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Contents

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REVIEW

Treatment of chronic hepatitis C in patients with HIV/HCV coinfection

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Abstract

Hepatitis C virus (HCV) infection is one of the most frequent causes of comorbidity and mortality in the human immunodeficiency virus (HIV) population, and liver-related mortality is now the second highest cause of death in HIV-positive patients, so HCV infection should be countered with adequate antiviral therapy. In 2011 began the era of directly acting antivirals (DAAs) and the HCV NS3/4A protease inhibitors telaprevir and boceprevir were approved to treat HCV-genotype-1 infection, each one in combination with pegylated interferon alfa (Peg-IFN) + ribavirin (RBV). The addition of the first generation DAAs, strongly improved the efficacy of antiviral therapy in patients with HCV-genotype 1, both for the HCV-monoinfected and HIV/HCV coinfected, and the poor response to Peg-IFN + RBV in HCV/HIV coinfection was enhanced. These treatments showed higher rates of sustained virological response than Peg-IFN + RBV but reduced tolerability and adherence due to the high pill burden and the several pharmacokinetic interactions between HCV NS3/4A protease inhibitors and antiretroviral drugs. Then in 2013 a new wave of DAAs arrived, characterized by high efficacy, good tolerability, a low pill burden and shortened treatment duration. The second and third generation DAAs also comprised IFN-free regimens, which in small recent trials on HIV-positive patients have shown comforting preliminary results in terms of efficacy, tolerability and adherence.

Key words: Hepatitis C virus infection; Human immunodeficiency virus infection; Anti-hepatitis C virus treatment; Directly acting antivirals; HIV/HCV coinfection; Chronic hepatitis C

Core tip: The combination pegylated interferon alfa + ribavirin has been used infrequently in patients with Human immunodeficiency virus/hepatitis C virus (HIV/HCV) coinfection because of its limited efficacy in these

patients, the high prevalence of medical and psychiatric comorbidities and the high incidence of serious adverse reactions. The introduction of directly acting antivirals has radically changed the scenario of the HIV/HCV coinfection treatment shown comforting preliminary results in terms of efficacy, tolerability and adherence. This paper provides a quick and comprehensive implementation guide to the managment of HIV/HCV patients in a historical moment in which it is not yet clear what is the best treatment.

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INTRODUCTION

The percentage of patients with human immunodeficiency virus (HIV) infection who contemporaneously carry the hepatitis C virus (HCV) ranges from 10% to 50% worldwide, reflecting a different diffusion of HCV infection and a different impact of the environmental factors responsible for HCV transmission in each single country[1-12].

The introduction of highly active antiretroviral therapy (ART) has increased by at least 20 years the average life expectancy of HIV-infected individuals and, consequently, the majority of HIV patients with chronic hepatitis C are at a higher risk of progressing to the more severe forms of the disease. At present HCV infection is one of the most frequent causes of comorbidity and mortality in the HIV population, and liver-related mortality is now the second highest cause of death in HIV-positive patients[13-15]. HIV infection unfavorably influences the natural history of HCV infection by increasing the rate of acute hepatitis C that progresses to chronicity, thus favoring the development of liver cirrhosis, hepatocellular carcinoma (HCC), liver decompensation and liver failure[13-20]. Therefore, optimized ART should be applied to reduce the unfavorable influence of HIV on HCV-related diseases. Also the HCV infection should be countered with adequate antiviral therapy.

The recent introduction of directly acting antivirals (DAAs) to treat chronic hepatitis C has enhanced the knowledge on the management and treatment of HIV/HCV coinfection.

ANTI-HCV TREATMENT FOR HIV-POSITIVE PATIENTS

The introduction of new, more effective and well-tolerated drugs for the treatment of patients with HIV infection has greatly improved the disease outcome of these patients[21]. In this context, however, the progression of HCV-related liver damage in HIV-positive patients, less evident in the pre-ART era because of the low average survival, has become a major life-threatening clinical condition[22]. Fewer advances were made in this period in the treatment of HCV[23], at that time based on the combination of pegylated interferon alfa (Peg-IFN) plus ribavirin (RBV), which was poorly tolerated and had a low rate of HCV eradication, especially in HIV-positive patients[24-26]. However, in 2011 the era of DAAs began and the HCV NS3/4A protease inhibitors (PIs) telaprevir (TPV) and boceprevir (BOC) were approved to treat HCV-genotype-1 infection, each one in combination with Peg-IFN + RBV[27-30]. These treatments showed higher rates of sustained virological response (SVR) than Peg-IFN + RBV[31-38] but reduced tolerability[39] and adherence due to the high pill burden. At the same time several pharmacokinetic interactions have been appeared between HCV NS3/4A protease inhibitors and antiretroviral drugs[40-43]. Finally in 2013 a new wave of DAAs arrived, characterized by high efficacy, good tolerability, a low pill burden and shortened treatment duration[44,45]. The second and third generation DAAs also comprised IFN-free regimens, which in small recent trials on HIV-positive patients have shown comforting preliminary results in terms of efficacy, tolerability and adherence[46,47]. Table 1 shows the SVR rate in therapy-naïve patients treated with the different combinations (Table 1). However, the new DAAs are still not available for HIV-positive patients in clinical practice and their high cost may be a handicap to their use in developing countries.

INTERFERON-BASED REGIMENS

Peg-IFN + RBV

The combination Peg-IFN + RBV, although considered the treatment of choice for chronic hepatitis C until 2011, has been used infrequently in patients with HIV/HCV coinfection because of its limited efficacy in these patients, the high prevalence of medical and psychiatric comorbidities and the high incidence of serious adverse reactions. In most studies on chronic hepatitis C monoinfected patients with HCV-genotype 1 or 4, an SVR of almost 50% was achieved. In the APRICOT[48] study, HIV/HCV coinfected patients were treated with Peg-IFN α-2a and a fixed dose of RBV (800 mg/d) for 48 wk and an SVR was obtained in nearly 40% of the cases, in 18% of those with HCV-RNA levels greater than 800000 copies/mL and in 61% of those with a lower HCV load. The current international guidelines suggest prolonging the 48-wk treatment to 72 wk, despite reduced tolerability, for patients with HCV-genotype 1 without a rapid virological response (RVR) (EACS)[49]. With the introduction of DAAs to treat chronic hepatitis C, Peg-IFN + RBV dual therapy should be considered obsolete at least for patients with HCV-genotype 1 or 4.

Peg-IFN + RBV + the first generation DAAs telaprevir or boceprevir

The addition of the first generation DAAs, TPV or BOC, to Peg-IFN + RBV strongly improved the efficacy of

Table 1 Sustained virological response rate in human immunodeficiency virus/hepatitis C virus coinfected patients naïve for anti-hepatitis C virus treatment

	Ref.	SVR rate in therapy-naive patients			
		Genotype 1	Genotype 2	Genotype 3	Genotype 4
Peg-IFN plus ribavirin	[85]	35.6% in 191 patients	72.4% in 152 patients		32.6% in 46 patients
Peg-IFN plus ribavirin + boceprevir	[32]	60.7% in 61 patients	-	-	-
Peg-IFN plus ribavirin + telaprevir	[51]	74% in 38 patients	-	-	-
Peg-IFN plus ribavirin + sofosbuvir	[44]		91% in 23 patients		
Peg-IFN plus ribavirin + simeprevir	[55]	79.2% in 52 patients	-	-	-
Peg-IFN plus ribavirin + faldaprevir	[59]	73.7% in 227 patients[1]	-	-	-
Sofosbuvir plus ribavirin	[60]	76% in 114 patients[2]	88% in 26 patients[3]	67% in 42 patients[3]	-
Sofosbuvir plus ribavirin	[62]	84% in 112 patients	90% in 19 patients	91% in 57 patients	84% in 31 patients
Sofosbuvir plus ledipasvir	[63]	100% in 13 patients	-	-	-
Paritaprevir-r/ombitasvir + dasabuvir + ribavirin	[65]	93.5% in 31 patients	-	-	-

[1]Therapy-naïve or relapser patients; [2]For 24 wk; [3]For 12 wk. SVR: Sustained virological response; Peg-IFN: Pegylated interferon alfa.

antiviral therapy in patients with HCV-genotype 1, both for the HCV-monoinfected and HIV/HCV coinfected, and the poor response to Peg-IFN + RBV in HCV/HIV coinfection was enhanced with the new combination therapy.

In patients with HIV/HCV coinfection, triple therapy including telaprevir 1125 mg every 12 h or 750 mg every 8 h is administered only for the first 12 wk, followed by a 36-wk Peg-IFN + RBV double therapy[50]. TPV pills should be taken with a fat meal to improve their absorption. In a randomized trial on HIV/HCV-genotype-1 coinfected patients naïve for anti-HCV treatment, the SVR rate was 74% for those treated with telaprevir-based triple therapy and 45% for the control group receiving Peg-IFN + RBV double therapy[51]. Adverse events commonly observed in TPV-based triple therapy included skin rash, pruritus, anemia, and ano-rectal discomfort. In addition, TPV may reduce the glomerular filtration rate and increase RBV concentrations by 55%, inducing in these cases severe anemia[39]. TPV cannot be administered with the ritonavir-boosted PIs used in HIV therapy due to the pharmacokinetic interactions. Thus, despite the enhanced efficacy of telaprevir-based triple therapy in eradicating HCV infection, the number of HIV/HCV patients eligible for this treatment is reduced due to its poor tolerability, interaction with ART and high pill burden.

Predictive factors of a favorable response to treatment, such as mild fibrosis, low HCV-RNA load, IL-28B CC genotype and Caucasian ethnicity should be assessed before starting telaprevir-based triple therapy.

Boceprevir is an NS3/4A protease inhibitor approved for treatment of genotype-1 chronic hepatitis C[52]. In these patients treatment with BOC + Peg-IFN + RBV begins after a 4-wk lead-in phase with Peg-IFN + RBV. BOC is stopped at week 36 and Peg-IFN + RBV continued until week 48. Cirrhotic patients or prior null responders should receive BOC + Peg-IFN + RBV until week 48. The SVR rates observed in therapy-naïve HIV + HCV co-infected patients receiving BOC + Peg-IFN + RBV or Peg-IFN + RBV in a phase II trial were 63% and 29%, respectively[32]. Adverse reactions included anemia, dysgeusia, nausea, and neutropenia. BOC cannot be administered with ritonavir-boosted PIs or non-nucleoside retro-transcriptase inhibitors because of the pharmacokinetic interactions[40]. Consequently, the use of the combination BOC + Peg-IFN + RBV for HIV/HCV coinfected patients is very limited.

Peg-IFN + RBV + a second wave DAA

In 2013, both sofosbuvir (SOF), a once-a-day oral DAA that inhibits the active site of the HCV NS5B polymerase with an anti-HCV pan-genotypic activity, and simeprevir (SMV), a once-a-day oral DAA that inhibits the HCV NS3/4A protease, were approved by the United States Food and Drug Administration (FDA) to be used in combination with Peg-IFN + RBV to treat patients coinfected with HIV/HCV genotype 1[44,45]. SOF was also approved to be used in combination with RBV to treat patients coinfected with HIV and HCV genotype 2 or 3[47]. SOF is particularly indicated for patients with HIV/HCV coinfection, since it is well tolerated and no pharmacokinetic interactions with the antiretroviral drugs have been documented[44].

In a small study from Porto Rico a combination of SOF plus Peg-IFN and RBV given to 23 patients with HIV/HCV coinfection (19 with HCV genotype 1) for 12 wk obtained HCV eradication in 91% of cases; no severe adverse event occurred and only 2 patients discontinued treatment, one due to anemia and one to altered mood[53].

SMV is a second generation HCV NS3/4A protease inhibitor for the treatment of patients with HCV-genotype-1 infection[54]. The combination of SMV plus Peg-IFN and RBV has been investigated in both in HCV-monoinfected[55-57] and HIV/HCV coinfected patients[58]. An overall 74% SVR rate was obtained in 106 patients coinfected with HIV and genotype-1 HCV treated with this triple therapy and a 79% SVR was achieved in the anti-HCV treatment-naïve patients. In this study, all patients received a 12-wk SMV + Peg-IFN + RBV treatment, followed by double Peg-IFN/RBV response-guided therapy for either 12 or 36 wk. Of note, 89% of naïve or prior relapser co-infected patients without cirrhosis met the inclusion criteria to receive response-guided therapy, which required that serum HCV RNA be undetectable at week 4. Fibrosis stage, HCV sub-genotype, IL28b genotype, and baseline CD4 count above or below 500 did not influence

the SVR rates. The impact of adverse events to SMV + Peg-IFN + RBV (rash, photosensitivity, pruritus, and nausea) was limited in the Phase Ⅱ/Ⅲ trials. However, phase Ⅲ studies, both on mono and coinfected patients, demonstrated that the addition of SMV to the combination Peg-IFN + RBV did not improve the response rate of the dual therapy in patients with HCV-genotype 1a with a baseline NS3 Q80K polymorphism. This polymorphism is detected in nearly one third of subjects infected with HCV-genotype 1a and in only 0.5% of those with HCV-genotype 1b. Screening at baseline for the presence of the NS3 Q80K polymorphism is recommended for patients with HCV-genotype 1a to exclude positive patients from treatment including SMV. Indeed, this polymorphism has a limited effect on SMV activity, but the resistance barrier of this drug appears to be lower in patients carrying the Q80K- variant, resulting in a more frequent emergence of additional mutations and in a higher rate of treatment failure. The United States FDA approval of SMV provides specific recommendations for interactions with commonly prescribed antiretroviral agents. SMV is also a component of several interferon-free combinations currently under study.

Faldaprevir (FDV) is a second generation oral, once-daily HCV NS3/4A protease inhibitor. In the START-Verso 4[59]multicenter study, an open-label randomized phase Ⅲ trial, HIV/HCV therapy-naïve or previous relapser patients with or without cirrhosis received either FDV 120 mg + Peg-IFN+RBV for 24 wk followed by Peg-IFN + RBV dual therapy for an additional 24 wk or FDV 240 mg + Peg-IFN + RBV for 12 wk followed by a 1:1re-randomization to either the same treatment for another 12 wk followed by a 24-wk Peg-IFN + RBV double therapy or to a 12-wk Peg-IFN+RBV double therapy. Due to drug to drug interactions, patients receiving efavirenz were placed in the 120 mg arm, and patients receiving darunavir/ritonavir or atazanavir/ritonavir were randomized to the 120 or 240 mg arms. Patients with an early treatment success stopped treatment at week 24, the others at week 48. Overall, 72% of patients achieved an SVR, the highest rates being observed in patients who had previously relapsed with Peg-IFN + RBV (83%) and in those with IL28b CC genotype (88%); HCV genotype, presence of cirrhosis and FDV dose and duration did not show any major impact on the SVR rate. Adverse events occurred in 7% of patients, neutropenia and bilirubin elevations being the most common grade 3 abnormalities; only 1% of these events were attributed to FDV. The research on this promising drug was stopped in 2014 for commercial reasons.

INTERFERON-FREE REGIMENS

Studies on HIV/HCV coinfected patients

IFN-free clinical trials on HIV/HCV coinfected patients are still in progress, but their preliminary data have shown that these treatments have a greater efficacy in eradicating HCV infection.

PHOTON-1 is the only study on IFN-free treatment of HIV/HCV coinfected patients published at present[60]. In this study, SOF + weight-based RBV were administered to 114 patients with HCV-genotype 1 naïve for anti-HCV treatment for 24 wk and to 68 therapy-naive patients with genotype 2 or 3 for 12 wk. The SVR rates were 76% in patients with HCV-genotype 1, 88% in those with genotype 2 and 67% in those with genotype 3.

In the phase Ⅱa COSMOS trial, the patients were randomized to SMV + SOF with or without RBV for 12 or 24 wk[61]. The preliminary data for patients in the 12-wk arm showed a 93% SVR rate in prior null-responders to Peg-IFN + RBV and 97% overall. Serious adverse events, anemia and bilirubin increase regarded only patients who received RBV.

In a recent study a combination of SOF plus RBV given for 12 wk to genotype-2 therapy-naive patients and for 24 wk to all other patients showed an 84% SVR in the 112 therapy-naïve genotype-1 patients, 90% in the 19 naïve genotype-2, 91% in the 57 naïve genotype-3, 84% in the 31 naïve genotype-4, 83% in the 6 therapy-experienced genotype-2 and 86% in the 49 experienced genotype-3 patients[62].

Ledipasvir (LDV) is an oral NS5A inhibitor administered once daily. In a small trial the combination LDV + SOF ± RBV was administered to HIV/HCV genotype-1 coinfected patients with no evidence of liver cirrhosis[63]. This regimen was well tolerated and the preliminary data showed a 100% SVR12 rate in 12 HCV-genotype-1 patients who were naïve for anti-HIV and anti-HCV treatment.

Daclatasvir (DCV) is an HCV NS5A replication complex inhibitor administered once daily. The safety and efficacy of the combination therapy with DCV + SMV ± RBV were evaluated in the LEAGUE-1 trial, a randomized open-label phase II study enrolling therapy-naïve and null-responder HIV/HCV patients with or without cirrhosis[64]. All patients with HCV-genotype 1b were given a 12-wk treatment with DCV + SMV ± RBV; at week 12 the patients were re-randomized to either an additional 12-wk treatment or to a 12-wk treatment-free follow up. The patients with HCV-genotype 1a received a 24-wk DCV + SMV + RBV treatment. In this study the SVR rate was 67% for patients with HCV genotype 1a and almost 82% for those with HCV genotype 1b.

Turquoise-I is a randomized, open-label study evaluating the safety and efficacy of a combination of paritaprevir/r, ombitasvir, dasabuvir and RBV in patients with HCV-genotype-1 chronic hepatitis and HIV infection[65]. The study is still ongoing, but the preliminary data show an SVR4 in 93.5% of 31 patients treated for 12 wk and in 96.9% of 30 patients treated for 24 wk; an SVR12 was obtained in 93.5% of 31 patients treated for 12 wk. Fatigue, insomnia and headache were the most common side effects, but no patient had a serious adverse event.

Studies on HIV/HCV patients started recently whose results are awaited

The efficacy of SOF + LDV is under evaluation in 100 patients with HIV/HCV-genotype-1 coinfection either untreated for HIV infection (CD4 > 500 cells/mm^3) or

with suppressed HIV-1 RNA replication with antiretroviral drugs. (ClinicalTrials.gov Identifier: NCT01878799). Still awaited are the results of the ALLY-2 study on the effect of the combination of SOF+ DCV given for 8 or 12 wk to HIV/HCV coinfected patients with HCV genotype 1, 2, 3, 4, 5 or 6.

The C-WORTHY is a study recently started to evaluate the safety and efficacy of the combination of the second generation HCV NS3/4A protease inhibitor MK-5172 + the second generation HCV NS5A inhibitor MK-8742 ± RBV for patients with HCV-genotype 1, both HIV positive and negative[66,67]. This study design underscores the emerging recognition that HIV-infected patients may not differ from the monoinfected in terms of the effectiveness of oral IFN-free DAA regimens.

A few trials on the efficacy of the combination DCV + SOF for HIV/HCV coinfected patients have recently started and the results are still awaited[68].

Recent studies on the efficacy of IFN-free DAA regimens for HCV-monoinfected patients, possibly extendible to HIV/HCV coinfection in the near future

Asunaprevir (ASV), a twice-daily NS3/4A protease inhibitor, used in combination with IFN + RBV or in IFN-free regimens, has shown promising results with fewer adverse effects. In HCV-monoinfected patients ASV was studied in a randomized, open-label, 24-wk-treatment study[69] where all 101 patients enrolled received DCV (60 mg) once daily and ASV as follows: 38 with genotype 1b also received ASV (200 mg) twice (DUAL A1) or once daily (DUAL A2), 36 with genotype 1a and 5 with genotype 1b also received ASV twice (QUAD B1) or once daily (QUAD B2) plus Peg-IFN/RBV and 18 patients with genotype 1a and 4 with genotype 1b also received ASV twice daily plus RBV (TRIPLE B3). An SVR12 was obtained in 78% of patients in DUAL A1, 65% in DUAL A2, 95% in QUAD B1, and 95% in QUAD B2. Most patients in the TRIPLE B3 arm developed a virological breakthrough, but aminotransferase elevation grade 3 or 4 was infrequent.

The BMS-791325 (BMS), a twice-daily non-nucleoside NS5B polymerase inhibitor, was investigated in the AI443-014 trial where the efficacy of the oral combination of DCV + ASV + BMS was assessed in a large group of HCV-genotype-1 patients, including cirrhotics[70]. The patients were randomized to DCV/ASV/BMS with BMS dosed at 75 mg or 150 mg. In this combination DCV was given twice daily. The SVR rates were above 90% in both groups. Only 2 of the 166 patients enrolled discontinued treatment due to adverse events.

The SYNERGY trial studied different combinations of DAA in order to achieve high SVR rates with shortened treatment duration in patients with HCV monoinfection[71]. In this study, besides some well-known drugs, the Authors also used two new molecules, GS-9669, a once-daily non-nucleoside HCV NS5B inhibitor, and GS-9451, a once-daily NS3/4A protease inhibitor. The patients were randomized to one of three arms: (1) SOF + LDV for 12 wk; (2) SOF + LDV + GS-9669 for 6 wk; and (3) SOF/LDV/GS-9451 for 6 wk. Patients with cirrhosis were excluded from arms B and C. All patients, with the exception of one in arm B, achieved an SVR. No patient discontinued therapy due to an adverse event. Worthy of note is that most individuals investigated were difficult-to-treat patients because of their Afro-American ethnicity, old age, HCV-sub-genotype 1a, IL28b genotype CT/TT or advanced liver fibrosis.

A 12-wk combination of DCV+SOF ± RBV was investigated in HCV-monoinfected patients with genotype 1, 2, or 3 in the AI444040 study[72]. The study included both therapy-naïve patients and previous non-responders to TVP- or BOC-based triple therapy; in both groups 98% of the patients achieved an SVR.

The Turquoise-Ⅱ is a multicenter, randomized, open-label study evaluating the efficacy and safety of a 12-wk or 24-wk treatment with paritaprevir/r + ombitasvir + dasabuvir + RBV in patients with HCV-genotype-1 chronic hepatitis or compensated liver cirrhosis[73]. An SVR12 was observed in 91.8% of patients treated for 12 wk and in 95.9% of those treated for 24 wk.

ART MANAGEMENT IN HIV/HCV COINFECTED PATIENTS DURING TREATMENT WITH DIRECTLY ACTING ANTIVIRALS FOR HCV INFECTION

Despite the remarkable virological response obtained with oral DAAs, the treatment of chronic hepatitis C in HIV patients remains complex and presents multiple challenges. The drug to drug interaction and the high prevalence of severe side effects influence the choice of the ART, and priority should be given to the antiretroviral drugs with fewer side effects and lesser interaction with the DAAs.

The drug to drug interaction in HIV/HCV coinfection mostly regards the use of HCV NS3 protease inhibitors, while the HCV nucleoside and non-nucleoside NS5B polymerase inhibitors and NS5A replication complex inhibitors seem to have minimal effects on the serum concentration of the HIV drugs. Both the anti-HCV and anti-HIV protease inhibitors and NNRTIs are metabolized by the cytochrome p450 pathway and, consequently, multiple complex drug to drug interactions develop that require management in highly experienced clinical centers. In addition, the knowledge on the interaction between anti-HCV protease inhibitors and anti-HIV drugs is in continuous development and even skilled clinicians should consult the www.hep-druginteractions.com web site.

TPV, BOC and SMV interact with CYP3A as inhibitors and substrates, with potential interaction and increased concentrations of drugs metabolized through this pathway and with a reduced TPV or BOC serum concentration due to drug-induced enzymatic activity[40,74]. A recent study assessed the pharmacokinetic interactions between BOC and the ritonavir (RTV)-boosted protease inhibitors atazanavir (ATV), lopinavir (LPV) and darunavir (DRV)

Table 2 Anti-human immunodeficiency virus drugs contraindicated during anti-hepatitis C virus treatment

	Contraindicated anti-HIV drugs				
	NRTI	NNRTI	IP	INI	CCR5 antagonist
Alpha interferon	-	-	-	-	-
Ribavirin	Didanosine, stavudine, zidovudine	-	-	-	-
Boceprevir	Didanosine, stavudine, zidovudine	Efavirenz	Lopinavir/r, atazanavir/r, darunavir/r	EVG/cobi/TDF/FTC[1]	Maraviroc[3]
Telaprevir	Didanosine, stavudine, zidovudine	Efavirenz[2]	Lopinavir/r darunavir/r fosamprenavir/r	-	Maraviroc[3]
Sofosbuvir	Didanosine, stavudine, zidovudine	-	Tipranavir/r	No data	-
Simeprevir	Didanosine, stavudine, zidovudine	Efavirenz, delavirdine etravirine, nevirapine	All protease inhibitors with or without ritonavir booster	EVG/cobi/TDF/FTC	No data
Daclatasvir	Didanosine, stavudine, zidovudine	Efavirenz[4], nevirapine, etravirine	Lopinavir/r[1] darunavir/r[1] fosamprenavir/r[1] tipranavir/r	-	-
Ledipasvir	Didanosine, stavudine, zidovudine	Nevirapine[1], etravirine[1]	Lopinavir/r[1] fosamprenavir/r[1] tipranavir/r	EVG/cobi/TDF/FTC[1]	No data
Dasabuvir/ ombitasvir/ paritaprevir-r	Didanosine, stavudine, zidovudine	Efavirenz, rilpivirine nevirapine[1] etravirine[1]	lopinavir/r[1] fosamprenavir/r tipranavir/r[1]	EVG/cobi/TDF/FTC[1]	No data

[1]No data, do not co-administer; [2]Increase the dose of telaprevir to 1125 mg three times a day; [3]Maraviroc 150 mg twice a day; [4]Increase the dose of daclatasvir to 90 mg a day. HIV: Hepatitis C virus.

in a randomized open-label study on 39 healthy adults. The protease inhibitor BOC decreased the exposure of all protease inhibitors; ATV/ritonavir did not significantly affect BOC exposure, whereas BOC was reduced by 45% and 32% when co-administered with LPV/ritonavir and DRV/ritonavir, respectively[75].

In a recent study no significant drug interaction between BOC and raltegravir was found in healthy volunteers[76].

The role of ritonavir in the drug interactions between TPV and ATV was recently investigated. In an open-label, sequential study on HCV/HIV coinfected patients on an RTV-boosted ATV-based antiretroviral regimen (300/100 mg every 24 h) and triple therapy (telaprevir, 1125 mg every 12 h, Peg-IFN + RBV) for genotype-1 chronic hepatitis C, the pharmacokinetic profiles were acquired before and after switching from RTV-boosted to unboosted ATV (200 mg every 12 h). The Authors found RTV responsible for the adverse interactions occurring when TPV and RTV-boosted ATV were administered together since the co-administration of TPV and unboosted ATV resulted in increased exposure of both drugs[77].

The co-administration with efavirenz led to a 20% reduction in the area under curve of TPV, thus requiring an increase in the dosage of TPV[74,78,79]. An open-label crossover study on healthy volunteers evaluated the bioequivalence of BOC and etravirine, an HIV non-nucleoside reverse transcriptase inhibitor, and a reciprocal interaction was demonstrated[80].

The study on the interaction between anti-HCV DAAs and antiretroviral drugs is at its real beginning and further investigation is needed to ensure the optimization of the contemporaneous administration of ART and anti-HCV therapy for HIV/HCV coinfected patients.

Choice of the best ART during treatment with DAAs

Precise knowledge of drug interactions and of the adverse events occurring during drug administration is indispensable in order to choose optimized ART and DAAs-based treatment for patients with HIV/HCV coinfection. Optimized ART should avoid possible interactions with protease inhibitors of HCV and improve drug tolerability and the patients' adherence. Table 2 shows a list of antiretroviral drugs incompatible with DAA administration. TPV can be safely administered in combination with RTV-boosted ATV, raltegravir, maraviroc, rilpivirine, etravirine or efavirenz (when administered with efavirenz, the TPV dosage should be 1125 mg every 8 h) and with tenofovir/emtricitabine or abacavir/lamivudine (www.hep-druginteractions.com). BOC can be safely administered in combination with raltegravir, rilpivirine or etravirine and with tenofovir/emtricitabine or abacavir/lamivudine. BOC can also be considered in combination with RTV-boosted ATV for patients with no previous HIV-treatment failure and no drug resistance[74,78,79,81]. BOC can be safely administered in combination with raltegravir, rilpivirine and with tenofovir/emtricitabine or abacavir/lamivudine.

Some new ART regimens including the integrase inhibitor raltegravir[82] or the entry inhibitor maraviroc[83] have been demonstrated to be safe and their use in HIV/HCV coinfection should be evaluated in clinical studies.

Concluding on this point, the management of HCV infection for HIV-positive patients is a complex issue. The physicians in care should carefully select the patients for the most suitable treatment and monitor them closely to evaluate the efficacy and tolerability of the drugs administered, their pharmacological interaction, the virus interaction and the patients' adherence.

CONCLUSION

Treatment of chronic hepatitis C for patients with HIV infection is essential to prevent the transition to liver

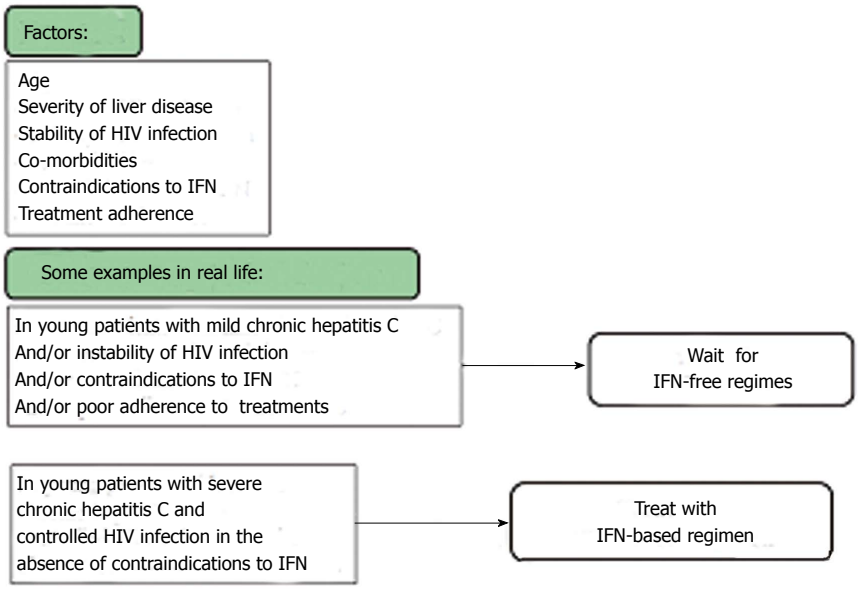

Figure 1 Factors influencing treatment decision for chronic hepatitis C in human immunodeficiency virus/hepatitis C virus-1 coinfection: treat or defer treatment. HIV: Human immunodeficiency virus; IFN: Interferon.

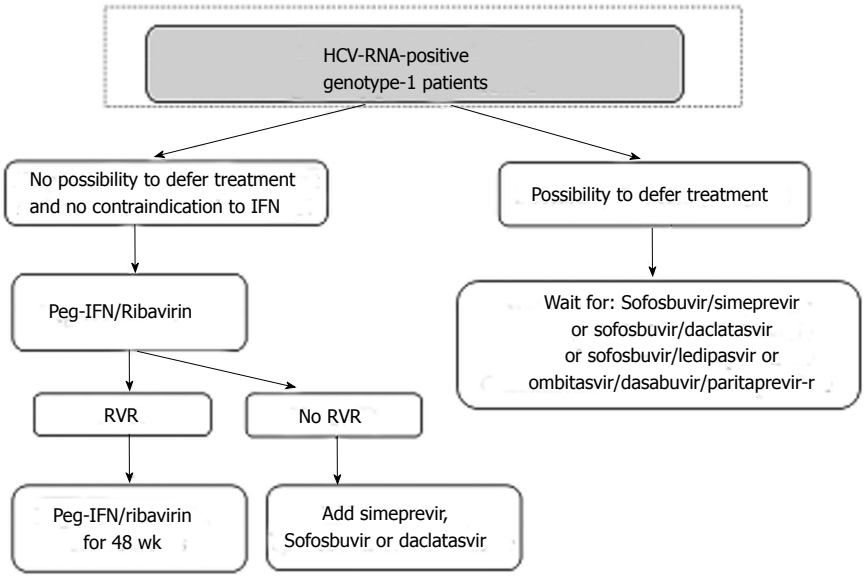

Figure 2 Treatment of chronic hepatitis C, hepatitis C virus-genotype 1 or 4, in patients with Human immunodeficiency virus/hepatitis C virus coinfection. RVR: Rapid virological response; IFN: Interferon. HCV: Hepatitis C virus.

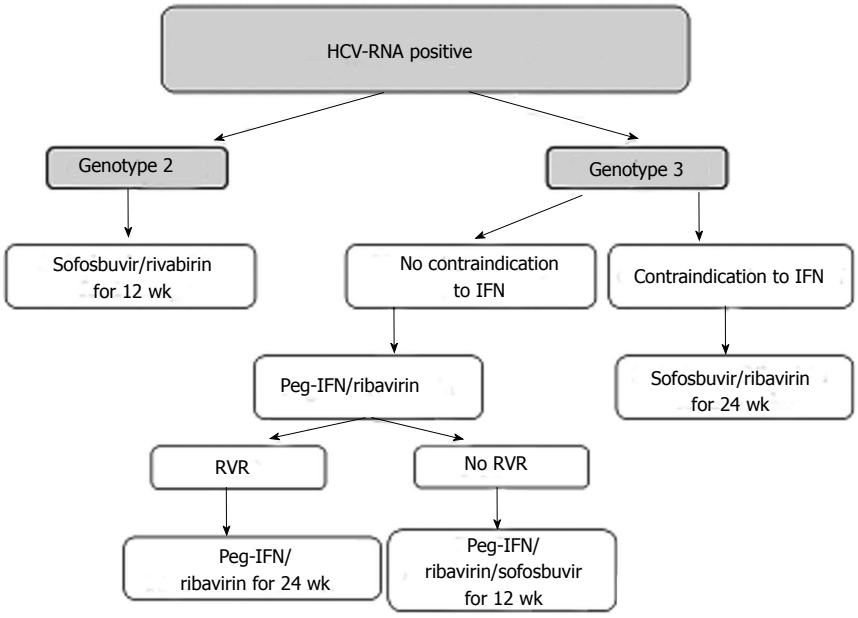

Figure 3 Treatment of chronic hepatitis C, HCV-genotype 2 or 3, in patients with human immunodeficiency virus/hepatitis C virus coinfection. RVR: Rapid virological response; IFN: Interferon.

cirrhosis, the development of HCC and liver failure. The poor tolerability of Peg-IFN + RBV double therapy and of triple therapy with Peg-IFN + RBV + boceprevir or telaprevir has been a serious obstacle to treating chronic hepatitis patients with HIV/HCV-genotype-1 coinfection. These treatment regimens compared to the new DAA-based therapies show lesser efficacy and tolerability because of the more frequent serious adverse events, a higher pill burden and longer period of treatment. Moreover, the first-generation PIs show more pharmacokinetic interactions with antiretroviral drugs than the second and third generation DAAs.

