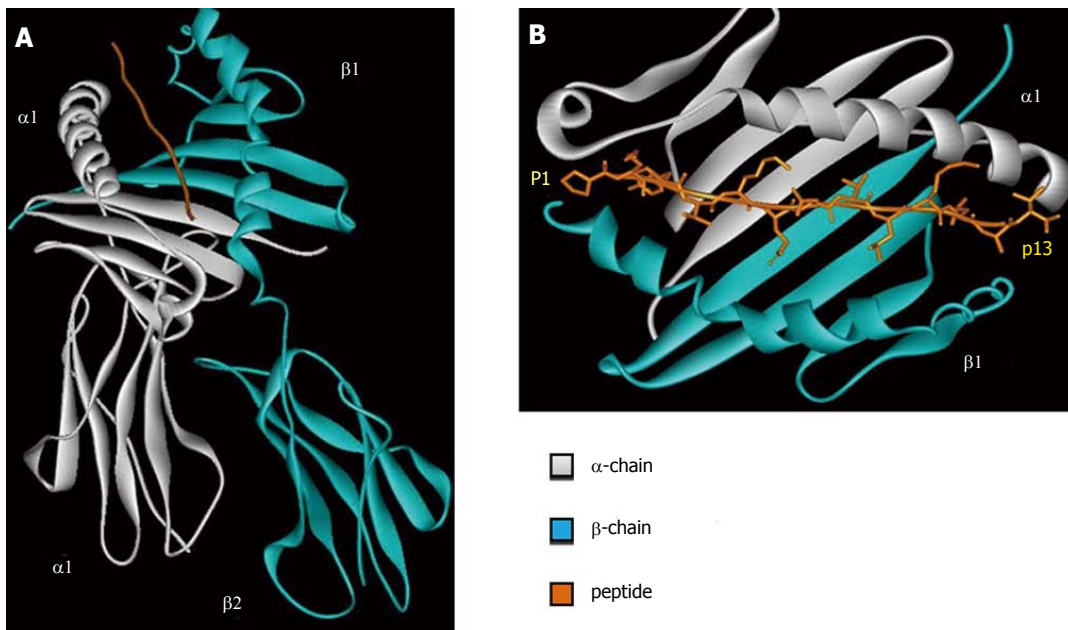


Figure 2 Human leukocyte antigen class II tridimensional structure. Crystal structure of the HLA-DR1 (PDB ID: 1DLH) in gray HLA α -chain, in blue HLA β -chain, in orange the peptide. A: HLA class II overall structure; B HLA class II peptide binding pocket. The figure has been made using WebLab Viewer Pro 3.7. HLA: Human leukocyte antigen.

and the presence of other immune associated genes increases the difficulty to select the genetic variant(s) responsible for the disease susceptibility. However, HLA alleles' association with particular immunological profile has been consistently assessed for different chronic viral infections including HIV.

In this context, heterozygosity for HLA class I molecules has been associated with HIV delayed disease progression and lower mortality in HIV infected patients^[27,28]. In addition, various HLA alleles have been associated

with an increase or decrease risk of HIV vertical and horizontal transmission and hypersensitivity to anti-HIV therapy^[29,30].

HLA IN HIV PROGRESSION

HLA/HIV association studies are useful to evaluate the host-pathogen interaction. HLA is important not only for the adaptive immune response but also for innate immune response. Polymorphisms within the

different HLA class I alleles generate structural changes in peptide-binding pockets. Amino acid changes in the peptide-binding pockets lead to the presentation of a different set of peptides to CTLs^[31-33].

The ability of particular HLA alleles to induce a viral selection could predict the HIV viral load. This could provide an “a priori” information about the disease progression^[34]. Evaluations of HLA supertypes, group of alleles that share specific peptide-binding preferences, simplify the association studies with different disease progression.

The study of EC sheds light on the contribution of Human Leukocyte Antigen B (HLA-B)*57:01 (Supertype B*58) allele with HIV delayed disease progression. This allele is able to recognize a conserved epitope of HIV Gag protein, leading to a higher CD4⁺ T-cell count and lower viral load in absence of Highly Active Antiretroviral Therapy (HAART). HLA-B*57:01 is also characterized by the presence of unique valine at position 97 that contributes to the formation of the C-pocket in the peptide-binding cleft^[31,33].

When a subject that do not carry HLA-B*57:01 allele is infected with a viral strain derived from a B*58 patient, it resembles the same CD4⁺ T-cell count and viremia of the B*58 patient. This observation suggests that HLA exerts a strong restriction on the viral replication and the viral mutant selected have a lower fitness^[35].

Other studies have associated HLA-B*27 and HLA-B*58 with a low viral load and higher CD4⁺ T-cell count. In this context, the selectivity exerted by CTL after antigen recognition by HLA class I molecule is responsible for delaying the HIV progression^[28,31,33,36-38].

Several HLA-B alleles have been associated with HIV rapid disease progression. Among them HLA-B*35 supertype contributes to a reduction of CTL peptide recognition and therefore leads to a non-efficient viral control^[28,37]. Further, supertype B*7 has been associated with high viral load, decrease CTL response and consequently rapid HIV progression towards AIDS^[28,31].

Multiple issues such as the viral strain variability within the subjects and the different genetic background of the population have limited the association studies related with HIV progression.

In this context, we performed a study in a defined cohort of children infected during a hospital outbreak with a monophyletic strain of HIV-1^[39]. The role of HLA amino acid polymorphisms determining specific characteristics of the HLA peptide-binding pocket has been assessed. In particular, HLA-B peptide binding pockets present a specific set of epitopes against which the subject can mount a HIV-specific immune response. According to previous observations, these findings might represent the basis of the HIV disease progression^[40-44].

As expected from previous immunogenetic studies, a large number of residues found in association with LTNP or progression to AIDS have been located in the HLA-B locus^[42-46]. Recently, we have further supported this notion with *in silico* identification of the HIV *gag*

protein epitopes. The study has been performed on the same outbreak cohort using HIV-1 viral sequences and HLA alleles. Peptides deriving from the HIV-1 sequences and recognized by the HLA allele combinations of the study subjects have been further analyzed.

Non-progressors recognized a higher number of epitopes compared to progressors in any HLA locus analyzed^[47]. This is in agreement with previous observations showing an important contribution of CTL immune response in controlling the HIV disease progression. In a nutshell, HLA class I molecules and the recognition of large set of CTL epitopes are the key factors for delaying HIV progression^[48-50].

CTL also determines escape mutants of the virus in different genes of HIV-1 such as Protease, Reverse Transcriptase (RT), Vpr and Nef^[38,51]. Different HLA alleles, such as HLA-B*580^[52], efficiently cross-recognize HIV-1 CD8⁺ T-cell epitopes leading to delayed progression^[53].

Recently, many studies have been focused on determining the HLA binding-peptides. The approaches are from direct measurement to the development of different Major Histocompatibility Complex (MHC) class I binding prediction systems^[54-56]. Different online databases are capable to extract epitopes obtained from experimental and *in silico* studies giving also the opportunity to predict HLA binding epitopes using any target protein sequence^[57-60]. The choice of the prediction system is very important and often the combination of more than one prediction system has shown the best performance^[56,61-63].

Once obtained the predicted epitope, it is always very useful to perform a comparison with literature data. Thanks to the large *in vitro* characterization of HIV epitopes, it has been determined that most of the *in silico* predicted epitopes are also described within the literature. This supports the efficiency of prediction methods related with the epitope discovery^[59,60].

Overall, HIV-specific T-cell response, and in particular CTL, plays a key role in controlling HIV infection^[40,41]. T-cell response depends on HLA molecules. Thus, the individual's variations in the HLA class I and II alleles has a profound effect on the outcome of infection and disease progression toward AIDS^[40,42].

NOVEL ASPECT IN HLA-HIV INTERACTION

HLA-B polymorphic variants 80I, 81A, 82L, 83R have been associated with LTNP^[46]. These positions interact with the peptide in the F-pocket of HLA-B^[46,64]. The LTNP associated pattern 80I, 81A, 82R, 83L is typical for HLA-B supertypes B58 and B27 which are already found associated with a slow progression to AIDS^[42]. The same amino acid positions are involved in the formation of the structurally related HLA serotyping epitope Bw4 and Bw6. When HLA-B alleles are classified accordingly with carrying Bw4/Bw6 epitope, we have shown a

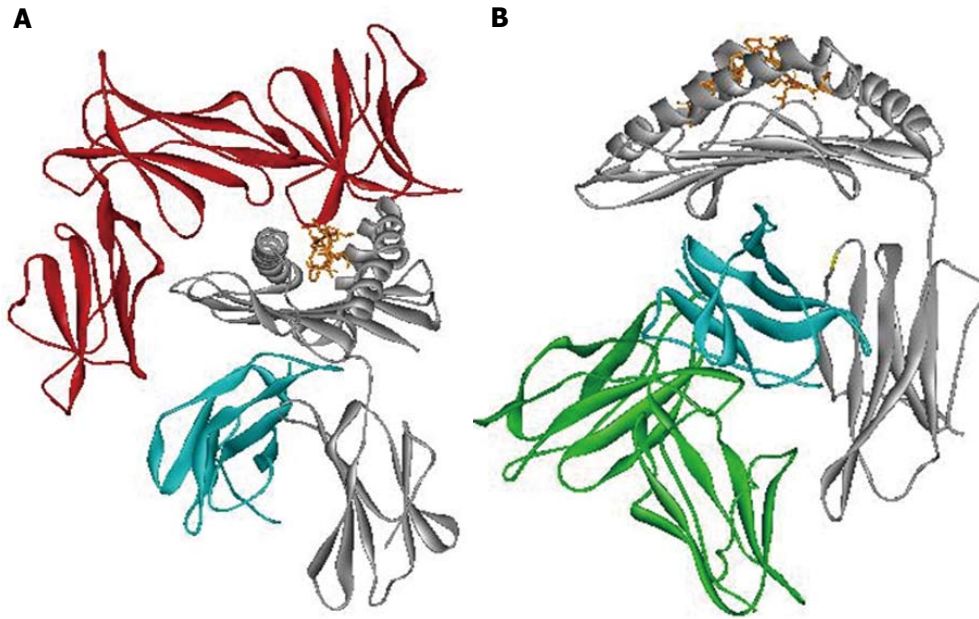


Figure 3 Human leukocyte antigen class I interaction with innate receptor. Crystal structure models of the HLA-B*57:01 interacting respectively with (A): KIR3DL1 receptor (red) (PDB ID: 3HV8); (B): LILRB1 receptor (green). HLA α -chain in gray, β 2-microglobulin in blue, the peptide in orange. The figure has been made using WebLab Viewer Pro 3.7. HLA: Human leukocyte antigen.

strong contribution of Bw4 homozygosity in delaying HIV progression^[46,65]. These results are in agreement with previous associations between Bw4 homozygosity and the control of HIV viremia^[66].

The importance of epitope Bw4 is due to different aspects. First, it has a direct interaction with the HLA bound peptide involved with CD8⁺ T cell recognition. Second, it is also a ligand for Killer Immunoglobulin Receptor 3DL1 (KIR3DL1), an NK's inhibitory receptor (Figure 3A)^[67,68].

This evidence suggests a strong contribution of the innate immune response in controlling HIV progression and confirming the key role played by HLA-B molecules^[2]. Recent studies evaluated the different contribution of KIR3DL1/HLA-B allele's interaction in modulating the innate immune system^[68-71]. The presence of HLA-B Bw4 epitope leads to a stronger interaction with all the different KIR3DL1 alleles. This is particularly evident within the HLA-B alleles belonging to the same supertype, in agreement with previous data^[72-75].

Different studies evaluate the contribution of Leukocyte Immunoglobulin-Like Receptor subfamily B member 1 (LILRB1) interaction with HLA class I in the context of several infections (Figure 3B)^[76-78]. Among HLA class I polymorphisms, we associated the HLA-B alpha 3 domain amino acid position 194 with different HIV progression^[46,65]. Amino acid position 194 of HLA-B has been found to take a part in the interaction with LILRB1 receptor (ILT2/LIR1/CD85j) when the Val variant is present^[69,78,79]. Moreover, Val 194 was in association with LTNP^[46,65]. Change in the strength of interaction between HLA-B alleles carrying Ile 194 and LILRB1 receptor might lead to rapid HIV progression. Previous

data suggests that the expression of LILRB1 receptor on the cell surface remains unchanged in subjects with different HIV progression^[80]. However, the presence of different amino acids at the polymorphic position 194 of HLA-B might modify the interaction with LILRB1. This might influence the LILRB1 strength of binding, as already reported for the LIR1-HLA-A interaction^[77]. These results show the influence of HLA allelic variation and conformation on LILR binding capability. These findings are according to recent studies particular in the HIV context^[77,78].

The contribution of the HLA-bound peptide seems to be the key point able to disrupt HLA interaction with the different immune receptors (Figure 4). In the context of HLA-B/KIR3DL1 interaction, the HLA-bound peptide position P8 is the main one that is able to disrupt KIR3DL1 binding. This has been previously observed in KIR3DL1 interaction with HLA-B*27:05 and HLA-B*57:01 alleles due to the conserved amino acid residue Glu282 of KIR3DL1 receptor^[68,81-85]. The strong influence of the HLA bound peptide in the modulation of the innate immune response, point out similarity between T-cell and NK cell immune response. Individual selection pressures exerted on HLA class I by T-cell and NK-cell might cause a competition between the two different immune responses. Therefore, depending on the HLA class I allelic variant and the antigenic peptide loaded on HLA molecule we might observe a beneficial NK or T-cell response with detrimental consequences for the other one^[86].

Altogether, the observations suggest that each peptide binding pocket position of the HLA class I molecule is capable of modulating innate and adaptive immune

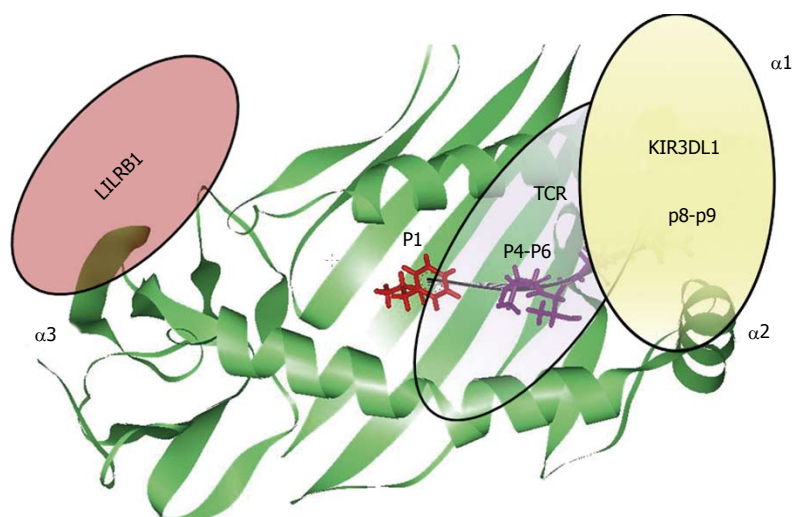


Figure 4 Peptide recognition. Schematic representation of the contribution of each HLA bound peptide position in the modulation of the interaction between HLA-B molecules (green) with TCR (violet) and KIR3DL1 (yellow), respectively. The figure has been made using WebLab Viewer Pro 3.7. HLA: Human leukocyte antigen; TCR: T cell receptor.

receptors leading to different immune responses (Figure 4).

Notably, identification of T-cell epitopes is actually made with the strategy of the reverse vaccinology. This strategy is based on HLA binding specificity and takes in consideration only the interaction with adaptive immune receptor. Future studies should be focused on the prediction of binding epitopes with wider characteristics. Peptides should be capable not only to be recognized by adaptive immune receptor, but also to modulate the innate immune receptor. These peptide characteristics could allow better fitting strategies for vaccination and diagnostics.

CONCLUSION

In conclusion, HLA molecules play a key role in modulating both adaptive and innate immune responses. The protective cytotoxic immune response is modulated by the interactions with TCR as well as other innate receptors^[31,33]. The modulation of innate immune responses depends also on the peptide-binding capability of HLA-B and on the interaction between HLA-B and NK's inhibitory receptors such as KIR3DL1 and LILRB1. The observed fine tune regulation might play a key role in the progression of HIV infection. The application of immune-informatics to immunogenic studies might shed new lights on the mechanisms behind the association of HLA genetic susceptibility to viral infections. This represents a powerful tool for novel design of vaccine and diagnostics, ensuring wider population coverage with the inclusion of genetically susceptible subjects.

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Early initiation of antiretroviral treatment: Challenges in the Middle East and North Africa

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Abstract

New World Health Organization guidelines recommend the initiation of antiretroviral treatment (ART) for asymptomatic patients with CD4+ T-cell counts of ≤ 500 cells/mm³. Substantial reduction of human immunodeficiency virus (HIV) transmission is addressed as a major public health outcome of this new approach. Middle East and North Africa (MENA), known as the area of controversies in terms of availability of comprehensive data, has shown concentrated epidemics among most of it's at risk population groups. Serious challenges impede the applicability of new guidelines in the MENA Region. Insufficient resources restrict ART coverage to less than 14%, while only one fourth of the countries had reportable data on patients' CD4 counts at the time of diagnosis. Clinical guidelines need to be significantly modified to reach practical utility, and surveillance systems have not yet been developed in many countries of MENA. Based on available evidence in several countries people who inject drugs and men who have sex with men are increasingly vulnerable to HIV and viral hepatitis, while their sexual partners - either female sex workers or women in monogamous relationships with high-risk men - are potential bridging populations that are not appropriately addressed by regional programs. Research to monitor the response to ART among the mentioned groups are seriously lacking, while drug resistant HIV strains and limited information on adherence patterns to treatment regimens require urgent recognition by health policymakers. Commitment to defined goals in the fight against HIV, development of innovative methods to improve registration and reporting systems, monitoring and evaluation of current programs followed by cost-effective modifications are proposed as effective steps to be acknowledged by National AIDS Programs of the countries of MENA Region.

Key words: Antiretroviral agents; HIV; CD4 counts; Co-infections; Regional health planning

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Core tip: The main purpose of the present review was to investigate the feasibility of new World Health Organization guidelines on earlier initiation of antiretroviral treatment in resource-limited settings, specifically in the Middle East and North Africa region.

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INTRODUCTION

In 2013, recommendations regarding the early initiation of antiretroviral treatment (ART) were published by World Health Organization (WHO) in consolidated HIV treatment and prevention guidelines. Initiation of ART among asymptomatic adults and adolescents with CD4+ T-cell counts of ≤ 500 cells/mm³, regardless of clinical stage is strongly recommended in this guideline^[1].

Previous guidelines (2010) suggested that treatment be initiated for patients with CD4 counts of ≤ 350 cells/mm³^[2]. Several recent studies provide evidence of substantially reduced risks of HIV transmission among serodiscordant couples, if ART is initiated when CD4 counts range from 350 to 550 cells/mm³^[3]. Hence new recommendations have a greater focus on public health issues concerning HIV transmission.

Although the new guidelines delineate the earlier initiation of ART as "highly cost-effective", adaptation of these recommendations is a significant challenge for many countries including those in the Middle East and North Africa (MENA). Evidence from most of the 23 countries of MENA has indicated a significant increase in HIV incidence and AIDS-related deaths in the recent decade; nonetheless, antiretroviral coverage was reported to be less than 14% by the end of 2011^[1-4].

From a social perspective, the cultural context that prohibits HIV related high-risk behaviors encourages ubiquitous stigma that affects access to available services in the MENA countries^[4-7]. Availability and allocation of resources, on the other hand, has been threatened by ongoing conflicts and high rates of migration^[8-12]. From a public health perspective, in many countries of the MENA lack of integrated data on HIV incidence rates and disease progression among known patients requires to be increased^[8]. In addition, scarcity of gender-sensitive programs and lack of systematic approach in development of national guidelines hinder

enactment of new treatment strategies in MENA^[4,5,13,14]. HIV epidemic is characterized by rising incidence and prevalence rates among key at-risk populations, specifically among people who inject drugs (PWIDs)^[12].

Hence the co-infections that are diagnosed more commonly among these population groups require particular attention in the implementation of the ART guidelines^[15,16]. Additionally, in many countries of the region where cost-effective tests for viral load and CD4+ T-cell counts are not available and retention in care is not well understood, monitoring of response to ART and disease progression inherits location-specific challenges in adapting ART guidelines^[17].

Previous studies that have investigated the quality of clinical practice guidelines in the region indicate that serious gaps; including outdated or inconsistent clinical contents, weak methodology in tailoring recommendations, and insufficient applicability^[13].

The development of HIV treatment protocols that are coordinated with international standards requires better knowledge of the epidemiological and clinical characteristics of patient populations in the region^[13,18]. Hence, we highlight the available evidence-based information that may benefit research scientists, clinicians, and health authorities of the region in their attempts at scaling up of HIV/AIDS programs including ART coverage.

RESEARCH

To conduct the present study, we searched PubMed, Embase, and local databases for all relevant articles published from the MENA region. Also, published results of incomplete cohorts and other related studies that investigated early initiation of antiretroviral regimens or initiation among at-risk populations, including articles not available before June 2013 were accessed through PubMed and Google search engines; online library of the International AIDS Society and Research Gate were also screened for possible research works. Publications with findings that are important in the context of HIV/AIDS responses among low and middle income countries in other parts of the world were also used for the review.