At present, treatment decisions range from waiting for all-oral second or third generation DAA regimens or treating with Peg-IFN/RBV double therapy + sofosbuvir, simeprevir or daclatasvir. A reliable guide in this difficult choice could be the entity of liver fibrosis, detected by liver biopsy or by a sensitive fibroscan assay, and other predictive factors of SVR such as the HCV viral load, the IL-28B genetic profile and the ethnic background (Figure 1). The patients' adherence, pharmacokinetic interactions between anti-HCV and antiretroviral drugs and sustainability in terms of cost-effectiveness are other important factors to be considered for a rational choice. The achievement of RVR during treatment remains the most sensitive predictor of SVR.

Currently, we deem it reasonable that HIV/HCV-genotype-1 therapy-naïve patients for whom treatment cannot be deferred should be treated with Peg-IFN + RBV dual therapy to establish whether they achieve an RVR during the first month of treatment (Figure 2). In positive cases double therapy should be administered for 12 mo, whereas for patients not achieving an RVR a second generation DAA (sofosbuvir, simeprevir, or daclatasvir) should be added, and this triple therapy administered for 3-6 mo[84] (Figure 2).

Once combinations of second/third generation DAAs are licensed for treatment of chronic hepatitis C in HIV/HCV coinfection, the patients with HCV genotype 1 for whom the treatment has been deferred and those with contraindications to IFN + RBV should be treated with an effective IFN-free DAA-based regimen (Figure 2). The same algorithm can be hypothesized for patients with HCV-genotype 4.

For patients with HIV/HCV genotype-2 coinfection, the treatment choice should be between a 24-wk low-cost Peg-IFN/RBV double therapy and a 12-wk high-cost treatment with sofosbuvir/ribavirin (Figure 3).

For patients with HCV-genotype 3, a 24-wk high-cost schedule with sofosbuvir/ribavirin or a 24-wk low-cost Peg-IFN/ribavirin double therapy seem reasonable (Figure 3).

The several ongoing trials will better define the role of the second and third generation DAAs in treating chronic hepatitis C in HIV/HCV coinfection, but in the meantime this review article may be of some help in making reasonable therapeutic choices.

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MINIREVIEWS

What psychiatric screening and monitoring might be needed with the new generation of hepatitis C treatments?

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Abstract

Psychiatric difficulties, including depression and alcohol use disorders, pose a challenge to treatment decision-making for chronic hepatitis C. This is especially made worse because interferon-alpha, as part of the standard of care, may exacerbate depressive symptoms and cause suicidal symptoms to appear. This requires a treatment setting that has the capacity to carry out psychiatric assessment and monitoring, and the capability to deliver patient education regarding these aspects of care. Psychiatric comorbidities create a challenging decision-making situation, especially since success rates for the most common hepatitis C genotype, genotype 1, hover around 40%. In recent years, new treatments have emerged. These significantly boost the likelihood of sustained viral response, including for genotype 1, and do not seem to have the side effects of interferon-alpha or ribavirin. Relevant data are reviewed to assess the degree that these new treatments might reduce the portion not eligible for treatment due to psychiatric comorbidities, and might reduce the emergence of psychiatric symptoms during treatment. Several organizations have recently released evidence-based treatment recommendation guidelines. It is apparent that interferon-alpha continues to be a standard of care, with the new drugs added to this recognized regimen in order to shorten treatment and to boost efficacy. Clinical settings must continue to assess appropriateness for treatment, including current or recent psychiatric comorbidities, and must continue to closely monitor patients for the emergence of psychiatric side effects. The newly developed hepatitis C treatments may affect the metabolism of several categories of psychiatric drugs, and so drug-drug interactions must also be considered and monitored. With many promising drugs under development, an all-pill regimen, with no interferon-alpha and no ribavirin, may emerge in the near future. This will greatly change the challenge of treatment decision-making, and should expand the portion of patients able to successfully complete a treatment regimen.

Key words: Depression; Therapy; Psychiatry; Review; Clinical

Core tip: Emerging hepatitis C treatment regimens, which include newer medications such as boceprevir, telaprevir, sofosbuvir, and simeprevir, hold promise to reduce the need for psychosocial screening and monitoring. Thus far, these medications do not seem to have the same psychiatric side effect profile as interferon-alpha.

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Based upon genotype prevalence data and efficacy data, several recently emerging treatment guidelines continue to call for first-line treatment that includes interferon-alpha in combination with one or more of these newer medications. Therefore, the need for psychiatric screening, monitoring, and support continues.

As interferon-alpha became a recognized standard-of-care treatment for chronic hepatitis C, it became apparent that psychiatric side effects complicated treatment decision-making[1]. Specifically, current or recent depression has been considered a contra-indication for interferon-based regimens because the interferon-alpha can provoke or increase depressive symptoms in those with depression or a history of depression[2]. The effect is possibly attributable to interferon's effect on serotonin pathways, but is more likely due to interferon-alpha's function as one of the many inflammatory cytokines which are recognized to cause flu-like symptoms and also cause "sickness behavior," experienced as depression[3,4]. It is hypothesized that acute inflammatory responses occur in response to infection and occur when tissue repair is needed, and that the cytokine-induced activation of anhedonia and low motivation serves the organism by allowing the body to devote physiological efforts to rest and healing rather than other activities[5].

Part of the interferon-induced depression phenomenology may include suicidality, making suicidality a treatment consideration before beginning therapy, and requires close monitoring during therapy[4]. Current or recent alcohol use has been another contra-indication: efficacy may be reduced in those drinking during treatment[6] and because those with a drinking history are perceived as having high risk of relapse under the distress of treatment[7].

Additionally, interferon therapies require that social circumstances needed to be assessed. This includes an appraisal of the ability of the patient to adhere to a challenging regimen across six or more months, the possible need to take leave from employment when experiencing side effects, and the need for stable residence, since interferon-alpha requires refrigeration. The revelation of the boosted efficacy of joint interferon-alpha and ribavirin treatment, circa 1994, brought more treatment success, but did not change this psychiatric aspect of the treatment decision matrix. Neither did the advent of pegylated interferon-alpha, circa 1998. Treatment guidelines have recommended a psychosocial evaluation for interferon-alpha candidates, with a period such as six months before re-assessment[8].

Because of these difficulties of treatment, and because chronic hepatitis C is a slowly progressing disease, the clinical decision often is to delay treatment, and "watch-and-wait." In the pegylated-interferon/ribavirin era, outcomes evaluations have determined that approximately 70% of otherwise eligible patients do not begin interferon-alpha treatment due to contra-indications, with a significant portion of reasons being psychiatric[9,10].

Further complicating the clinical picture is the fact that ribavirin, while boosting rates of sustained viral response when combined with interferon-alpha, is a teratogen. Therefore, great care and attention must be used when considering hepatitis C treatment for women of child-bearing age, and when delivering this treatment. Before beginning therapy, a pregnancy test must be conducted, and the woman must decide whether she will be able to maintain two types of birth control throughout treatment, and to conduct repeat pregnancy tests, as well as maintaining adherence to the interferon-alpha/ribavirin regimen[11].

For quite some time, the standard of care has been to carefully consider the psychiatric profile of a potential interferon-alpha candidate. Patients with current or recent psychiatric difficulties can be referred for a course of psychiatric treatment, perhaps with re-assessment in six months, or be started on interferon-alpha therapy as long as supportive care and monitoring are in place. This requires a great deal of the hepatology or gastroenterology clinical setting: these settings must have the capacity or resources to carry out psychiatric evaluations, to provide psychiatric treatment or link a patient with psychiatric care, and to monitor psychiatric symptoms throughout therapy. Patients must also be willing to accept the treatment decision to not begin a potentially curative therapy, and follow other medical advice such as abstention from alcohol.

Across time, clinicians have become more confident in treating patients who had current or recent psychiatric difficulties. Risks have been addressed by using a multidisciplinary team[12-14], by conducting regular psychiatric monitoring[15], and by prophylactic antidepressant treatment[16]. Although clinicians have become competent at detecting and addressing these complicating factors, the inherent difficulties have driven the development of newer drugs that do not carry these challenges.

In recent years, many newer drugs have been developed. In the spring of 2011, boceprevir and telaprevir received Food and Drug Administration (FDA) approval for hepatitis C virus (HCV) treatment. In winter 2013, sofosbuvir and simeprevir received FDA approval. Daclatasvir has been approved as of July 2014 by the European Medicines Agency, and an application for approval of ledipasvir was submitted to the FDA in February 2014.

These drugs, and others under investigation, may resolve the difficulties of interferon-alpha-based therapy. Generally, since they do not behave as inflammatory cytokines, they do not share the side effect of inducing flu-like symptoms, depression, or suicidality. The new drugs do not require refrigeration, and they are not known to have the teratogenic risk of ribavirin. FDA prescribing information for the recent drugs that are thus far FDA-approved, including sofosbuvir, simeprevir, telaprevir, and boceprevir, do not note psychiatric symptoms as recognized side effects. Since they can be used in

combination with interferon-alpha and with ribavirin, or both, the prescribing information for each of these new medications does note the risks associated with the entire regimen, as approved.

Will these newer therapies make the focus upon psychiatric status a thing of the past? Will the new therapies make the psychiatric assessments and psychiatric care, and lifestyle assessments, such as the assessment of residential stability or pregnancy monitoring, a thing of the past? Ideally, the new generation of medications would eliminate these challenging treatment considerations, and far fewer treatment candidates should be delayed by psychiatric concerns.

This question can be answered by reviewing recently updated treatment guidelines. In March 2014, a guideline was develop and released jointly by the American Association for the Study of Liver Disease and the Infectious Diseases Society of America[17]. In April 2014, guidelines were released by the World Health Organization[18] and the European Association for the Study of the Liver[11]. The Veterans Affairs (VA) National Hepatitis C Resource Center and Office of Public Health released a treatment consideration guide in March 2014, with an update in May 2014[19].

These guidelines incorporate recent efficacy evidence for hepatitis C treatment. At this point in time, recommended care has not yet reached the point of being able to decrease concerns over psychiatric or social factors.

The guidelines are very consistent in recommending that the majority of patients with chronic hepatitis C, who are candidates for treatment, should still be treated with a regimen that includes interferon-alpha. The innovation provided by the recently developed medications is that one or more of these should be added in order to boost the likelihood of achieving a sustained viral response. The most common hepatitis C genotype is type 1, at possibly 46% of cases worldwide[20]. Genotype 3 may account for approximately 30% of cases world-wide, genotype 2 may account for approximately 9% of cases, genotype 4 may account for 8%, and genotype 6 may account for 5% of cases.

The VA guideline is organized by genotype, then by other parameters such as whether the patient is treatment-naïve, and whether or not cirrhosis is present. This guideline suggests that treatment-naïve patients with genotype 1 and no notable contra-indications be treated with a regimen of pegylated interferon-alpha combined with ribavirin, and also combined with either sofosbuvir or simeprevir.

Thus, the greatest numbers of patients coming under consideration, those with genotype 1 who are treatment naïve, are still advised to receive a regimen that includes interferon-alpha and ribavirin, and so includes the treatment challenges inherent with those regimens. Treatment-naive patients with genotype 3 may be started on a regimen of ribavirin combined with sofosbuvir for 24 wk, with an alternative, 12-wk regimen including pegylated interferon along with the ribavirin and the sofosbuvir. Therefore, for genotype 3, the second-most prevalent genotype, the concern about pregnancy remains when following recommended care. It thus remains the case that, for a majority of treatment-naïve patients, a hepatitis C treatment setting must have the capacity to carry out psychosocial assessment, education, intervention, and monitoring, even though a new generation of much more benign drugs have been developed and are receiving FDA approval.

One problem affecting some of these new drugs is that they may affect the metabolism of psychiatric drugs[21]. Because of this possibility, the clinical care team will need to monitor for any drug-drug interactions for drugs that the patient may have already been prescribed, or may consider taking while being treated for hepatitis C. For example, FDA prescribing information notes that sofosbuvir may interact with anti-epileptic medications, such as carbamazepine and phenytoin, which are both used for the treatment of bipolar disorder and other psychiatric conditions. Telaprevir has a longer list of potential interactions with psychiatric drugs, including anti-epileptics, some antidepressants, and some benzodiazapenes. Kiser *et al*[21] note possible interactions with some of the atypical anti-psychotics, as well. Treatment may call for close monitoring, or the patient may want to discontinue a drug for the length of hepatitis C treatment, or the patient might switch to another drug, with no interaction risk, for the noted indication.

Drawing upon the same set of available efficacy data, the Veterans Affairs guidelines are very concordant with those from the other noted organizations. Overall, when considering the epidemiology of hepatitis C genotype and the first line of treatment suggested by recently developed guideline statements, interferon-alpha with ribavirin continues to be a mainstay of treatment, with the innovation being the boosted rates of sustained viral response when adding the newly approved drugs.

Since interferon-alpha and ribavirin will continue to be mainstays of care, treatment settings will continue to be required to accommodate the problem of psychiatric comorbidities in their clinical populations, and to be able to address treatment-based psychiatric side effects including depressive symptoms and suicidality. A strong emphasis on patient education continues to be required to convey information regarding regimen adherence, dosing, timing, drug-drug interactions, and the problem of the teratogenicity of ribavirin.

The AASLD/IDSA 2014 recommendations[17] note that "evaluation by a practitioner who is prepared to provide comprehensive management, including consideration of antiviral therapy, is recommended for all persons with current (active) HCV infection." They proceed further on this issue to note that such comprehensive care is not common for settings diagnosing and treating liver disease, but strategies, such as co-localization of care and collaborative care arrangements, can be developed to meet this recommended style of comprehensive care.

At the same time, evaluation of new drugs, including combinations of new drugs, is actively being pursued, largely with the goal of an all-pill regimen that avoids

interferon-alpha, and also avoids, where possible, ribavirin. With FDA approval for four new drugs thus far, and several more under evaluation, the pragmatics of providing effective, evidence-based treatment for chronic hepatitis C, with a much more benign patient experience, may be much easier in the near future. In many cases, a "watch-and-wait" approach remains appropriate, as the treatment options may soon increase dramatically. "Watch-and-wait" may be acceptable for much of the patient population as long as the decision-making process has been patient-centered[22].

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MINIREVIEWS

Mechanistic insights on immunosenescence and chronic immune activation in HIV-tuberculosis co-infection

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Abstract

Immunosenescence is marked by accelerated degradation of host immune responses leading to the onset of opportunistic infections, where senescent T cells show remarkably higher ontogenic defects as compared to healthy T cells. The mechanistic association between T-cell immunosenescence and human immunodeficiency virus (HIV) disease progression, and functional T-cell responses in HIV-tuberculosis (HIV-TB) co-infection remains to be elaborately discussed. Here, we discussed the association of immunosenescence and chronic immune activation in HIV-TB co-infection and reviewed the role played by mediators of immune deterioration in HIV-TB co-infection necessitating the importance of designing therapeutic strategies against HIV disease progression and pathogenesis.

Key words: Cluster of differentiation 38; Human immunodeficiency virus-tuberculosis co-infection; Immunosenescence

Core tip: The mechanistic aspects associated with increased expression of senescence and immune activation markers cluster of differentiation (CD) 38, CD69, CD57, human leukocyte antigen-DR, and the down-regulation of functional molecules, *viz.*, CD28, CD27, CD40L and CD127 on human immunodeficiency virus-specific T cells appear to be crucial in the immunopathogenesis of HIV-tuberculosis (HIV-TB) co-infection. *Mycobacterium tuberculosis* appears to play a major role in accelerating HIV disease progression, by directly or indirectly facilitating factors associated

with immune senescence. Measures to ameliorate immunosenescence and immune activation appear to stem from identification of novel targets of downstream senescence signaling. Restoration of molecules associated with T-cell homeostasis, differentiation, cell survival and proliferation abilities of HIV-specific CD8$^+$ T cells is key to foster functional immune responses.

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INTRODUCTION

The hallmark of human immunodeficiency virus type 1 (HIV-1) disease is the destruction of cluster of differentiation (CD) 4$^+$ T cells eventually leading to the failure of functional attributes of the host immune system in containing viral establishment. The key target cells of HIV infection are CD4$^+$ T cells, dendritic cells (DCs), monocytes/macrophages, thymocytes and microglial cells[1,2]. HIV enters these cells *via* binding primarily but not limited to target receptors/coreceptors CD4, chemokine coreceptor 5 (CCR5) and CC-chemokine receptor 4 (CXCR4)[3-5]. The CCR5-tropic HIV (macrophage-tropic R5 strain) appears to predominate primarily during the onset of infection, and eventually the CXCR4 HIV (T cell-tropic X4 virus) takes over to establish a chronic phase leading to eventual destruction of CD4$^+$ T cells harnessed by the onset of opportunistic infections and neoplasms[6].

HIV reportedly evades the immune system through several ways to effect direct or indirect killing of infected and uninfected cells[7]. HIV facilitates CD4$^+$ T cell depletion primarily *via* accelerated destruction, chronic immune activation (CIA) and also by impairing the regeneration of new T cells from existing T-cell precursors[8]. Evidence suggests that monocytes and macrophages although may not be significantly affected in HIV-infected individuals, their role as reservoirs for HIV-1 provides homes for long-term survival following infection and can therefore be transmitted to bystander T cells[9,10]. DCs contribute even more to HIV-1 pathogenesis and studies have reported reduced levels of peripheral blood DCs in HIV-1 patients and changes in their phenotypic and functional properties[11]. Evidence also suggests that HIV could alter the expression of costimulatory molecules as well as chemokine receptors[11]. HIV-1 infected DC in contact with T cells fail to provide optimal feedback to T cells partly due to impaired release of IL-12, which in turn fail to provide optimal survival signals for DCs owing to impairment in the expression of CD40L on T cells. Furthermore, sustenance of T-cell proliferation is also impaired due partly to decreased secretion of IL-2 by activated T cells[12,13].

IMMUNOSENSESCENCE AND CHRONIC IMMUNE ACTIVATION - KEY CULPRITS OF HIV DISEASE PROGRESSION

Immunosenescence is a common biological phenomenon occurring in elderly individuals, and represents gradual deterioration of the immune system leading to attenuated responses to infections and vaccinations[14]. Roy Walford was the first to use the term "immunosenescence" in 1969. He believed that normal ageing in humans and animals is related to deficient immune functions[15]. Like any other cells in the body, immune cells undergo senescence. Immune senescence is characterized by changes in T-cell subsets, molecular alterations and often involves atrophy of lymphoid organs, eventually culminating in the decline of T- and B-cell functions[16]. Recent studies have shown that immunosenescence can occur involving both the adaptive and innate arms of the immune systems[17]. However, the major immune cells severely affected by immunosenescence are the T cells, which ultimately result in compromised responses to antigens and increased rates of differentiation of naïve T cells to terminally-differentiated T cells[18,19].

Immunosenescence is marked by accelerated degradation of immune system with increased turn-over of senescent T cell phenotypes showing remarkable ontogenic defects[20]. The cells possess reduced life-span with shorter telomere lengths, reduced proliferation abilities, dysfunctional cytokine-secreting abilities, deficient anti-viral responses (exhausted effector T cells), and suppression of T-cell responses due to expansion of suppressor T cells and up-regulation of multiple negative immune receptors[21-25]. Currently, there is increasing evidence of the expansion of senescent T cells expressing surface markers such as CD28, CD27, CD57 and CD127, especially in HIV and cytomegalovirus (CMV) infections[26-30]. This suggests that persistent viral infections (PVIs) can induce the expansion of senescent T cells *via* a mechanism called "replication senescence" or "Hayflick phenomenon", also defined as the decrease in the ability of a cell to proliferate, with significant mark of terminal differentiation[31,32].

Interestingly, immunosenescence also appears to occur in younger individuals with underlying malignancies and autoimmune conditions. An overwhelming body of evidence shows that persistent microbial infections with highly sustained levels of chronic antigenic stimulation, especially with HIV and CMV, could lead to functional impairment of Ag-specific T cells including proliferative abilities[33]. Furthermore, premature senescence of CD4$^+$ and CD8$^+$ T cells is well-characterized in chronic HIV infection with evidence of up-regulated surface markers and functions similar to that seen in elderly HIV-uninfected individuals[34,35]. Chronic HIV-infected patients have also shared some similarities in T-cell dysfunction with that of 'healthy' aging elderly[36,37]. Interestingly, the persistence of immune activation is exceptionally notable in chronic HIV disease both in mono-infected and co-infected with

other infectious agents such as HCV, HBV and MTB, despite that highly-active antiretroviral therapy suppressed viral replication in these subjects[38-40]. This phenomenon appears to be attributed to the up-regulation of immune activation markers namely ki-67, CD38, human leukocyte antigen - DR (HLA-DR), and CD69 on HIV-specific $CD4^+$ and $CD8^+$ T cells[41,42]. Of these, CD38 expression has been reported to serve as a reliable marker of disease progression and acquired immunodeficiency syndrome (AIDS)-associated mortality[43].

Markers of CIA apart from T cells, are also expressed in a plethora of other immune cells such as monocytes, DCs, and natural killer (NK) cells[44]. Elevated immune activation of T cell appears to be one of the potent predictors of HIV disease progression[45,46] as highly sustained immune activation may contribute to rapid disease progression by impairing the ability of the immune system to respond to antigens[47], suggesting that CIA could be a key player in HIV pathogenesis and indirectly predicts progression to non-AIDS related morbidity and mortality[34]. Accumulating line of evidence also suggests that increased expression of CD57 and reduced levels of CD127 in patients with CIA highly correlated with T-cell dysfunction and senescence[26,27,48,49] supporting the notion of potential association between CIA and immunosenescence, especially in T cells.

IMMUNOSENESCENCE AND HIV-TB CO-INFECTION

Current investigations in human HIV/TB co-infection have provided several fundamental principles to understand how these distinct pathogens additively interact to accelerate the rates of disease progression. Although the precise mechanism of co-pathogenesis still remains elusive, it has widely been shown that both TB and HIV exert substantial influence on the host immune system. Hence, investigations underpinning the influence of TB in HIV/TB co-infection, and the importance of T-cell responses to elucidate the mechanisms underlying the failure of the immune system resulting from the dreadful interaction between HIV and TB are urgently required.

While it is increasingly becoming clear that persistent HIV disease facilitates the onset of CIA and consequently to premature senescence[20,24,28,45,47,50], existing hypotheses suggest that MTB exacerbates HIV disease by enhancing viral transmission and entry into immune cell by causing alternations in signal transduction, cytokine modulation; overcoming anti-viral responses with overwhelming HIV promoting responses; and facilitating HIV amplification by rendering the formation of granuloma[51-54]. The up-regulation of immunosenescence markers on T cells appears to accelerate the depletion of functional T cells, hastening a shift to terminally-differentiated T cells with altered immune functions[55], and hence we speculate that this potentially might facilitate the onset of AIDS, and disseminated and extra-pulmonary TB infections. Based on this mechanistic viewpoint it is also possible to correlate immunosenescence with CIA in HIV-TB co-infection.

CD38 AND HLA-DR - IMMUNE ACTIVATION MARKERS IN HIV-TB CO-INFECTION

CD38 and HLA-DR have been widely used to deduce the activation status of various immune cells, apart from other markers such as CD27, CD28, Ki-67 and CD69[41,42]. CD38 is a glycoprotein receptor found on the surface of T cells, B cells and NK cells with key roles in signal transduction and calcium mobilization associated with their activation[56]. On the other hand, HLA-DR is an major histocompatibility complex class II molecule that presents antigens to APCs and acts as a marker of T-cell stimulation and activation[56-58]. Numerous literatures have established that immune activation is a direct measure of HIV disease progression[45,59-61], which has previously been shown with CD38 expression on $CD8^+$ T cells[43,46,62]. Multiple studies have also shown that concomitant with HCV, HBV, and MTB can directly impact HIV disease progression with excessive T-cell activation in the peripheral blood[40,63-65]. Increased expression of CD38 on both $CD4^+$ and $CD8^+$ T-cells of HIV-TB co-infected subjects has been described relative to HIV mono-infection[63,66,67]. This was also consistent with existing evidences that explain the association of HIV/TB co-infection with sustained levels of peripheral activation in immune compartment following pathogenic persistence[67-69]. Indeed, TB infection fosters immune activation as evident from up-regulated CD38 expression on T-cell subsets as compared to uninfected subjects[66,70]. Besides this, CD38 expressions in both the $CD4^+$ and $CD8^+$ T-cell subsets have been inversely correlated with $CD8^+$ T-cell counts and HIV plasma viral load, and that enhanced CD38 expression could lead to rapid HIV disease progression[66,70]. The mechanism whereby MTB appears to attenuate the expression of HLA-DR, particularly on innate cells such as macrophages and DCs, is *via* the synthesis of bacterial proteins (such as 19kD lipoprotein and lipoprotein rG), which subsequently cause impaired antigen presentation and processing, potentially affecting the downstream signaling for HLA-DR expression on T cells[71-74]. In addition, MTB can evade phagocytosis by macrophages and eventually delay the onset of adaptive immune responses[75,76]. Active MTB infection can suppress the expression of HLA-DR *via* innate receptors (*i.e.*, TLR2), gene repression (*i.e.*, histone deacetylation), and cytokine-mediated inhibition (*i.e.*, IFN-γ) in infected individuals.

HOW DOES HIV-TB CO-INFECTION ENGINEER THE DIFFERENTIATION OF SENESCENT PHENOTYPES?

Cellular differentiation is the process by which a less specialized cell becomes a more specific phenotype with

unique functions. Upon antigenic stimulation, naïve T cells are activated and undergo differentiation into various subsets that possess distinct functionalities. CCR7 and CD45RA are two markers of T-cell differentiation but many others do exist, with two intriguing co-stimulatory molecules, CD27 and CD28[16]. Others have proposed a model of differentiation using co-expression of CD27 and CD28 to subdivide $CD8^+$ T cells into three distinct subsets based on their proliferation history, *viz*., early ($CD28^+CD27^+$), intermediate (CD28-$CD27^+$), and late ($CD28^-CD27^-$) T-cell subsets[77]. Subsequent research also showed that intermediate-differentiated $CD4^+$ T-cell subsets lose CD27 prior to CD28 ($CD28^+CD27^-$)[36,78]. CD27 and CD28 has also been reported to indicate the stage of T-cell activation and proliferation[79,80]. Lowered expression of CD28 indicates immunosenescence, marked by shortened telomeres and diminished replicative abilities[81], whereas CD27 has been recently characterized as a modulator of T-cell functions, and has been suggested as a better correlate of proliferative potentials[55]. Late-differentiated subsets have been associated with strong cytotoxic potentials, and gradual up-regulation of CD57 expression suggesting a closer relationship between senescence and differentiation[77]. It has also been established that persistent infections might lead to loss of CD27 and CD28, which reflects that more proliferation cycles have taken place in response to pathogens, eventually leading to increased T-cell activation and advanced stages of differentiation[82,83]. Hence, HIV-TB co-infection appears to have a synergistic effect in down-regulating CD27 and CD28 in accelerated rate as in HIV mono-infection.

Research also shows that persistent HIV infection impacts the differentiation of $CD8^+$ T-cell subsets resulting in the over-presentation of intermediate-differentiation stage[84,85]. This could largely be due to a block in maturation of $CD8^+$ T cells engineered by HIV to maintain chronicity leading to ineffective cytokine and cytotoxic responses following antigenic stimulation[85-87]. Hence it is speculated that MTB may be involved in accelerating T-cell differentiation despite the blockade of maturation exerted by HIV, leading to biased distribution of advanced stage of differentiation.

ROLE OF CD57 AND CD127 IN IMMUNE CELLULAR SENESCENCE

CD57 is a marker of senescence that has been associated with *in vitro* replicative senescence, or proliferation incompetence in both $CD4^+$ and $CD8^+$ T cells of healthy elders as well as PVIs[48,88,89]. Extensive investigations have also been carried to decipher the functional role of CD57 in apoptosis, activation-induced cell death, senescence and overwhelming cytokine and cytolysin responses. Outside HIV infection, CD57 has also been associated with various diseases and cancers[88]. Both HIV and MTB alone have been shown to facilitate the expansion of $CD57^+CD8^+$ T cells with wide range of functionality changes and contributing to the immunpathogenesis of each disease progression[48,90]. Furthermore, expansion of $CD57^+CD8^+$ T cells upon stimulation by MTB have more extensive cytokine and cytolytic potential with secretion of TNF-α and IL-6[53], and this abnormality of modulation may eventually promote HIV manifestation in co-infected individuals.

Based on our understanding, immunosenescence is best characterized by T cells showing increased CD57 and decreased CD28 expressions, also known as "late-differentiated" or senescent cells, despite several studies have proposed that co-expression of CD57 and CD27 may be a better correlate compared to CD28 as an indicator of replicative senescence[55,87]. It is also evident that loss of CD27 and CD28 with concurrent up-regulation of CD57 descriptively represents increase of replicative inability when T cells differentiate further[24,91]. Co-infection appears to foster the expansion of late "senescent" $CD8^+$ T cells ($CD57^+$ CD28/$CD27^-$) compared to early "senescent" $CD8^+$ T cells ($CD57^-$CD28/$CD27^+$), whereas HIV mono-infection has over-presentation of intermediate "senescent" $CD8^+$ T cells ($CD57^-CD28^-/CD27^-$). Hence, given that late "senescent" $CD8^+$ T cell is associated with decreased telomerase activity with the shortest telomere length, and a reduction in activation-induced activation[92], there appears to be more turnover of late-differentiated $CD8^+$ T cells in co-infected individuals with expanded expression of CD57, which suggests that more T cells have reached the stage of true senescence[93].

CD127 has been indicated in activation, homeostasis, differentiation, and cell survival of different T cell populations[94,95]. Decrease of CD127 expression has been associated with HIV disease progression[49,96]. The importance of maintenance CD127 for T-cell survival, especially during chronic HIV infection has also been suggested[27]. The down-regulation of CD127 may ensue due to several mechanisms, one involving HIV infection where there is a dysfunctional cytokine response when excessive IL-7 may cause an inhibitory effect on CD127 expression; while the other may be due to imbalance of IL-7 levels in the peripheral circulation[97,98].

CONCLUSION

Despite the disparity in pathogenesis and natural history HIV-TB disease, current literature suggest that both the pathogens harness a higher quantum of symbiotic impact on each other leading to accelerated rates of deterioration of host's immune responses. Existing understanding of pathogen interaction based on immunology research has contributed to genesis of several novel hypotheses to precisely address how contemporaneous manifestations of HIV aggravate TB disease progression and vice versa[51]. However, these evidences have not been conclusively successful in deciphering the mechanism underlying the role of MTB and HIV in accelerating immune deterioration. A better understanding of immunosenescence, and the development of strategies aimed to rejuvenate T cells, especially in PVIs will direct to improved quality of life of infected individuals. In addition, extension of knowledge

on immunosenescence to precisely identify therapeutic targets and surrogate biomarkers to validate senescence phenomena as clinical endpoints may be key to better healthcare requirements.

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MINIREVIEWS

Current molecular methods for the detection of hepatitis C virus in high risk group population: A systematic review

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Abstract

Hepatitis C virus (HCV) is an emerging infection worldwide and the numbers of persons infected are increasing every year. Poor blood transfusion methods along with unsafe injection practices are potential sources for the rapid spread of infection. Early detection of HCV is the need of the hour especially in high risk group population as these individuals are severely immunocompromised. Enzyme Immunoassays are the most common detection techniques but they provide no evidence of active viremia or identification of infected individuals in the antibody-negative phase and their efficacy is limited in individuals within high risk group population. Molecular virological techniques have an important role in detecting active infection with utmost specificity and sensitivity. Technologies for assessment of HCV antibody and RNA levels have improved remarkably, as well as our understanding of how to best use these tests in patient management. This review aims to give an overview of the different serological and molecular methods employed in detecting HCV infection used nowadays. Additionally, the review gives an insight in the new molecular techniques that are being developed to improve the detection techniques particularly in High Risk Group population who are severely immunocompromised.

Key words: Molecular detection; Enzyme immunoassay; High risk group population; Nucleic acid amplification assays; Polymerase chain reaction

Core tip: The review focuses on the current molecular diagnostic techniques that are being used to detect hepatitis C virus worldwide. Special emphasis is given on the detection techniques that can be used to screen the individuals with repeated blood transfusion history; particularly thalassaemic individuals, intravenous drug users and persons on hemodialysis.

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INTRODUCTION

Hepatitis C virus (HCV) infection is a global health problem which has affected around 170 million people worldwide and is one of the major causes of deaths related to liver cirrhosis and hepatocellular carcinoma[1]. HCV can be classified to seven major genotypes and 80 subtypes[2-4]. HCV genotypes vary in patterns of geographical distribution and therapeutic response. However, the geographical and genetic diversity of this RNA virus is constantly evolving because of rapid globalization. In India, HCV infection has been reported in 0%-21% population and responsible for 14%-26% cases of chronic liver disease[5]. HCV infection is mostly transmitted through transfusion of blood or blood products. A high prevalence of HCV is found in many high-risk groups (HRG) exposed to blood or blood products like intra venous drug users (IDUs), patients with pediatric hematologic malignancies and those with thalassemia and hemophilia. India reported a higher percentage of blood donors (1%-1.5%) than in any developed country[6,7].

An increasing burden of HCV related liver complications has been estimated particularly taking into account those who were infected before safety precautions of blood transfusions happened. A major concern is careful screening of blood and blood related products, but in developing countries like in India, the regulations for strict checking of blood and blood related products came to place only in 2001[8,9]. Recent surveys have reported that testing of blood and blood related products are poorly regulated in India[10]. In United States, data showed that death related to HCV exceeded than those by HIV. Though novel antiviral therapies are recently in the horizon with enhanced efficacy and fewer side effects but the challenge remains in detecting HCV at an early stage.

During HCV infection, though attempts are made to diagnose and differentiate acute from chronic hepatitis C infection, it is often not possible to distinguish between the two phases. The infection may be recognized only when it becomes chronic[11,12]. The serologic diagnostic tests used as first step for detecting the infection cannot distinguish between acute and chronic infection[13]. Investigations for patients with HCV infection include serological assays for antibodies to hepatitis C (anti-HCV) and molecular assays for detection of viral RNA.

The importance of low cost molecular diagnostic assays are especially important for the developing nations as they are already burdened with increasing number of hepatitis C patients who are generally economically backward. The advent of molecular diagnostic approaches has allowed for the development of nucleic acid assays that are more sensitive and specific than antibody based technologies. The linking of these assays with appropriate detection systems, therefore, makes them highly desirable for detecting HCV RNA in patient samples. Molecular techniques not only help to detect HCV RNA but confirm active state of infection, *i.e.*, the virus is in replicating state in the patient's body. In individuals falling in high risk diagnosis of HCV can give false negative results as these patients are already immuno-suppressed, in this scenario, molecular testing remains the best choice for detection.

This review aims to give an overview of the different serological and viral genome based laboratory tests which has become instrumental in the management of HCV infection to diagnose viral infection, and more importantly guide treatment decisions which could be of enormous help to clinicians.