Major search key words and phrases included, but were not limited to, the following: "early antiretroviral initiation + 2013-14", "ART and HIV-Hepatitis C (HCV)", "ART and HIV-hepatitis B (HBV)", "ART and HIV-tuberculosis (TB)", "ART cost-effectiveness", "HIV retention in care", "HIV antiretroviral adherence", "HIV and drug resistance", "HIV-HCV PWIDs", "HIV/AIDS MENA region", "PWIDs in MENA", "Men who have sex with men (MSM), Female sex workers (FSWs) in MENA region"; all the keywords were specifically searched for in publications from countries of the MENA region.

ART INITIATION BASED ON CD4 + T-CELL COUNTS

Available antiretroviral drugs are prescribed to delay

immunologic failure and control HIV-related diseases rather than to eradicate the virus^[19]. CD4+ T-cell counts are the most utilized, and probably available, immunologic marker for clinicians to decide for initiation of ART^[20]. This test also serves as a predictor of rates of AIDS and non-AIDS related causes of death^[21,22]. However, many resource-limited settings still depend on the WHO clinical staging system for decision-making^[17].

According to a recent short report, only six of the countries in MENA had reportable data on patients' initial CD4 counts at the time of diagnosis in 2011, and eight countries had provided similar data in 2009. In most countries over 50% of the patients were ART eligible meaning they had CD4 counts of ≤ 350 cells/mm³ at the time of diagnosis^[5,18]. Development or improvement of standard and organized surveillance systems in settings where CD4 measurements are available seems a priority in the region^[18,23]. In resource-limited countries, WHO clinical staging if carefully adapted in practice should provide as an accurate substitute to accelerate response in these areas^[17].

IMPLEMENTING ART UPTAKE AMONG AT-RISK POPULATIONS

Concentrated HIV epidemics have been reported among at least one of the high-risk population groups (PWIDs, female/male sex workers, MSM) in all countries of the region^[11]. However, at least in ten countries out of 23, HIV surveillance is not available among most at risk groups and sample size estimations of most at risk groups using primary data acquisition has been established only in Pakistan^[18]. Overall, HIV prevalence, and concurrent high-risk behaviors of injecting drug users have been widely investigated within MENA countries^[24,25]. MSM and FSWs are the two other major groups; in recent years their HIV risk behaviors have been better understood in several countries of the region^[11]. Evidence also suggests that the disproportionate prevalence of HIV among men is related to unknown sexual behaviors and drug use as overlapping high-risk activities^[5]. Nevertheless, no studies are available concerning response to ART or patterns of treatment adherence among HIV infected patients who are affiliated with these high-risk groups; nor is there sufficient evidence from the other countries regarding improved outcomes due to the early initiation of ART among PWIDs and other high risk groups^[20].

PWIDs

From 13 MENA countries that have published evidence-based data on PWIDs, epidemics are reported as being concentrated in Pakistan, Iran, Afghanistan, Egypt, Morocco and Libya^[26]. While increasing trends are reported in Pakistan, Iran and Egypt, Libya has recently been facing the highest HIV prevalence (87%) among

PWIDs in the region^[27].

The burden of disease related to blood-borne infections among PWIDs remains high and raises global concerns^[4,11,28]. The MENA region is no exception, and expansion of harm reduction programs is essential for effectively fighting HIV. HCV and HBV are also not targeted appropriately despite higher transmission rates compared to HIV^[26].

Management of HBV or HCV co-infections in HIV patients has been a matter of controversy for years^[20]. Although ART regimens have shown benefits in several studies, despite moderate levels of liver toxicity among non-injectors^[23,29], serious hepatic complications impede initiation of ART among PWIDs^[24]. Study of a large cohort of more than 60 thousand people living with HIV/AIDS in Europe and North America has led to the proposal that liver-related causes of death, including hepatitis and liver failure, are the most common causes of mortality among PWIDs during and after the first year of ART initiation^[15]. For example, Nevirapine-based regimens may lead to elevated CD4 levels in HIV-HBV co-infected patients^[29], however, the very complicated immunopathology of viral hepatitis co-infections in these patients requires evidence-based decisions that are suitable for the epidemic in the MENA Region^[24]. We underscore the HIV and hepatitis co-infection prevalence among general and high-risk populations in various MENA countries in Table 1.

Bridging populations

New guidelines also suggest that ART should be initiated among HIV patients who are in serodiscordant relationships, to reduce risk of transmission^[1]. This recommendation is based on recent findings that indicate substantial decrease (89%) in virus transmission from infected patients to their uninfected partners, who were prescribed ART with CD4 counts of 350 to 550 cells/mm³ of blood^[3]. A recent study from Iran provides valuable data on potential concentrated epidemics among the non-injecting sexual partners of PWIDs^[7,30]; this study provides insights into the possibility of overlapping transmission between high-risk groups such as PWIDs and FSWs; considering the disproportionately high male-to-female prevalence and the lack of confirmatory surveys in the region, we cannot rule out the possibility of current or future hidden sub-epidemics among FSWs and MSM in certain countries of the region^[18,30,31]. The existing gap between identified and estimated numbers of people infected with HIV may potentially feed the increasing vertical transmission of the virus in the near future^[4,5,32].

Women in monogamous relationships with PWIDs or MSM, are perceived to have low rates of high-risk activities, but are at high risk of transmitting HIV, and probably also HBV and HCV to their newborns^[26,30]. On the contrary these women do not have consistent access to HIV counseling and lack the willingness to test for HIV^[4,33]. Hence, HIV awareness should be developed

Table 1 Evidence-based prevalence rates of human immunodeficiency virus, hepatitis B and C co-infections in 13 countries of the Middle East and North Africa region

Country - city names (yr)	Target population (n) ¹	HIV prevalence	Methodology	HCV ² (Isolated or co-infection prevalence)	HBV ² (Isolated or co-infection prevalence)
Afghanistan - three cities (2009) ^[56,56]	FSWs (n = 520)	0.19%	Cross-sectional	Isolated: 1.92%	Isolated: 6.54% (range: 3% to 17.5%) Isolated: 7.1%
	PWIDs	7.1% (1% to 18.4%)	Cross-sectional	Isolated: 40.3% Co-infection: 94.9%	
Bahrain - Bahrain (2002-2006) ^[57]	HCV infected patients (n = 183)	0.5%	Retrospective cohort	HCV-HBV co-infection: 3.3%	-
Egypt - Cairo (2010) ^[58]	Blood donors-family members (n = 15017)	0.01%	Cross-sectional	Isolated: 4.3%	Isolated: 1.7%
Iraq - Karbala (2009) ^[59]	Healthcare workers (n = 124)	0	Cross-sectional	0	0
Iran - Tehran (2007) ^[60]	PWIDs (n = 899)	10.7%	Cross-sectional	Isolated: 34.5% Co-infection: 80.6%	Isolated: 50.7% Co-infection: 7.8%
Jordan (2009) ^[61]	Blood donors (n = 8190)	0	Cross-sectional	Isolated: 0.9%	Isolated: 1.4%
Lebanon - Beirut (2008) ^[62]	Inmates (n = 580)	0.17%	Cross-sectional	Isolated: 3.43%	Isolated: 2.4%
Libya - Tripoli (2013) ^[27]	PWIDs (n = 328)	87%	Respondent-driven sampling	Co-infection: 83%	Co-infection: 4%
Morocco - Casablanca (2006-2010) ^[63]	HIV infected patients of a referral clinic (n = 504)	All patients were infected.	Cohort	5.4%	Co-infection: 5.2%
Pakistan - Lahore (2011) ^[64]	Inmates (n = 4915)	2.01%	Cross-sectional	Co-infection: 73.74%	0.01% (HBV/HCV Co-infections: 77.78%)
Saudi Arabia (2009) ^[65]	PWIDs (n = 344)	Not checked	Cross-sectional	Isolated: 38% (HBV-HCV: 3.5%)	Isolated: 12% (HBV DNA)
	Rehab center inmates-drug users (n = 115)	4%	Cross-sectional	-	Isolated: 4%
Tunisia (2007) ^[67]	HIV infected patients (n = 362)	Not applicable	Cross-sectional	Co-infection: 39.7% (For PWIDs: 87.4%)	-
Yemen - Hodeidah (2010) ^[68]	Blood donors (n = 1483)	0.14%	Cross-sectional	Isolated: 0.79%	Isolated: 2.35%

¹Sample size; ²Lab evaluations: Anti-HIV antibody (enzymatic immunoassay or western blot); Anti-HCV antibody; HBs antigen (enzymatic immunoassay). HCV: Hepatitis C virus; HBV: Hepatitis B virus; FSWs: Female sex workers; PWIDs: People who inject drugs.

in parallel with HIV testing facilities to successfully implement HIV clinical guidelines in MENA region.

FSWs that also comprise a proportion of sexual partners of both PWIDs and men with same-sex behaviors are also a potential bridging population in transmitting HIV to the general population in MENA^[11,30,34]. Notably rates of syphilis (weighted prevalence: 7.2%) in Iran^[35], and of viral hepatitis reported among non-injecting FSWs in Afghanistan is relatively high^[36].

HIV AND TB IN MENA REGION

Latest WHO estimates of the burden of disease caused by TB in the Eastern Mediterranean Region indicate a stable trend in TB mortality, prevalence, and incidence; these estimates introduce the region as a medium-burden area. Since 2010, New incidence of TB among HIV-infected patients in the region has remained steady [11 (10-12) thousand in 2012], however, no improvements are evident in case detection rates during this same period. In addition, lack of detection and surveillance systems suggests that these estimates

may not fully capture actual incidence^[37].

On the other hand, the number of reported patients with multi-drug resistant TB (MDR-TB) has tripled from 2007 to 2010 globally. Estimates show that 56% these MDR-TB patients have been successfully treated in the Eastern Mediterranean Region, and despite better outcomes compared to other regions, this number is much lower than WHO's defined goal of 75% for 2015^[37]. Country-specific studies highlight the urgent need for addressing MDR-TB, specifically among patients who are re-treated with anti-TB drugs^[38-40]. The situation is becoming especially critical in Saudi Arabia due to high influence of travelers and migrating laborers from TB endemic areas, and also in Somalia which reports the highest prevalence of MDR-TB in the region^[41,42]. Similar situations cannot be precluded in other countries, where sufficient measures have not been taken into account.