LABORATORY INVESTIGATION

The investigation of HCV diagnosis starts with serological assays for detecting antibodies to HCV followed by molecular assays for detecting HCV RNA (Figure 1). Initial diagnosis of HCV infection is classically done by serologic methods either by determining anti HCV antibody by EIAs or by immunoblot assays and by determining the presence of HCV RNA. The advent of simple rapid immunoassays has significantly reduced the risk of HCV transmission, but concern remains for patients in high risk groups[13-16]. Studies have shown that false negative results in rapid tests might arise in patients who are severely immunocompromised such as those co-infected with HIV[17], in patients on hemodialysis, IDUs, thalassaemic. In these patient groups molecular detection by reverse transcription polymerase chain reaction (RT-PCR) remains the best method for detection.

RAPID IMMUNOASSAYS FOR DETECTION OF HCV IN SERUM OR PLASMA

Rapid immunoassay tests are based on the principle to detect HCV antigens from core, NS3, NS4 and NS5 regions of the virus. In western countries, these tests are used besides nucleic acid testing, and used only as point of care tests, but in developing countries these tests are solely relied in commercial places for detection of HCV[18]. Commercial kits like OraQuick rapid HCV antibody test use device that delivers HCV antibody test results in 20 min using a single drop of whole blood. The kit was approved for laboratory use in United States from June 2010. The OraQuick is very accurate, with sensitivity and specificity performance that meets the standards for FDA approval[19]. Though rapid kits have been extensively used for surveillance purposes, they are not well suited for high risk groups and immunocompromised patients[20,21]. In developing countries like India, WHO has recommended certain kits for rapid testing for surveillance purposes.

ENZYME IMMUNOASSAY FOR HCV DETECTION IN SERUM OR PLASMA

Enzyme immunoassays are the most common screening test for HCV [enzyme immunoassay (EIA), microparticle EIA, chemiluminescence immunoassay (CIA)] that detects anti-HCV antibodies in plasma or serum. These assays are

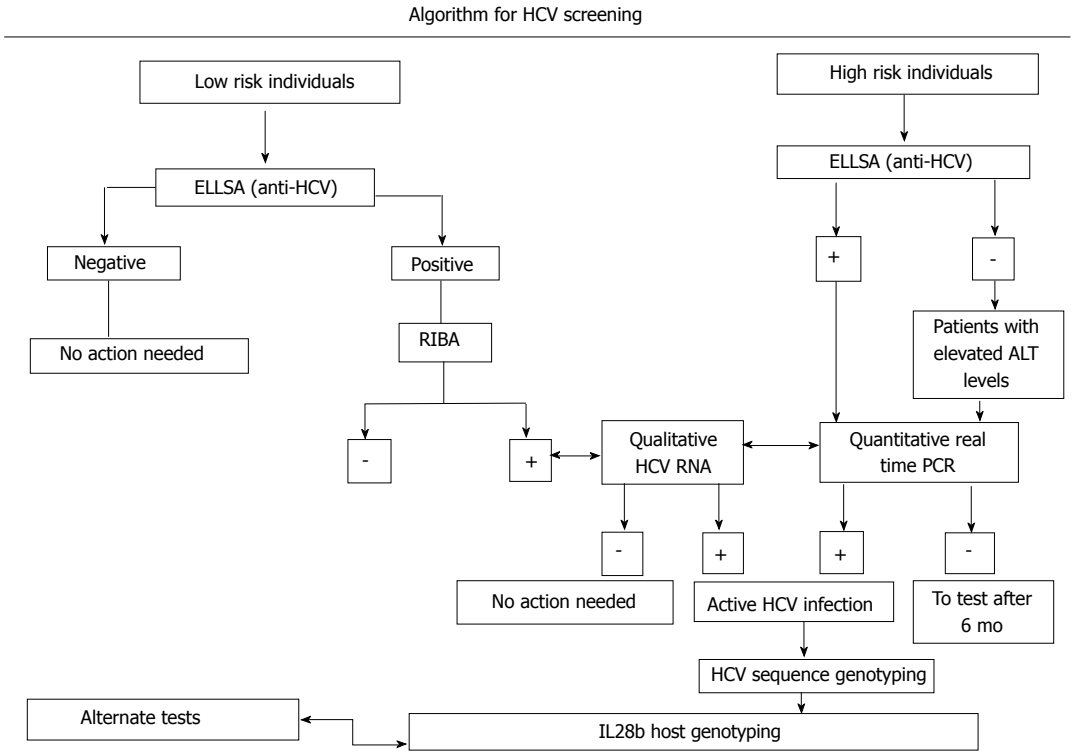

Figure 1 Algorithm for hepatitis C virus screening and detection. HCV: Hepatitis C virus; PCR: Polymerase chain reaction.

relatively easy to use, does not require expert technicians, automation is simple, have a low variability and are inexpensive.

Three generations of EIA antibody testing have been developed since 1989. In the first generation of EIA developed in 1992, c100-3 epitope from the non-structural NS4 regions was incorporated. A newer and better second generation EIA-2 was developed next which contained HCV antigens from core, NS3, NS4 regions[22-24]. The third generation of EIA developed contained modified antigens from core, NS3, and a slightly modified antigen from NS5 region. The incorporation of these antigens increased the overall sensitivity to 97%, which was better than the second generation assays. The mean time to seroconversion in the improved third generation kits has gone down to 2-3 wk as compared to 4-6 wk in the second generation kits. EIA methods have several advantages as the kits are relatively inexpensive and highly sensitive too, but one of the disadvantages is that it may give false positive results in routine blood donors and asymptomatic adults. For this reason, Centres for Disease control and prevention has recommended the supplementary tests like RIBA or PCR based methods to confirm positive ELISA tests unless the signal-to-cut-off ratio is above a predetermined threshold[25].

False-negative EIA especially occur in patients with major immunosuppression (advanced HIV infection or organ transplantation recipients), patients with chronic renal failure on long-term hemodialysis, and patients with acute or early HCV infection have been reported in HCV EIA[26].

In developing countries like India, WHO recommends the use of 3rd generation HCV EIA kits. Several commercial kits which employ the structural and non-structural antigens *i.e.*, core, E1, E2, NS3, NS4 and NS5 are in use. The 3rd generation kits are better than the previous versions with improved specificity and sensitivity. The use of EIA kits in India is limited as it requires expertise in handling and in developing countries like India where the health care resources are already burdened; it becomes very difficult to reach the people[27].

RECOMBINANT IMMUNOBLOT ASSAY FOR HCV ANTIGEN DETECTION

In the recombinant immunoblot assay (RIBA) assays, multiple HCV antigens are individually displayed on a nitrocellulose strips as bands. In positive HCV infection, RIBA results show two reactive bands, in intermediate infection show one positive band. Since positive cases in RIBA, show two bands they are considered more sensitive than EIA. However, they are not considered as independent gold standard as two tests contain similar antigens to detect HCV antibody[28-30].

DETECTION OF HCV CORE ANTIGEN IN PLASMA OR SERUM

HCV core antigen testing was developed as an alternative to nucleic acid testing (NAT assays). The HCV core antigen detects viral antibodies within the sero-conversion period and can be used to utilize to monitor antiviral therapy. But till now is not commercially used as detection

system[31]. The Architect HCV Ag assay (Abbott) which is a quantitative core antigen based assay, is commercially available in Europe. The assay is chemiluminesce based immunoassay in an automated platform[32]. In these assay, micro particles are coated with a monoclonal antibody against HCV core antigen. Studies have shown that HCV core antigen could be detected within first two weeks of acute infection. The core antigen based testing has a sensitivity ranging from 80% to 99% and a specificity ranging from 96% to 100%. The core antigen based assay could be an important detection technique in individuals who are in High Risk Group, because the core protein is the most conserved viral antigen amongst HCV types and, was therefore, a likely antigenic probe. Also the core protein is one the first protein which is synthesized and therefore could be an attractive target for molecular detection in high risk group patients who are immunocompromised. Studies have demonstrated two major B-cell epitopes at the N-terminus of core: amino acids (aa) 5-23 and 39-74 using peptide reactivity to sera from patients with chronic HCV can be used as antigenic determinants[33,34].

NAT FOR DETECTION OF HCV RNA

Molecular diagnostic assays are an integral part in the management of HCV patients. Both qualitative and quantitative HCV molecular assays are used in the diagnosis of acute and chronic infection. The principle of qualitative HCV assays includes viral RNA isolation, complementary DNA (cDNA) synthesis, PCR amplification and detection of PCR amplicons. Qualitative HCV RNA test detects the presence of HCV circulating in the blood and is among the most sensitive tests available. Since HCV is a RNA virus, reverse transcription PCR is used to detect viral RNA[35-37]. The viral genome is 9.6kb long, contains a single open reading frame that is translated to produce a single protein product, which is then further processed to produce functional proteins for viral replication and propagation[36]. At the 5' and 3' ends of the viral RNA are the untranslated region (UTR) that are not translated into proteins but are important to translation and replication of the viral RNA. Most of the commercial and in-house PCR amplification strategies are targeted against the 5' UTR region as there is more than 90% sequence identity among different HCV genotypes, with some segments nearly identical among different strains[37-39]. The secondary and tertiary structures of this region are also largely conserved and this is one of the first regions which is transcribed of the first regions which is transcribed.

Other than the 5' UTR region, the core and the 3' UTR region are also targeted for PCR based detection of HCV[40-44]. A recent study showed that detection based on the sequence of the core region could reliably identify subtypes as well as major genotypes since the sequence divergence was greater than the divergence of the 5'UTR sequence. Though there are other regions like the E1, E2, NS2, which can be used as detection targets for PCR amplification but they are not in much use as there is a lack of conservation in the primer binding sites[45-50].

Detection of viral RNA is useful in diagnosing HCV infection prior to sero-conversion, distinguishing active from resolved infection, and diagnosing chronic hepatitis carriers who are HCV antibody negative, especially among HRGs. Nucleic acid testing is recommended: (1) for confirmation of HCV RNA in cases where patients are HCV seropositive; (2) to confirm the presence of HCV viremia in patients who are seronegative but immunocompromised such as HIV infected individuals; (3) in babies who are born to HCV positive mothers- as antibody testing in babies can give false positive results upto 18 mo of age; and (4) for determining the baseline value before starting the anti-viral therapy. Molecular detection of HCV includes both qualitative and quantitative assays. The qualitative HCV RNA testing is very popular due to its higher sensitivity, but a major disadvantage of the qualitative assays is that it only determines the presence or absence of HCV RNA. On the other hand, quantitative HCV RNA determines the HCV RNA level and thus provides prognostic information for treatment. Nowadays, there are several widely used commercial tests which are used to detect the presence of HCV RNA in patient's serum. One of the commercial assays used is the Cobas Amplicor HCV version 2.0 (Roche Molecular Diagnostics, Pleasonton, CA, United States) based on a standard RT-PCR is available for the qualitative measurement of HCV RNA. The lowest detection limit is 50 IU/mL whatever the HCV genotype[51,52]. Another assay commercially used is versant HCV qualitative assay (Siemens Healthcare Diagnostics, Deerfield, IL, United States) which is based on transcription mediated amplification technique. In this assay, first viral RNA is isolated from the patient's serum and then amplified by utilizing two enzymes (reverse transcriptase and T7 RNA polymerase). These amplicons are further detected *via* hybridization protection assay (HPA) in which only hybridized probes remain chemiluminescent and are detected in a luminometer. Analytical sensitivity is 10 IU/mL for most genotypes and 5.3 IU/mL for genotype 1[53].

QUANTITATIVE ASSAY

HCV quantitative assay is used to determine the number of international units of HCV RNA per millimeter of serum or plasma (IU/mL) in known HCV positive patients.

Recently, real time PCR based detection systems have become widely available and are considered as the detection method of choice by many clinicians. The advantages of this technique are that they have a very low limit of detection, have a broad dynamic range. Several companies now market the real time PCR assays: the COBASs Ampliprep/Cobas TaqMan assay (CAP/CTM, Roche Molecular Diagnostics) and the real-time HCV assay (also named AccuGenes HCV, Abbott Molecular Inc., Des Plaines, IL, United States). These assays have the advantage of having a broad dynamic range of amplification, thus improving the limits of detection (LOD) to 10 IU/mL, and linear quantification up to 10^7-10^8 IU/mL[54,55].

The quantitation of HCV viral RNA in Cobas Amplicor is performed using the HCV Quantitation

Standard. The HCV quantitation standard is a non-infectious armoured RNA construct of HCV sequences with identical primer binding sites as the HCV RNA target and a unique probe binding region that allows HCV Quantitation Standard amplicon to be distinguished from HCV target amplicon. The HCV Quantitation Standard is pipetted into each individual sample and control at a known copy number and is and is then amplified by PCR. The COBAS TaqMan HCV Test, v2.0 uses reverse transcription and PCR amplification primers against the highly conserved 5' untranslated region of the HCV genome[56].

The Versant HCV quantitative test (Siemens Healthcare Diagnostics) which is HCV RNA assay based on signal amplification by branched DNA (bDNA). In this assay, single stranded DNA molecules are present; which acts as probe DNA molecules. Next an extender DNA molecule is added. Once the capture and extender molecules are in their proper place they are hybridized and the sample is added. The bDNA assay version 3.0 has been reported to have a lower detection limit of 615 IU/mL to 8 million IU/mL whatever the HCV genotype[57].

The advantage of RT-PCR is that it allows continuous monitoring of amplicon kinetics during the exponential phase before the amplification reaches its plateau. This allows for a good correlation between the initial numbers of template copies whereas in qualitative assays based on PCR, amplicon detection was at the end[56,58]. Thus the use of quantitation techniques have greatly enhanced the sensitivity and reliability in detection techniques.

VIRAL GENOTYPING ASSAYS

There are at least seven genotypes and over 80 subtypes of HCV. Different assays are used to determine genotype such as sequencing and hybridization[2]. Most genotype assays use amplification of specific region of viral genome by PCR followed by direct DNA sequencing. While a variety of techniques are used, the gold standard for HCV genotyping is nucleotide sequencing, which can be done by using core (C), envelope (E1), or the non-structural (NS5B) regions which can be amplified by reverse transcription followed by polymerase chain reaction[59-63]. Most diagnostic assays commonly target the 5' UTR but in research settings core and or NS5B region is usually sequenced as this region is more conserved amongst all genotypes. Genotypes are very useful for determining the duration of treatment regimens and predicting treatment response[64-68].

EMERGING MOLECULES TECHNIQUES

One of the emerging diagnostic assays is nanoparticle based diagnostic assay. Quantum dot and gold based nanoparticle based diagnostic assay[69-71]. Quantum dots are nanoparticles made of semiconductors that emit light at different spectra; the emission is dependent on the size which greatly increases the ability to multiplex[72-74]. Another novel technique being developed recently is the use of aptamers as capture molecules. Aptamers are short, single stranded oligonucleotide that can fold into specific 3-dimensional structures and recognize target molecules such as small chemicals, proteins, and even cells[75]. These techniques have been used for various diagnostic applications because of their ability to bind their targets with high affinity and specificity.

CONCLUSION

Molecular diagnostic testing for HCV has provided a crucial tool for addressing significant controversies in HCV management. NATs for detecting HCV RNA remain the mainstay for detecting HCV infection in individuals in high risk group population. Nucleic acid test not only helps to detect HCV RNA but confirms active state of viral infection, *i.e.*, the virus is in replicating state in the patient's body. However, in developing countries due to financial constraints and lack of technical expertise in clinical settings, these tests are difficult to perform and time consuming. In these settings, the most widely employed screening tests are the HCV rapid immunoassays. However, it is the need of the hour to effectively design strategies to detect HCV infection even in sero-conversion period.

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LETTERS TO THE EDITOR

Ledipasvir and sofosbuvir: Interferon free therapy for hepatitis C virus genotype 1 infection

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Abstract

Hepatitis C virus (HCV) has infected more than 200 million people around the globe. From 2001-2011, interferon plus ribavirin remained the standard of care for patients with HCV infection. The therapy had a limited response with a number of side effects. Recently, results for phase III trials of ledipasvir and sofosbuvir combination therapy have been announced. In treatment naïve patients, 12 wk of therapy with ledipasvir and sofosbuvir showed a sustained virological response (SVR) rate of 99%. In treatment experienced patients, 12-24 wk of therapy with ledipasvir and sofosbuvir in the absence or presence of ribavirin showed an SVR rate of 94%-99%. In cirrhotic patients the rate of SVR was 86% and 99% for 12 and 24 wk of therapy, respectively. The ledipasvir and sofosbuvir therapy showed very good results in different subgroups of patients regardless of patient's race, alanine aminotransferase levels, sex and host genetic factors. The combination therapy was well tolerated with no emergence of resistant mutants. The most common adverse effects were nausea, headache and fatigue. With the availability of interferon free therapy with minimal adverse effects, it will be easy to decrease the future morbidity and mortality caused by HCV infection.

Key words: Hepatitis C; Interferon; Ledipasvir; Sofosbuvir; Genotype

Core tip: The interferon based therapy for hepatitis C patients has a limited response with a number of adverse effects. The ledipasvir and sofosbuvir combination therapy showed a sustained virological response (SVR) rate of 99% in treatment naïve patients. The rate of SVR was 94%-99% in treatment experienced patients, while in cirrhotic patients the rate of SVR was 86%-99%. The treatment response was not affected by ethnicity or host genetic factors.

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TO THE EDITOR

Hepatitis C virus (HCV) infection is a major health problem around the globe, with more than 200 million people infected worldwide. Although the rate of HCV infection is continuously declining, the rates of HCV associated morbidity and mortality are continuously increasing.

From 2001-2011, interferon and ribavirin therapy remained the standard of care for patients living with HCV. The therapy had a limited response with a number of side effects. The major adverse effects associated with interferon administration were flu like symptoms, cytopenia and depression, whereas ribavirin therapy causes fatigue, anemia, rash and pruritus. The major objective of recent treatment regimens is to eliminate the interferon and ribavirin from the treatment regimen so that the adverse effects of therapy can be reduced and the therapy become available for patients who are ineligible for the interferon and ribavirin therapy.

Sofosbuvir is a nucleoside analogue that can inhibit the HCV polymerase, approved by the Food and Drug Administration for the treatment of patients living with HCV. Ledipasvir is an inhibitor of HCV NS5A protein, showing antiviral activity against HCV genotype 1 infection.

In a phase Ⅱ clinical trial, 100 patients with HCV genotype 1 infection who were treatment naïve or previously treated with protease inhibitors were enrolled at a centre in the United States. The patients were given a fixed-dose combination of sofosbuvir (400 mg) and ledipasvir (90 mg). In cohort A, 60 treatment naïve, non-cirrhotic patients who were given sofosbuvir plus ledipasvir (8 wk), sofosbuvir plus ledipasvir along with ribavirin (8 wk), or sofosbuvir plus ledipasvir (12 wk) showed an SVR rate of 95%, 100%, and 95% respectively. In cohort B, 40 previous non-responders to protease therapy were included. They were given sofosbuvir plus ledipasvir (12 wk) or sofosbuvir plus ledispavir along with ribavirin (12 wk), and the sustained virological response (SVR) rate was 95% and 100%, respectively[1]. The sofosbuvir-ledipasvir combination therapy cured most of patients with HCV genotype 1 infection, irrespective of their treatment history. Further investigations were required to optimize the treatment duration and the role of ribavirin in treatment response.

In a phase Ⅲ clinical trial, 865 previously untreated patients were enrolled and they were randomly divided into four groups. Group 1 received ledipasvir and sofosbuvir for 12 wk and showed an SVR rate of 99%. Group 2 received ledipasvir and sofosbuvir along with ribavirin for 12 wk and showed an SVR rate of 97%. Group 3 received ledipasvir and sofosbuvir for 24 wk and showed an SVR rate of 98%. Group 4 received ledipasvir and sofosbuvir along with ribavirin for 24 wk and showed an SVR rate of 99%. The study concluded that the 12 wk therapy with ledipasvir and sofosbuvir was highly effective for patients living with HCV genotype 1 infection. No additional benefit was observed by the addition of ribavirin or by the extension of therapy to 24 wk[2].

In another phase Ⅲ trial, 440 previously treated patients were enrolled, 20% of whom had cirrhosis. The patients were given ledipasvir and sofosbuvir in the presence or absence of ribavirin from 12 or 24 wk. The rate of SVR achieved was 94%-99%. In patients with cirrhosis the rate of SVR was 86% (ledipasvir-sofosbuvir) and 82% (ledipasvir-sofosbuvir plus ribavirin) with 12 wk of treatment, while the rate of SVR was 99% (with both regimens) in patients having 24 wk of treatment. The study concluded that the single tablet of ledipasvir–sofosbuvir showed a better rate of SVR even in the patients who were not responders to the interferon based therapy[3].

The ledipasvir and sofosbuvir therapy produced very good results in different subgroups of patients regardless of patient's race, alanine aminotransferase levels, sex and host genetic factors. The combination therapy was well tolerated. No S282T variant was observed. The most common adverse effects were nausea, headache and fatigue[2-4].

A total of 1952 patients were enrolled in three different phase III trials of ledipasvir and sofosbuvir, out of which 97% showed SVR[2-4]. Out of the remaining 3%, half of them withdrew consent or were lost to follow-up. Undetectable viral RNA was not achieved in only two patients. The rate of relapse was observed in only 2% after stopping therapy. The rate of relapse was also linked with the treatment duration. The rate of relapse was observed in 5%, 2% and 0.2% of patients who received 8 wk, 12 wk and 24 wk of treatment, respectively[5].

With the availability of oral, short duration, interferon free therapy with minimal adverse effects, the future morbidity and mortality associated with HCV infection will decrease. The major problem with the therapy is its cost. The cost of 12 wk therapy with sofosbuvir alone is $84000 and the addition of ledispavir will further increase the cost[5]. The high cost of the therapy will affect the goal of providing safe and effective treatment for millions of patients living with HCV around the globe.

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EDITORIAL

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 (HBsAgs) are present on HDV virions. The envelopment process of HDV occurs through interactions between the HDV ribonucleoprotein (RNP) complex and HBV HBsAgs. 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

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 (HBsAgs) 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 HBsAgs (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 HBsAgs 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-HBAgs for viral

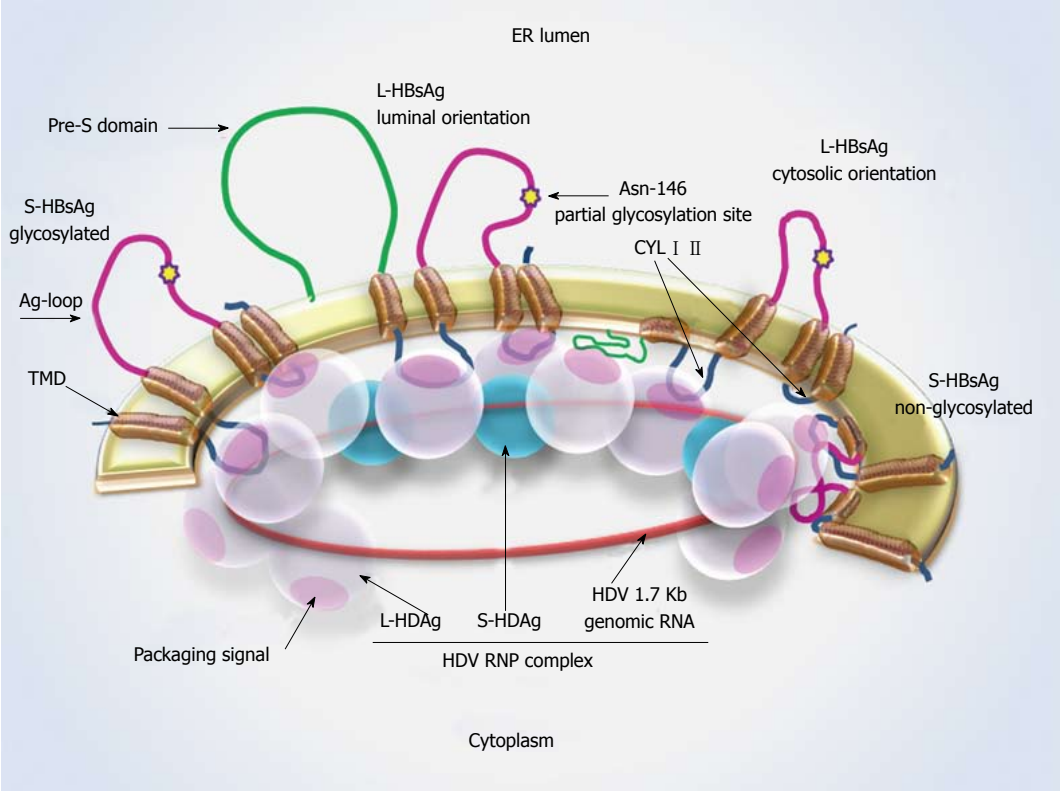

Figure 1 Hepatitis D virus-ribonucleoprotein complex interaction with S-hepatitis B virus surface antigen. Schematic representation of L- and S-HBsAg locations in the ER membrane and the interaction sites with the HDV-RNP complex. The Asn-146 glycosylation site is highlighted by a star. About half of the residues remain non-glycosylated at this site. A cytosolic orientation has been suggested for the non-glycosylated Ag loop, which may interfere with HBsAg-HDAg interaction. L-HDAg mediates HDV assembly at the late stage of viral replication through forming connections with HDV-RNA, S-HDAg and HBsAg. Ag loop: Antigenic loop; CYL I, II: Cytosolic loop I, II; ER: Endoplasmic reticulum; L-/ S-HBsAg: Large and small hepatitis B virus surface antigens; L-/ S-HDAg: Large and small hepatitis D virus proteins; RNP: Ribonucleoprotein; TMD: Transmembrane domain.

assembly, HDV needs S-HBsAg for its *in vitro* virion packaging and L-HBsAg for infectivity[18,19]. Therefore, it is very likely that most of the HDAg binding sites are located on the S domain of HBsAg[20]. While an intact HBsAg is not able to interact with L-HDAg, a denatured form of HBsAg is competent for such interactions suggesting that L-HDAg has no connection to the external domains of HBsAg but rather to the domains inside the particles[21]. The cytoplasmic orientation of the CYLs of the S protein, provides a reasonable condition for these sites to interact with pre-mature HDV virions in the cytosol (Figure 1)[22].

The importance of S-HBsAg residues 24 to 28 and 56 to 80 for HDV secretion has been shown in previous studies[23,24]. Also, a C-terminal truncation of HBsAg by 50 amino acids inhibits HDV envelopment and secretion[25]. Based on these data and also from our recent observation indicating a high rate of amino acid selection at CYLs of S-HBsAg in HBV isolates from HBV/HDV infected patients (own unpublished observations), these domains are expected to make a significant contribution in HDV packaging. Of special importance are tryptophan residues at positions 196, 199 and 201 at the C-terminal domain of S-HBsAg, which are suggested to have a central localization in binding interface with HDV-RNP complex[26].

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 deferent 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-alpha-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-κB activation, which results from ER stress (induced by HBsAgs) or TNFα 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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REVIEW

New advances on glial activation in health and disease

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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, 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

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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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 end-feet 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 Ca^{2+} 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 filapodia 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 capabilitie[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-beta 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 stres[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-κB	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-kappaB: Nuclear factor kappa-light-chain enhancer of activated B cells; AD: Alzheimer's disease; HIV: Human immunedeficiency 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, IFNβ, TNF-α, TGF-β, GM-CSF, BAFF, IL-1β, 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, IFNβ, 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 (Aβ) peptides activate astrocytes, which increase production of inflammatory mediators[148]. Furthermore, astrocytes are able to remove and degrade Aβ, 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-inflam matory 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 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-1β 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 Aβ 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 Aβ 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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REVIEW

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

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.

da Cunha J, Maselli LMF, Stern ACB, Spada C, Bydlowski SP. Impact of antiretroviral therapy on lipid metabolism of human immunodeficiency virus-infected patients: Old and new drugs. *World J Virol* 2015; 4(2): 56-77 Available from: URL: http://www.wjgnet.com/2220-3249/full/v4/i2/56.htm DOI: http://dx.doi.org/10.5501/wjv.v4.i2.56

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 regimes 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)
	Didanozine (ddl)	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)
Fusion inhibitors	Enfuvirtide, T-20	Fuzeon® Hoffmann La Roche (2003)
Integrase strand transfer inhibitors	Dolutegravir (DTG)	Tivicay® GlaxoSmithKline (2013)
	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 (IFNγ) originating from lymphocytes and macrophages. IFNγ levels are elevated at early stages of infection and are also correlated with the presence of hypertriglyceridemia[41,42]. Tumor necrosis factor-α (TNFα) is another potent pro-inflammatory mediator whose concentrations increase in HIV-1 infected ART-naïve patients. TNFα 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

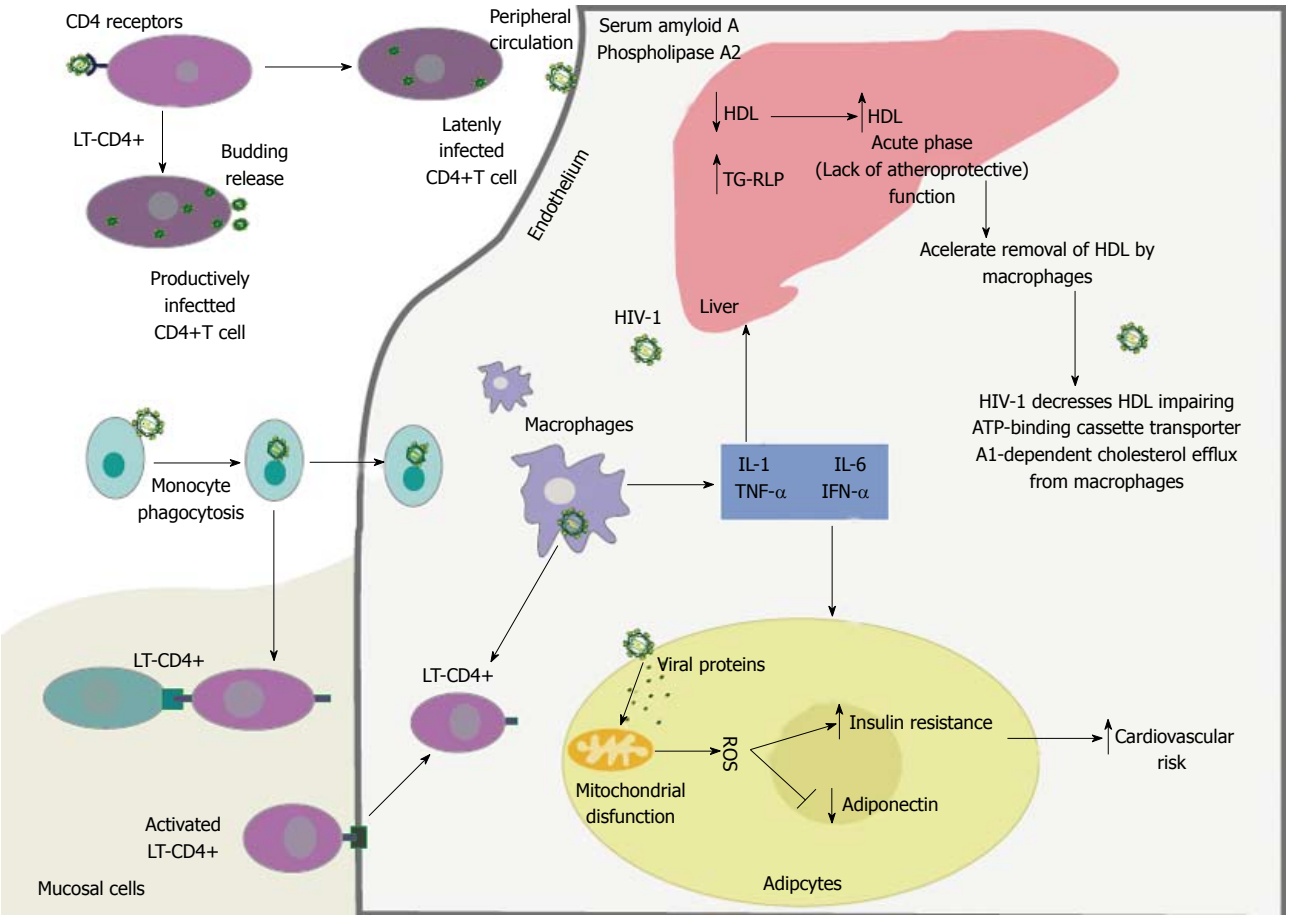

Figure 1 The human immunodeficiency virus type 1, upon entering peripheral circulation, will infect lymphocytes and macrophages. The viral proteins gp120 and gp41 of HIV-1 bind to the CD4+ receptor and coreceptors, C-C chemokine receptor type 5 and C-X-C chemokine receptor type 4, on the surface of these 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*[211]. 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; TNFα: 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-PPARγ) 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-PPARγ 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
	Didanozine (ddl)	↑↑ 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
Fusion inhibitors	Enfuvirtide, T-20	Neutral effect	No effect
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-PPARγ. The decrease in RXR-PPARγ 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

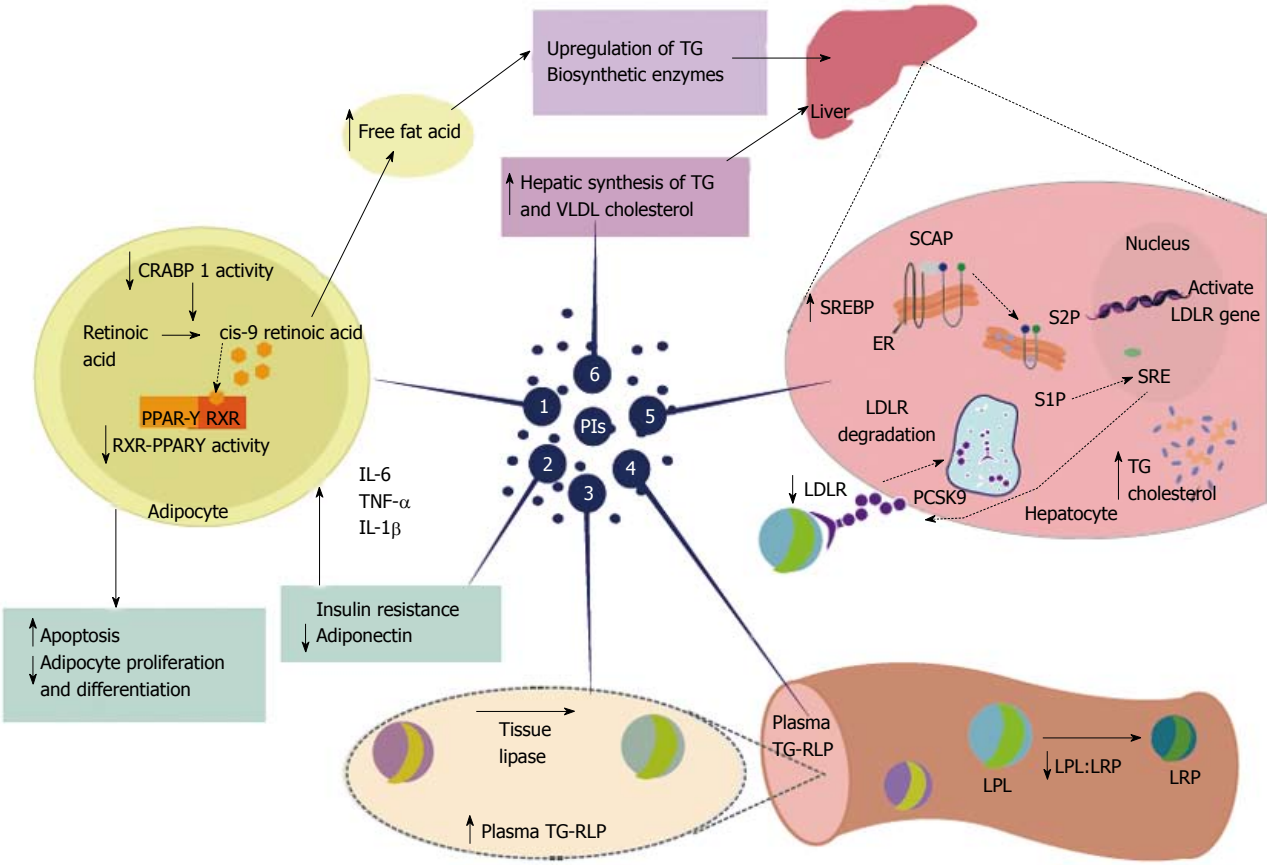

Figure 2 Highly active antiretroviral therapy-associated dyslipidemia is especially evident with the use of protease inhibitors. Protease inhibitors (PIs) promote a decrease in plasma high-density lipoprotein cholesterol and increased overall cholesterol, triglycerides(TG), and low-density lipoprotein 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*[211]. 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-1β: Interleukin 1β; 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-PPARγ: 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 (apoAI) 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 zidovudineto 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, sulfonylureas, 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 lipoatrophy 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 lipoatrophy 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 lipoatrophy. 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 CYPA4 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 acety lhydrolase, 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

Festinavir (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 (EC_{50}) 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 festinavir 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. Albuvirtide (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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REVIEW

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

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 olignucleotides. Prevention strategies such as surveillance, physical prevention, and vaccine development form the second part of the review.

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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 iden tified 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 treat ment, 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	*in vitro*/*in vivo*	Inhibit viral absorption/penetration	No obvious side effects	Weak oral activity	[21-30]
Flavonoids	*in vitro*	Inhibit viral RNA/protein synthesis	Low escape mutants	Mechanism not clear	[18,31–35]
Alkaloids	*in vitro*/*in vivo*	Inhibit protein synthesis	No obvious side effects	Mechanism not clear	[36-38]
Deferoxamine	*in vitro*/*in vivo*	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 administrated 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-hydro xyisoflavone[34], chrysin and its phosphate esther[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	*In vivo*	Prevents attachment by binding to viral capsid	High oral availability	Varied capacity of inhibition	[47-49]
BPROZ	*In vitro*	Prevents attachment by binding to viral capsid	High oral availability	Resistant mutants	[49-54]
Soluble and anti-SCARB2/PSGL-1	*In vitro*	Prevents attachment	N/A	N/A	[55-57]
Lactoferrin	*In vitro / in vivo*	Prevents entry by binding to VP1/ cellular receptor	No obvious side effects (animal)	Mechanism not clear	[58-62]
Suramin	*In vitro*	Prevents attachment	May inhibit other multiple stages of HEV71 life cycle	Mechanism not clear	[63]
Peptides (SP40)	*In vitro*	Prevents attachment by binding to glycosaminoglycans	Small size, high activity/specificity, low toxicity	Low bioavailability	[64-66]
Post-infection					
Rupintrivir	*In vitro /in vivo*	Inhibits viral 3C protein	Low quantity, low toxicity, high barrier for drug resistace	Lack efficacy in natural infection	[67,68]
DTrip-22	*In vitro*	Inhibits viral 3D polymerase activity	Broad spectrum activity	N/A	[69]
Aurintricarboxylic acid	*In vitro*	Inhibits viral 3D polymerase activity	N/A	N/A	[76-81]
NITD008	*In vitro / in vivo*	Inhibits viral 3D polymerase activity	More potent than ribavirin in vivo	May have toxicity issue, resistant mutants	[82,83]
Sorafenib	*In vitro*	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 infec tion[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) E_2 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 *Pischia 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 αS1-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	*In vitro / in vivo*	Broad spectrum, low cost	Incomplete attenuation	[143-149]
Inactivated vaccine	*In vitro / in vivo / clinical trial*	Inability to replicate	High cost	[150-162]
Subunit vaccine	*In vitro / in vivo*	Safe to use	Low immunogenicity	[153,154,163-178]
Synthetic peptides	*In vitro / in vivo*	Small and safe to use	Low immunogenicity, escape mutants	[94,179-181]
Virus-like particles	*In vitro / in vivo*	Safe to use	Unstable, need purification, high cost	[162,182-185]
DNA vaccine	*In vitro / in vivo*	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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REVIEW

Viral hepatitis and human immunodeficiency virus co-infections in Asia

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Abstract

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

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 *et al*[15], 2012
	15.3	IDU	B3	Utsumi *et al*[6], 2013
	7.0	Men with IDU	B3, D1, B2, C1	Fibriani *et al*[16], 2014
Myanmar	8.7	Homosexual men		Zaw SK *et al*[17], 2013
Thailand	13.0	HB vaccination women		Aurpibul *et al*[18], 2012
	3.3			Peters *et al*[19], 2013
	11.9			Tsuchiya *et al*[20], 2013
Vietnam	28.0	IDU	B4, B2, C1,	Dunford *et al*[21], 2012
	15.2	CSW	C5	
	10.3			Sereno *et al*[22], 2012
East Asia				
China	4.9	Older children ethnicity		Zhou *et al*[23], 2010
	6.3	Sexual		He *et al*[24], 2011
	7.2	Sexual		Maimaiti[25], 2012
	6.1			Chen *et al*[26], 2013
Japan	8.8	Homosexual men		Gatanaga *et al*[27], 2007
	6.4	Homosexual men		Koike *et al*[28], 2008
	7.9	Homosexual men	Ae	Fujisaki *et al*[29], 2011
	6.0	Homosexual men		Yanagimoto *et al*[30], 2012
South Asia				
India	9.0	Hetero sexual		Saravanan *et al*[31], 2007
	11.3	Hetero sexual men		Saha *et al*[32], 2013
	1.5		D > A > C	Saravanan *et al*[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 prognoses 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 (*e.g.*, TDF, 3TC)	TDF-based regimen	Combination of TDF with FTC or 3TC	HBeAg 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*

[1]Lamibudin 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 mono-infection[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/mm^3, 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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MINIREVIEWS

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

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 trails (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/μL against 42.0%-48.0% of those with CD4 > 100 cells/μL 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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MINIREVIEWS

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

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Abstract

Dengue is an arboviruses due to single-stranded 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

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 *Stegomya*).

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 filed 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 (*Aedes aegypti* and *Aedes albopictus*)	Mosquitoes (genus *Culex* but also Aedes albopictus)	Mosquitoes (genus *Culex*)	Ticks (genus *Ixodes*)	Mosquitoes (*Aedes aegypti*, *Aedes albopictus*)	Ticks (*Dermacentor andersoni*)
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 fist 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

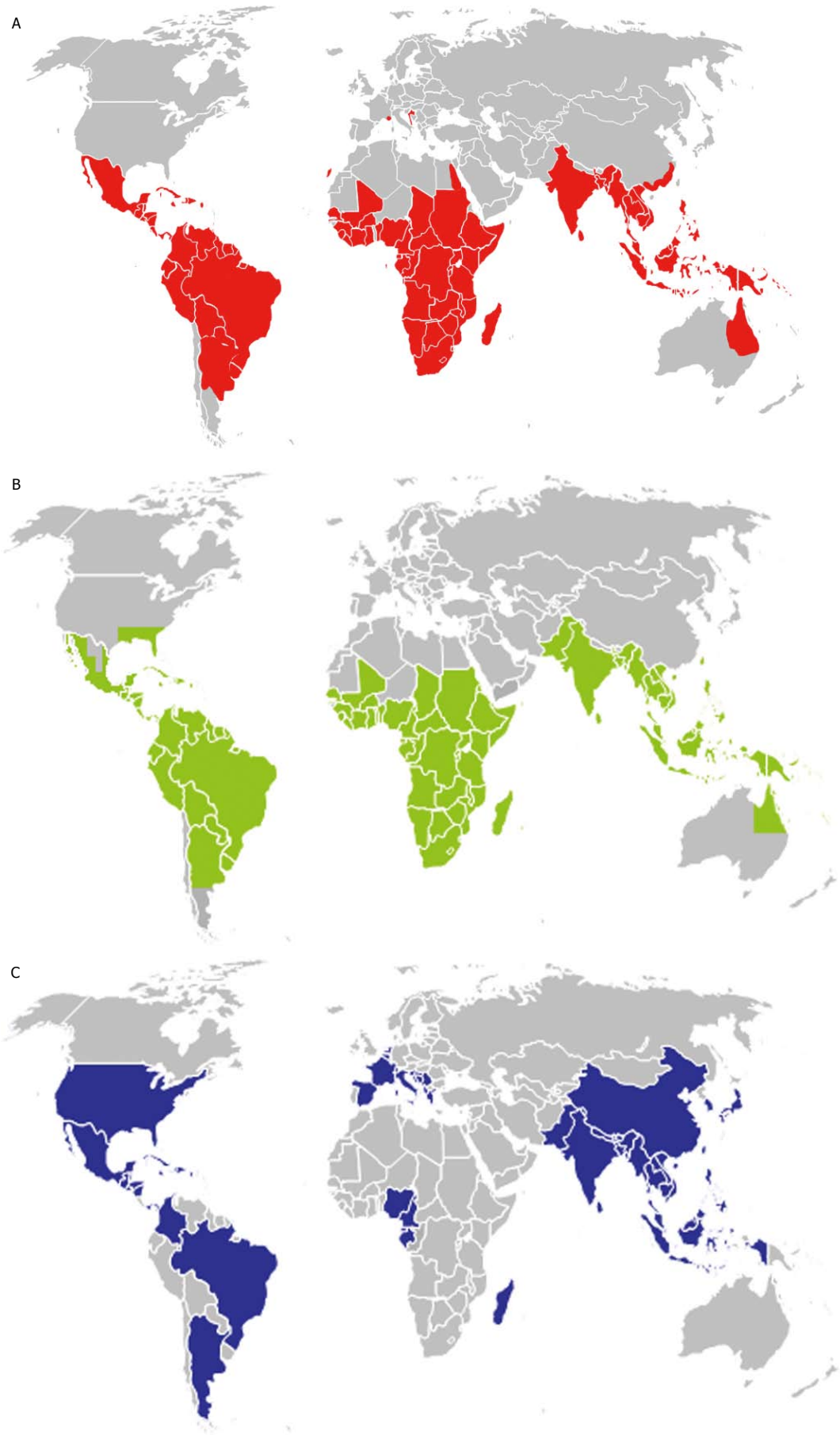

Figure 1 Overall distribution of dengue cases (endemic or epidemic) worldwide (A) and perimeter of expansion of the two main vectors of dengue viruses, *Aedes aegypti* (B) and *Aedes albopictus* (C).

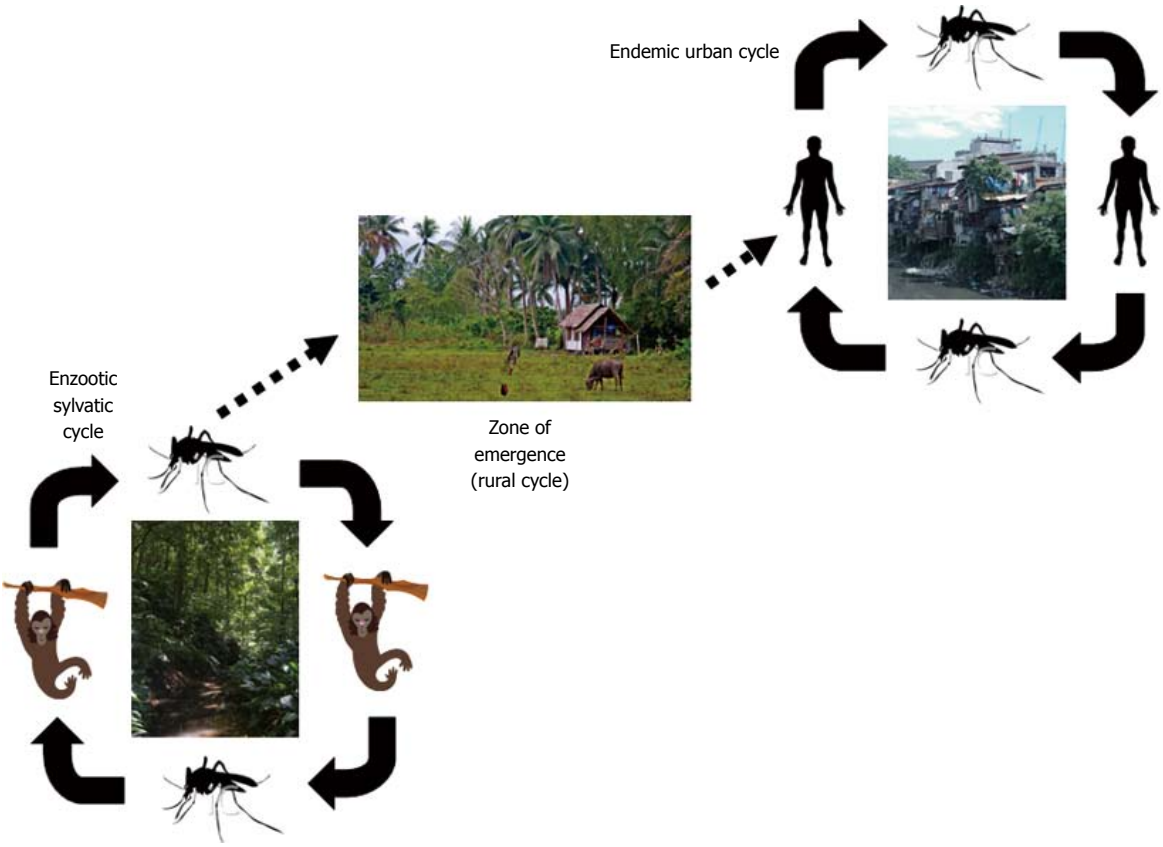

Figure 2 Simplified representation of the sylvatic and urban/peri-urban cycles of dengue that models the natural spread of dengue viruses through bites by infected mosquitoes.

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 consists 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.

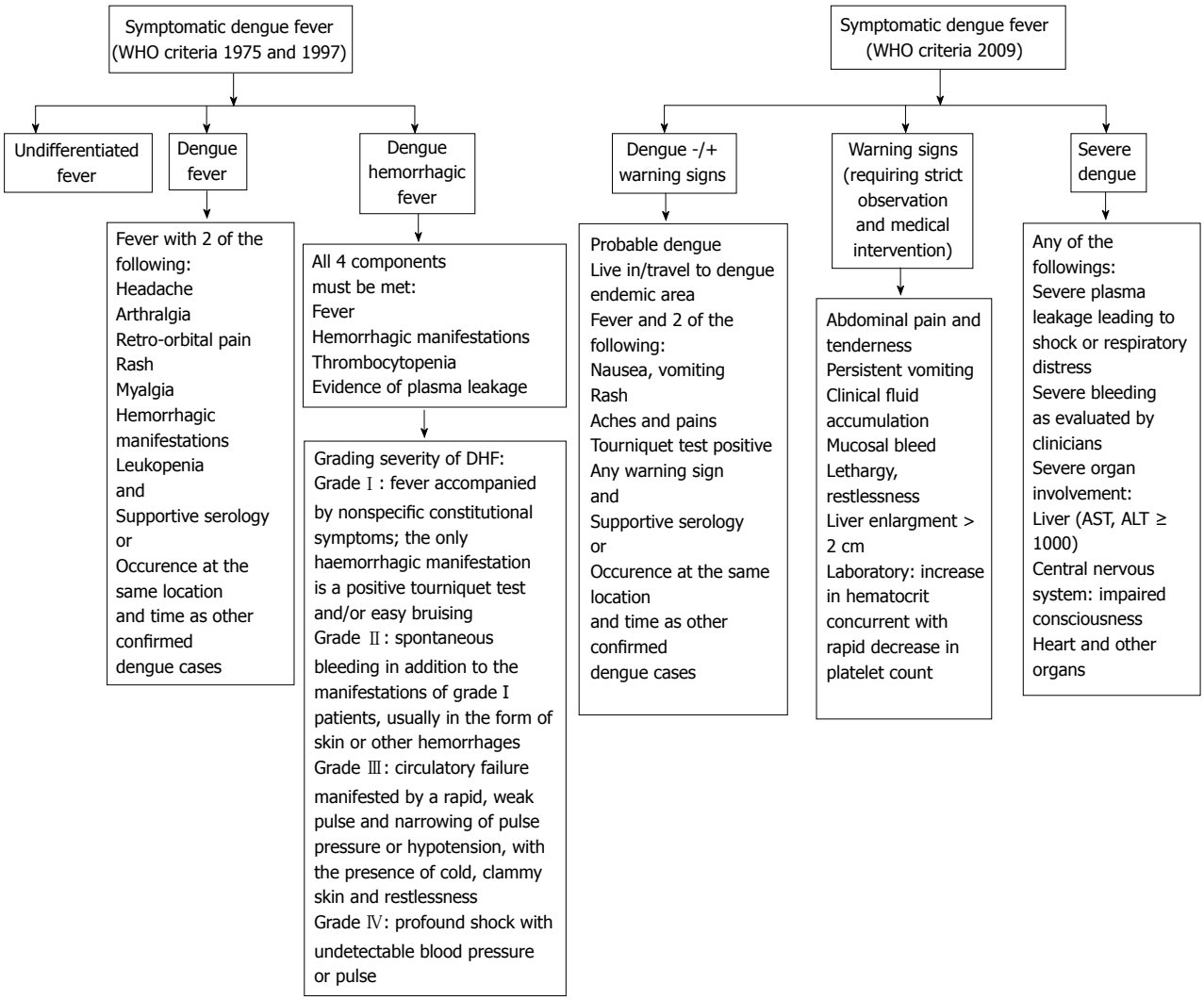

Figure 3 Successive classifications of dengue clinical presentations according to the World Health Organisation definitions. WHO: World Health Organisation. AST: Aspartate transaminase; ALT: Alanine transaminase.

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 ant 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 10^8 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.*, bacterical 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 arbovirosis, 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 describer 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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MINIREVIEWS

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

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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 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

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.

Grifoni A, Montesano C, Colizzi V, Amicosante M. Key role of human leukocyte antigen in modulating human immunodeficiency virus progression: An overview of the possible applications. *World J Virol* 2015; 4(2): 124-133 Available from: URL: http://www.wjgnet.com/2220-3249/full/v4/i2/124.htm DOI: http://dx.doi.org/10.5501/wjv.v4.i2.124

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/mm^3) 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

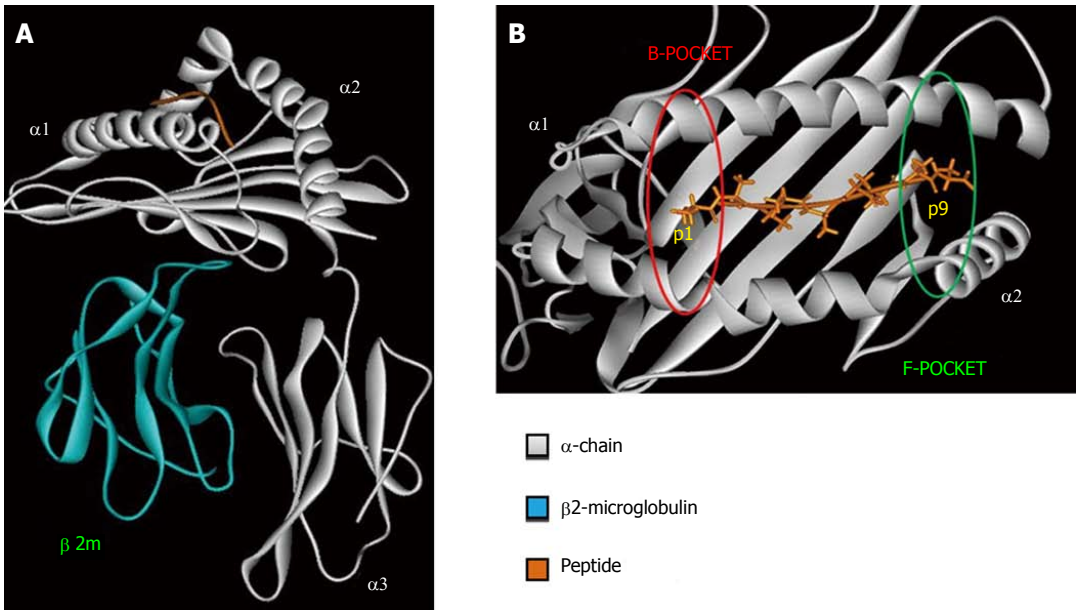

Figure 1 Human leukocyte antigen class I tridimensional structure. Crystal structure of the HLA-B*57:03 (PDB ID: 2YPK); HLA α-chain in gray, β2-microglobulin in blue, the peptide in orange. A: HLA class I overall structure; B: HLA class I peptide binding pocket. In red is shown the HLA pocket B, in green 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.

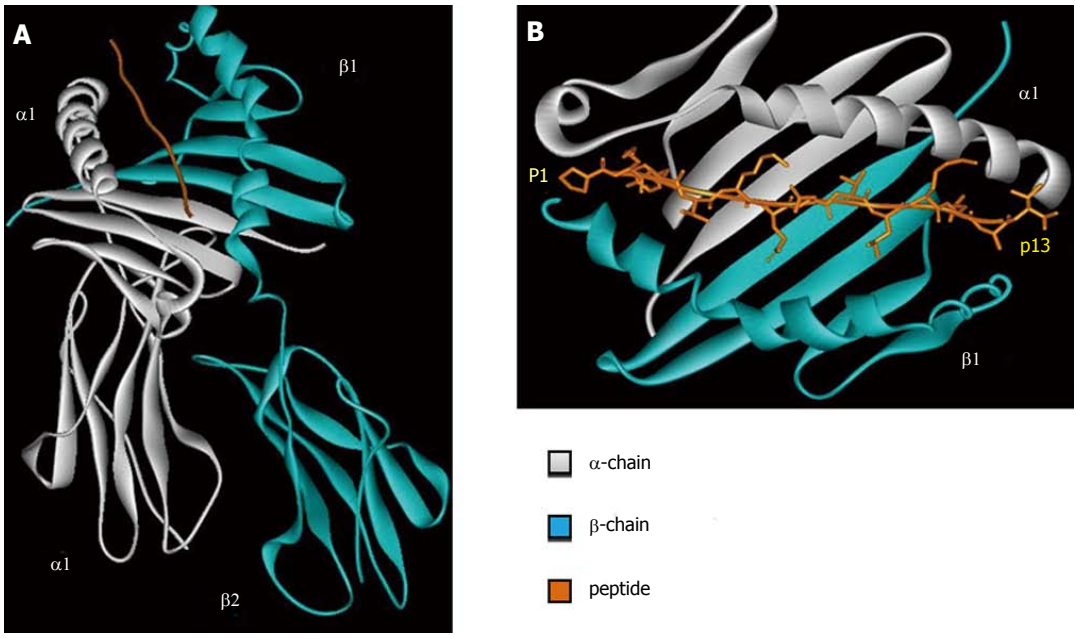

Figure 2 Human leukocyte antigen class II tridimensional structure. Crystal structure of the HLA-DR1 (PDB ID: 1DLH) in gray HLA α-chain, in blue HLA β-chain, in orange the peptide. A: HLA class II overall structure; B HLA class II peptide binding pocket. The figure has been made using WebLab Viewer Pro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

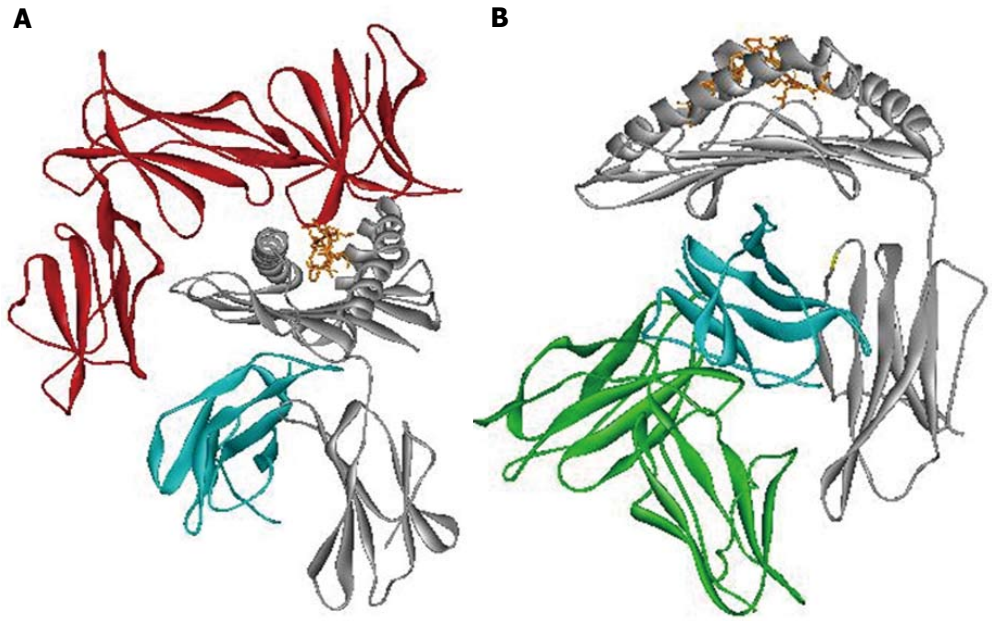

Figure 3 Human leukocyte antigen class I interaction with innate receptor. Crystal structure models of the HLA-B*57:01 interacting respectively with (A): KIR3DL1 receptor (red) (PDB ID: 3HV8); (B): LILRB1 receptor (green). HLA α-chain in gray, β2-microglobulin in blue, the peptide in orange. The figure has been made using WebLab Viewer Pro 3.7. HLA: Human leukocyte antigen.

strong contribution of Bw4 homozygosis in delaying HIV progression[46,65]. These results are in agreement with previous associations between Bw4 homozygosis 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

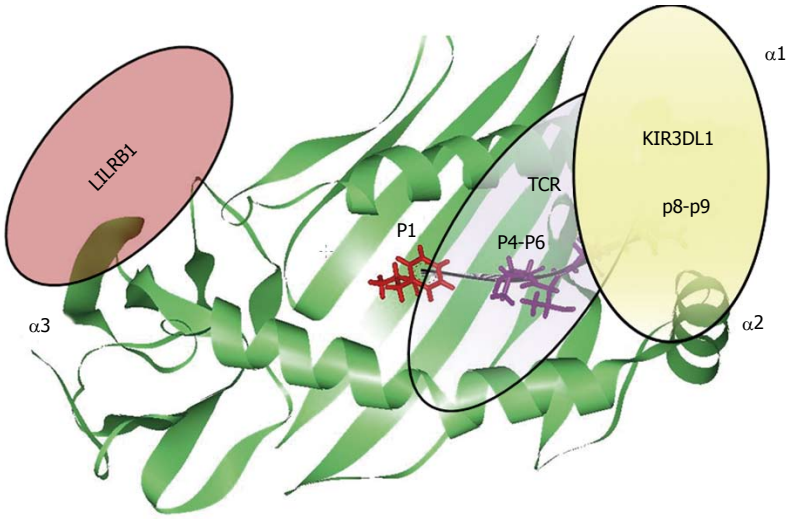

Figure 4 Peptide recognition. Schematic representation of the contribution of each HLA bound peptide position in the modulation of the interaction between HLA-B molecules (green) with TCR (violet) and KIR3DL1 (yellow), respectively. The figure has been made using WebLab Viewer Pro 3.7. HLA: Human leukocyte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

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 $\leqslant$ 500 cells/mm^3. 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

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/mm^3, 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/mm^3[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/mm^3[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/mm^3 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/mm^3 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)[36,56]	FSWs (*n* = 520)	0.19%	Cross-sectional	Isolated: 1.92%	Isolated: 6.54% (range: 3% to 17.5%)
	PWIDs	7.1% (1% to 18.4%)	Cross-sectional	Isolated: 40.3% Co-infection: 94.9%	Isolated: 7.1%
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)
(2012)[66]	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%

[1]Sample size; [2]Lab 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 distri bution, 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 counties. 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

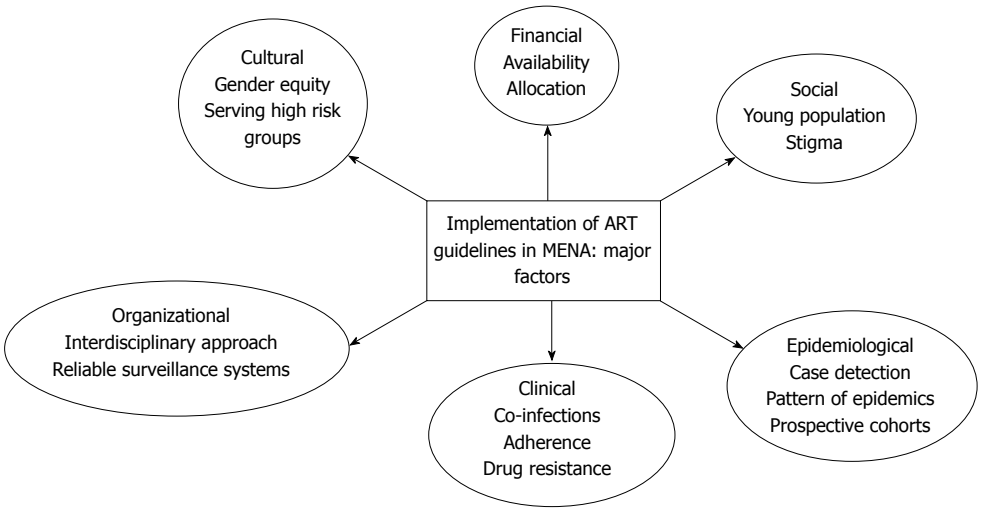

Figure 1 Major challenge for development of human immunodeficiency virus treatment guidelines in Middle East and North Africa. MENA: Middle East and North Africa; ART: Antiretroviral treatment.

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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MINIREVIEWS

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

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 TECHONOLOGIES 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

[1]Per TVOR; [2]Per 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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META-ANALYSIS

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

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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 μmol/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 μmol/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

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 metionine 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

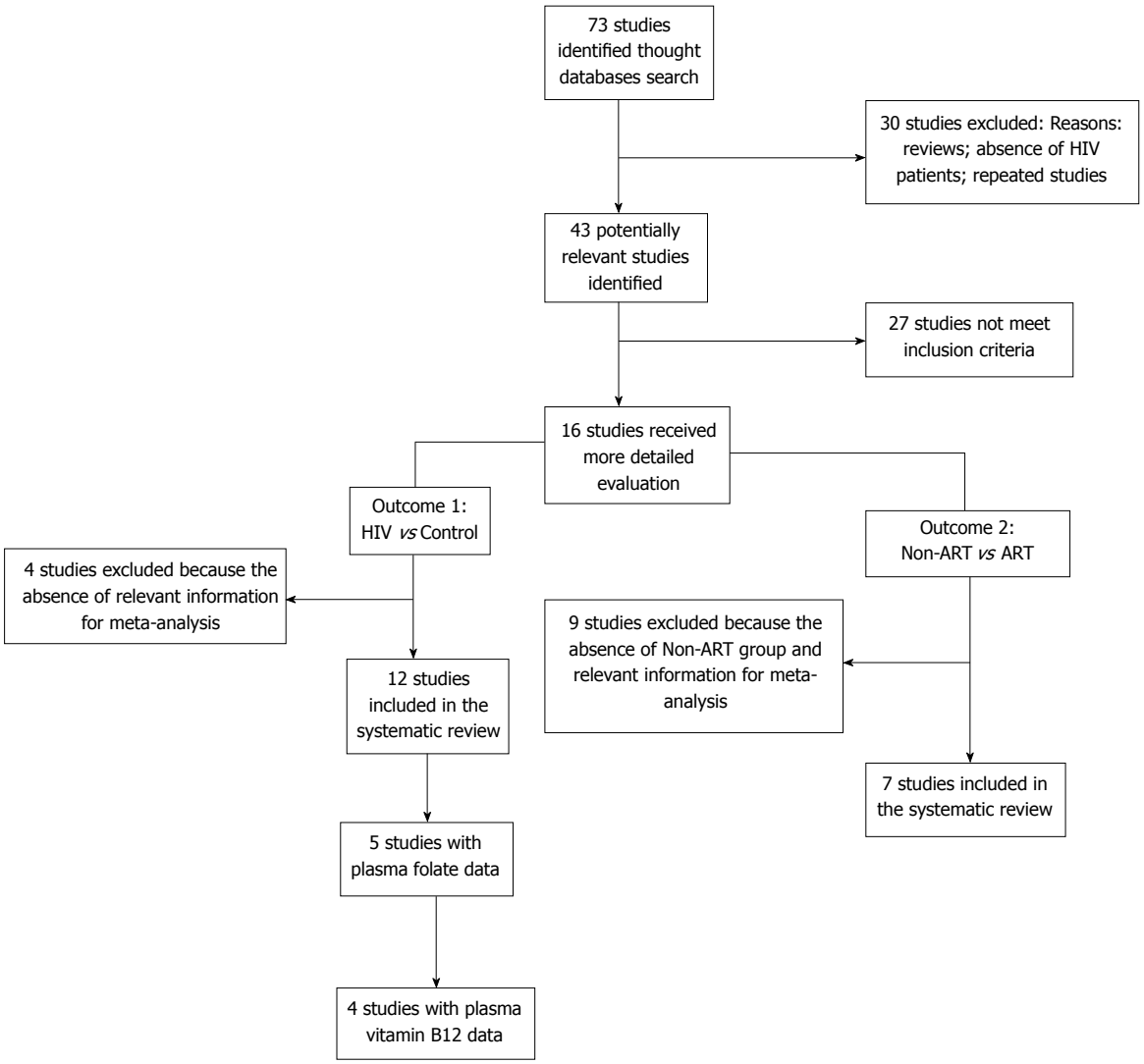


Figure 1 PRISMA flow diagram of the study selection process. After careful discussion between the 3 reviewers, two outcomes were identified and included in the meta-analysis. HIV: Human immunodeficiency virus; ART: Antiretroviral therapy.