New guideline recommends initiation of ART among all patients with active TB regardless of CD4 counts or clinical stage^[1]. Comparison of migrant and non-migrant patients by the Antiretroviral Therapy Cohort Collaboration revealed different AIDS-defining events

in the two groups, with migrants from MENA displaying significantly higher rates of TB in the first year after initiation of ART^[15]. Immune response to tuberculosis is blunted following ART initiation, and this suppression persists for years due to decreased alveolar macrophage activity and specific cellular responses^[43]. Hence, patients that receive ART remain susceptible to TB, a challenge that requires further attention in TB endemic countries of MENA.

Simultaneous treatment of HIV and TB, as recommended by WHO, remains controversial because of patients who develop immune reconstitution syndrome (IRIS) after ART initiation, and show higher mortality rates. The reason why IRIS affects certain patients is not thoroughly understood, but may be linked to monocyte-derived responses^[44]. Our expanding knowledge about human leukocyte antigens associations observed among non-seroconverting partners of HIV patients should uncover a role for genetic factors in the future^[45]. Context-specific decisions on the timing for initiation of ART among TB-infected patients are highly dependent on future findings of large cohorts among different high-risk groups from various ethnic backgrounds. Meanwhile, a scaling up of HIV testing and counseling and of efforts at detecting cases with MDR-TB should be considered a priority^[36,46].

ADHERENCE AND DRUG RESISTANCE

Our knowledge about adherence to ART in MENA is limited to a few studies^[47,48], which show that the role of stigma and discrimination, specifically among female patients, poses a major barrier to adherence^[47]. A cross-sectional study conducted in Iran shows adherence rates of 65.5% (self-report) and 60.4% (pill count) during a three month follow-up; however, the short term follow-up and the limited number of patients evaluated in a single center setting limit our ability to generalize these findings. Results of this study indicate that living with family members, shifting to new ART regimens, and the stage of the disease are major correlates of higher adherence rates^[48].

Although most evidence on the antiretroviral resistance profiles of ART-naïve and ART-treated patients is limited to a few studies^[47-50], the critically high rates of resistance to antiretroviral regimens requires immediate attention in the countries of the region, with the goal of increasing availability of viral load tests and modification of ART regimens, based on genotype sequencing^[51]. Policymakers should consider the cost-effectiveness of early ART initiation compared to scaling up of educational programs that target ART adherence, or to reaching out for the new and cheaper means of viral load measurement^[52].

Studies conducted in resource poor settings emphasize that documentation of ART adherence through patient self-reports derived from administration of standard questionnaires, is not costly and helps

to improve clinical outcomes (CD4 and viral load). Healthcare managers can also monitor ART distribution, availability of services, and counseling practices by utilizing metrics from these screenings^[53]. Documentation about adherence is helpful in decreasing transmitted drug resistance, which in-turn may require inevitable high expenditure in the future in these countries.

CONCLUSION

Serious challenges need to be tackled before adapting new clinical guidelines in current healthcare settings in MENA countries. The general response of MENA countries to HIV epidemics although effective has not been proportionate to the increasing needs. Insufficient human and financial resources, presence of punitive laws and social stigma are barriers for implementation of prevention, treatment or care programs in this region. Additionally, the lack of consistent documentation, registration and reporting systems that is required for monitoring and evaluating the present situation of HIV impedes appropriate response.

We recommend the following as major areas requiring action by health authorities in MENA countries for successful adaptation of ART guidelines: (1) To manage registration of HIV prevalence and incidence, and to design innovative data registry systems where necessary; (2) To investigate and monitor the genetic sequence of the most common HIV strains, and drug resistance profiles of infected patients (*e.g.*, among different high-risk groups); (3) To conduct cohort studies to investigate clinical outcomes among large groups of patients to empower health policymakers and clinicians with relevant data; (4) To develop standard adherence measurement tools to promote adherence and to document findings required for proper resource allocation; (5) To design disease databases for prevalent HIV co-infections to help in potential revisions required in national guidelines based on local patient needs; and (6) To recognize the concomitant role of social stigma and access to illicit drugs (opiates or stimulants) in the future pattern of HIV epidemic in the region.

Figure 1 summarizes the major areas that we suggest they need to be targeted by healthcare systems for development of new HIV treatment guidelines. We also suggest original research and implementation projects can be of prominent help in MENA. Based on computational models early initiation of ART could be of high impact in reducing immunologic failure^[54]. In MENA region such innovative models could be of assistance among the networks of infected people to make size estimations and map distribution where the majority also has high-risk activities^[32]. A practical approach could be to strengthen case detection systems through finding vulnerable people in each patient's network to reduce the time to diagnosis in newly identified patients. This will reduce the probability of transmitting

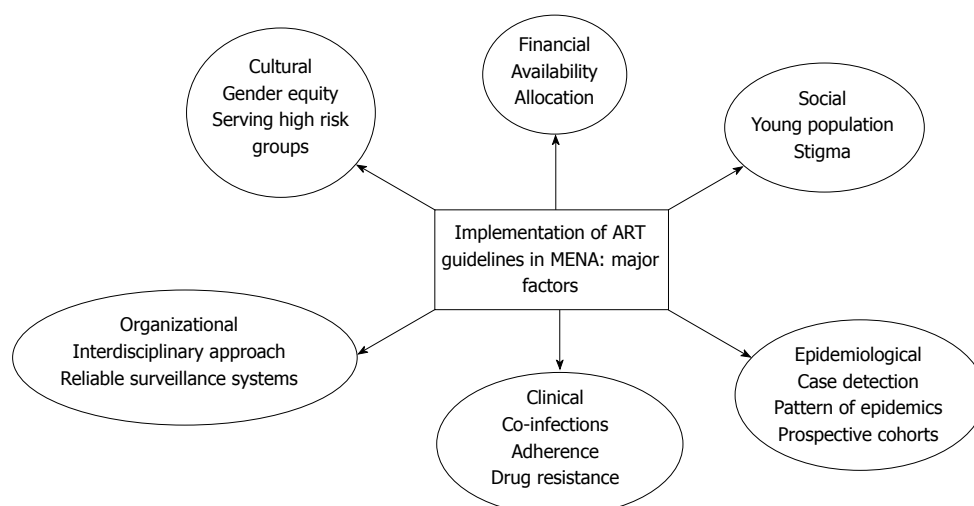


Figure 1 Major challenge for development of human immunodeficiency virus treatment guidelines in Middle East and North Africa. MENA: Middle East and North Africa; ART: Antiretroviral treatment.

drug-resistant strains which could be a major concern in coming years. Recent clinical trials are also suggesting new treatment protocols; as an example alternating regimens that provide patients with intermittent combinations of antiretroviral agents are supposed to reduce the probability of multi-drug resistance^[55]. These new protocols may be of prominent significance in the future of HIV epidemic in MENA region; however, their feasibility is yet to be established in settings with limited access to ART.

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Cost and safety of assisted reproductive technologies for human immunodeficiency virus-1 discordant couples

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Abstract

Due to significant advances in the treatment of human immunodeficiency virus type-1 (HIV-1), HIV-1 infection gradually has become a treatable chronic disease. Successfully treated HIV-positive individuals can have a normal life expectancy. Hence, more and more HIV-1 discordant couples in Taiwan and the rest of the world are seeking fertility assistance. Pre-treatment of highly active antiretroviral therapy (HAART) combined with sperm washing and RT-polymerase chain reaction examination for HIV-1 viral load has become the standard procedure to assist them to conceive. However,

in order to reduce the transmission risk to the lowest level for the couple and to diminish the cost of health care for the insurance institutes or government, *in vitro* fertilization (IVF)-intracytoplasmic sperm injection (ICSI) therapy provides the ideal solution for HIV-1 discordant couples with infected men. Intrauterine insemination (IUI) theoretically introduces more than 10^7 times of sperm counts or semen volume to uninfected women vs IVF-ICSI. However, since some regimens of HAART may significantly decrease the sperm motility, compared to IVF-ICSI, IUI only produces 1/5 to 1/2 pregnancy rates per cycle. Given the risk of seroconversion of HIV infection which actually happens after successful treatment, IVF-ICSI for these HIV-1 seropositive men is more cost-effective and should be the first line treatment for these cases.

Key words: Highly active antiretroviral therapy; human immunodeficiency virus-1 discordant; Seroconversion; Intrauterine insemination; Intracytoplasmic sperm injection

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Core tip: For human immunodeficiency virus type-1 (HIV-1)-infected men and uninfected women, highly active antiretroviral therapy, sperm washing and HIV-1 viral load check by RT-polymerase chain reaction have become the standard procedure to enable conception. Although the risk of seroconversion of HIV infection is very low, it remains possible. Intrauterine insemination may introduce more risk of HIV-1 transmission and also possesses less chance of pregnancy compared to *in vitro* fertilization-intracytoplasmic sperm injection (ICSI). Therefore, ICSI may be the preferred choice.

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INTRODUCTION

According to the World Health Organization (WHO)'s data and statistics, more and more people were newly infected with human immunodeficiency virus type-1 (HIV-1), *i.e.*, 2.1 million, in 2013. In the beginning, HIV-1 couples were often discouraged from planning a pregnancy due to its poor prognosis. Nowadays, due to many advances in highly active antiretroviral therapy (HAART) in the last 10 years, the expected age at death of a 35-year-old man could be extended up to 80 years of age^[1]. As a result, many seropositive couples are now looking for ways to safely conceive their own babies.

However, pregnancy by natural conception in HIV-negative women with HIV-infected partners may result in 4.3% seroconversion^[2]. In the stage of lower HIV-1 load, the rate of HIV-1 transmission per coital act could be as low as 0.1% in HIV-1 discordant couples^[3]. This has implications for HIV-1 prevention and for projecting the effects of HAART in this situation. However, the conception rate after a single coital act prior to ovulation is relatively low because the semen volume and spermatozoa motility decreases in HIV-1-infected patients under HAART^[4].

The clinical use of semen washing was first reported in 1992 by Semprini *et al.*^[5] and since then, assisted reproductive technologies, including intrauterine insemination (IUI), *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI), have widely combined the use of semen washing to help HIV-1-infected discordant couples with an HIV-1-infected man. About 10 years ago, the database of Centers for Reproductive Assistance To HIV couples in Europe reported over 500 infants born following this procedure in 4989 cycles of assisted conception^[6]. More recently, assisted reproductive technology has proved to reduce the risk of HIV-1 transmission of uninfected women and helped these discordant couples to conceive^[7-11].

Therefore, in 2010, the ASRM Committee on Ethics modified their guidelines concerning assisted reproductive technology for these HIV-1 discordant couples as follows^[12]: (1) in couples in which the man is HIV-infected, the use of sperm preparation techniques coupled with either inseminations or IVF with ICSI has proven to be highly effective in avoiding seroconversion of uninfected women and offspring; and (2) fertility clinics, to the extent it is economically and technically feasible, should offer services to HIV-infected individuals and couples who are willing to use risk-reducing therapies. In this article, we would like to discuss which assisted reproductive technology is the most effective and theoretically the safest way to conceive for the couples and most economical for the insurance companies or the government.

HIV-1 SEMEN WASHING

HIV-1 transmission *via* artificial insemination using donor sperm was first reported in 1985^[13] and the risk

remained high, especially in untreated urethritis^[14]. In 1992, Semprini *et al.*^[5] reported a simple method to eliminate the leukocytes from HIV-infected semen for intrauterine insemination. The method is a three-step system: (1) filtering the liquefied semen through a gradient; (2) washing the recovered spermatozoa to remove seminal plasma; and (3) swim-up to collect highly motile spermatozoa. A testing of the final sample by using a polymerase chain reaction (PCR) assay would assure the clearance of HIV-1 virus throughout the washing procedure.

However, the viral burden is an issue. The amount of HIV-1 present in the original semen sample affects the efficiency of the above procedure^[15]. So, in 2004, the Harvard group further decreased HIV-1 RNA copy numbers from 1300 by gradient/swim-up to 200 by double-tube gradient^[16]. Moreover, the amount of motile sperm recovered is superior to Semprini's method. In 2005, Loskutoff *et al.*^[17] used a novel washing method by combining multiple density gradients and trypsin addition for removing HIV-1 from semen, significantly reducing HIV-1 load without affecting sperm quality. Moreover, in 2006, Kato *et al.*^[11] also used an improved swim-up method to collect HIV-free spermatozoa from the semen of HIV-positive males. They demonstrated complete removal of HIV-1 RNA and proviral DNA by nested PCR assay.

Regarding the efficiency of sperm washing in removing HIV-1, the key depends on the seminal viral load. Fiore *et al.*^[15] demonstrated that 5×10^4 copies/mL were generally considered as the upper limit for the standard washing methods. From their study, in semen samples containing 1 and 3×10^6 copies/mL, persistence of viral RNA after standard washing procedures was observed in some of the aliquots tested. In light of this finding, pre-treatment with HAART before sperm washing is rational for these HIV-1 discordant couples.

ASSISTED REPRODUCTIVE TECHNOLOGIES IN HIV-1 DISCORDANT COUPLES (MALE HIV-1 POSITIVE)

Originally, these sperm washing techniques were applied in IUI and some of them even presented with relatively high pregnancy rates (24%-52%)^[7,18]. As we know, different conditions (*e.g.*, women's age) and policies (*e.g.*, high cancellation rates) resulted in different IUI results. Over the past 30 years, our data showed around 10% pregnancy rates for IUI^[19,20] and 45% pregnancy rates for IVF^[21,22]. Since 2003, we have performed IUI in these HIV-1 discordant couples, resulting in 10% clinical pregnancy rates (data not shown) and only 26% in fresh IVF cycles (Table 1). One reason is that some cases are of advanced age and therefore sometimes no embryos or even no oocytes could be obtained. On the other hand, the frozen-thawed cycles have normal pregnancy rates (45%).

Table 1 Results of *in vitro* fertilizations in human immunodeficiency virus type-1 discordant couples with a human immunodeficiency virus type-1-infected male partner at National Taiwan University Hospital from 2005-2014

Results	n
Couples	38
Fresh cycles	72
Age	35.9 ± 4.9
Oocytes retrieved	11.3 ± 7.7
Total 2PN fertilized	6.4 ± 5.1
Clinical pregnancies ¹	19 (26.4%)
Miscarriage	4 (21.1%)
Ectopic pregnancies	2 (10.5%)
Babies born	18
Thawed cycles	20
Clinical pregnancies ²	9 (45.0%)
Miscarriage	0
Ectopic pregnancies	0
Babies born	12
Accumulated pregnancies	23 (60.5%)
Seroconversions	0

¹Per TVOR; ²Per ET.

Semprini and Fiore^[6] favored IUI in the treatment of HIV-1 discordant couples and concluded that "IVF carries a higher pregnancy rate per cycle, but requires ovarian hyperstimulation, egg retrieval under sedation and carries a 20% risk of multi-fatality". Multiple pregnancy is no longer an issue in modern IVF practice since single embryo transfer^[23,24] or elective single embryo transfer was developed^[25,26].

Although combined pre-treatment with HAART, sperm washing and RT-PCR could provide a relatively safe sperm sample for conception in HIV-1 discordant couples, it is not completely virus-free as our HIV-1 assay detection limit is 40 copies/mL at present^[27]. Using more sperm may translate into more volume used or more viruses transmitted. On average, we introduce 40 million spermatozoa into the uterus in IUI, use 0.4 million spermatozoa in the culture dishes for IVF and only one sperm for ICSI. Unless the sperm sample for IUI is extremely concentrated, the volume (means the virus count or the transmission risk of HIV-1) of ICSI will be far less than 1/400000 compared to the IUI procedure.

Furthermore, when HIV-1 RNA is not detectable, is it risk free? Previously, Zhang *et al.*^[28] demonstrated over 50% cases with positive proviral DNA, even HIV-1 RNA less than 50 copies/mL. Therefore, fewer spermatozoa used to conceive (*e.g.*, ICSI) will have the lowest risk of HIV-1 transmission.

COST/BENEFIT

From a model of antenatal screening for HIV-1 infection in Australia, is it cost-effective in a setting of very low prevalence? The answer is "YES" if the prevalence of HIV-1 > 0.004372%^[29]. The expense of massive screening to avoid a new vertical transmission has to be

calculated. Here, HIV-1 discordant couples have already decided to get pregnant. Now, the open question is whether IUI or ICSI is safer?

IVF treatment with ICSI provides 2-5 times higher pregnancy rates compared to IUI, meaning less frequent exposure to HIV-1 in ICSI cycles. Moreover, in single reproductive assistance, IUI involved more than 4×10^7 times of sperm or virus exposure, which may result in the tragedy of HIV-1 seroconversion. That might be a strong reason to choose IVF-ICSI in addition to the theoretical risk. In 1997, Columbia University began offering IVF-ICSI but not IUI to HIV-seropositive men to limit viral exposure to a few motile sperm cells^[30]. Although some worried about more ovarian hyperstimulation syndrome (OHSS) in IVF protocols than in IUI treatment^[9], as a matter of fact that was wrong. The controlled ovarian stimulation protocols are the same and flushing medium into the follicle could remove the most granulosa cells. As we can freeze all embryos and/or use a GnRH agonist to trigger ovulation, the OHSS rates were relatively low in our IVF program. As mentioned, some doctors also criticized the higher multiple pregnancies in IVF cases^[6] which was also actually wrong. Single embryo transfer could assure singleton pregnancy but IUI could not.

If we consider the cost difference between IUI and IVF, it is very trivial (\$2000-15000 USD per IVF course) when compared to the medical fee of long-term HAART (\$28861-40804 USD per person-year) in a new HIV-1 seroconversion^[31]. If we consider the sedation for oocyte pickup, the psychological stress, including more trials and longer waiting of IUI, should also be considered when comparing IVF with ICSI treatment.

CONCLUSION

Modern HAART has prolonged the life expectancy of HIV-1 infected men; moreover, modern assisted reproductive technologies also have helped these couples to conceive successfully and safely. Pre-treatment with HAART, standard sperm washing procedure and controlled ovarian stimulation plus IUI/IVF may provide a promising way to improve pregnancy outcome in these couples. IVF treatment with ICSI in recent years may have given patients higher pregnancy rates and less risk of HIV-1 transmission. Furthermore, accurate HIV-1 assays or even embryo biopsy to verify the status of HIV-1 infection may be the future efforts.

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Elevated homocysteine levels in human immunodeficiency virus-infected patients under antiretroviral therapy: A meta-analysis

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Data sharing: All data generated during the project will be made freely available *via* correspondent author e-mail (deminice@ig.com.br). Data will be maintained for a minimum of 10 years. There are no security or licensing related to the expected data, and all data used in the project will be generated directly as a result of the project, without any pre-existing data being used.

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Abstract

AIM: To evaluate the association between the levels of homocysteine (Hcy), folate, vitamin B12 in human immunodeficiency virus (HIV)-infected patients who were treated with antiretroviral therapy (ART) or not treated with ART.

METHODS: The PubMed and Scielo databases were searched. Eligible studies regarding plasma Hcy level in HIV-infected patients were firstly identified. After careful analysis by two independent researches, the identified articles were included in the review according to two outcomes (1) Hcy, folate and vitamin B12 blood concentration in HIV-infected subjects *vs* health controls and; (2) Hcy blood concentration in HIV-infected subjects under ART *vs* not treated with ART. RevMan (version 5.2) was employed for data synthesis.

RESULTS: A total of 12 studies were included in outcome 1 (1649 participants, 932 cases and 717 controls). Outcome 1 meta-analysis demonstrated higher plasma Hcy (2.05 $\mu\text{mol/L}$; 95%CI: 0.10 to 4.00, $P < 0.01$) and decreased plasma folate concentrations (-2.74 ng/mL; 95%CI: -5.18 to -0.29, $P < 0.01$) in HIV-infected patients compared to healthy controls. No changes in vitamin B12 plasma concentration were observed between groups. All studies included in the outcome 2 meta-analysis (1167 participants; 404 HIV-infected exposed to ART and 757 HIV-infected non-ART patients) demonstrated higher mean Hcy concentration in subjects HIV-infected under ART compared to non-ART HIV subjects (4.13 $\mu\text{mol/L}$; 95%CI: 1.34 to 6.92, $P < 0.01$).

CONCLUSION: This meta-analysis demonstrated that the levels of Hcy and folate, but not vitamin B12, were associated with HIV infection. In addition, Hcy levels were higher in HIV-infected patients who were under ART compared to HIV-infected patients who

were not exposed to ART. Our results suggest that hyperhomocysteinemia should be included among the several important metabolic disturbances that are associated with ART in patients with HIV infection.

Key words: Antiretroviral therapy; Homocysteine; Folate; Vitamin B12; Human immunodeficiency virus; Acquired immune deficiency syndrome

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Core tip: Although antiretroviral therapy (ART) has changed dramatically the speciation of life of human immunodeficiency virus (HIV)-infected patients, it has increased the incidence of chronic diseases, especially cardiovascular diseases. Nowadays, elevated levels of homocysteine have been considered to be an independent risk factor for cardiovascular disease development. Our study demonstrated that the levels of Hcy and folate, were associated with HIV infection, especially for those exposed to ART.