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 access 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 (μmol/L)		HHcy	Folate (ng/mL)/Vitamin B12 (pg/mL)	
				Control	HIV		Control	HIV
Castagna *et al*[29]	Italy	20/14	CS and CC of some patients	10.8 ± 3.8	6.0 ± 2.2	-	-	-
Muller *et al*[30]	Norway	15/21	CS	9.2 ± 7.3	9.0 ± 5.0	-	-	-
Naisbitt *et al*[31]	England	33/33	CS	11.9 ± 4.7	14.5 ± 5.6	-	-	-
Bernasconi *et al*[32]	Swaziland	80/73	COS	7.6 ± 3.6	8.7 ± 4.1	5% control; 12.3% HIV	-	-
Vilaseca *et al*[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 ± 7.5/ B12 455 ± 160[1]	Folate 12.6 ± 6.7/ B12 481 ± 181[1]
de Larrañaga *et al*[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 *et al*[23]	Spain	128/235	CS	7.5 ± 7.8	14.3 ± 12.9	6.2% control;	-	B12 368.6 ± 219
Raiszadeh *et al*[35]	United States	127/249	COS	7.2 ± 2.7	7.4 ± 2.7	13.4% control; 16.90%	-	-
Vigano *et al*[36]	Italy	19/23	CS	9.0 ± 5.0	11.0 ± 8.0	-	Folate 6.9 ± 1.7	Folate 4.7 ± 1.5
Abdollahi and Shoar[37]	Iran	58/58	CC	12.6 ± 1.1	27.1 ± 10.2	91.4% HIV	-	-
Borges-Santos *et al*[38]	Brazil	20/12	CC	13.9 ± 5.5	9.8 ± 1.6	-	Folate 7.5 (6.3-9.0)[2]/B12 288 ± 130	Folate 1.9 (1.4-6.6)[2]/B12 367 ± 139
Deminice *et al*[39]	Brazil	10/23	CS	6.6 ± 1.5	9.4 ± 2.7	0% controls; 30.4% HIV	Folate 11.7 ± 3.4/ B12 713.1 ± 110.1	Folate 7.0 ± 2/B12 514.6 ± 99.3

[1]Folate and vitamin B12 determined in 56 controls and 69 HIV-infected patients only. Values in Hcy, folate and vitamin B12 are expressed as mean ± DP; [2]Values expressed as range (25th-75th percentiles). CC: Case-control; CS: Cross-sectional; COS: Cohort study; Hcy: Homocysteine; HHcy: Hyperhomocysteinenemia; 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 μmol/L higher than the plasma Hcy levels in uninfected controls (95%CI: 0.13-4.01, $P < 0.01$; Figure 2).

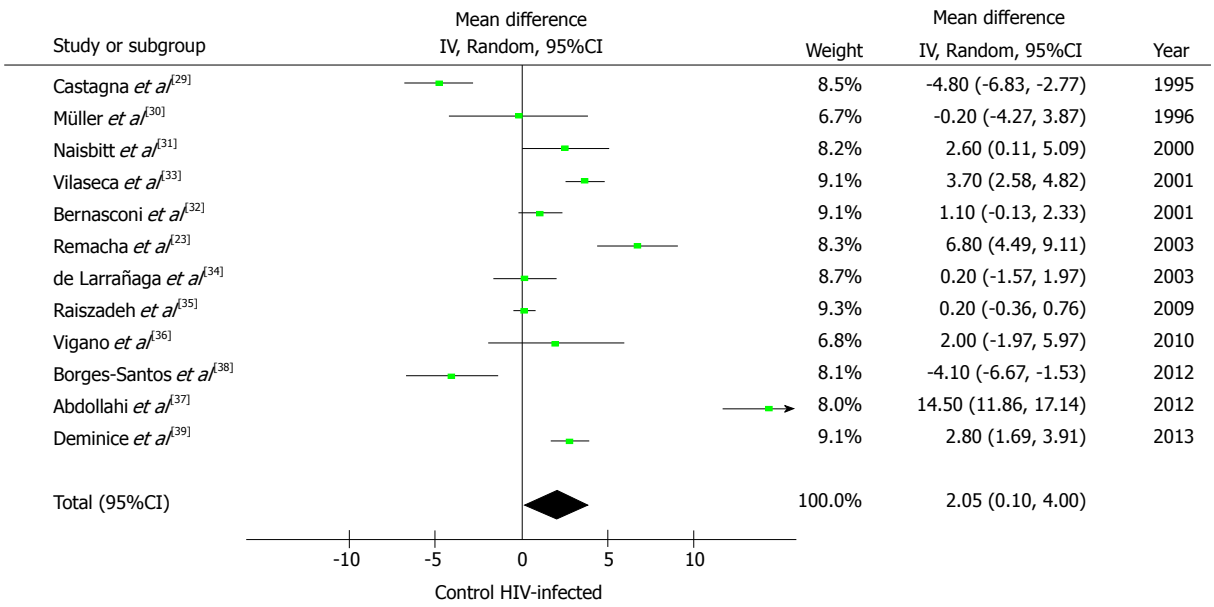
Subgroup analyses on folate and vitamin B12 plasma concentrations between test groups (Figure 3) demonstrated plasma folate levels were -2.74 ng/mL decreased in HIV-infected patients compared to uninfected controls (95%CI: -5.18 - -0.29, $P < 0.01$), but no significant difference in the B12 concentration 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 μmol/L; 95%CI: 1.34-6.92, $P < 0.01$; Figure 4).

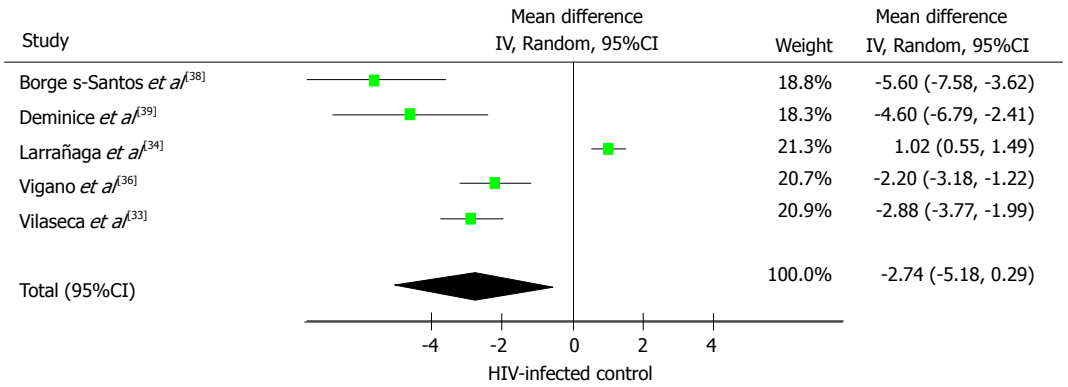
DISCUSSION

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

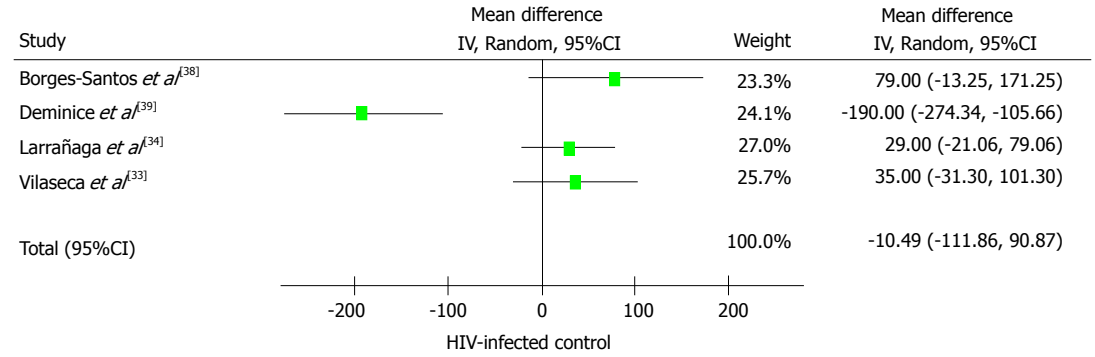


Heterogeneity: Tau^2 = 10.50; Chi^2 = 212.29, df = 11 (P < 0.00001); I^2 = 95%
Test for overall effect: Z = 2.06(P =0.04)

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 (μmol/L) and 95%CI. HIV: Human immunodeficiency virus.



Heterogeneity: Tau^2 = 7.25; χ^2 = 117.99. df = 4 (P < 0.00001); I^2 = 97%
Test for overall effect: Z = 2.19 (P = 0.03)



Heterogeneity: Tau2 = 9267.92; χ^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 (μmol/L)		HHcy	ART
				ART	Non-ART		
Mercié *et al*[42]	France	78/304	COS	12.5 ± 4.8	11.5 ± 4.3	-	-
de Larrañaga *et al*[34]	Argentina	31/128	CS	9.7 ± 7.1	8.0 ± 6.1	-	-
Remacha *et al*[23]	Spain	128/235	CS	17.3 ± 13	8.2 ± 7.8	-	Patients under ART (taking > 3 antiretroviral drugs)
Martí-Carvajal *et al*[41]	Venezuela	14/40	CS	10 ± 7.5	9.2 ± 6.7	48.6% non-ART; 45.5% ART	-
Currier *et al*[42]	United States	40/41	COS	9.6 ± 5	8.0 ± 6.1	-	Patients on ART including a PI continuously for 2 yr
Raiszadeh *et al*[35]	United States	127/249	COS	7.5 ± 2.8	7.3 ± 2.4	-	-
Coria-Ramirez *et al*[43]	Mexico	69/69	CC	24.8 ± 14.6	7.9 ± 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: Hyperhomocysteinenemia; PI: Protease inhibitors; ART: Antiretroviral therapy.

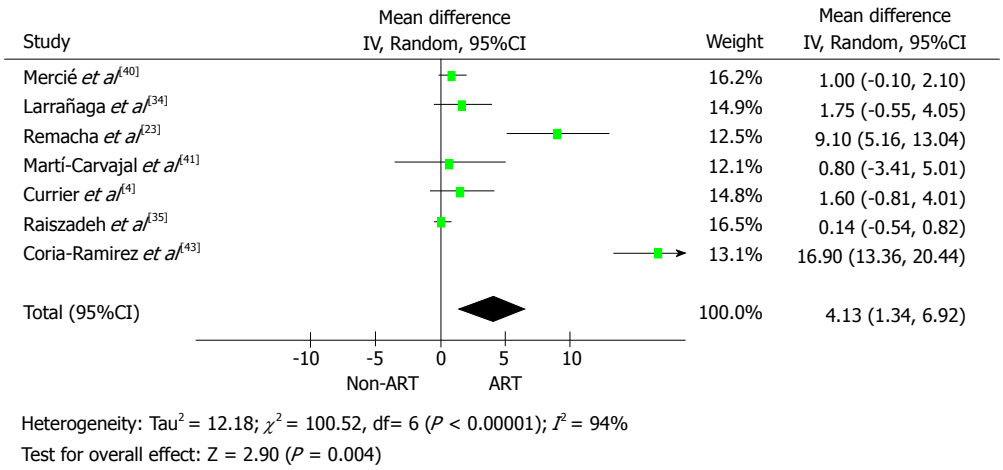


Figure 4 Meta-analysis of blood homocysteine concentration in human immunodeficiency virus-infected exposed and non-exposed to antiretroviral therapy. Calculation based on random effects model. Results are expressed as weighted mean difference of homocysteine (μmol/L) and 95%CI. ART: Antiretroviral therapy.

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 μmol/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 μmol/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 μmol/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 μmol/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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WJV World Journal of Virology

Contents

Quarterly Volume 4 Number 3 August 12, 2015

EDITORIAL

REVIEW

MINIREVIEWS

SYSTEMATIC REVIEWS

World Journal of Virology
Volume 4 Number 3 August 12, 2015

Contents

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EDITORIAL

Novel antigen delivery systems

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Abstract

Vaccines represent the most relevant contribution of immunology to human health. However, despite the remarkable success achieved in the past years, many vaccines are still missing in order to fight important human pathologies and to prevent emerging and re-emerging diseases. For these pathogens the known strategies for making vaccines have been unsuccessful and thus, new avenues should be investigated to overcome the failure of clinical trials and other important issues including safety concerns related to live vaccines or viral vectors, the weak immunogenicity of subunit vaccines and side effects associated with the use of adjuvants. A major hurdle of developing successful and effective vaccines is to design antigen delivery systems in such a way that optimizes antigen presentation and induces broad protective immune responses. Recent advances in vector delivery technologies, immunology, vaccinology and system biology, have led to a deeper understanding of the molecular and cellular mechanisms by which vaccines should stimulate both arms of the adaptive immune responses, offering new strategies of vaccinations. This review is an update of current strategies with respect to live attenuated and inactivated vaccines, DNA vaccines, viral vectors, lipid-based carrier systems such as liposomes and virosomes as well as polymeric nanoparticle vaccines and virus-like particles. In addition, this article will describe our work on a versatile and immunogenic delivery system which we have studied in the past decade and which is derived from a non-pathogenic prokaryotic organism: the "E2 scaffold" of the pyruvate dehydrogenase complex from *Geobacillus stearothermophilus*.

Key words: Vaccines; Antigen display; Delivery systems; E2 scaffold; Immune response

Core tip: Several promising strategies of vaccination have been proposed over the past years to treat and/or prevent infectious and cancer diseases. These include live attenuated or inactivated viral vaccines, recombinant viral vectors, DNA vaccines, subunit vaccines, nanoparticle carriers, and lipid-based delivery systems such as liposomes and virosomes. Although some of these suffer from certain limitations (*e.g.*, safety concerns, weak immunogenicity, adverse side-effects associated with adjuvants), recent advances in vaccine technology have

provided further insights for guiding vaccine design. Here, we review the current status of antigen delivery systems with emphasis on a versatile and immunogenic vaccine delivery candidate: the "E2 scaffold".

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LIVE ATTENUATED AND INACTIVATED, RECOMBINANT SUBUNIT VACCINES

Currently, the majority of vaccines licensed for human uses include live-attenuated and inactivated or killed vaccines[1]. They came from disease-causing viruses or bacteria manipulated *in vitro* to reduce or attenuate the pathogenicity, without altering the antigenic properties. Vaccines are manufactured using several different methods[2]. They may contain live microorganisms attenuated by repeated passages in cell-culture or animal embryos; inactivated (viral) or killed (bacterial) microorganisms that have lost the ability to replicate by physical, chemical or radiation treatments; inactivated toxins and conjugated subunits[3] (Table 1). Live attenuated vaccines currently available on the market include those against measles, mumps, rubella, varicella, influenza, rotavirus, and smallpox. Most of them are formulated as dry solids. Commercially available killed or inactivated vaccines, toxoids and subunit vaccines include several products, most of them being formulated in liquid dosage forms to treat other diseases, *e.g.,* rabies, meningitis, diphtheria, tetanus, poliomyelitis, *Haemophilus influenzae* type b, pertussis and hepatitis B. These vaccines are able of eliciting both humoral and cell-mediated immune responses[4]; however, some safety, stability, and efficacy concerns must be considered when developing these vaccines. Live attenuated vaccine can eventually mutate into a more virulent form capable of causing diseases[5], whereas inactivated or killed vaccines and protein subunit vaccines generally generate weak immune responses often requiring the use of adjuvants[6]. Many live attenuated vaccines are capable of eliciting virus-specific T cell and B cell responses and long-term immunity by mimicking the natural infection, and therefore they usually do not require the use of adjuvants. However, for some viruses vaccines have been very difficult to develop, due to the absence of tissue culture systems that allow for efficient propagation and production in a scalable setting. They tend to be more difficult and expensive to store and to distribute, since viability must be maintained, often requiring formulation approaches for stabilization[7]. On the other hand, killed/inactivated vaccines have a number of disadvantages. The major challenge is that since cells are never infected with the live microbe, these vaccines are generally not effective at eliciting a full adaptive immune response. They do not give rise to pathogen-specific cytotoxic T cells, thus often requiring multiple booster shoots and co-administration with adjuvants to increase antigenicity and to create long-term immunity, with subsequent local reactions at the vaccine site. However, for the absence of living pathogens these types of vaccines are usually safe compared to live attenuated vaccines.

Overall, these technologies have allowed to achieve the successes of vaccinology in the last century and to produce the vaccine formulations available on the market. However, many new vaccines are needed and for them new strategies have to be found[8]. In this context, the development of novel delivery technologies aimed to design safer and more effective vaccines is a relevant topic.

DNA VACCINES

DNA vaccines have emerged as a safer alternative to live and inactivated vaccines for treating human and animal infections, allergy, autoimmune disorders and cancer diseases[9]. They exhibit several advantages over traditional strategies in terms of safety, stability, ease of manufacturing, and immunogenicity (Table 1). As DNA-based plasmid vaccines are non-live, non-replicating, non-spreading vaccines, there is a little or no risk of mutation or reversion to the virulent form as with viral vectors, therefore raising fewer safety concerns. They are easy to manufacture and to manipulate compared with live attenuated vaccines, and the DNA product is highly stable and easily stored, without requiring refrigeration procedures. DNA vaccines can activate innate immunity and both arms of the adaptive immune response without inducing anti-vector antibodies unlike viral vector particles, thus being theoretically suitable for repeated booster shots. Furthermore, recent innovations in plasmid host strain and vector engineering increased plasmid manufacturing quality and yield, transgene expression levels, transfection efficiency, for a safer and more effective gene platform compared to first generation vectors[10,11]. Essentially, plasmid DNA vaccines consist of purified vectors that combine an eukaryotic region - which includes a strong enhancer/promoter for the expression of transgene coding for antigenic/therapeutic proteins or peptides in mammalian cells and the transcript termination/polyadenylation (poly A) sequence for mRNA transcript stabilization - with a prokaryotic region that provides selection and propagation in host bacteria. Although the exact mechanism by which DNA vaccines work still remains unclear, recent advances have provided a deeper understanding of the molecular and immunological mechanisms of action of these vectors[12-14]. Generally, once the DNA plasmid is administered *via* intradermal, intravenous, intraperitoneal, subcutaneous, nasal or intramuscular route, the plasmid is internalized into the host cells (myocytes and antigen-presenting cells), it translocates to the cellular nucleus where the host cellular machinery initiates the transgene transcription followed by the cytoplasmic translation of the transgene into protein. Plasmid-encoded proteins may be processed in transfected

Table 1 Overview of the different vaccine formulations

Vaccine type	Description	Advantages	Disadvantages	Immunogenicity	Examples
Live attenuated vaccines	Living weakened microbes that generally show reduced pathogenicity	Induce a protective immune response by activating both B and T cell responses; induce long-term immunity; do not require adjuvants; unable to spread and cause infection	They can revert towards virulent forms or can be insufficiently attenuated for immunosuppressed individuals with risk of infection; difficult to produce in a scalable setting; heat-labile; quality and safety requirements	Humoral and cytotoxic immune responses	Smallpox; yellow fever; rabies; measles; mumps; rubella; typhoid; influenza; rotavirus; varicella
Killed/inactivated vaccines	Bacteria (killed vaccines) or viruses (inactivated vaccines) inactivated by chemical or physical treatments	Due to the absence of living pathogens they do not revert towards virulent forms and can be used in immunodeficient hosts; not heat-labile	Repeated booster shots and adjuvants (with subsequent local reactions at the vaccine site) are required to optimally trigger the adaptive immune system and generate long-term immunity; do not give rise to cytotoxic T cells; poor induction of mucosal immunity; difficult to produce in a scalable setting; quality and safety requirements	Humoral immunity	Diphtheria; tetanus; pertussis; haemophilus influenzae type b; poliomyelitis; rabies; meningitidis; Japanese encephalitis; cholera; hepatitis A; hepatitis B
Toxoids vaccines	Purified exotoxins chemically inactivated into toxoids that retain the ability to induce toxin-neutralizing antibodies	Safe and stable. There is no possibility of reversion to pathogenicity or spread of live microbe to other animals	Poorly immunogenic; need adjuvants and large amounts or multiple doses to ensure efficient activation of the adaptive immune response and generation of long-last immunity; local reactions at vaccine site	B cell activation (T cell dependent)	Diphtheria, tetanus, and pertussis toxoids; acellular pertussis vaccines; anthrax secreted proteins
Subunit/ polysaccharide vaccines	Antigenic components of pathogens: partly or fully purified protein antigens or capsular polysaccharides	Can be chemically linked to protein carrier	Variable degree of immunogenicity; need adjuvants (and often multiple doses); frequent local reactions at the injection site	T-dependent and/ or T-independent immune responses	Hepatitis B and Haemophilus influenzae type b; influenza; meningococcus, pneumococcus, and Haemophilus influenzae type B polysaccharides
Plasmid DNA	Genetically engineered vectors expressing antigens of interest	Inability to revert to pathogenic forms; activation of innate and adaptive immune responses; highly stable; easy storage and transport; large-scale production; optimization of plasmids and transcript is possible	Not-useful for non-protein immunogens; lower immunogenicity in human compared to mice; low transfection efficiency	Activation of antigen-specific B cells, CD4+ and CD8+ T cells	Infectious haematopoietic necrosis virus; West Nile virus; melanoma; growth hormone releasing hormone
Vectored vaccines	Live recombinant viral and bacterial vectors expressing heterologous antigens	Ability to induce specific humoral and cellular immune responses; high transduction efficiency; highly effective in dividing and non-dividing cells; production of high levels of antigens inside target cells; sustained gene expression; vector itself can provide an adjuvant effect	High expense; toxic side effects; limits on transgene size; potential for insertional mutagenesis; anti-vector immunity; difficult to manufacture and store	B cell, CD4+ and cytotoxic CD8+ T cell activation	Adenovirus; adeno-associated virus; retrovirus; lentivirus; Herpes simplex virus; *Salmonella*
Nanoparticles	Nano-scale size materials made of polymers, proteins or lipids used as carrier systems (*e.g.*, PLGA, liposomes, virosomes, Virus-like particles)	Ability to induce humoral and cellular immune responses; increased antigen uptake, processing and presentation; controlled/sustained release of vaccine target; depot effect; targeted delivery; adjuvanticity; high encapsulation; improved cargo bioavailability; transport efficiency; enhanced permeability; biodegradability and biocompatibility	Challenges in vaccine formulation, production, stabilization. Immunotoxicity can occur	B-cell, CD4+ and cytotoxic T-cell responses	Hepatitis A virus; influenza; human papilloma virus; hepatitis B virus; hepatitis E virus

somatic cells *via* the TAP-dependent, endogenous pathway for the presentation on MHC class I molecules, whereas soluble/secreted plasmid product may simultaneously gain access to the major histocompatibility complex (MHC) class II exogenous pathway in phagocytic cells, for the activation of B cells, CD4+ and CD8+ T lymphocytes[15]. Many reports emphasized on the ability of DNA vaccines to induce immune responses against a variety of infectious agents and cancers in preclinical animal models and more recently in clinical trials[16,17]. Until now, four animal DNA products have been licensed for veterinary uses, demonstrating the well tolerated and safety profile of DNA vaccination. Although there are no US/FDA approved DNA vaccine for human uses, several DNA delivery strategies have been developed and improved in order to increase DNA vaccine performance, including the use of adjuvant plasmids expressing immunostimulatory molecules, such as costimulatory molecules, signaling proteins, cytokine, and chemokines[18]. In addition, the use of mixed vaccines in prime-boost immunization strategies or in simultaneous delivery approaches resulted in an improved immunogenicity in several preclinical models against different pathogens such as HIV-1[19]. Genetically engineered DNA can be administered by different methods following different routes, including physical approaches and viral and non-viral delivery systems[20]. However, so far in human application the efficiency of DNA vaccination has not been so encouraging[21].

GENE DELIVERY SYSTEMS: RECOMBINANT VIRAL AND BACTERIAL VECTORS

A huge amount of delivery systems based on recombinant viruses have emerged recently and have been widely employed as highly evolved natural vehicles for gene therapy and for vaccine purposes[22]. Viral-based delivery systems consist of genetically engineered replication-defective viruses carrying a therapeutic gene expression cassette cloned into the viral backbone (Table 1). Viral vaccine vector systems, such as adenovirus (type 2 and 5), adeno-associated virus, retrovirus, lentivirus, poxvirus, alphavirus, herpes simplex virus (HSV), offer several potential advantages over traditional vaccines, even though each of them show some limitations and side effects[23,24]. Viral vectors can produce high levels of antigens directly within the host cells; they can efficiently deliver antigens to specific subsets of immune cells [such as antigen-presenting cells (APCs)] and potentially act as adjuvant. They can be administered in different combination with other vaccines resulting in enhanced immune responses. However, some issues must be taken into consideration when using viral vectors for vaccination, including potential integration, transcriptional activation of oncogenes, pre-existing immunity against the viral vector, and limitations in transgenic capacity size. Several recombinant viral vectors, both RNA and DNA viruses, have been used and widely investigated as vaccines being able to express the antigenic/therapeutic protein *in vivo* and to stimulate potent specific humoral and cellular immune responses[25]. RNA viral vectors, such as retrovirus and lentivirus, allow long-term expression of the transgene, while DNA viral vectors allow expression in episomal form. Viral vectors based on adenovirus, adeno-associated virus, retrovirus, lentivirus and HSV represent those currently used in clinical trials, with adenovirus being the most commonly used, whereas others are under development[26].

More recently, vaccine based on alphavirus vector has been considered a particular attractive option. All alphavirus vectors take advantage of extremely efficient RNA replication resulting in almost 200000 RNA copies from each RNA template[27].

Although replication-deficient particles provide a high level of safety, there is still a marginal risk of the generation of replication-proficient particles through non-homologous recombination. To minimize this risk, split helper vector systems with capsid and envelope genes expressed from separate vectors have been produced[28]. Furthermore, the potential of alphavirus causing epidemics has raised additional concern. Regarding efficiency, recent alphavirus-based vaccines have been subjected to clinical trials. Disappointingly, no clinical benefit was found, indicating that these types of vaccines require further optimization.

In addition to viral vectors, recombinant bacterial carriers, derived from lactic acid bacteria, *Salmonella* and *L. monocytogenes* strains, have been used extensively as delivery systems being able to stimulate both systemic and mucosal immune responses[29,30].

NANOPARTICLE DELIVERY SYSTEMS

Nanoparticle delivery systems offer several advantages over traditional vaccines. Due to their physicochemical characteristics - nanoparticle size, surface charge, biomaterials composition, hydrophobicity/hydrophilicity - and immunostimulatory properties, nanoparticles-based formulations have extensively been investigated as vaccine and drug delivery systems, adjuvants, nucleic acid delivery platforms, and nanocarriers for imaging approaches[31-34]. Nanoparticle systems can be designed to optimally present antigens in their native conformations to the immune system in controlled, slow release formulations promoting their targeting to specific immune populations with attachment of targeting moiety. They can be engineered to improve antigenicity of the delivered antigens and thus acting as adjuvants. Moreover, by co-delivering antigen and adjuvant to the same antigen presenting cells, these nanocarriers can enhance immunogenicity of vaccines. The antigen multimeric display on the surface of some nanoparticle systems allows cross-linking of the B cell receptor, leading to an enhanced antibody response. Moreover, some of these nanoparticles can be designed for promoting the cytosolic delivery of antigens, enhancing cross-presentation *via* MHC-I pathway and thus leading to cytotoxic T-cell responses. In addition to increased antigen uptake, processing and presentation, nanocarriers also

offer the opportunity to encapsulate or entrap a variety of compounds, preventing their degradation, improving their solubility and half-life, providing site-specific targeting and a sustained release of compounds. Most of nanocarriers are biodegradable, biocompatible for different routes of administration (parenteral and non-parenteral administrations), exhibits low toxicity and stability, and they are able to induce strong humoral and cellular immune responses without anti-vector immunity[35-37].

Nanoparticle delivery systems comprise a wide variety of nano-scale size materials (< 1 μm) including solid particulate delivery systems and emulsion delivery systems. Solid nanoparticles include synthetic or biodegradable polymers (nanospheres and nanocapsules) - such as poly(lactic-co-glycolic acid) (PLGA), chitosan, hydrogel capsules, poly (phosphazenes), polyanhydrides, poly(alkylcyanoacrylate) (PACA) and poly(methyl methacrylate) (PMMA) nanoparticles - solid lipid nanoparticles (SLNs), liposomal delivery systems, virosomes, immune stimulating complexes (ISCOMs), virus-like particles (VLPs), non-degradable nanoparticles, colloidal iron-based preparations and many others, while emulsions include heterogenous liquid systems suitable for the entrapment of hydrophobic drugs, such as nanoemulsions and nanoliposomes (details in[31-33,35]). Some formulations have proceeded to clinical trials and are commercially available, whereas many others are under preclinical development[31].

POLYMERIC NANOPARTICLES

Polymer-based nanoparticle delivery systems (polymeric nanoparticles, polymeric micelles, dendrimers) have emerged as promising and innovative candidates to diagnose, monitor, treat, and prevent infectious, inflammatory and cancer diseases due to their excellent features - including biocompatibility and biodegradability, enhanced permeability, stability, low toxicity, improved cargo bioavailability, controlled/sustained release of vaccine targets, depot effect, high encapsulation and transport efficiency, targeted delivery[38]. Polymeric nanoparticles (NPs) consist of polymeric colloidal nanoparticles prepared from biodegradable and biocompatible, natural or synthetic polymers, ranging in sizes from 10 nm to 1 μm. A wide variety of diagnostic and therapeutic compounds (such as hydrophilic and hydrophobic drugs, proteins, peptides, nucleic acids, biological macromolecules) can be entrapped or encapsulated within the polymeric matrix with good efficacy, protecting them from enzymatic degradation and thus improving their bioavailability, or adsorbed or chemically conjugated on their surface for antigen and targeted delivery. NPs can be made from many different polymer types including natural or synthetic polymers such as poly-d,l-lactide-co-glycolide (PLGA), polylactic acid (PLA), poly-ε-caprolactone (PCL), chitosan, gelatin, poly-alkyl-cyano-acrylates (PAC), gamma polyglutamic acid (γ-PGA), hyaluronan [or hyaluronic acid (HA)][34,35,39]. However, the most commonly studied polymers for parenteral and mucosal drug and antigen delivery are biodegradable and biocompatible synthetic polymers - such as PLGA and PLA - since they provide biological compatibility with less toxicity[40]. According to the structural organization, biodegradable nanoparticles are usually distinguished in nanospheres, where molecules are homogenously dispersed, adsorbed or dissolved within the polymeric matrix, and nanocapsules, where a polymeric wall surround a vesicular core containing the agent of interest. Several methods have been developed to produce structurally stable optimized NPs, including encapsulation and adsorption of drugs, proteins, and nucleic acids[39,40]. NPs can be prepared by polymerization of monomers following emulsion-based methods or by dispersion of polymers following nanoprecipitation (solvent displacement), salting out, or solvent evaporation methods[39,40]. A huge amount of preclinical studies have emphasized the utility of PLGA/PLA-based nanoparticles as drug and antigen delivery systems. It has been reported that PLGA/PLA-based nanocarriers, carrying immunostimulatory molecules and/or vaccine antigens, confer antigenicity and immunogenicity to a large variety of antigens, being able to increase antigen-specific humoral and cellular immune responses[40]. In addition, PLGA-based nanoparticles are able to specifically delivery vaccine compounds to antigen-presenting cells such as dendritic cells, enhancing cross-presentation and thus promoting CTL responses[41]. PLGA nanoparticles are frequently used for encapsulating and successfully delivering a variety of anticancer drugs (reviewed in[39]). Problems of stability, cytotoxicity and conservation may represent constrains that require further optimized formulations[42].

LIPID-BASED ANTIGEN DELIVERY SYSTEMS: THE LIPOSOME FAMILY

Liposomal carrier systems

Liposomes and liposomal-based delivery systems represent a promising technology to deliver a variety of compounds to target sites. Various kinds of lipid vesicles belong to the liposome family, including LPD (liposomes-protamine-DNA complexes), polymerized targeted-liposomes, PEGylated liposomes, archaeosomes, ISCOMs (immune stimulating complex), virosomes, niosomes and many other, which are classified according to their structures, composition, and preparation[43]. Essentially, they are spherical, uni- or multi-lamellar, nano or micro-sized vesicles composed of a phospholipid bilayer capable of encapsulating or incorporating bioactive molecules. Hydrophilic water-soluble compounds can be entrapped within the aqueous hollow cavity, whereas hydrophobic molecules can be intercalated into or attached on the phospholipid bilayer. Several methods of liposome preparation techniques including manufacturing process and process controls have been developed, although all the methods share a common general procedure[43]. Liposome formulations with optimized properties - such as high stability, long blood circulation half-life (GM glycolipid

or PEG polymer-coated liposomes), enhanced target efficiency and activity (immunoliposomes), controllable and prolonged release properties, low toxicity, improved adjuvant and immunostimulatory properties - can be achieved by modulating the lipid membrane composition (neutral, anionic, and cationic lipid species), the liposome size, the net charge and the hydrophilicity of the liposomal surface, and/or by encapsulating additional adjuvants ("conventional" and second-generation liposomes, the stealth technology[44-46]). Since liposomes were first described in 1960, these nanoparticulate carriers were investigated for various purposes - including industrial, pharmaceutical, clinical and therapeutic applications (from vaccination to cancer treatment, gene therapy with cationic liposomes, and diagnostic imaging), due to their adjuvant activity, immunostimulatory properties, safety, biodegradability, and tolerability, following intramuscular, subcutaneous, oral, or intravenous administrations[44,46]. Many reports emphasized on the utility of liposomes as adjuvanted vaccine candidates and drug delivery systems, due to their ability to induce specific immune responses toward the encapsulated or surface-attached antigen, and to treat various diseases, including cancers, infectious, and auto-immunity (reviewed in[46]). Currently, several liposomal formulations are commercially available and clinically approved[44-46].

Virosomes as vaccine and delivery system

In 1975, using preformed liposomes, Almeida *et al*[47] first generated lipid vesicles (named virosomes) containing the envelope proteins, Hemagglutinin and Neuraminidase, purified from influenza virus. Essentially, virosomes are lipid-based semi-synthetic complexes (approximately 150-200 nm in diameter) comprising of functional viral envelope glycoproteins protruding from the surface of a phospholipid bilayer membrane. These lipid vesicles closely mimic the native viral envelope but are devoid of the nucleocapsid including the viral genome of the parenteral virus they are derived from, thus they are not able to replicate. Functionally reconstituted glycoproteins retain the receptor binding property and the pH-dependent membrane fusion activities of the native viral proteins. These functional characteristics have been exploited in the design of vaccine adjuvant and carrier system to deliver molecules[48-51]. After the first description of influenza virosomes, different envelope glycoproteins have been reconstituted to produce virosomes with full biological fusion activity, through detergent solubilization and detergent removal procedures[48,51,52]. Several methods have been described to manufacture virosomes, including antigen loading, and DNA-binding to cationic-virosomes for gene delivery. Essentially, these procedures rely on the use of lipids (egg-derived, purified viral membrane lipids: first-generation virosomes or synthetic phospholipids: second-generation vaccines), envelope proteins (plant-expressed or purified from the inactivated parental virus), and heterologous compounds (details in[51]). A variety of compounds, including antigens, nucleic acids, drug molecules, cancer chemotherapeutic agents, tumor-associated antigen, antibody (targeted-virosomes), can be encapsulated within the aqueous lumen of virosomes, and adsorbed or cross-linked to their surface[53]. Virosomes are qualified for administration *via* different routes (intramuscular, intradermal, intranasal, vaginal routes); they ensure a rapid uptake of the delivered molecule by immune cells (APCs and B cells), for MHC class I and class II presentation. Heterologous antigens exposed on the surface primarily evoke humoral immune responses, while the encapsulation approach give rise to CTL responses; thus, virosomes activate both arms of the adaptive immune response[48]. In addition, due to the presence of the antigenic viral glycoproteins, virosomes can be used as vaccine adjuvant and carrier system to induce immune responses against the viral envelope and the unrelated antigen, being suitable for prophylactic and therapeutic immunizations[46,54]. First-generation virosomes and virosomal adjuvanted formulations are currently applied in commercial vaccines (Hepatitis A vaccines: Epaxal and Epaxal junior; Influenza vaccines: Inflexal V and FluAd). Moreover, several promising virosome vaccine candidates (Malaria, HCV, breast cancer, HIV, Candida vaccines) are currently in preclinical and in clinical development[51].

VIRUS-LIKE PARTICLE DELIVERY TECHNOLOGY

Virus like particles (VLPs), also called pseudovirions, are composed of one or more viral structural proteins (capsid and/or envelope proteins) that retain the ability to self-assemble into multimeric structures (or subviral particles) when expressed *in vitro* using recombinant protein expression systems - including plant, yeast, bacteria, viral vectors, insect cells (baculovirus technology), and mammalian cells[55-57]. They form highly organized monomeric or oligomeric structures with a well-defined geometry (usually icosahedral or rod-like) and diameter ranging approximately from 20 to 120 nm, closely mimicking the native virus but unable to replicate since they lack the infectious viral genome. Thus, VLP-based vaccines offer a safer and more appealing alternative to live, attenuated and inactivated vaccination strategies. Intrinsic characteristics of VLP - such as the particulate nature and the size, the highly ordered and repetitive structure, the charge surface - coupled with immunogenic properties and adjuvanticity, make them particularly attractive as vaccine candidates, targeted drug carriers and antigen delivery systems for prophylactic and therapeutic applications: from vaccination against viral, bacterial, parasitic and fungal infections to gene therapy, immunotherapy against a variety of chronic diseases, including allergies, neurodegenerative and autoimmune disorders, cancers (VLPs targeting self-antigens)[55,57]. Particulate delivery systems similar in size and geometry to pathogens, such as VLPs, are efficiently uptaken by professional antigen-presenting cells for both MHC class

I and II presentation; they efficiently reach lymphoid organs where they can directly interact with immune cells. Most importantly, the highly repetitive surface structures (PAMPs) can induce maturation of antigen-presenting cells (DCs, B cells) by triggering TLRs and cross-linking B cell receptors. These properties increases the ability of VLPs to stimulate strong B and T cell-mediated immune responses[58]. Subviral particles, genetically engineered plant viruses, insect-derived virus-like particles, are suitable as presentation scaffold and adjuvant platform for multimeric display of foreign antigens in a correct, ordered and highly repetitive three-dimensional configuration, to optimally present B and T-cell epitopes and activate immune cells. Antigenic determinants (continuous or conformational immunological epitopes) can be incorporated into adequate permissive insertion sites at high density per particle by genetic fusion (chimeric VLPs) or by *in vitro* chemical conjugation (conjugated VLPs), without compromising the correct folding of VLPs, leading to optimized formulations[59]. Currently, several VLP-based vaccine candidates for human diseases are under clinical development including those directed against Influenza A virus, Norwalk virus, Ebola and Marburg viruses, Hepatitis C virus, HIV and Malaria. To date, VLP-based vaccines for human papilloma virus (HPV), hepatitis B virus (HBV), and hepatitis E virus (HEV) have already been licensed and are commercially available worldwide[59].

The current HPV vaccines are based on virus-like particles (VLPs). The first HPV vaccine to be licensed was Gardasil (Merck and Co., Inc.) - approved by the FDA in 2006 - a quadrivalent (HPV types 6, 11, 16 and 18) VLP-based vaccine made of the recombinant HPV major capsid protein L1 produced in *S. cerevisiae*. In 2009 the FDA approved Cervarix, a bivalent (HPV types 16 and 18) vaccine commercialized by GlaxoSmithKline (GSK). Both the HPV VLP vaccines have shown to have a sustained prophylactic efficacy in clinical trials against infection and genital disease, generating a long-lasting antibody response[60]. VLP vaccines combine many of the advantages of the whole-virus vaccines and recombinant subunit vaccines. In addition, compared to individual proteins or peptides, they closely mimic the organization and conformation of authentic native viruses, leading to a more efficacious activation of the adaptive immune system. They can elicit a protective response without requiring multiple booster shoots, thus significantly reducing the vaccine costs. VLPs do not need attenuation or inactivation - as the live attenuated and killed/inactivated vaccines - avoiding all the possible side effects of inactivation treatments on the epitope modifications. Moreover, but lacking the viral genome VLPs potentially yield safer vaccine candidates compared to whole-virus vaccines. However, some technical challenges need to be considered for VLP production[56], essentially related to the limitations of the size of the expressed antigens and the choice of the expression systems. VLPs are normally expressed in bacteria, and therefore VLP assembly and stability, solubility, yield, endotoxin-free production, and composition may be potentially affected by all the concerns related to the prokaryotic expression machinery. Baculovirus/insect cell systems allow high expression levels. However, co-production of enveloped baculovirus contaminants may significantly impact the vaccine efficiency, and even though VLPs expressed in mammalian cells undergo complex post-translational modifications, this system show high production costs, low controllability and productivities. Currently, researchers are actively investigating methods to produce cheaper optimized VLP-based vaccines with increased half-life.

"E2 SCAFFOLD" AS A VERSATILE VACCINE DELIVERY SYSTEM

The E2 protein scaffold represents a versatile antigen delivery system (E2DISP) where antigenic determinants can be exposed on the surface of an icosahedral dodecahedral nanoparticle[61,62]. The scaffold is composed of the E2 acetyltransferase protein derived from the pyruvate dehydrogenase (PDH) multienzyme complex of *Geobacillus stearothermophilus*. The PDH complex belongs to the family of 2-oxo acid dehydrogenase multienzyme complexes that catalyse the irreversible oxidative decarboxylation of 2-oxo acids. They comprise multiple copies of three different enzymes, and in the case of PDH of *Geobacillus stearothermophilus*, two of these enzymes, E1 and E3, assemble over the surface of a large structural scaffold formed by the multi-domain core enzyme, E2, a specific dihydrolipoyl acetyltransferase. The E2 polypeptide chain is composed of three independently folded domains separated by flexible linker regions: a lypoil domain (LD) of 9.5 kDa, a peripheral (E1 and/or E3) subunit-binding domain (PSBD) of 5.3 kDa and a catalytic acetyltransferase core domain (CD) of 28 kDa (Figure 1A). The E2 CD forms trimers that assemble to generate a pentagonal dodecahedral protein scaffold resembling a virus-like particle (VLP) with icosahedral symmetry, composed of 60 identical E2 subunits (60-mer), that is 24 nm in diameter, with a molecular weight of 1.5 MDa, with an outer and inner domains of 240 Å and 50 Å, respectively[63] (Figure 1A). In the field of antigen display, the acetyltransferase core domain (CD) of the E2 protein is of great potential utility (E2DISP) (Figure 1B). Two engineered plasmids, pET-HE2DISP and pET-E2DISP, allow to insert exogenous oligonucleotides coding for the antigen of interest at the 5′ end of gene encoding the E2 CD, and thus to display foreign peptides/proteins as N-terminal fusions to CD (Figure 1B). Due to the stability and ability of this thermophilic protein to assembly *in vitro*[64], it is possible to display 60 copies of heterologous polypeptides on the surface of the E2 macromolecular scaffold, still capable of self-assembly to the 60-mer. This property is particularly suitable for vaccine design. There is no limitation to the size of peptide displayed, given the ability of the E2 CD to naturally present 60 lipoyl domains plus 60 copies of the E1 (150 kDa) or E3 (100 kDa) enzymes. Domingo *et al*[61] demonstrated that a green fluorescent

Table 2 Preclinical studies based on E2 formulations

E2 construct	Description	Route	Immune response	Ref.
Gag(p17)-E2	HIV-1 Gag p17 matrix protein	sc	Mice immunized with Gag(p17)-E2 mounted a strong and sustained Ab response; the isotype of induced Abs was biased toward IgG1; CD8+ T cells primed with E2 particles were able to exert lytic activity and to produce IFN-γ	[65]
BS1-E2	Mimotope 1 from HIV-1 bridging sheet domain (BS)	IM[1]/sc[1]	The E2-BS1 fusion peptide showed good antigenic results; a moderate neutralizing antibody response was found against two HIV-1 clade B and one clade C primary isolates	[67]
Env(V3)-E2	HIV-1 SF162 Env V3 loop peptide 291-336 from gp120 (HXB2 numbering)	Env-E2: IM[1]; pDNA[2]: ID[1]	Env(V3)-E2 induced potent binding Ab and T-cell responses in mice, as well as autologous NAbs in rabbits, when co-immunized with pDNA; co-immunization with pDNA and E2 multimers generated potent immune responses after only two immunizations	[19]
Env(MPER)-E2	HIV-1 SF162 Env MPER peptide 649-689 from gp41 (HXB2 numbering)	Env-E2: IM[1]; pDNA[2,3]: ID[1]	MPER (membrane proximal external region) displayed on E2 focused Ab responses toward conserved region of HIV-1 Envelope when co-administered with pDNA lacking hypervariable loop regions	[66]
(1-11)-E2	Peptide 1-11 of beta-amyloid	sc	(1-11)E2 vaccine induced fast-rising, robust and persistent Ab responses to beta-amyloid; the Ab response was characterized by a marked prevalence of IgG1 over the IgG2a isotype	[68,69]

[1]Routes of administration for rabbit immunizations; [2]pDNA: codon-optimized HIV-1 SF162 plasmid DNA encoding gp160 full-length; [3]Lacking hypervariable regions. sc: Subcutaneous; IM: Intramuscular; ID: Intradermal administration; Env: Envelope; gp: Glycoprotein; Ab: Antibody; NAbs: Neutralizing antibodies.

protein (EGFP) displayed on the E2 surface folded into its active form. We and others have successfully expressed and refolded several HIV-1 antigens and protein domains[19,65-67]. In addition, peptides 1-11 and 2-6 of beta-amyloid were displayed as N terminal fusions of the E2 core domain[68,69]. N-terminal fusion proteins are displayed without constraint on the surface of the E2 60-mer particles. Efficient expression was achieved in *Escherichia coli* (*E. coli*) cells. If soluble, proteins are purified as a large soluble aggregate, according to previously described methodologies[64] with a yield of pure E2 particles of about 15 mg/L of cell culture. Insoluble aggregates can be purified from inclusion bodies (IBs)[70]. It was shown that solubility and stability of HIV-1 Env-E2 fusion proteins substantially increased when they were refolded in the presence of the E2 wild type (E2wt) core protein, with no precipitation[19,66]. In details, pure HIV-1 Env-E2 IBs can be solubilized in presence of 6 M GuHCl (guanidine hydrochloride) and then refolded in the presence of E2 wild-type core protein (E2 monomers without the N-terminal HIV-1 fusion) in step-down dialysis by slow removal of the denaturant in the presence of oxidizing agents and low molecular weight additives, as schematically shown in Figure 1C. HIV-1 Env(V3)-E2 construct was refolded with equimolar amounts of E2wt, requiring a 1:1 ratio of Env-E2 fusion protein: E2wt to remain fully soluble[19]. Solubilized particles typically have more than 50 EU/mL of *E. coli*-derived endotoxin (lipopolysaccharide, LPS) as a result of expression in this system. Endotoxin levels can be reduced to less than 0.05 EU/mL using standard biochemical techniques[71]. The resulting vaccines are non-replicative multimeric particles formed by exogenous antigens inserted on the surface of E2 60-mer scaffold protein that is able to confer high immunogenicity to the displayed determinants.

We previously described that epitopes displayed on the surface of E2 scaffold are able to elicit both B and T cell responses, demonstrating that E2 particles can reach both MHC class I and class II compartments for the processing and presentation of the displayed epitopes[72-74], and we have investigated this system in various preclinical studies demonstrating the immunogenicity of E2-based vaccine formulations (resumed in Table 2). In particular, using this system, we demonstrated that mice immunized with the HIV-1 Gag (p17) protein displayed as an N-terminal fusion to the E2 CD [Gag (p17)-E2] mounted a strong and sustained humoral immune response. High titers of specific-antibodies were induced even in the absence of any adjuvants, and priming of transgenic mice with Gag(p17)-E2 particles induced antigen-specific cytotoxic CD8+ T cells able to produce IFN-γ[65]. Moreover, a moderate neutralizing antibody response was found in rabbits immunized with an E2 scaffold displaying a peptide mimotope of the HIV-1 gp120 bridging sheet[67].

Furthermore, E2 multimeric scaffolds displaying HIV-1 neutralizing antigens, such as the HIV-1 Envelope (Env) V3 loop from gp120 glycoprotein, was able to elicit potent binding antibodies and T-cell responses in mice, as well as autologous neutralizing antibodies in rabbits, when co-immunized with an HIV Env glycoprotein (gp160) expression plasmid DNA[19]. Interestingly, co-immunization of plasmid DNA vaccine with E2 multimeric scaffolds appeared to be more effective in eliciting rapid, specific, and sustained autologous neutralizing antibody responses as well as antigen-specific CD8+ T cells producing IFN-gamma, compared to standard DNA-prime/protein-boost regimen. On this line, the E2 scaffold displaying the membrane proximal external region (MPER) from HIV-1 Env gp41 glycoprotein - N-terminally fused to E2 core domain - was able to focus humoral immune responses

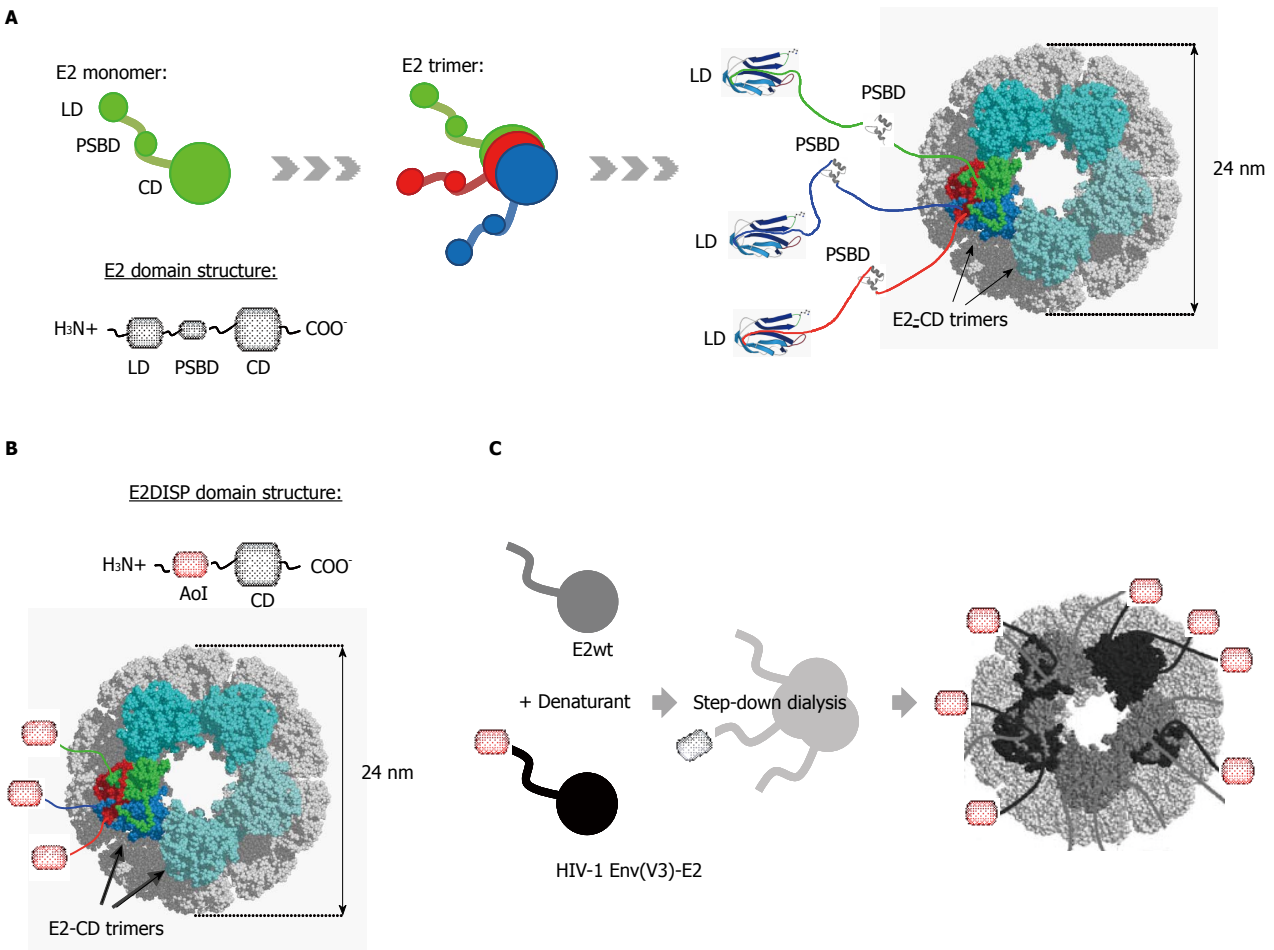

Figure 1 E2 acetyltransferase component from *Geobacillus stearothermophilus* pyruvate dehydrogenase complex. A: Schematic illustration of the native E2 chain with: lypoil domain (LD), peripheral subunit-binding domain (PSBD), and catalytic acetyltransferase core domain (CD). E2 CD forms trimers that assemble to generate a pentagonal dodecahedral scaffold(60-mer) with icosahedral symmetry. Trimers are in cyan with monomers of one trimer shown in green, red and blue; B: E2 core from E2DISP acetyltransferase system displaying an antigen of interest (AoI) N-terminally fused to CD; C: Schematic illustration of *in vitro* refolding of insoluble E2 displaying HIV-1 Envelope V3 in presence of E2 wild-type (E2wt).

toward constant region of Env when co-administered with a plasmid DNA vaccine encoding gp160 lacking immunodominant regions[66]. The E2 scaffold was also found to be suitable for the display of neurodegenerative disease-associated targets. Peptide 1-11 of beta-amyloid displayed as N-terminal fusion on the surface of E2 nanoparticle, the (1-11)E2 vaccine, induced fast-rising, robust and persistent antibody responses to beta-amyloid. E2 vaccination polarized the immune response toward the production of the Th2 cytokine Interleukin-4, without inducing a T cell response to β-amyloid[68]. Moreover, LPS-free (1-11)E2-based vaccines induced anti-amyloid-β antibodies even in the absence of adjuvant, or more interestingly, when formulated in adjuvants licensed for use in human vaccines[69].

In addition, Ren *et al*[75] developed an E2-based drug delivery systems for hydrophobic molecular transport of the antitumor compound doxorubicin in attempt to treat cancer diseases. Finally, we recently provided experimental evidence to the possible application of E2 scaffold as antigen delivery system for mucosal immunization and taking advantages of genome-wide approach we were able to dissect the type of T cell response induced by E2 particles (Trovato *et al*, manuscript in preparation).

Overall, E2 scaffold was shown to be a versatile and immunogenic delivery system, being able to display in a properly configuration antigenic/therapeutic peptides or proteins and to elicit humoral and cellular immune responses upon different ways of administrations.

These properties make the E2DISP system an attractive option for vaccine delivery. Theoretically, there is no limitation to the size of peptide displayed on the E2 surface, given the potential of the E2 core domain to naturally accommodate 187 amino acid residues in the form of the two folded protein domains (LIP and PSBD domains) and two flexible linkers (Figure 1A). Displaying full-length protein as antigen may be a convenient option compared to peptide to provide optimal epitope diversity for antibody production and T cell induction. In this context, the E2DISP delivery may be particularly favorably to other types of antigen display systems - such as the Hepatitis B surface antigen vector that has a limit of approximately 36 amino acids[76] or the chimeric human papilloma virus-simian/human immunodeficiency virus virus-like particle vaccine that can only accept approximately 60 amino acids of foreign

antigen[77]. Repetitive presentation of an epitope in highly organized structures - as with E2 nanoparticle - can increase the ability of particulate delivery systems to stimulate stronger immune responses by triggering and cross-linking specific B cell antigen receptors. Within this context, the E2 nanoparticle may be particularly useful as repetitive antigen delivery system due to its potential to display up to 60 copies of an antigen of interest per particle. Moreover, the E2DISP delivery may function as presentation scaffold for multiple displays of antigens, all on the same E2 particle, in their native form to properly activate both the humoral and cellular branches of the immune response. The ability of E2-based vaccines to generate both CD8+ T cell responses and antibodies may represent an advantage over protein subunit vaccines, which primarily evoke humoral responses, and recombinant viral vectors being more effective at generating cellular immune responses.

Bacterial expression is the most common expression system employed for the expression and purification of heterologous recombinant proteins - as for the production of the E2 nanoparticles. However, proteins expressed in a prokaryotic-based system are not correctly modified - in terms of protein phosphorylation and glycosylation - and might precipitate in the form of inclusion bodies, thus affecting the protein folding. Moreover, as result of expression in *E. coli* cells, recombinant proteins are generally contaminated with the lipopolysaccharide (LPS) component of the outer cell membrane. Such a toxic component triggers secretion of pro-inflammatory cytokines, and it often requires extensive and expensive removal during protein purification, thus affecting the final yield. It was shown that solubility and stability of recombinant E2 scaffolds that precipitate into the insoluble fraction could increase when they are refolded *in vitro* from denaturing conditions in presence of the E2wt core protein. In addition, treatment by phase separation with Triton X-114 detergent leads to an endotoxin reduction of less than 0.05 EU/mL. However, alternative organisms and expression systems could be more useful for the expression and production of E2 nanoparticles in order to circumvent all the problems related to the *E. coli* expression machinery.

We previously explored the potential of the E2 antigen display system as an HIV-1 vaccine candidate. It was shown that E2-based multimeric vaccines displaying the V3 loop or the MPER region from the HIV-1 Envelope are able to focus and to direct antibody responses to conserved neutralization determinants. However, the V3 epitope displayed on the surface of E2 scaffold is not effective in generating broadly neutralizing antibodies (NAbs), and we can only generate low levels of neutralizing antibodies that are MPER-specific[19,66]. Clearly, this current E2-based immunogen requires further optimization for advancement. A major goal of HIV-1 vaccine development is to find strategies for inducing high levels of broad-spectrum neutralizing antibodies. We hypothesize that the E2-mediated immune responses can likely be further enhanced using molecular modeling to determine the appropriate regions of the E2 protein to serve as insertion sites for key neutralization determinants in order to improve presentation and thus immunogenicity of HIV-1 regions in this system.

Overall, the potential of this system is that it exhibits stability and no toxicity, it is able to induce sustained humoral and cellular antigen-specific immune responses without anti-vector immunity, and thus low-cost, non-replicating, non-integrating, non-pathogenic E2 vaccines could be designed and combined with other approaches to advance the field of vaccinology.

CONCLUSION

Vaccines play a pivotal role in host protection against infectious diseases and have significantly reduced mortality worldwide. However most of vaccine candidates have failed to completely protect individuals from emerging and re-emerging diseases/agents, with many diseases, such HIV/AIDS, tuberculosis, and malaria, being not yet preventable by vaccination. Hence, the development of new vaccine formulations is of fundamental priority. Several strategies have been developed over the years in order to achieve this goal, and the recent advances in the field of vaccine technology may provide valuable insights for the rational design of next-generation vaccine delivery systems. Historically, vaccinology has relied on the use of live attenuated, killed/inactivated, toxoid and subunit vaccines with most of them currently available on the market. Many live attenuated vaccines are able to stimulate humoral as well as cell-mediated immune responses, by mimicking the natural infection. However, some concerns still remain to be addressed when using attenuated/inactivated vectors as vaccines, including safety, instability and weak immunogenicity. Alternative strategies have been developed to provide safer and more effective vaccines. Recombinant DNA technology could be a useful approach, mainly due to the ability of DNA vaccines to elicit different types of immune response, providing many advantages over traditional vaccines in terms of safety, stability, costs of production, and ease of manufacturing. However, until now DNA vaccines have not been successful in non-human primates and humans. Recombinant viral vectors represent an attractive tool to deliver antigen and to stimulate stronger immune responses than DNA vaccines, with the majority of current clinical trials for gene therapy using viral vectors; however, biosafety and pre-existing immunity concerns must be taken into account when using viral vectors as vaccine. Nanoparticle-based delivery systems have arisen as promising vaccine candidates over traditional vaccines, mainly due their ability to elicit robust immune responses without toxicity and anti-vector immunity, even though these formulations suffer of problems of stability and conservation. Given this scenario, we have been studying in the past decade a delivery system based on a protein scaffold formed by a 60-mer assembled over the domain of the E2 component of the PDH complex from *Geobacillus stearothermophilus*. The E2 scaffold represents a versatile vaccine delivery candidate, being able to trigger both arms

of the adaptive immune response, combining good safety and stability with strong immunogenicity.

In conclusion, in this review we have described the advancement obtained in the recent past on the topic of antigen delivery systems for new vaccine formulations. Studies aimed to compare in controlled assay conditions should be performed in a near future in order to identify the most promising vaccination strategies.

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EDITORIAL

Can antiretroviral therapy be tailored to each human immunodeficiency virus-infected individual? Role of pharmacogenomics

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Abstract

Pharmacogenetics refers to the effect of single nucleotide polymorphisms (SNPs) within human genes on drug therapy outcome. Its study might help clinicians to increase the efficacy of antiretroviral drugs by improving their pharmacokinetics and pharmacodynamics and by decreasing their side effects. *HLAB*5701* genotyping to avoid the abacavir-associated hypersensitivity reaction (HSR) is a cost-effective diagnostic tool, with a 100% of negative predictive value, and, therefore, it has been included in the guidelines for treatment of human immunodeficiency virus (HIV) infection. *HALDRB*0101* associates with nevirapine-induced HSR. *CYP2B6* SNPs modify efavirenz plasma levels and their genotyping help decreasing its central nervous system, hepatic and HSR toxicities. Cytokines SNPs might influence the development of drug-associated lipodystrophy. *APOA5*, *APOB*, *APOC3* and *APOE* SNPs modify lipids plasma levels and might influence the coronary artery disease risk of HIV-infected individuals receiving antiretroviral therapy. *UGT1A1*28* and *ABCB1 (MDR1) 3435C > T* SNPs modify atazanavir plasma levels and enhance hyperbilirubinemia. Much more effort needs to be still devoted to complete large prospective studies with multiple SNPs genotyping in order to reveal more clues about the role played by host genetics in antiretroviral drug efficacy and toxicity.

Key words: Pharmacogenomics; Pharmacokinetics; Antiretroviral drugs; Adverse effects; Human immunodeficiency virus infection; Single nucleotide polymorphisms

Core tip: Pharmacogenetics may play an important role in

the near future for the treatment of human immunodeficiency virus-infection, as exemplified by the *HLAB*5701* genotyping to prevent the abacavir-associated hypersensitivity reaction. Diverse other single nucleotide polymorphisms have been described as related to certain pharmacokinetic characteristics and adverse effects of antiretroviral drugs. In this Editorial we summarize the current knowledge on this rapidly evolving field.

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INTRODUCTION

Antiretroviral therapy (ART) has become so effective that human immunodeficiency virus (HIV) infection is not any more the deadly plague of the past, but a chronic, easy to handle condition. Although ART is much less toxic nowadays than it was in the past, it is still not free of side effects. The choice of the most effective and safe ART regimen is the daily task of HIV clinicians throughout the world. An aim that has been made easier by the existence of ART guidelines that are updated yearly by different agencies and societies.

Another approach, much more cumbersome, is the use of pharmacogenetics to prescribe ART. The same antiretrovirals administered at the same doses produce different antiviral effects and toxicities in different individuals, suggesting that genetic factors of the host may also play a role. The term pharmacogenetics refers to the effect of polymorphisms within human genes on drug therapy outcome. Single-nucleotide polymorphisms (SNPs) are sequence variations in human DNA with single nucleotide changes occurring at an allele frequency greater than 1%. Nucleotide changes occurring with a lower frequency are referred to as mutations.

SNPs are candidates for a causal role for a given phenotype when they are associated with changes in protein function, which occurs more likely when the SNP is located in an exon, a DNA protein-coding region, and lead to changes in the encoded amino acid. However more than 95% of SNPs are located in non-coding gene regions, such as those of the promoter, untranslated, introns and intergenic regions. Such non-exonic SNPs can still alter protein function or expression by changes in gene transcription, mRNA splicing, mRNA stability or alterations in translation and conformation of the protein. Therefore, pharmacogenetics gives ground to individualized therapy.

This genetic tool might help clinicians to enhance ART efficacy by improving the pharmacokinetics and pharmacodynamics of antiretroviral drugs and by decreasing their side effects[1-10]. The use of *HLAB*5701* genotyping to avoid the abacavir-associated hypersensitivity reaction (HSR) is a cost-effective diagnostic tool, which have a negative predictive value of 100% for all ethnic groups and, consequently, it has been included in all ART guidelines[11,12]. Unluckily, pharmacogenetics cannot offer so bright solutions to other ART problems at present, although it might still be of some help to the clinician, however.

A major problem of the SNP-phenotype association studies in the field of ART is the lack of reproducibility. This might be related to the relatively small size of the populations genotyped, the lack of statistical power of the study or a selection bias. Other times the SNP association of the observed effect is found only within a specific ethnic group but not in others. Also, some of the reported positive associations might have been obtained after multiple statistical comparisons, giving place to potentially spurious associations due to chance. Likewise, only positive results are usually reported, which means that some published associations may not have been overtly refuted by other authors that found no such a relationship. On the other hand, a SNP-phenotype association might not be necessarily due to the functional effect of the gene variant, but to the presence of other variant on the same chromosome in linkage disequilibrium, combination that is referred to as a haplotype. Finally, most of the pharmacogenetic studies are retrospective or cross-sectional. A large prospective study on a multiethnic population, with simultaneous genotyping of multiple SNPs known to be relevant in the general population, would be much more informative.

In the following lines we will focus on the most frequent associations of genetic variants with the pharmacokinetic changes and toxicity of antiretroviral drugs, the most relevant of which are summarized in Table 1.

ABACAVIR-ASSOCIATED HSR

As mentioned above, the use of *HLAB*5701* genotyping to avoid the abacavir-associated HSR is the ideal example of a genotype-phenotype correlation in HIV medicine. The involvement of host genetic factors was first suggested by the observation of abacavir-associated HSR in members of the same family. Later, several groups demonstrated a strong association between abacavir and the haplotype comprising *HLAB*5701*, *HLA-DR7* and *HLA-DQ3* genotypes[11].

The clinical utility of *HLAB*5701* genotyping was confirmed in a large, randomized, double-blind, international, multiethnic prospective study. HIV-infected patients with a positive *HLAB*5701* genotype were excluded from abacavir prescription (prospective screening group) while other HIV-infected patients received abacavir without *HLAB*5701* genotyping (control group). Patients with clinically suspected HSR underwent a confirmatory skin-patch testing (immunologically confirmed HSR). Prospective *HLAB*5701* screening eliminated immunologically confirmed HSR with a negative predictive value of 100% and significantly reduced the rate of clinically suspected HSR from 7.8% to 3.4%[12].

Table 1 Summary of most relevant genetic determinants of antiretroviral drug pharmacokinetics and toxicity

Drug/drug class	Gene, allele(s)/SNPs	SNP	Reported associations	Additional observations	Ref.
Abacavir	*HLA-B*5701*	2395029	↑ risk of HSR	Cost effective test and included in all ART guidelines	[11-13]
Tenofovir	*ABCC2 (MRP2)1249G > A*	2273697	↑ risk of renal proximal tubulopathy in French populations	To be confirmed in other populations	[14,15]
Lamivudine, Zidovudine	*ABCC4 (MRP4) 3724G > A, 4131T > G*	2273697 3742106	↑ intracellular exposure of stavudine triphosphate	Uncertain clinical significance	[15,53]
NRTIs	*TNFα238G > A*	361525	Earlier onset of lipoatrophy	Negative findings reported by others	[16-20]
Stavudine, NRTIs	*IL1β + 3954C > T*	1143634	↓ risk of lipodystrophy in Spanish populations	To be confirmed in other populations	[20]
NRTIs	*MMP1-16071G > 2G*	1799750	↑ risk of lipodystrophy in Spanish populations	To be confirmed in other populations	[21]
Stavudine, Zidovudine	*TS* ↓ expression and *MTHFR 1298 A > C* ↑ activity genotypes	1801131	↑ risk of lipodystrophy and peripheral neuropathy in Spanish populations	To be confirmed in other populations	[24,25]
NRTIs	*LPS-binding protein (LBP) T > C*	2232582	↑ risk of lipodystrophy in Spanish population	To be confirmed in other populations	[22]
NRTIs	Mitochondrial DNA (haplogroup T): *MTND1*LHON4216C, MTND2*LHON4917G, 7028C > T, 10398G > A, 13368G > A*	28357980	↑ risk of peripheral neuropathy	Tissue specific mitochondrial DNA depletion may also play some role in NRTI toxicity	[7,26,27]
NRTIs	*HFE845G > A*		↓ risk of peripheral neuropathy	Negative findings reported by others	[28,29]
NRTIs	*CFTR 1717-1G > A, IVS8 5T, SPINK-1 112C > T*		↑ risk of pancreatitis	Reported also in the general population	[30]
Nevirapine	*HLA-DRB1*0101*		↑ risk of HSR and hepatotoxicity	CD4 cell % > 25% associated with ↑ risk	[31,32]
Nevirapine	*HLA-cw8*		↑ risk of HSR in Italian and Japanese populations		[33,34]
Nevirapine	*CYP2B6 983T > C*	28399499	↑ risk of HSR in Malawian and Ugandan populations	Stevens-Johnson syndrome or toxic epidermal necrolysis, but no other HSR	[37]
Nevirapine, Efavirenz	*ABCB1 (MDR1) 3435C > T*	1045642	↓ risk of hepatotoxicity		[35,36]
Efavirenz	*ABCB1(MDR1) 3435C > T*	1045642	↓ plasma exposure	Negative findings reported by some authors	[51-53]
Efavirenz	*CYP2B6 *1/*1 haplotype*		↓ plasma concentrations	In patients receiving antituberculosis treatment	[45]
Efavirenz	*ABCB1 (MDR1 3435C > T*	1045642	↑ HDL-cholesterol in Spanish populations	To be confirmed in other populations	[60]
Efavirenz	*CYP2B6 516G > T, 983T > C*	3745274 28399499	↑ plasma exposure and ↑ risk of CNS side effects	Reports of successful efavirenz dose individualization	[39,42,44, 46,48,49]
Efavirenz	*CYP2A6 48T > G, UGT2B7 735A > G*	28399433 28365062	↑ plasma concentrations in Black and White, but not in Hispanic individuals from the United States	To be confirmed in other populations	[47]
Efavirenz, Nevirapine	*CYP2B6 516G > T, 983T > C*	28399499	↑ plasma exposure in African populations	To be confirmed in other populations	[43]
NNRTIs	*ABCA1/Hepatic Lipase (LIPC)*/Cholesteryl Ester Transfer Protein (*CETP*)	4149313 173539 3764261	↑ LDL-cholesterol in Spanish populations	To be confirmed in other populations	[61]
PIs	*ABCA1 2962A > G*		↑ risk of hyperlipidemia		[60]
PIs	*CETP 279A > G*		↑ risk of hyperlipidemia		[60]
PIs	APOA5-1131T > C, 64G > C	662799	↑ risk of hyperlipidemia		[60,62]
Antiretrovirals	*APOE/LDL Receptor (LDLR)*	405509 2228671	↑ risk of trunk fat gain in Spanish populations	To be confirmed in other populations	[23]
PIs	*APOC3 482 C > T, 455 C > T, 3238 C > G*	2854117 2854116 5128	↑ risk of hyperlipidemia		[18,63]
PIs	*APOE ε2 and ε3* haplotypes		↑ risk of hyperlipidemia		[18]
Antiretrovirals	*Insulin Receptor Substrate 1 (IRS1)*	1801278	↑ risk of limbs lipoatrophy in Spanish populations	To be confirmed in other populations	[23]
Raltegravir	*UGT1A1*28/*28*		↑ modestly plasma levels	Clinically no significant	[57]
Atazanavir, Indinavir	*UGT1A1*28*		Unconjugated hyperbilirubinemia and jaundice		[54,55]

Atazanavir	*ABCB1 (MDR1) 3435C > T*	1045642	Unconjugated hyperbilirubinemia and jaundice	↑ plasma levels	[57]
Atazanavir	*ABCB1 (MDR1) 2677 G > T*	2032582	↑ intracellular/plasma concentration ratios	For GG homozygous as compared with GT and TT genotypes	[58]
Nelfinavir	*CYP2C19*2 (681G > A)*	4244285	↑ drug exposure in Italian and multiracial Americans	To be confirmed in other populations	[39]
Indinavir	*CYP3A5*3 (A6986G)*		↑ oral clearance	To be confirmed in other populations	[53]
Maraviroc	*CCR5WT/Δ32*		No effect on virologic response	Clinically not significant	[7]

SNP: Single-nucleotide polymorphisms; HSR: Hypersensitive reaction; ART: Antiretroviral therapy; CNS: Central nervous system; NRTIs: Nucleoside reverse transcriptase inhibitors; NNRTIs: Non-nucleoside reverse transcriptase inhibitors; PIs: Protease inhibitors.