Deminice R, Silva TCV, de Oliveira VHF. Elevated homocysteine levels in human immunodeficiency virus-infected patients under antiretroviral therapy: A meta-analysis. *World J Virol* 2015; 4(2): 147-155 Available from: URL: <http://www.wjgnet.com/2220-3249/full/v4/i2/147.htm> DOI: <http://dx.doi.org/10.5501/wjv.v4.i2.147>

INTRODUCTION

The introduction of antiretroviral therapy (ART) has changed the spectrum of human immunodeficiency virus (HIV) infections, reducing the risk of opportunistic infections and substantially reducing mortality rates^[1]. Although ART has changed HIV infection from an acute to a chronic disease, this therapy has increased the incidence of cardiovascular disease (CVD) among HIV-infected subjects^[2], associated with the presentation of risk factors, such as dyslipidemia, insulin resistance, and lipodystrophy, among others. Development of these risk factors may be due to HIV infection itself or ART-associated toxicity^[3]. Epidemiological studies have demonstrated an increased incidence of myocardial infarction, atherosclerotic disease, and mortality in HIV-infected patients compared to noninfected individuals, especially when exposed to ART^[4-7].

Since McCully *et al.*^[8] firstly demonstrated elevated incidence of homocystinuria in patients with severe atherosclerosis and arterial thrombosis, elevated levels of homocysteine (Hcy) have been considered to be an independent risk factor for CVD development^[9,10]. Recently, several studies have demonstrated an association between hyperhomocysteinemia (HHcy) and a spectrum of diseases, including neurodegenerative diseases, diabetes, chronic kidney disease, and fatty liver disease^[11-15]. Much of this association has been

attributed to the characteristics of Hcy, which is a potent toxic agent that may increase oxidative stress and promote neurotoxicity, endothelial dysfunction, and accelerate the atherosclerotic process^[16-19]. Hcy is an amino acid formed exclusively by demethylation of methionine^[10]. In Hcy synthesis, methionine is activated by ATP to form S-adenosylmethionine (SAM). SAM acts primarily as a universal methyl donor in the synthesis of methylated compounds such as neurotransmitters (epinephrine, norepinephrine), DNA, RNA, phosphatidylcholine and creatine^[11]. A subproduct of these methylation reactions is S-adenosylhomocysteine, which is hydrolyzed to adenosine and Hcy^[10]. Hcy can be remethylated to form methionine by the action of the enzyme methionine synthase which uses *N*^{5,10}-methylene-THF-reductase (MTHFR) as a methyl donor. Vitamins B12 and folate are co-factors in this reaction. The catabolism of methionine is performed by transsulfuration, with Hcy reacting with serine to form cystathionine in an irreversible reaction catalyzed by cystathione-β-synthase and dependent of vitamin B6^[16].

Owing to their involvement in the Hcy remethylation pathway, vitamin B12 and folate deficiencies have been associated with elevated Hcy concentrations^[20,21]. However, studies examining this association in HIV patients have reported conflicting results. Folate and vitamin B12 deficiencies have been reported in HIV-infected patients due to low intake and/or malabsorption^[22,23]. Remacha *et al.*^[23] demonstrated that HIV-infected subjects with low serum vitamin B12 and red blood cell folate concentrations had HHcy. In contrast, studies found no changes in folate and vitamin B12 levels during follow-up after ART^[24-26].

Given the inconsistency of the existing literature and the insufficient statistical power of primary studies, we conducted a meta-analysis to clarify the relationship between the levels of Hcy, folate, and vitamin B12 in HIV-infected patients. We also investigated the relationship between Hcy levels in HIV-infected patients who were treated with ART or not treated with ART.

MATERIALS AND METHODS

The PubMed and Scielo databases were searched for English- or Spanish-language articles, by using the following keywords: "homocysteine," "HIV-infected," and "AIDS". Any case-control, cross-sectional, or cohort study that assayed the blood concentrations of Hcy, vitamin B12, or folate in HIV-infected patients was analyzed. After careful analysis by two independent researchers, identified articles were included in the review if they satisfied the following criteria: (1) contained human clinical outcomes (rather than outcomes from animal experiments); (2) used a case-control, cross-sectional trial or cohort design; (3) contained quantitative information regarding Hcy, vitamin B12, or folate plasma or serum concentrations; and (4) included a healthy

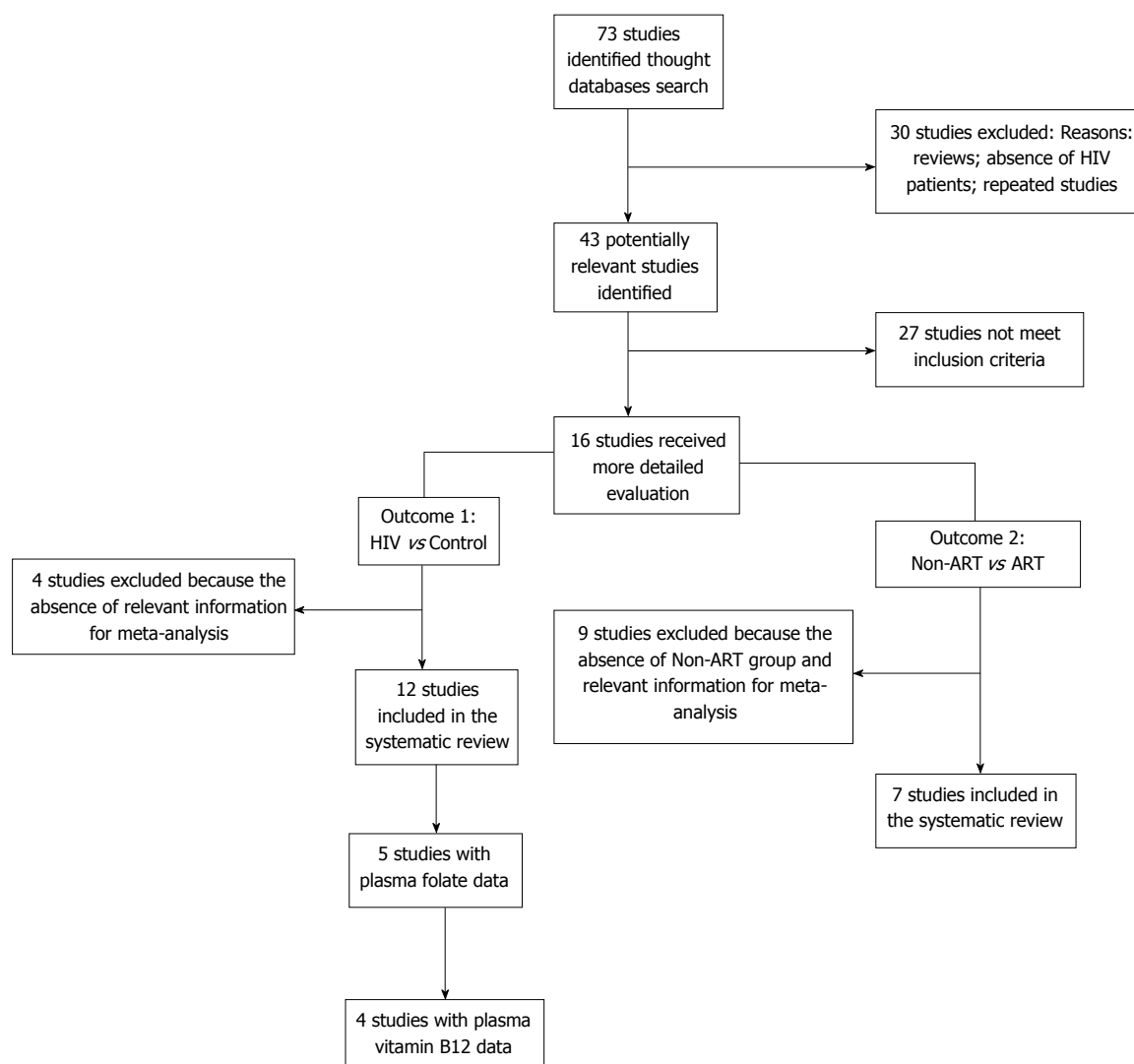


Figure 1 PRISMA flow diagram of the study selection process. After careful discussion between the 3 reviewers, two outcomes were identified and included in the meta-analysis. HIV: Human immunodeficiency virus; ART: Antiretroviral therapy.

control group. Relevant articles that were cited in the publications were reviewed and included in the meta-analysis if they satisfied the inclusion criteria.

The search was conducted considering two main outcomes: (1) Hcy blood concentrations in HIV-infected subjects compared to healthy controls; and (2) Hcy blood concentrations in HIV-infected individuals who were treated by ART compared to HIV-infected individuals who were not treated by ART (HIV-infected ART vs non-ART groups, Figure 1). A subgroup of outcome 1 was created to determine the vitamin B12 and folate plasma concentrations in HIV-infected individuals compared to healthy controls. The selection process is described in Figure 1.

For all articles included in the meta-analyses for the two outcomes, the following data were extracted: authors and year of publication; country where the study was conducted; study design; number of subjects in the study; plasma/serum concentrations of Hcy, vitamin B12, percent of each group with HHcy; and folate in HIV-infected and control subjects. Inclusion of studies in

the meta-analyses was discussed by the 3 authors. All meta-analysis procedures were conducted as described by Stroup *et al*^[27].

Statistical analysis

Effects of HIV infection on the Hcy blood concentration were quantified by performing meta-analyses for the two outcomes described above with the RevMan software package (version 5.0). In outcome 1, two subgroup analyses were performed to assess the impact of HIV on the folate and vitamin B12 plasma concentrations. RevMan was used to calculate the weighted mean difference. The 95%CI was employed for presenting the statistical results for continuous outcomes. Weighted percentages were based on the sample sizes of respective studies. Differences with a P value < 0.05 were considered to be statistically significant. Study heterogeneity was evaluated by the I^2 statistic. All meta-analyses were considered to be of high heterogeneity ($I^2 > 75\%$), and the random-effects model was used^[28]. All data were analyses with

Table 1 Characteristics of studies included in the outcome 1

Ref.	Country	Sample size (Control/HIV)	Study design	Hcy ($\mu\text{mol/L}$)		HHcy	Folate (ng/mL)/Vitamin B12 (pg/mL)	
				Control	HIV		Control	HIV
Castagna <i>et al</i> ^[29]	Italy	20/14	CS and CC of some patients	10.8 \pm 3.8	6.0 \pm 2.2	-	-	-
Muller <i>et al</i> ^[30]	Norway	15/21	CS	9.2 \pm 7.3	9.0 \pm 5.0	-	-	-
Naisbitt <i>et al</i> ^[31]	England	33/33	CS	11.9 \pm 4.7	14.5 \pm 5.6	-	-	-
Bernasconi <i>et al</i> ^[32]	Swaziland	80/73	COS	7.6 \pm 3.6	8.7 \pm 4.1	5% control; 12.3% HIV	-	-
Vilaseca <i>et al</i> ^[33]	Spain	170/69	COS	6.2 (4.0-10.4) ²	9.9 (5.5-23.3) ²	50.7% HIV	Folate 19.1 \pm 7.5/ B12 455 \pm 160 ¹	Folate 12.6 \pm 6.7/ B12 481 \pm 181 ¹
de Larrañaga <i>et al</i> ^[34]	Argentina	31/128	CS	9.0 (7.2-13.0) ²	9.0 (6.5-12.7) ²	12.9% control; 16.4% HIV	Folate 2.5 (2.1-3.1) ² /B12 309 (268-477) ²	Folate 3.6 (2.5-5.6) ² /B12 337.4 (222-493) ²
Remacha <i>et al</i> ^[23]	Spain	128/235	CS	7.5 \pm 7.8	14.3 \pm 12.9	6.2% control;	-	B12 368.6 \pm 219
Raiszadeh <i>et al</i> ^[35]	United States	127/249	COS	7.2 \pm 2.7	7.4 \pm 2.7	13.4% control; 16.90%	-	-
Vigano <i>et al</i> ^[36]	Italy	19/23	CS	9.0 \pm 5.0	11.0 \pm 8.0	-	Folate 6.9 \pm 1.7	Folate 4.7 \pm 1.5
Abdollahi and Shoar ^[37]	Iran	58/58	CC	12.6 \pm 1.1	27.1 \pm 10.2	91.4% HIV	-	-
Borges-Santos <i>et al</i> ^[38]	Brazil	20/12	CC	13.9 \pm 5.5	9.8 \pm 1.6	-	Folate 7.5 (6.3-9.0) ² /B12 288 \pm 130	Folate 1.9 (1.4-6.6) ² /B12 367 \pm 139
Deminice <i>et al</i> ^[39]	Brazil	10/23	CS	6.6 \pm 1.5	9.4 \pm 2.7	0% controls; 30.4% HIV	Folate 11.7 \pm 3.4/ B12 713.1 \pm 110.1	Folate 7.0 \pm 2/B12 514.6 \pm 99.3

¹Folate and vitamin B12 determined in 56 controls and 69 HIV-infected patients only. Values in Hcy, folate and vitamin B12 are expressed as mean \pm DP; ²Values expressed as range (25th-75th percentiles). CC: Case-control; CS: Cross-sectional; COS: Cohort study; Hcy: Homocysteine; HHcy: Hyperhomocysteinemia; HIV: Human immunodeficiency virus.

a biostatistician support.

RESULTS

Outcome 1

The initial search was independently executed by two reviewers, resulting in the selection of 73 articles. Reviews, repeated studies, and studies conducted in the absence of a control group identified 43 relevant studies. Screening by title and abstract was conducted in accordance with inclusion criteria. After extensive discussions between the authors, 16 articles were identified and included in the meta-analyses. Studies were included in either outcome 1 or 2, as described in Figure 1.

Twelve studies were included in the meta-analysis for outcome 1. Table 1 describes the characteristics of the 12 studies included in outcome 1. Four of the 16 selected studies were excluded because of the absence of a control group^[40,41,42,43]. These studies included 1649 participants (932 HIV-infected patients and 717 healthy controls). Nine of the 12 studies reported that the mean Hcy concentration was greater in HIV-infected individuals compared to controls. Only five^[33,34,36,38,39] and four^[33,36,38,39] of the included studies described quantitative folate and vitamin B12 data, respectively (Table 1).

A meta-analysis performed on outcome 1 indicated that the plasma Hcy levels in HIV-infected patients were 2.05 $\mu\text{mol/L}$ higher than the plasma Hcy levels in uninfected controls (95%CI: 0.13-4.01, $P < 0.01$;

Figure 2).

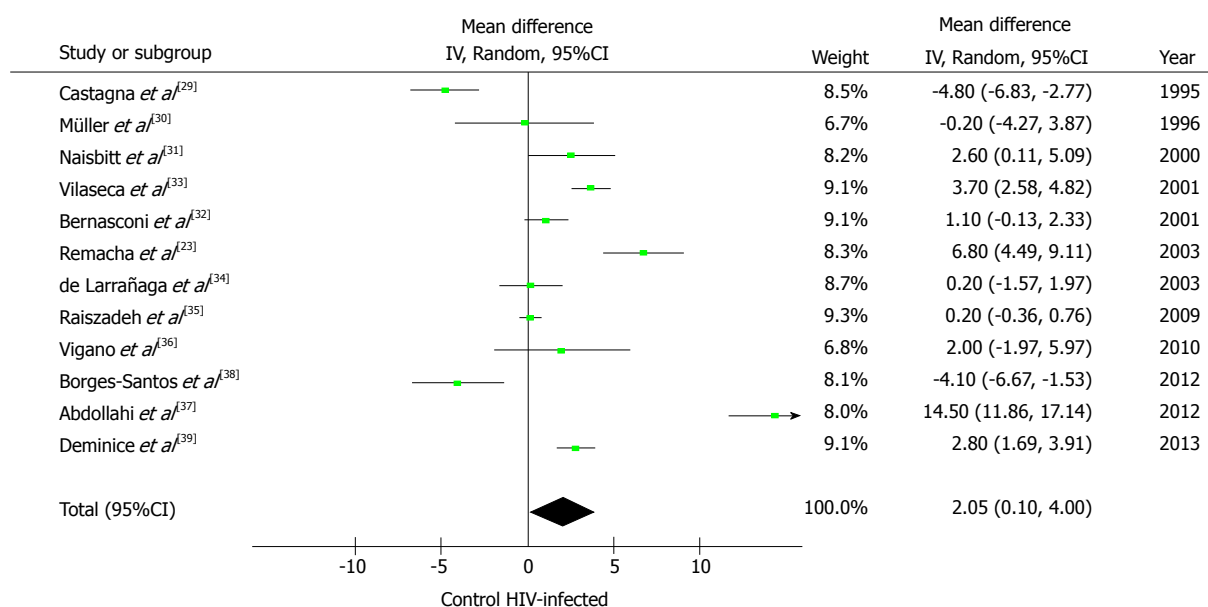
Subgroup analyses on folate and vitamin B12 plasma concentrations between test groups (Figure 3) demonstrated plasma folate levels were -2.74 ng/mL decreased in HIV-infected patients compared to uninfected controls (95%CI: -5.18 - -0.29, $P < 0.01$), but no significant difference in the B12 concentration between the two groups. Funnel plot analysis did not show evidence of substantial publication bias for the association between Hcy level and HIV infection.

Outcome 2

Nine of the 16 studies selected were excluded from outcome 2 because of the absence of an HIV-infected non-ART group^[29,30-33,36-39] (Figure 1). Table 2 describes the characteristics of the 7 studies included in outcome 2. These studies included 1167 participants (404 patients in the HIV-infected ART group and 757 patients in the HIV-infected non-ART group). All studies included in the meta-analysis for outcome 2 described a higher mean Hcy concentration in the HIV-infected ART group compared to the HIV-infected non-ART group (4.13 $\mu\text{mol/L}$; 95%CI: 1.34-6.92, $P < 0.01$; Figure 4).

DISCUSSION

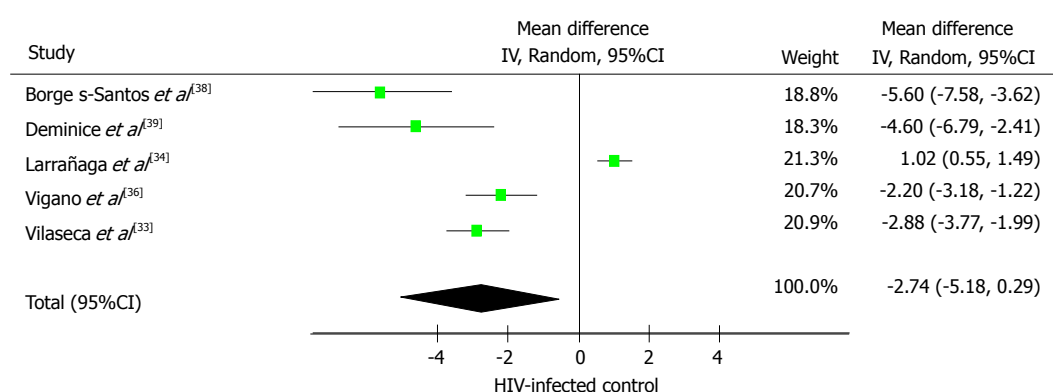
Overall, we found that HIV infection and ART were significantly associated with elevated plasma Hcy levels. The pooled mean Hcy concentration was greater in HIV-infected subjects compared to healthy controls. Hcy blood concentrations were elevated among HIV-infected



Heterogeneity: $\tau^2 = 10.50$; $\chi^2 = 212.29$, $df = 11$ ($P < 0.00001$); $I^2 = 95\%$

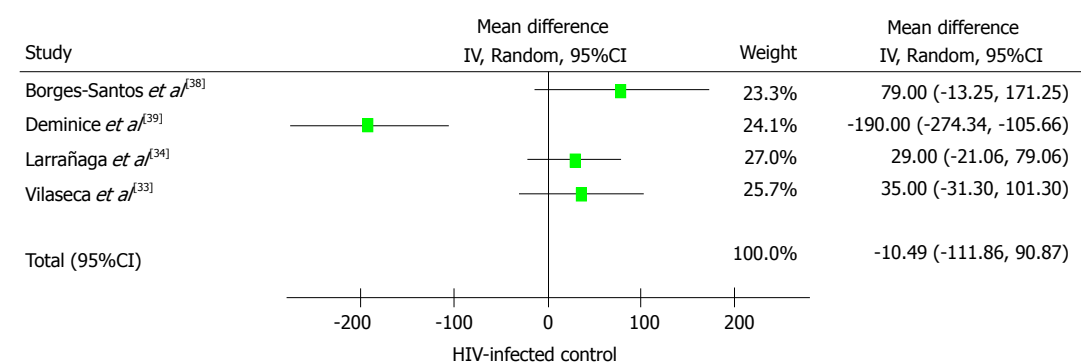
Test for overall effect: $Z = 2.06$ ($P = 0.04$)

Figure 2 Meta-analysis of blood homocysteine concentration in human immunodeficiency virus-infected subjects compared with healthy controls. Calculation based on random effects model. Results are expressed as weighted mean difference of homocysteine ($\mu\text{mol/L}$) and 95%CI. HIV: Human immunodeficiency virus.