A recent meta-analysis has quantified the utility of *HLAB*5701* testing[13]. The pooled odds ratio to detect abacavir-induced hypersensitivity on the basis of clinical criteria was 33.07 (95%CI: 22.33-48.97), while diagnostic odds ratio for detection of immunologically confirmed abacavir hypersensitivity was 1141 (95%CI: 409-3181). The meta-analysis also found that prospective *HLA-B*5701* testing significantly reduced the incidence of abacavir-induced hypersensitivity.

These results strongly support the clinical value of *HLAB*5701* screening to avoid this condition. Therefore, *HLAB*5701* genotyping has proved to be cost-effective and is already included as a routine tool in all ART guidelines.

TENOFOVIR-ASSOCIATED RENAL PROXIMAL TUBULOPATHY

Tenofovir, the most widely prescribed antiretroviral nowadays, has shown to produce renal proximal tubulopathy and bone toxicity in the long run. Tenofovir is introduced in the renal proximal tubular cell by the human organic anion transporters 1 and 3. Multidrug resistance-associated proteins (ABCC/MRP) 2 and 4 are located in the apical membranes of the proximal renal tubules and transport different drugs from the tubular cells to the urine. Variations in the genes that encode ABCC2 (MRP2) and ABCC4 (MRP4) proteins might block tenofovir excretion, enhancing intracellular tenofovir levels and increasing the risk of renal tubular toxicity.

In fact, *ABCC2 (MRP2)1249G > A* SNP has been linked to tenofovir-associated renal proximal tubulopathy in HIV-infected French patients[14], a genetic association that needs to be confirmed in other populations. However, this finding needs further explanation because tenofovir is not a substrate for ABCC2, although this genetic variant might be in linkage disequilibrium with other SNPs in genes coding for unidentified factors that might exacerbate tenofovir toxicity[15].

NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS-ASSOCIATED LIPODYSTROPHY

British and Australian researchers have reported an association of the *TNFα238G > A* SNP with the earlier onset of lipoatrophy in Caucasian HIV-infected patients under nucleoside reverse transcriptase inhibitors (NRTI)[16,17], findings that have not been reproduced by others and need further confirmation[18-20]. *IL1β + 3954C > T* SNP, which decreases TNF-α plasma levels, have been associated with protection against lipodystrophy in HIV-infected Spanish individuals on stavudine[20].

Metalloproteases (MMPs), involved in extracellular matrix remodeling, can modulate adipocyte differentiation[8]. *MMP1 - 16071G > 2G* SNP induces increased MMP-1 plasma levels and has also been associated with lipodystrophy[21]. Increased lipopolysaccharide (LPS) plasma levels have been found in HIV-infected subjects. Lipopolysaccharide-binding protein (LBP), which transports LPS, has been linked to obesity and metabolic perturbations. *LPS-binding protein (LBP)T > C* SNP has been associated with lipodystrophy in Spanish HIV-infected individuals[22].

Specific SNPs in *APOE* and *LDL receptor (LDLR)* genes (rs 405509 and rs 2228671) have been related to trunk fat gain in HIV-infected individuals on ART. *Insulin Receptor Substrate 1 (IRS1)* SNPs (rs 1801278) has been associated with increased risk of limbs lipoatrophy in the same Spanish Caucasian cohort[23]. Low-expression thymidylate synthase SNPs have also been associated with lipodystrophy in HIV-infected patients exposed to estavudine[24].

NRTI-ASSOCIATED PERIPHERAL NEUROPATHY AND PANCREATITIS

Low-expression thymidylate synthase SNPs have been related to increased stavudine triphosphate intracellular levels[24]. Methylenetetrahydrofolate reductase (*MTHFR) 1298 A > C* SNP has been associated with decreased activity of this enzyme and abnormalities of folate metabolism. The conjunction of a low-expression thymidylate synthase plus a MTHFR genotype in HIV-infected patients exposed to stavudine has been associated with the development of peripheral neuropathy and lipodystrophy in HIV-infected individuals[24,25]. Mitochondrial haplogroup T *MTND1*LHON4216C* and *MTND2-*LHON4917G* genotypes and mitochondrial haplogroup T and *7028C > T, 10398G > A,* and *13368G > A,* SNPs were independently linked to increased susceptibility to

NRTI-associated peripheral neuropathy[7,26,27].

Iron transport is dysregulated in HIV infection and disorders of iron metabolism are linked to mitochondrial dysfunction and other neurodegenerative disorders. Hemochromatosis (*HFE*) gene SNPs alter the structure of HFE protein dysregulating intestinal iron absorption and its cellular transport. The carriage of the hemochromatosis *(HFE) 845G>A* SNP decreased the risk of NRTI-associated peripheral neuropathy, although this finding could not be reproduced by others[28,29].

Cystic fibrosis transmembrane conductance regulator (*CFTR*) and serine protease inhibitor Kazal-1 (*SPINK-1*) mutations have been reported to increase the risk of pancreatitis in the general population. *CFTR 1717-1G > A*, *IVS8 5T*, and *SPINK-1 112C > T* SNPs are also frequent among HIV-positive patients suffering from acute pancreatitis, what suggests that these mutations might increase the susceptibility to pancreatitis if the patients are exposed to environmental risk factors such as thymidine NRTIs (stavudine, didanosine)[30].

NON-NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS-ASSOCIATED HSR AND HEPATITIS

Carriage of the class II allele *HLA-DRB1*0101* has been linked with nevirapine-associated hepatotoxicity and HSR (but not with isolated rash) in HIV-infected Western Australians, especially in those individuals with a CD4 cell count > 25%[31]. A similar association with cutaneous hypersensitivity has also been reported for nevirapine and efavirenz in French Caucasian patients regardless of the CD4 values[32].

Additional HLA alleles (*HLA-cw8/HLA-B14*) have been recently associated with nevirapine hepatotoxicity in Sardinian[33] and Japanese[34] HIV-infected patients. On the other hand, *ABCB1 (MDR1) 3435C > T* SNP has been found to decrease the risk of nevirapine-associated hepatotoxicity in multiethnic South African and American individuals[35,36].

Likewise, an association between the *CYP2B6 c.983T > C* SNP and the development of nevirapine-induced Stevens-Johnson syndrome or toxic epidermal necrolysis, but not other hypersensitivity reactions, has been described in Malawian and Ugandan HIV-infected individuals[37]. Considering that this SNP is found in a small part of African populations, but not in Caucasians, these findings would point out to an ethnic-specific predisposing factor.

EFAVIRENZ DISPOSITION AND CENTRAL NERVOUS SYSTEM SIDE EFFECTS

The cytochrome P450 (CYP) enzyme CYP2B6, primarily expressed in the liver, is involved in the biotransformation of efavirenz. CYP2B6 is one of the most polymorphic CYP genes in humans and its variants have shown to affect transcriptional regulation, splicing, mRNA and protein expression and catalytic activity[38]. *CYP2B6 516G > T, 983T > C, 785A > G* and *21563C > T* SNPs have been associated with greater efavirenz plasma exposure and the development of more severe central nervous system (CNS) effects in different HIV-infected populations, including African and Thai patients[39-46].

Likewise, increased efavirenz concentrations were associated with *CYP2A6 -48T > G* and with *GG* homozygosity for *UGT2B7 735*, a SNP of the microsomal enzyme uridine 5′-diphospho-glucuronosyltransferase (UGT), in Black and White, but not in Hispanic individuals from the United States[47].

Also, *CYP2B6 *6/*6* and *6/*26* carriers have been found to be associated with extremely high plasma concentrations of efavirenz in Japanese patients receiving standard doses of the drug[48]. Efavirenz doses were substantially reduced down to 200 mg/d in these patients without loss of antiviral efficacy and improvement in CNS symptoms. In addition, *CYP2B6 516G > T* genotyping has been found to reduce treatment costs, even considering only the sparing related to efavirenz dose reduction[49]. These two reports constitute examples of practical applications of genotyping and how pharmacogenomics may be useful for the management of HIV-infected individuals receiving antiretroviral drugs.

On the other hand, there are conflicting results about the effect of *ABCB1(MDR1) 3435C > T* SNPs in decreasing efavirenz plasma exposure[50-52], and an independent association between low efavirenz plasma concentrations and the *CYP2B6 *1/*1* haplotype has also been found in patients receiving antituberculosis drugs[45].

SNPs in other CYP enzymes such as *CYP3A5* SNPs have also been associated with faster clearance of other antiretroviral drugs such as indinavir[53].

ATAZANAVIR AND INDINAVIR-ASSOCIATED HYPERBILIRRUBINEMIA

The most common side effect of atazanavir is hyperbilirubinemia (observed in 20%-50% of patients exposed to this drug), a mostly minor disturbance that in 6% of cases can reach the range of clinical jaundice. Bilirubin needs to be conjugated with glucuronic acid to be excreted in the bile. This step is mediated by the microsomal enzyme UGT, which can cause unconjugated hyperbilirubinemia when its activity is reduced. Fifteen UGT isoforms with different substrate specificities, including the bilirubin-specific isoform UGTA1, have been identified. *UGT1A1*28* SNP has been associated with hyperbilirubinemia in HIV-infected Swiss and Spanish Caucasian individuals starting atazanavir or indinavir[54,55], and this SNP might modify raltegravir plasma levels as well[56].

Likewise, the P-glycoprotein, an efflux pump coded by the *ABCB1 (MDR1)* gene, is one of the most important transporters, especially expelling protease inhibitors outside the cell. *ABCB1 (MDR1)* SNPs might therefore influence atazanavir plasma concentration and, in fact,

ABCB1 (MDR1) 3435C > T SNP has been associated with increased atazanavir plasma levels and hyperbilirubinemia in Spanish patients[57]. Also, the intracellular/plasma concentration ratio of atazanavir was higher in *GG* carriers compared with those with *GT* and *TT* genotypes of the *ABCB1 2677 G>T* SNP in an Italian study[58].

PROTEASE INHIBITOR AND EFAVIRENZ-ASSOCIATED LIPIDIC ABNORMALITIES AND CORONARY ARTERY DISEASE RISK

Hyperlipidemia is usually associated with ritonavir-boosted protease inhibitor therapy, but also with efavirenz use. *ABCA1* SNPs have been linked to hyperlipidemia in HIV-infected patients treated with protease inhibitors or efavirenz. Thus, *ABCA1 2962A > G* SNP has been associated with increased HDL-cholesterol plasma levels after efavirenz treatment in Spanish patients[59] and after ritonavir-boosted protease inhibitor therapy in the Swiss HIV cohort[60]. The contribution of other SNPs associated with plasma lipid levels in the general population has also been extensively studied in the same Swiss cohort and in other populations. *APOA5*, especially the *-1131T > C* and *64G > C* SNPs, *APOC3,* especially the *482 C > T*, *455 C > T* and *3238 C > G* SNPs, and *APOE*, especially the *APOE ε2 and ε3* haplotypes and *APOB* SNP have been shown to contribute to increased plasma triglyceride, HDL-cholesterol and/or LDL-cholesterol levels during ART[18,60-63].

ABCA1, Hepatic Lipase (LIPC) and Cholesteryl Ester Transfer Protein *(CETP)* gene variant*,* especially the *279A > G SNP*, were favorably associated with HDL-cholesterol when ART included non-nucleoside reverse transcriptase inhibitors (NNRTI). However an unfavorable effect on total-cholesterol and triglyceride levels was observed when ART included protease inhibitors[62].

Recently, a large meta-analysis has shown the role in HIV-infected patients on ART of 23 SNPs associated with coronary artery disease (CAD) in the general population. The authors report that the effect of unfavorable genetic background was similar to traditional CAD risk factors and certain adverse antiretroviral exposures. The authors concluded that genetic testing might provide prognostic information complementary to the family history of CAD[64].

DISCUSSION AND CONCLUSION

The field of pharmacogenetics is just beginning, but it will help the clinician to tailor and individualize ART for each HIV-infected patient. The gold standard to reach is currently the *HLAB*5701* genotyping, which has proven to be highly efficacious to prevent the abacavir-associated HSR and, consequently, it has been included as a routine tool for the care of HIV-infected patients in all ART guidelines.

In this short review we have focused more on the possible role of pharmacogenetics to prevent ART side effects than in pharmacokinetics. However, the reader must be aware of the value of pharmacogenetics to modulate the pharmacokinetic parameters of antiretroviral drugs. For instance, efavirenz dosage can be tailored for each individual knowing his/her *CYP2B6* SNPs carriage, as *CYP2B6* genetic variants seem to substantially modify efavirenz absorption and plasma levels. Moreover, genotyping has even shown to be a cost-effective measure, as the costs of the determination are compensated by savings related to efavirenz dose reduction and management of side-effects. Therefore, the clinician might adjust efavirenz doses to achieve maximal antiviral efficacy with minimal side effects.

The same train of thought can be applied to *UGT1A1*28* and *ABCB1* genotypings, to control the plasma and intracellular concentrations of atazanavir and to decrease the atazanavir-associated hyperbilirubinemia without modifying its antiviral effect.

The practical usefulness of other genetic testings is less clear at present, pending on the confirmation of the results observed in different studies and the discovery of new genetic variants associated with the pharmacokinetics and side-effects of antiretroviral drugs. Therefore, much more effort is needed to complete large size prospective studies with multiple SNPs genotyping, to reveal more clues about the role played by host genetics in ART response.

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EDITORIAL

Is the use of IL28B genotype justified in the era of interferon-free treatments for hepatitis C?

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Abstract

In 2009, several groups reported that interleukin-28B (IL28B) genotypes are associated with the response to peginterferon plus ribavirin therapy for chronic hepatitis C virus (HCV) infection in a genome-wide association study, although the mechanism of this association is not yet well understood. However, in recent years, tremendous progress has been made in the treatment of HCV infection. In Japan, some patients infected with HCV have the IL28B major genotype, which may indicate a favorable response to interferon-including regimens; however, certain patients within this group are also interferon-intolerant or ineligible. In Japan, interferon-free 24-wk regimens of asunaprevir and daclatasvir are now available for HCV genotype 1b-infected patients who are interferon-intolerant or ineligible or previous treatment null-responders. The treatment response to interferon-free regimens appears better, regardless of IL28B genotype. Maybe other interferon-free regimens will widely be available soon. In conclusion, although some HCV-infected individuals have IL28B favorable alleles, importance of IL28B will be reduced with availability of oral interferon free regimen.

Key words: Hepatitis C virus; Interleukin-28B; Interferon; Japan; Sustained virologic response

Core tip: Genome-wide association studies have revealed that interleukin-28B (IL28B) genotypes are associated with the response to interferon therapy for chronic hepatitis C. The mechanism of this association is not yet clear. Although many hepatitis C virus (HCV)-infected individuals have IL28B favorable alleles, in the near future, HCV-infected patients in Japan may be treated with interferon-free regimens, which avoid the adverse events caused by interferon plus ribavirin therapy.

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INTRODUCTION

Chronic hepatitis C virus (HCV) infection is a major cause of end-stage liver diseases and hepatocellular carcinoma (HCC) in Japan and the United States[1-4]. Chronic hepatitis C is an important health problem worldwide[5]. The eradication of HCV by interferon-including treatment could lead to the following benefits[6]: (1) fibrotic regression[7-9]; (2) reduction of HCC occurrence and recurrence[10-12]; (3) reduction of other complications, including liver failure, liver-related death[13,14] and liver-unrelated death[15]; and (4) improved quality of life[15]. A sustained virologic response (SVR), which is defined as HCV RNA negativity 24 wk after completion of antiviral therapy, could have beneficial effects in HCV-infected patients. In the era of direct-acting antivirals (DAA) against HCV, regimens including interferon remain important treatments for HCV eradication[5,16-33], although interferon-free regimens should be available worldwide soon[34]. In this review, we focused the distribution of interleukin-28B (IL28B) status in Japanese patients currently infected with HCV, and their treatment.

INTERLEUKIN-28B GENOTYPES

In 2009, several groups reported that a genetic polymorphism near the *IL28B* gene, which encodes interferon-lambda-3 (IL28B genotypes), was associated with the response to peginterferon plus ribavirin therapy for chronic hepatitis C in a genome-wide association study[35-37]. The IL28B minor genotype plays a crucial role in interferon resistance[38]. The host genetic polymorphism may be useful for predicting drug response[37,39]. IL28B major or minor genotype, respectively, could predict better or poor response to interferon therapy in patients infected with HCV. An association between inosine triphosphatase (ITPA) genetic variants and treatment-induced anemia has been reported in HCV-infected patients treated with peginterferon plus ribavirin[40-42]. ITPA major genotype could predict profound anemia induced by peginterferon plus ribavirin treatment in HCV-infected patients. A genetic polymorphism of interferon-lambda-4 has also been associated with the treatment response to interferon-including regimens for chronic hepatitis C infection[43-45]. Similar to IL28B genotypes, interferon-lambda-4 major or minor genotype, respectively, could predict better or poor response to interferon therapy in HCV-infected patients.

Mechanism of the association between the IL28B genotype and treatment response

Recently, Aoki *et al*[46] reported that serum IL28B levels are increased in patients with chronic hepatitis C, regardless of the IL28B genotype. They also suggested that serum IL28B is a biomarker of the activity and fibrosis of liver disease; however serum IL28B is not correlated with the responsiveness to peginterferon plus ribavirin therapy[46]. The same group reported that IL28B genotype affects IL28B production but that the outcome of peginterferon plus ribavirin treatment depends on the amount of IL28B protein[47].

Hepatic interferon-stimulated genes (ISGs) have been significantly associated with the IL28B polymorphism, and expression level of hepatic ISG was significantly higher in patients with the minor genotype than those with the major genotype[48,49]. Lagging *et al*[50] found that the favorable IL28B variants were associated with lower baseline plasma interferon-gamma-inducible protein-10 (IP-10), although high baseline levels of IP-10 predicted a slower first phase decline in HCV RNA and poor outcome following interferon plus ribavirin therapy in patients with chronic hepatitis C[51-53]. We also reported that IL28B genotypes and hepatic STAT1-nuclear translocation are independent predictors of treatment response[54]. IL28B overexpression in HepG2 cells induces ISGs that have been associated with the progression of HCV-related pathogenesis and antiviral activities against HCV[55]. Sugiyama *et al*[56] reported that the A (TA) dinucleotide repeat rs72258881 is associated with the transcriptional activity of IL28B. A functional polymorphism (rs4803217) in the 3′ untranslated region (UTR) of IL28B has been shown to influence the AU-rich element (ARE)-mediated decay (AMD) of IL28B mRNA and binding of HCV-induced microRNAs during infection[57]. At the present, we do not know the precise mechanisms between IL28B variants and treatment response to interferon. Additional studies investigating these mechanisms are needed.

DISTRIBUTION OF IL28B GENOTYPES IN JAPANESE PATIENTS INFECTED WITH HCV

Kobayashi *et al*[58] analyzed IL28B genotypes in 1518 Japanese patients infected with HCV and reported that TT at rs8099917 and CC at rs12979860 as IL28B major genotypes were detected in 77.7% and 76.8% of patients, respectively, and that TG/GG at rs8099917 and CT/TT at rs12979860 as IL28B minor genotypes were detected in 22.3% and 23.2% of patients, respectively. Although there are some discrepancies between these two sets of genotypes, the linkage disequilibrium between two IL28B polymorphisms at rs8099917 and rs12979860 is strong in Japanese HCV patients[58]. In 2010, Akkarathamrongsin *et al*[59] found that genotyping by both rs8103142 and rs11881222 indicated that 77.9% and 22.1% of the patients had the major and minor genotypes, respectively. In 2011, we also reported that TT and TG/GG at rs8099917 as IL28B major and minor genotypes, respectively, were detected in 65.6% and 34.4% of HCV-infected patients, respectively[38]. Kurosaki *et al*[60] reported that TT and TG/

GG at rs8099917 as IL28B major and minor genotypes, respectively, were detected in 69.6% and 30.4% of HCV genotype 1-infected patients, respectively.

Thomas *et al*[61] reported that HCV clearance was observed much more frequently than expected (53%) in the CC IL28B genotypes at rs12979860, although the proportion of individuals with CT/TT IL28B genotypes at rs12979860 who cleared the virus (28%) was similar to a general population expectation, because HCV clearance occurs in approximately 30% of HCV-infected patients. Approximately 65%-70% of Japanese patients infected with HCV had the IL28B major genotype. In 2011, telaprevir, a first-generation HCV NS3/4A protease inhibitor with peginterferon plus ribavirin was introduced as treatment for HCV genotype 1 infection in Japan[22,45], and in 2013, simeprevir, a second-generation HCV NS3/4A protease inhibitor with peginterferon plus ribavirin was also made available in Japan[27,62]. We next examined the current status of IL28B genotypes in Japanese patients infected with HCV.

CURRENT DISTRIBUTION OF IL28B GENOTYPES IN JAPANESE PATIENTS INFECTED WITH HCV

The IL28B genotype is a strong predictor of treatment response in HCV-infected patients treated with interferon-including regimens. We examined the current status of the IL28B genotype rs8099917 distribution of the outpatients infected with HCV. Blood samples were obtained from 432 HCV-infected outpatients (mean age: 59.9 years, male/female: 224/208, HCV genotypes 1/2/3/unknown: 314/102/1/15) in our hospital. The IL28B genotype at rs8099917 was determined by TaqMan SNP genotyping assay using the Step One real-time PCR system (Applied Biosystems, Foster City, CA, United States). Clinical backgrounds, including the present status of HCV RNA positivity, were also examined. Written informed consent was obtained from all patients, and the study protocol was approved by the Ethics Committee of Chiba University, School of Medicine (number 508). Some patients had been included in previous studies[38,42,54,63-66].

Of the 432 patients, 301 and 131 had the IL28B major and minor genotypes, respectively (Figure 1A), and 87.7% were treated at least once with an interferon-including regimen, resulting in 184 SVR, 184 non-SVR, and 64 untreated/others, respectively. Of the 314 patients with HCV genotype 1, 218 and 96 had the IL28B major and minor genotypes, respectively (Figure 1B), and 122, 143, and 49 patients had SVR, non-SVR/untreated, or other, respectively. Of the 143 patients with HCV genotype 1 with non-SVR or untreated, 85 and 58 had the IL28B major and minor genotypes, respectively, and 22 (25.9%) of the 85 patients with HCV genotype 1 and the IL28B major type are now interferon-intolerant or ineligible. Of the 118 patients with HCV genotype non-1, 83 and 35 had the IL28B major and minor genotypes, respectively, and 62, 41, and 14 patients had SVR, non-SVR/untreated, or other, respectively. In the 41 patients with HCV genotype non-1 with non-SVR or untreated, 27 and 14 had the IL28B major and minor genotypes, respectively (Figure 1C), and 10 (37%) of the 27 patients with HCV genotype 1 and the IL28B major type are now interferon-intolerant or ineligible. The distribution of IL28B genotypes is not significantly different between HCV genotype 1 and non-1 (P = 0.947; Figure 1B and C).

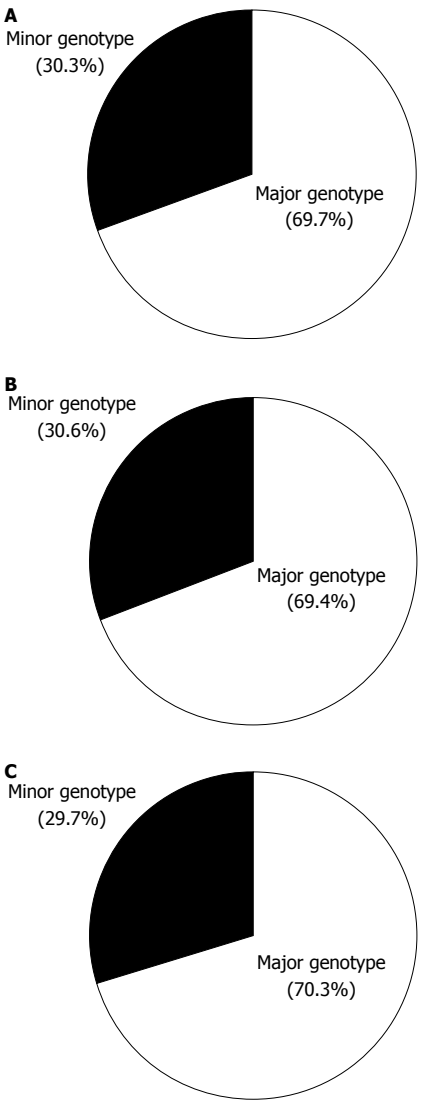

Figure 1 Distribution of interleukin-28B genotypes in Japanese patients infected with hepatitis C virus between February 2010 and April 2014. (A) Total patients (n = 432), (B) HCV genotype 1 patients (n = 314), and (C) HCV non-genotype 1 patients (n = 118). The white and black parts indicate the IL28B major and minor genotypes, respectively. IL28B: Interleukin-28B; HCV: Hepatitis C virus.

Thus, the patients infected with HCV genotypes 1 and non-1, who had IL28B minor genotypes in 40.6% (58/143) and 34.1% (14/41), respectively, should be treated. Further, some patients who had the IL28B major genotype are interferon-intolerant or ineligible. Regarding the current status of IL28B genotype rs8099917 distribution, we re-confirmed that the HCV-infected population in Japan should be treated with interferon-free regimens,

although interferon-including regimens may be effective in certain patients. The rs8099917 TT genotype may be significantly independently predictive of rapid virologic response, which is the single best predictor of SVR, in Asian HCV genotype patients[67].

CONCLUSION

In Japan, interferon-free 24-wk regimens of asunaprevir, a HCV NS3/4A inhibitor, and daclatasvir, a HCV NS5A inhibitor, can now be used for HCV genotype 1b-infected patients who are interferon-intolerant or ineligible, or previous-treatment null-responders[68-70]. In the near future, interferon-free 12-wk regimens of sofosbuvir plus ribavirin for HCV genotype 2-infected patients will be available[71]. Interferon-free 12-wk regimens of sofosbuvir, a HCV NS5B nucleotide polymerase inhibitor, and ledipasvir, a HCV NS5A inhibitor, for HCV genotype 1-infected patients will also be available[72]. The response to the treatment with interferon-free regimens appears to have no association with IL28B genotypes. In conclusion, although some HCV-infected individuals have IL28B favorable alleles, importance of IL28B will be reduced with availability of oral interferon free regimen.

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EDITORIAL

Middle-East respiratory syndrome coronavirus: Is it worth a world panic?

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Abstract

In 2012 Middle-East respiratory syndrome coronavirus (MERS-CoV) was evolved in the Arabian Peninsula. Tremendous and successful efforts have been conducted to discover the genome structure, epidemiology, clinical signs, pathogenesis, diagnosis and antiviral therapy. *Taphozous perforatus* bats are the incriminated reservoir host and camels are the currently confirmed animal linker. The virus resulted in less than 1000 infected cases and 355 deaths. The case fatality rate of the MERS-CoV is high, however, many survivors of MERS-CoV infection showed inapparent infections and, in several cases, multiple co-infecting agents did exist. Although MERS-CoV appears to be a dangerous disease, it is argued here that a full assessment of current knowledge about the disease does not suggest that it is a truly scary killer.

Key words: Coronavirus; Camels; Disease threat to humans; Middle-East respiratory syndrome coronavirus; Mortality rate

Core tip: Middle-East respiratory syndrome coronavirus (MERS-CoV) emerged as a novel human coronavirus in 2012. Although it induces a high level of case fatality, fatal infections were recorded mainly in immune compromised patients and co-infections were frequently recorded. Camels are the currently known natural animal host and are susceptible to mild non-fatal infections. There is a growing evidence that the virus has been circulating in camels for decades in the Middle East, Africa and possibly other areas where camel herds are present. The fact that the virus has existed for decades, together with the absence of large-scale human mortalities from unknown respiratory infections, gives a first indication that MERS-CoV is not a particularly dangerous virus.

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As of March 31, 2015, 1102 laboratory-confirmed cases of Middle-East respiratory syndrome coronavirus (MERS-CoV) infection have been reported by WHO with the case fatality rate reaching 37.7% (416/1102). Most cases (973/1102; 88%) were reported in Saudi Arabia and other countries of the Arabian Peninsula. Cases reported outside the Arabian Peninsula have been reported to have a direct or indirect link to the Arabian Peninsula, mostly through recent travel. MERS-CoV belongs to the C lineage of the genus Betacoronavirus. The *Taphozous perforatus* is a probable natural reservoir of MERS-CoV. Bat guano, saliva and/or urine are assumed to contaminate food and water resources of animals in areas with palm orchards and this may constitute an indirect source of transmission to camels, people and possibly to other animals. Dipeptidyl peptidase 4 (DPP4) (or so-called CD26) has been proved to be the functional receptor for MERS-CoV reviewed in[1].

Although all ages are affected, the most severe cases of MERS-CoV infection have generally been recorded in aged patients with underlying conditions. Mild and asymptomatic cases have also increased recently[2]. Co-infection with other pathogens including influenza A, parainfluenza, herpes simplex and pneumococcus was reported[3]. The clinical epidemiology of MERS-CoV cases has some similarities to human seasonal flu. Like seasonal flu, MERS-CoV infections resulted in respiratory illness in the majority of patients, with the disease affecting all ages, but being most severe in the elderly and immuno-compromised people. The fact that approximately half of the lethal cases of MERS-CoV involved mixed infections and/or immuno-compromised patients, and the fact that many subclinical and inapparent infections have been reported, suggest that the virus may not lead to catastrophic consequences.

Additionally, the annual mortalities associated with MERS-CoV are surprisingly lower than many other viruses that induce acute viral infections, *e.g.*, seasonal influenza, 250000 to 500000 deaths[4]; rotavirus, 453000 child deaths[5]; measles, 145700 deaths[4]; rabies, 55000 deaths[6]; yellow fever virus, 30000 deaths (mostly in Africa)[4]; dengue fever virus, 25000 deaths mostly among children[7]; respiratory syncytial virus (RSV), 66000 to 199000 deaths in children less than 5 years old[8]; Lassa fever, 5000 deaths (in West Africa)[9].

It is worth mentioning that MERS-CoV has been isolated from camels in Saudi Arabia, Qatar, Oman, UAE and Egypt and the presence of MERS-CoV antibodies have been reported in camels from Saudi Arabia, Qatar, Oman, UAE, Egypt, Kenya, Nigeria, Ethiopia and the Canary Islands-Spain. Sera from sheep, goats, cattle, buffaloes, pigs, chicken and wild birds have all been found to be negative for MERS-CoV. It is interesting to note that the virus is prone to replicate efficiently in primate, pig and goat cell cultures, a finding which necessitates the screening of large numbers of these types of animals[1]. Overall, however, people in direct contact with camels are at most risk of contracting MERS-CoV infection, other possible indirect contact includes the consumption of of unpasteurized camel milk, even though only a few primary cases of MERS-CoV can be linked to an established direct contact with camels. The detection of MERS-CoV in camel serum samples archived over a period of decades is reassuring. MERS-CoV positive sera has been found in samples from 1983 in Somalia and Sudan[10], from 1993 in Saudi Arabia[11], from 1997 in Egypt[10], from 1996 in Kenya[12] and from 2003 in the UAE[13]. These findings imply that the MERS-CoV has been circulating for at least 31 years in the Horn of Africa countries and for at least 21 years in Saudi Arabia, without causing large-scale fatalities in humans. This conclusion can be further supported since, even in the absence of knowledge about MERS-CoV there have not been hundreds or thousands of cases of patients dying from unknown respiratory infections during this period. There is some hope, therefore that MERS-CoV may not be as a dangerous virus as was first feared. The ancestral virus strain might has been experienced mutations during this long period, which have rendered it able to cross the species barrier. It is not clear, however, whether the currently circulating virus strains have acquired additional mutations which have render them able to be easily transmissible from human to human.

Most viral diseases that affect cloven-hooved animals appear to be less virulent to camels, which typically develop only inapparent or mild clinical signs. This may be due to the camels' robust immune system. It is also worth mentioning that coronavirus infection in camels leads to mild respiratory symptoms that may reflect restricted virus proliferation and consequently low virus shedding. The possibility that there is another animal linker needs to be investigated. Taking into consideration that palm dates are consumed extensively in the Arabian Peninsula, the role of an animal linker that may harbour the MERS-CoV and contaminate palm dates needs to be investigated.

A small cohort serosurvey that was conducted in Saudi Arabia did not report MERS-CoV antibodies in slaughterhouse workers who were in close contact with camels, sheep, goat and cattle[14]. Further large scale MERS-CoV serosurveys in a range of populations such as those who have no contact with animals, health-care workers, people with close contact with camels in countries where camels are bred and traded, especially in the Arabian Peninsula, Eastern Africa and Asia, are needed to explore the exact morbidity rate of MERS-CoV.

Coronaviruses are continuously evolving, but major genetic differences have not yet been recorded among human cases. Nonetheless, elucidation of the genetic diversity of MERS-CoV strains from camels in Africa, and other parts of the world where camels are found, should be undertaken as a matter of urgency. MERS-CoV is assumed currently to constitute only a mild to moderate

risk to human health, but it remains important not to underestimate the potential risk of the virus.