Heterogeneity: $\tau^2 = 7.25$; $\chi^2 = 117.99$, $df = 4$ ($P < 0.00001$); $I^2 = 97\%$

Test for overall effect: $Z = 2.19$ ($P = 0.03$)



Heterogeneity: $\tau^2 = 9267.92$; $\chi^2 = 24.65$, $df = 3$ ($P < 0.0001$); $I^2 = 88\%$

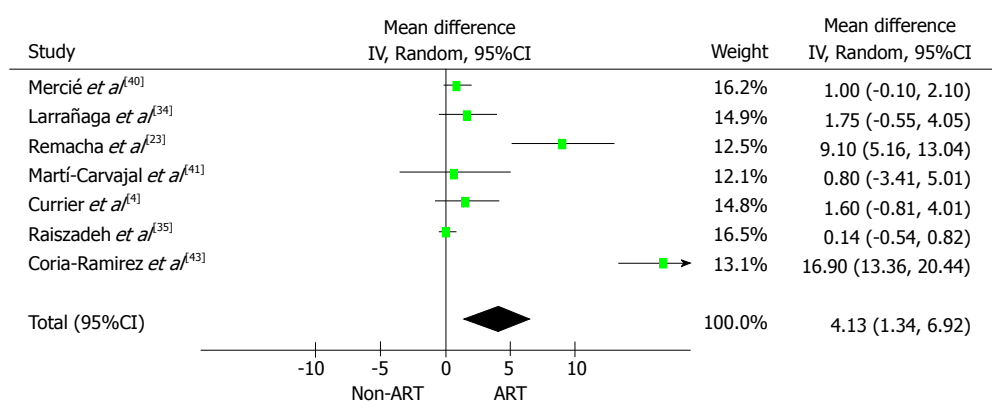
Test for overall effect: $Z = 0.20$ ($P = 0.84$)

Figure 3 Meta-analysis of serum folate and vitamin B12 levels in human immunodeficiency virus-infected subjects compared with healthy controls. Calculation based on random effects model. Results are expressed as weighted mean difference of folate (pg/mL) and vitamin B12 (pg/mL) and 95%CI.

Table 2 Characteristics of studies included in the outcome 2

Ref.	Country	Sample size (Control/HIV)	Study design	Hcy ($\mu\text{mol/L}$)		HHcy	ART
				ART	Non-ART		
Mercié <i>et al</i> ^[42]	France	78/304	COS	12.5 \pm 4.8	11.5 \pm 4.3	-	-
de Larrañaga <i>et al</i> ^[24]	Argentina	31/128	CS	9.7 \pm 7.1	8.0 \pm 6.1	-	-
Remacha <i>et al</i> ^[23]	Spain	128/235	CS	17.3 \pm 13	8.2 \pm 7.8	-	Patients under ART (taking > 3 antiretroviral drugs)
Martí-Carvajal <i>et al</i> ^[41]	Venezuela	14/40	CS	10 \pm 7.5	9.2 \pm 6.7	48.6% non-ART; 45.5% ART	-
Currier <i>et al</i> ^[42]	United States	40/41	COS	9.6 \pm 5	8.0 \pm 6.1	-	Patients on ART including a PI continuously for 2 yr
Raiszadeh <i>et al</i> ^[35]	United States	127/249	COS	7.5 \pm 2.8	7.3 \pm 2.4	-	-
Coria-Ramirez <i>et al</i> ^[43]	Mexico	69/69	CC	24.8 \pm 14.6	7.9 \pm 3.4	7.3% non-ART; 79.9% ART	Patients who began ART and maintained the treatment for 6 mo

CC: Case-control; CS: Cross-sectional; COS: Cohort study; Hcy: Homocysteine; HHcy: Hyperhomocysteinemia; PI: Protease inhibitors; ART: Antiretroviral therapy.



Heterogeneity: $\tau^2 = 12.18$; $\chi^2 = 100.52$, $df = 6$ ($P < 0.00001$); $I^2 = 94\%$

Test for overall effect: $Z = 2.90$ ($P = 0.004$)

Figure 4 Meta-analysis of blood homocysteine concentration in human immunodeficiency virus-infected exposed and non-exposed to antiretroviral therapy. Calculation based on random effects model. Results are expressed as weighted mean difference of homocysteine ($\mu\text{mol/L}$) and 95%CI. ART: Antiretroviral therapy.

patients who were exposed to ART compared to patients who were not exposed to ART. In addition, HIV-infected patients presented decreased plasma levels of folate, but not vitamin B12. These findings provide consistent evidence that Hcy and folate levels are associated with HIV infection, especially when patients are also receiving ART.

Studies in rodents and *in vitro* have demonstrated that HHcy may be associated with decreased nitric oxide bioavailability and endothelial dysfunction^[44], altered cellular methylation, formation of Hcy adducts (e.g., Hcy-thiolactone), and oxidative stress. These perturbations are linked to cell toxicity^[16], in addition to atherosclerosis and thrombotic processes^[17-19]. In humans, a total Hcy level of 14.3 $\mu\text{mol/L}$ or greater was independently associated with a relative risk of mortality (54% for all-cause mortality and 52% for cardiovascular mortality)^[21,45]. Humphrey *et al*^[46] demonstrated that each increase of 5 $\mu\text{mol/L}$ in Hcy levels increased the risk of cardiovascular events by approximately 20%. However, previous studies have provided conflicting results regarding circulating Hcy levels in HIV-infected patients.

In nine of the 12 studies included in the meta-analysis of outcome 1, the mean Hcy concentrations were greater in HIV-infected subjects compared to levels in healthy controls. Our meta-analysis demonstrated that the plasma Hcy levels in HIV-infected patients were 2.05 $\mu\text{mol/L}$ higher compared to levels in healthy controls. This observation is particularly relevant considering the heterogeneity of patients included in those studies with regard to the disease stage, ART status, comorbidities, and gender, among others. Considering the relationship between the Hcy level and ART status, we observed that the Hcy levels were higher by an average of 4.13 $\mu\text{mol/L}$ in HIV-infected subjects who were exposed to ART compared to HIV-infected patients who were not exposed to ART.

ART is a causal factor of increased cardiovascular risk in HIV-infected subjects. ART promotes different metabolic disturbances, including hepatic and neurotoxicities, lipodystrophy syndrome, hyperlactatemia, hyperlipidemia, and insulin resistance^[47,48]. The present study demonstrates that HHcy can be included on this list. Different classes of antiretroviral drugs and time exposed to the treatment may generate different ART-

associated adverse metabolic effects^[47,49]. However, a lack of data in the revised papers prevents us from determining the contributions of different drug classes and durations of ART on Hcy levels (Table 2).

Although the precise mechanisms by which HIV infection and ART affect Hcy metabolism are not known, the vitamin B12 and folate levels have been shown to affect Hcy levels in HIV-infected patients^[23,50]. Specifically, Hcy levels are inversely related to the daily intake of folate and vitamin B12 in general population^[20,21]. That is because Hcy remethylation to methionine by methionine synthase requires folate^[10]. However, this association appears to be different in the context of HIV infection. Remacha *et al.*^[23] identified HHcy in 100% and 51.5% of HIV-infected patients who had erythrocyte folate concentrations below the 2.5th and 10th percentiles, respectively. In our results, decreased levels of plasma folate, but not vitamin B12, were associated with elevated Hcy levels in HIV-infected subjects. Deminice *et al.*^[39] demonstrated that folate intake was higher in HIV-infected patients compared to healthy controls. Coria-Ramirez *et al.*^[43] noted that nutritional abnormalities, such as decreased vitamin B12 and folate intake, were not responsible for the high incidence of HHcy observed in HIV-infected patients. Taken together, these findings suggest that disturbances in Hcy metabolism and the Hcy levels observed in HIV-infected patients may not be due to nutritional status. Instead, it may be that HIV and/or ART complications are linked to disturbances in Hcy metabolism. One possibility is that ART impairs the metabolism of Hcy, such as its remethylation or transsulfuration because both pathways may be affected^[43]. However, to the best of our knowledge, here are no studies demonstrating ART modify Hcy metabolism enzymes. It is probably because liver biopsies are an invasive procedure with a relatively high risk of complications. Animal models could enable studies on ART and Hcy metabolism however; few studies have tested ART in animal models.

This study has some limitations that need to be considered. First, only a few of the included studies analyzed HIV-infected patients in the context of Hcy metabolism. This fact contributed to the lack of clinical homogeneity among subjects of the included studies. Most of the studies did not consider the nature or duration of the ART that was administered. Most studies excluded the effects of comorbidities and diseases that may have affected Hcy levels (*e.g.*, kidney disease). Second, many studies did not include information regarding vitamin levels, or assessed folate plasma levels of determining folate status. Plasma folate levels may not be the best parameter for assessing folate deficiency or intake^[43,51,52]. Erythrocyte folate or serum methylmalonic acid levels are better indicators of folate status, because they reflect folate turnover over the preceding 2 to 3 mo^[23,43]. Lamarre *et al.*^[52] recently showed that formate levels provide important information regarding folate metabolism, and that increased Hcy levels can be caused by defects in the

remethylation and transsulfuration pathways. Finally, some studies have demonstrated an association of the MTHFR polymorphisms and HHcy in different cases and diseases. However, this association was not considered in our included studies.

In conclusion, the levels of Hcy and folate, but not vitamin B12, were associated with HIV infection. Hcy levels were higher in HIV-infected patients who were exposed to ART compared to HIV-infected patients who were not exposed to ART. Finally, HHcy can be included among the several important metabolic disturbances that are associated with ART in patients with HIV infection.

COMMENTS

Background

Although antiretroviral therapy (ART) has changed dramatically the speciation of life of human immuno deficiency virus (HIV)-infected patients, it has increased the incidence of metabolic disorders as dyslipidemia, insulin resistance, lipodystrophy and others. Elevated homocysteine (Hcy) levels have been considered to be an independent risk factor for cardiovascular disease development. However, few studies have been considered Hcy levels in HIV-infected patients.

Research frontiers

Elevated Hcy levels have been considered to be an independent risk factor for cardiovascular disease development. Recently, several studies have demonstrated an association between hyperhomocysteinemia (HHcy) and a spectrum of diseases, including neurodegenerative diseases, diabetes, chronic kidney disease, and fatty liver disease. However, few researchers have focused in study Hcy levels HIV-infected patients.

Innovations and breakthroughs

The data demonstrated that the levels of Hcy and folate, were associated with HIV infection, especially for those exposed to ART. That is the first meta analysis carried out in search of a relationship between plasma homocysteine levels and HIV infection.

Applications

HHcy is associated to ART can be included among the several important metabolic disturbances that are associated with ART in patients with HIV infection.

Terminology

Hcy is an S-containing amino acid formed exclusively by demethylation of methionine. Hcy has gained attention in medical field because its instability and toxicity, especially in elevated concentrations.

Peer-review

The topic of manuscript is interesting and valuable. The logical thinking is reasonable and convincing. The reference collection and analysis process described in detailed, it is easy to follow.

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