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REVIEW

Prion-induced neurotoxicity: Possible role for cell cycle activity and DNA damage response

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Abstract

Protein misfolding neurodegenerative diseases arise through neurotoxicity induced by aggregation of host proteins. These conditions include Alzheimer's disease, Huntington's disease, Parkinson's disease, motor neuron disease, tauopathies and prion diseases. Collectively, these conditions are a challenge to society because of the increasing aged population and through the real threat to human food security by animal prion diseases. It is therefore important to understand the cellular and molecular mechanisms that underlie protein misfolding-induced neurotoxicity as this will form the basis for designing strategies to alleviate their burden. Prion diseases are an important paradigm for neurodegenerative conditions in general since several of these maladies have now been shown to display prion-like phenomena. Increasingly, cell cycle activity and the DNA damage response are recognised as cellular events that participate in the neurotoxic process of various neurodegenerative diseases, and their associated animal models, which suggests they are truly involved in the pathogenic process and are not merely epiphenomena. Here we review the role of cell cycle activity and the DNA damage response in neurodegeneration associated with protein misfolding diseases, and suggest that these events contribute towards prion-induced neurotoxicity. In doing so, we highlight PrP transgenic *Drosophila* as a tractable model for the genetic analysis of transmissible mammalian prion disease.

Key words: Neurodegenerative disease; Protein misfolding; Prion; Transmissible; Cell cycle; DNA repair; Chromatin; PrP transgenic *Drosophila*

Core tip: It is important to understand the cellular and molecular mechanisms of protein misfolding-induced neurotoxicity in order to combat conditions such as Alzheimer's, Huntington's, Parkinson's, and motor neuron disease, tauopathies and prion diseases. Here, we review the role of cell cycle activity and the DNA damage

response in neurodegeneration associated with protein misfolding diseases, including prion diseases. In doing so, we highlight PrP transgenic *Drosophila* as a tractable model of transmissible mammalian prion disease. Our review provides a new impetus to the study of prion diseases, which are increasingly seen as an important paradigm for neurodegenerative conditions in general.

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INTRODUCTION

While many diseases can cause degeneration of nervous system tissue, including human immunodeficiency virus infection and acquired immune deficiency syndrome, multiple sclerosis or rabies, the designation of protein misfolding neurodegenerative disease is typically assigned to those induced by aberrant folding and aggregation of disease-specific host proteins. These conditions, which include Alzheimer's disease, Huntington's disease, Parkinson's disease, motor neuron disease, tauopathies and prion diseases, are invariably fatal as there are no known treatments[1,2]. Each of these conditions is characterised by the misfolding of a disease-specific protein[3] and accumulation of misfolded protein in the brain is central to the pathological process that typically manifests as synaptic loss, neuronal dysfunction, with resultant clinical symptoms. Prion diseases include scrapie of sheep, bovine spongiform encephalopathy (BSE) of cattle, together with Creutzfeldt-Jakob disease (CJD) and fatal familial insomnia (FFI) in humans[4]. Prion diseases are an important paradigm for protein misfolding neurodegenerative conditions in general since Alzheimer's, Huntington's, Parkinson's and motor neuron disease, as well as tauopathies all possess features of prion-like transmission in experimental settings, evidenced by transcellular spread of misfolded disease-specific protein[5]. However, prion diseases are unique since they are transmissible between individuals of the same and different species, that sometimes occurs unintentionally. Protein misfolding neurodegenerative diseases typically cause clinical disease late in life and are therefore a major concern to society because of the increasing size of the ageing population. In addition, prion diseases are a significant concern to food security since they occur in animals destined for human consumption. Understanding the mechanism of neurotoxicity induced by protein misfolding will allow the design of strategies to alleviate the burden of these conditions.

Many aspects of prion-induced neurotoxicity remain incompletely understood. During prion diseases the normal host protein PrPC is converted into the abnormal form, PrPSc, the transmissible prion agent[4,6] (Figure 1). This conversion event appears to be an essential requirement

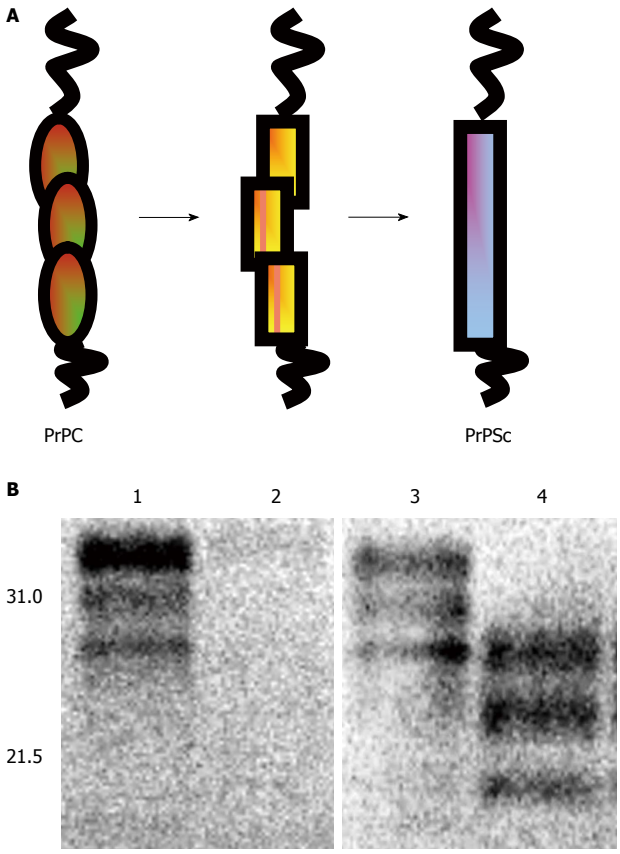

Figure 1 Conversion of PrPC into PrPSc. A: Schematic diagram of the conversion of PrPC into PrPSc. A major structural event occurs in the C-terminal domain of PrPC as it converts from a predominantly α-helical form into one enriched for β-sheet. This conformational change may involve the formation of intermediate structures of the protein; B: Western blot detection of ovine PrP. VRQ/VRQ sheep brain homogenate from animals that were scrapie-free (tracks 1 and 2) or scrapie-infected (tracks 3 and 4) were pre-treated with (tracks 2 and 4) or without (tracks 1 and 3) PK at 32 μg/mL at 37 ℃ for 30 min and the products analysed by SDS/PAGE, and Western blot probed with anti-PrP monoclonal antibody 683. Molecular weight markers (in kDa) are shown on the left (Reproduced by kind permission of CAB Reviews).

for prion disease neurotoxicity, evidenced by the failure of exogenous PrPSc to cause pathology in brain tissue devoid of PrPC[7,8] and the reversal of neurodegeneration when PrPC expression is ablated during prion infection[9-11]. The essential requirement for PrP expression in prion-induced neurotoxicity may suggest that an intermediate in the conversion of PrPC to PrPSc is the neurotoxic agent[12,13]. Alternatively, neurotoxicity may result from an interference with the normal biosynthesis and metabolism of PrPC mediated by the presence of PrPSc[14]. For example, PrP can accumulate in the cytosol in a misfolded form when proteasomal activity is compromised[15,16] and cytosolic PrP has been reported to be neurotoxic in some neurons[17-20]. A feature of prion-induced neurotoxicity is its effect on protein synthesis. For example, it has been shown that accumulation of PrPSc in cells[21] and mice[22] with an ongoing prion infection triggers over-activation of the PERK/eIF2a branch of the unfolded protein response. This in turn leads to persistently high levels of phosphorylated eIF2a and consequently a block of protein translation. Pharmacological inhibition of PERK can reverse the prion disease-induced

block in protein synthesis and alleviate this toxic phenotype despite the continued accumulation of PrPSc[23].

The value of these discoveries would be amplified by a more complete understanding of the sequence of cellular events that occur during the early stages of prion disease. This applies particularly to those acting prior to the onset of, and which may lead to, inhibition of protein synthesis. Knowledge in this area will be of fundamental importance to the understanding of prion biology *per se* and facilitate the search for early acting genetic modifiers of the neurotoxic process associated with these conditions. Interestingly, a number of reports have documented cell cycle activity and the DNA damage response (DDR) in post mitotic, terminally differentiated neurons during various neurodegenerative diseases[24-27], which represent potential candidates for such early acting pathways. This appears paradoxical since these are events traditionally associated with dividing cells. Here we discuss a potential role of cell cycle activity and DDR in prion-induced neurotoxicity. In support of this viewpoint, we present a novel *Drosophila* model of transmissible mammalian prion disease that provides a new animal system to study protein misfolding disease, one that combines the robust tools of experimental prion disease and fly genetics.

DDR

During the cell cycle, proliferating cells replicate their DNA and undergo division. This process is a highly organised series of cellular events that are tightly coordinated through the phase-specific expression of positive and negative regulatory proteins. Various quality control checkpoints operate to ensure faithful progression through the cell cycle. In addition to DNA replication errors, all cells whether proliferating or not, are constantly exposed to stimuli that can induce damage to DNA. These genotoxic stimuli may arise from exogenous events such as exposure to irradiation or carcinogens, or alternatively from endogenous events such as intracellular metabolism and associated reactive oxygen species (ROS)[28-30].

DNA damage in metazoan cells is deleterious: it may initiate mutagenesis or chromosomal re-arrangements that result in de-regulated cell cycle activity and neoplasia, or aberrant gene expression concomitant with cellular dysfunction and senescence or cell death[31-33]. In order to avoid these hazardous effects, cells have evolved a variety of molecular mechanisms for the repair of DNA damage[34]. For example, base excision repair (BER) is used to correct oxidative lesions[35] while nucleotide excision repair (NER) can excise UV light-induced thymidine dimers[36]. Single strand breaks (SSBs) in DNA, which may occur through ROS-mediated lesions or intermediates in BER, are repaired by polB and various ligases[37]. Double strand breaks (DSBs), that can arise through failures in DNA transcription or replication, are repaired by two different mechanisms: non-homologous end joining (NHEJ), which is error prone, or homologous recombination (HR), which is error-free but is restricted to the S/G_2 phase of the cell cycle in dividing cells[38]. DSBs in DNA arise relatively infrequently, though are particularly hazardous as they can induce a significant loss of genomic integrity[39].

The maintenance of genome integrity is critical to organismal function and survival. As a consequence, cells co-ordinate an elaborate set of mechanisms that function in the surveillance and repair of DNA lesions with cell cycle progression. These integrated pathways are collectively referred to as the DNA damage response (DDR). In proliferating cells, checkpoint control mechanisms mediate cell cycle arrest to allow DNA repair when damage is detected, although senescence or apoptosis may ensue in the case of extensive lesions[40-42]. In contrast, post mitotic terminally differentiated neurons appear to display a lower capacity for DNA repair than proliferating cells, and they are thought to accumulate and tolerate comparatively high levels of DNA damage, since they are unable to replace damaged cells by division[43,44]. However, increasing evidence suggests that cell cycle activity and DDR are features of post mitotic neurons in neurodegenerative conditions[25-27,45,46]. For example, post mitotic neurons, when exposed to genotoxic stimuli, can replicate DNA and initiate apoptosis associated with cell cycle activation[47]. In addition, evidence of cell cycle activity and DNA damage can be found in natural and experimental hosts undergoing protein misfolding diseases, such as Alzheimer's disease[48-51]; amyotrophic lateral sclerosis[52,53]; Huntington's disease[54,55] and Parkinson's disease[56-58].

THE CONTRIBUTION OF DNA DAMAGE AND DDR TO NEUROTOXICITY

Neurons like all other cell types are subject to a variety of stimuli that can potentially induce deleterious DNA damage. In dividing cells DNA damage activates cell cycle arrest concomitant with DDR so that the integrity of the cellular genome is maintained between successive generations. A major cell cycle checkpoint control operates at the G_2/M interface to allow for DNA damaged during replication to be repaired prior to mitosis. Since post mitotic neurons are unable to divide, the expression of cell cycle associated genes in these cells may promote the DDR and facilitate access to DNA for repair in order to maintain genome integrity and appropriate regulation of gene expression. An emerging view is that structural modulation of chromatin associated with these processes, together with genome integrity, have a major influence on the neurotoxic process in post mitotic neurons during neurodegenerative disease[27,59]. In this context, important unanswered questions include: Do the same processes and events also occur in protein misfolding neurodegenerative diseases? And if so, what precisely are the molecular mechanisms that confer neurotoxicity and that culminate in neuronal dysfunction and neurodegeneration?

Chromatin is a repeat structure of nuclear DNA and histone proteins with nucleosomes representing the fundamental core unit[60,61]. The structure of chromatin

is strongly influenced by post translational modifications of the histone proteins through the addition of various chemical groupings including phosphate, acetyl or methyl moieties[62]. In addition, sequence variants of core histone proteins (*e.g.*, H2A.X) exist that further enhance chromatin structural diversity[63]. Chemical modification of histones, or the inclusion of their sequence variants, influence nucleosome-DNA or inter-nucleosome interactions and thereby regulate the degree of chromatin compaction and consequentially DNA transcriptional activity. Heterochromatin is relatively compacted and transcriptionally silent, whereas euchromatin is a more relaxed and open structure that is permissive for gene activation[64-67]. Chromatin structure and its modulation are therefore fundamental features in the maintenance of DNA integrity and regulation of gene expression.

DNA contained in compacted chromatin is relatively well protected from genotoxic stimuli and is typically inaccessible to transcription and DDR machinery. During DDR, chromatin undergoes transient dis-aggregation at the sites of DNA lesion to facilitate access of repair and cell cycle checkpoint proteins[68-70]. In some cases of DNA repair, chromatin modulation may be quite extensive and extend over several kilobases[71]. Since open chromatin is evident in regions of actively transcribed DNA, heterochromatin relaxation in response to DDR can trigger aberrant gene expression of normally silenced regions of the genome. Indeed, it has been shown that wide spread loss of heterochromatin occurs in *Drosophila* and mouse tauopathy models (*tau* transgenics), and human Alzheimer's disease, and that this is associated with aberrant gene expression in CNS neurons[72]. Conversely, genetic rescue of *tau*-induced heterochromatin loss substantially reduced *tau*-induced neurodegeneration in *Drosophila*. It has been postulated that post mitotic neurons undergoing DDR and associated changes in chromatin organisation, may have the potential to revert to a de-differentiated state, and that this might be linked to activation of apoptotic pathways[73,74]. Mechanistically, oxidative stress and subsequent DNA damage were identified as causes of heterochromatin loss in *tau* neurotoxicity[72]. These studies suggest an etiological progression from neurotoxic stimuli to chromatin-mediated gene regulation and subsequent neurodegeneration.

General instability of the cellular genome, as a consequence of damage to mitochondrial or nuclear DNA, or to chromatin, is also a potential cause of neurotoxicity[75]. Since post mitotic terminally differentiated neurons are unable to divide, these cells are forced to endure genotoxic insults. However, if the level of DNA damage exceeds the capacity of the DDR, or if DDR function is compromised, mutations and incorrect repair may lead to inappropriate DNA metabolism and, de-regulated gene expression or harmful mutations[32]. This view is supported by the correlation between neurodegeneration and sensitivity to DNA damage and/or DDR deficiencies[76-81]. DNA damage that compromises mitochondrial function could lead to disturbances in the cellular energy balance and have a detrimental effect on neuronal function including synaptic defects, as occurs in various inherited neurological disorders[82]. Since the brain has a high metabolic activity neurons are thought to be particularly prone to oxidative stress, a recognised cause for DNA damage. Oxidative stress and mitochondrial dysfunction are increasingly implicated in protein misfolding-induced neurodegeneration although the molecular events of this association have not yet been defined[83]. Mitochondria are the principal source of cellular ROS and mitochondrial DNA is particularly sensitive to ROS-mediated damage[84]. The mutation rate of mitochondrial DNA, which lacks histone proteins, is > 15 fold higher than that of nuclear DNA[85]. Mutations in mitochondrial DNA can perturb the expression and function of oxidative phosphorylation complexes and thereby precipitate mitochondrial dysfunction, which in turn may lead to accelerated ROS generation[86,87].

Many studies have shown that ageing, a major risk factor for neurodegenerative disease, is associated with an accumulation of DNA lesions in the mature brain. DNA lesions may additionally arise from an age-dependent reduction in DNA repair capacity[88] and contribute to a reduction in genome integrity[43,89]. These DNA lesions, which are envisaged to occur in individual neurons, may result in the expression of mutant proteins that either fold or traffic incorrectly. This will result in an increasing demand on the cellular protein quality control machinery that functions to detect and triage these molecules, a situation already exacerbated in the case of protein misfolding diseases. In this situation, activation of the unfolded protein response may occur in order to attempt to maintain protein homeostasis[21,22]. The effects of aberrant misfolded protein accumulation that arise in protein misfolding diseases presumably enhance DNA damage and accelerate the loss of genome integrity and thereby promote the onset of neurodegenerative disease.

CELL CYCLE-ASSOCIATED PROTEINS WITH A ROLE AT THE SYNAPSE

Mature nerve cells are derived from neural progenitors that undergo proliferation, exit the cell cycle and mature into terminally differentiated neurons. Under normal circumstances, post mitotic neurons do not participate in any further cell cycle activity. Any attempt by post mitotic neurons to undergo cell cycle re-entry is considered to be detrimental to these cells. However, it has become evident that terminally differentiated neurons express a variety of proteins with important roles in cell cycle regulation that have a normal function in diverse post mitotic neuronal events under physiological conditions[90]. Significantly, some of these cell cycle-associated proteins localise to synapses in post mitotic neurons. For example, the Orc2-5 core subunits of the origin of recognition complex (Orc), which is key to initiating DNA replication, are highly expressed in differentiated

mammalian neurons. Orc3 and Orc5 are enriched in the postsynaptic dendritic compartment, and regulate the dendritic filopodia and spine formation[91]. The anaphase-promoting complex/cyclosome (APC/C), an E3 ubiquitin ligase, locates to both pre- and postsynaptic sites in post mitotic neurons, regulating synaptic terminal growth and differentiation as well as synapse formation and function (reviewed in[92]). Other cell cycle associated gene products implicated in regulating synaptic function include the PI3Kinase family member ataxia telangiectasia mutated (ATM), which in post mitotic neurons associates with synaptic vesicle proteins[93], and Cyclin E that acts as a repressor of the synaptic regulator Cdk5[94].

While it is accepted that these various proteins, initially discovered as central to cell division, can have additional roles in post mitotic cells, it remains unclear whether dysregulation of their expression or function is linked to neurotoxicity and cell death in protein misfolding neurodegenerative diseases[24]. One suggestion has been that synaptic loss early in neurodegenerative conditions, results in upregulation of cell cycle-associated gene expression in a bid to maintain synaptic function and plasticity, but that this might lead to inappropriate action of these proteins in the nucleus, promoting neuronal dedifferentiation and apoptosis[24]. For example, shuttling of Cdk5 from the nucleus to cytoplasm has been postulated as critical for the breakdown of the post mitotic state in neurons[95]. Alternatively, it is conceivable that dysregulation of cell cycle-associated proteins at the synapse and concomitant sub-optimal synaptic communication may lead to increased metabolism as neurons struggle to remain within their homeostatic activity range. This in turn could lead to increased production of ROS, with an ensuing cycle of genotoxicity and associated dysregulation of gene expression.

CELL CYCLE ACTIVITY AND DDR IN PRION-INDUCED NEUROTOXICITY?

It is not yet established whether cell cycle activity and DDR are features of prion-mediated neurotoxicity. Evidence this might be the case derives from observations of mammalian models of prion disease. For example, nuclear accumulation of proliferating cell nuclear antigen (PCNA) and phosphorylated histone H2A.X proteins, which in other cell types are indicative of DNA replication and/or repair, have been detected in CNS neurons of mice that model familial CJD and FFI prion diseases[96]. In addition, the brains of scrapie-affected hamsters show evidence of cell cycle activity with an increase in the proteins polo-like kinase (PLK) 1 and cyclin B1, and a decrease of PLK3 and Cdc25C[97]. PLKs, which function as key regulators of the cell cycle and its checkpoint response to genotoxic stress, are regulated by synaptic activity in post mitotic neurons[98]. Prion infectivity experiments *in vivo* have shown that mice deficient in BER activity displayed an accelerated clinical course of prion disease as compared to wild type animals[99]. These various animal models of prion disease are supportive of the view that DNA damage plays a pivotal role in prion-induced neurotoxicity. It will be important to verify this is the case, in order to determine the extent of commonality in the mechanism(s) of neurotoxicity between different neurodegenerative conditions and prion diseases. This is underlined by the fact that bona fide prion diseases are seen as important paradigms for other protein misfolding diseases, and common underlying mechanisms would suggest the possibility of common therapeutic strategies for these presently invariably fatal diseases. However, prion diseases are difficult to study in their natural hosts, such as ruminants and humans, because these diseases can take many years to develop, resulting in progress being slow and cumbersome[4]. In addition, the natural forms of prion diseases tend to occur in outbred populations that render genetic analysis of complex biochemical pathways difficult. Even in the more tractable experimental system of mouse models, the significant expenses of time and husbandry restrict the scope of genetic experimentation for dissection of prion disease mechanisms.

A *DROSOPHILA* MODEL OF TRANSMISSIBLE MAMMALIAN PRION DISEASE

In order to circumvent the difficulties associated with the genetic analysis of prion diseases in their natural hosts, we have established *Drosophila* as a new tractable animal model of transmissible mammalian prion disease. Importantly, because of the high evolutionary conservation of most cellular signaling pathways and processes, our *Drosophila* model system allows exploitation of the power of fly genetics to probe the mechanisms of prion-induced neurotoxicity.

We have used pUAST/PhiC31-mediated site-directed germ line transformation to generate *Drosophila* transgenic for topological and polymorphic variants of ovine PrP under expression control of the bipartite *UAS-GAL4* system[100-102]. The topological variants of ovine PrP were targeted to the plasma membrane, to the cytosol, or for secretion. Site-specific PCR using genomic DNA from ovine PrP transgenic flies as substrate, together with DNA sequence analysis, was used to confirm that a single copy of each PrP transgene had been inserted at a single site in the genome of each appropriate fly line. Expression control of ovine PrP in *Drosophila via* the *UAS-GAL4* system allowed the prion protein to be targeted to defined cell populations during a specific period of development and ageing. For example, *UAS*-ovine PrP flies crossed with the *elav-GAL4* driver fly line achieves efficient expression of cell-surface anchored ovine prion protein in all neurons of *Drosophila*[100,102].

Our *Drosophila* model allowed us to test the hypothesis that exogenous ovine prions can induce toxicity in flies transgenic for ovine PrP. Remarkably, adult *Drosophila*, which express ovine PrP pan neuronally and that are

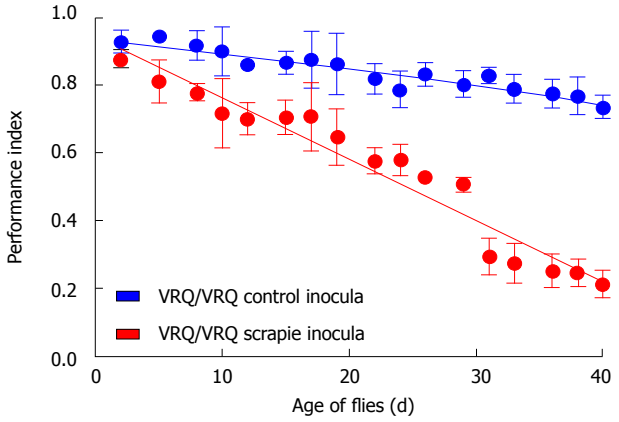

Figure 2 Prion-exposed ovine PrP transgenic *Drosophila* show enhanced locomotor defect. *Drosophila* with pan neuronal expression of ovine VRQ(cyt) were fed VRQ/VRQ scrapie-free (blue circles, blue line) or scrapie-infected (red circles, red line) sheep brain homogenate at the larval stage of development. The locomotor activity of adult flies was assessed by a negative geotaxis climbing assay. The performance index is shown for each genotype of fly per time point (Reproduced with permission, from Thackray *et al*[100] 2014. © the Biochemical Society).

exposed to ovine prions at the larval stage, show a neurotoxic phenotype as compared to control non-transgenic flies that have been similarly exposed to prion inocula. The prion-induced neurotoxicity in PrP transgenic *Drosophila* is evidenced by an accelerated decline in locomotor activity[100,101,103] (Figure 2). In addition, we have used protein misfolding cyclic amplification (PMCA) to show that this prion-induced phenotype is accompanied by accumulation of proteinase K (PK)-resistant PrPSc in fly brains[100]. The presence of PrPSc is a pathogonomic feature of prion diseases. However, the most sensitive hallmark of transmissible prion diseases, is the transmission of these conditions to new hosts, since in some prion-infected hosts, neuropathology can develop in the apparent absence of PrPSc and conversely, PrPSc can accumulate in the absence of neuropathology[4,104]. Importantly therefore, we have demonstrated that the prion-induced fly phenotype is transmissible to PrP transgenic *Drosophila*[100,101,103]. In mammalian hosts, prion-mediated toxicity has been shown to be inextricably linked to prion replication[4,12,105] and these two events only occur in PrP expressing hosts. In our experiments, scrapie-infected sheep brain material did not induce toxicity in control non-PrP transgenic flies, and head homogenate from these prion-exposed control flies did not transmit any toxicity to fresh PrP transgenic recipient flies. Collectively, these data are consistent with the formation of transmissible prions in *Drosophila* transgenic for PrP expression. Furthermore, while the conversion of PrPC to PrPSc has been reported to occur either at the cell surface or within the endocytic pathway[106-108], our novel studies in *Drosophila* show that PrP targeted to the plasma membrane, to the cytosol, or for secretion, can participate in the generation of prion-induced toxicity.

Our observations validate PrP transgenic *Drosophila* as a new animal model to study the mechanisms of prion-induced neurotoxicity. One of the key benefits of this model system is its rapid and highly reproducible progression to symptomatic stages. This opens the door to a detailed cellular and molecular analysis of the sequence of changes that occur from immediately after infection until symptoms of neurotoxicity become overt. To this end we have performed a functional genomic analysis of prion-infected *Drosophila* transgenic for ovine PrP, membrane bound by a glycosylphosphatidyl-inositol (GPI) anchor in order to search for biochemical pathways and genetic modifiers of prion-induced neurotoxicity[109]. Our preliminary RNA-Seq-based analysis has revealed that during the early phase of prion infection in PrP transgenic *Drosophila*, the expression of genes associated with cell cycle re-entry and DNA damage repair were up-regulated in the fly brain. This observation is indicative of cell cycle activity and DDR in the early phase of prion-induced neurotoxicity. Significantly, during the early phase of prion infection in our fly model, cell cycle activation genes (*e.g.*, PCNA) and double-stranded DNA repair genes (*e.g.*, H2Av) are up-regulated, as also seen in brains of prion-diseased mice[96]. Importantly, we found that this response precedes a dramatic down-regulation of genes associated with protein synthesis, including those involved with eIF2a and mTOR pathways. These are interesting observations in light of the reports of translational defects in prion-infected mice[22]. Our novel observations show that prion infection in *Drosophila* has the potential to re-capitulate prion-induced events in mammalian hosts. Our data further suggest that cell cycle re-entry and inhibition of protein synthesis are temporally linked events in prion-induced neurotoxicity. In this context our hypothesis (Figure 3) is that neurotoxicity in post-mitotic neurons, stressed by prion replication, arises through aberrant cell cycle re-entry that contributes to the effect of sustained inhibition of protein synthesis and eventual neuronal dysfunction.

CONCLUSION

Prion diseases are an important paradigm for protein misfolding neurodegenerative diseases. It is important to establish the sequence and causal links of cellular events that underlie prion-induced neurotoxicity. This will help determine how protein misfolding and aggregation causes neurotoxicity and how this devastating process may be alleviated. Emerging evidence suggests that cell cycle activity and the DNA damage response are cellular processes that may be involved in prion-induced neurodegeneration, as appears to be the case in other neurodegenerative diseases. With the power of *Drosophila* genetics now in play, many important questions can be systematically addressed. Important questions to be answered include what is the temporal order of the cellular events that are responsible for the progression of prion-induced neurotoxicity. In addition, what is the relationship between the accumulation of cell-cycle related proteins in prion-infected post mitotic neurons, the suppression of translation and resultant neurotoxicity? Future research

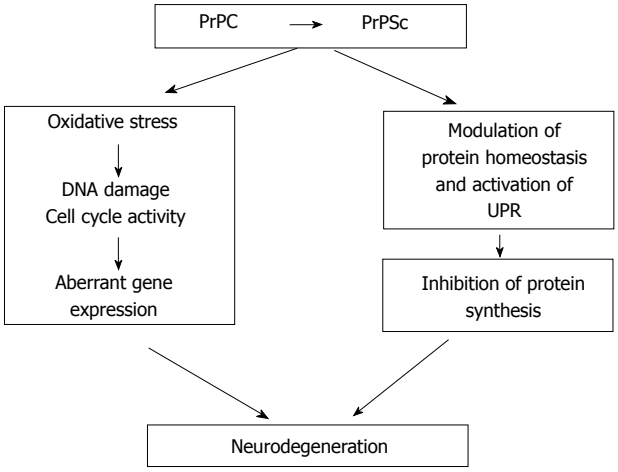

Figure 3 Hypothetical model for prion-induced neurodegeneration. The conversion of PrPC into PrPSc is an essential requirement for the neurotoxicity that occurs during prion disease. Neurodegeneration in post mitotic neurons, stressed by prion replication, may arise through various cellular events including aberrant cell cycle re-entry and sustained inhibition of protein synthesis. These two processes may operate in parallel or may potentially represent temporally linked events. In both cases, aberrant cell cycle re-entry may contribute to the effect of sustained inhibition of protein synthesis evident in prion-induced neurotoxicity.

in this area will be enhanced by the use of a *Drosophila* model of transmissible mammalian prion disease.

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REVIEW

Pharmacogenetics as a tool to tailor antiretroviral therapy: A review

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Abstract

Highly active antiretroviral therapy (HAART) has substantially changed human immunodeficiency virus (HIV) infection from an inexorably fatal condition into a chronic disease with a longer life expectancy. This means that HIV patients should receive antiretroviral drugs lifelong, and the problems concerning with a chronic treatment (tolerability, side effects, adherence to treatment) have now become dominant. In this context, strategies for the treatment personalization have taken a central role in optimizing the therapeutic response and prevention of adverse drug reactions. In this setting, the study of pharmacogenetics features could be a very useful tool in clinical practice; moreover, nowadays the study of genetic profiles allows optimizations in the therapeutic management of People Living With HIV (PLWH) through the use of test introduced into clinical practice and approved by international guidelines for the adverse effects prevention such as the genetic test HLA-B*5701 to detect hypersensitivity to Abacavir. For other tests further studies are needed: CYP2B6 516 G > T testing may be able to identify patients at higher risk of Central Nervous System side effects following standard dosing of Efavirenz, UGT1A1*28 testing before initiation of antiretroviral therapy containing Atazanavir may aid in identifying individuals at risk of hyperbilirubinaemia. Pharmacogenetics represents a research area with great growth potential which may be useful to guide the rational use of antiretrovirals.

Key words: Pharmacogenetics; Pharmacogenomics; Single nucleotide polymorphism; Pharmacokinetics; Highly active antiretroviral therapy; Polymorphism; Phenotype; Pharmacodynamic

Core tip: The wide availability of drugs and therapeutic regimens for the human immunodeficiency virus infection treatment and the presence of associated adverse effects related to interindividual variability leads the clinician to look for an individualized therapy as much as possible. Pharmacogenetics can provide useful tools for this purpose and can propose models of genetics tests that, however, need to be further studied. This paper aim is to provide a critical and understandable review of published literature and a guidance about future

prospects in this field.

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INTRODUCTION

The concept of personalized therapy is of increasing interest in the management of antiretroviral therapy and PLWH [people living with *human immunodeficiency virus* (HIV)], especially now, because we must confront with a chronic therapy that can count on a large number of possible combinations, but also on a number of individual issues of effectiveness, toxicity, tolerability and convenience. Pharmacogenomics, as well as the need for specific diagnostic tests for therapy customization, drug-drug interactions and TDM (Therapeutic Drug Monitoring) are emerging topics that, in a long-term management of HIV infection, will need to be explored during the doctor-patient interview.

Pharmacogenetics deals with the role of genes in response to drugs and it is well known that there is a variability between individuals in drugs response due to hereditary genetic factors.

The purpose of pharmacogenetics is to identify candidate genes, define the differences in the candidate genes among individuals, and to correlate the phenotype's changes - defined by a specific drug response - with the patient's genotype.

These studies, using technologies of high capacity for DNA analysis (such as DNA microarrays or DNA chips), have been extended, more recently, to the whole human genome analysis, taking into account the possibility that the drug response is influenced by a multitude of genes, not just those ones that code for proteins directly involved in the drug action, but also by the genes power to alter this response, exactly called "modifiers". The development of this research gave impetus to the development of pharmacogenomics.

"SNPs" (single nucleotide polymorphism) are the result of a single pair of bases substitution in DNA sequence. They are very common and present in every 1000 base pairs. The entire genome contains 3000000-10000000 SNPs and of these, one million and eight hundred thousand were characterized by SNP consortium. It is believed that each gene has between five and ten SNPs, although only less of 1% has got biological significance. This biological significance may come from substitution of an amino acid in a protein or by alterations in the expression of the protein due to SNPs in the promoter region. Polymorphisms affect the concentration and the half-life of the drug in the blood.

Drugs with concentrations and half-life in the blood higher than the average population indicate a decreased drug metabolism. However, a reduction of the drug concentration and half-life in the blood is indicative of a high metabolism of the drug. In the first case the adverse effects are increased, in the second case therapeutic effects are reduced. This example shows the importance of pharmacogenomics and the analysis of the whole genome to discover the complex mechanisms that determine the response to drugs.

The potential of pharmacogenomics is to identify patients with the same diagnosis but genetically different about the response to drugs in terms of efficacy anwd adverse reactions. Patients with an unfavourable pharmacogenetic profile must be treated with alternative drugs or different doses. Individuals with a pharmacogenetic profile compatible with a favorable response can be treated with medication and conventional doses. The results of genetic testing may be used by the physician to choose which drug can be used for the patient's treatment, to optimize the dose and to minimize the risk of side effects. The utility of the pharmacogenetic test, therefore, consists in the possibility of being able to evaluate the response of a patient to a certain drug on the basis of a genetic test routine, to customize the therapy.

Pharmacogenomics is thus an important key to achieve a predictive medicine aim to provide personalized therapy: the right drug at the right dose for the right patient.

Highly active antiretroviral therapy (HAART) against HIV infection has considerably increased life expectancy and in this statement there is its great application in clinical daily. These drugs, however, have the disadvantage of relatively high incidence of side effects and a lack of response to therapy by some subjects. Pharmacogenomics could be particularly useful in the case of drugs, such as antiretrovirals, which have a narrow therapeutic index associated with pharmacokinetic and pharmacodynamic variables.

An example of pharmacogenomics, applied in the context of antiretroviral treatment and more generally in the pharmacological field, is evident in the use of the screening test for the HLAB5701 in clinical practice, able to detect hypersensitivity to Abacavir, recommended by international guidelines as a preparatory test to use this drug in an HIV patient's treatment regimen. This paper aim is to provide a critical and understandable review of published literature and a guidance about future prospects in this field.

PHARMACOGENETICS AND HAART TOXICITY

Nucleoside reverse transcriptase inhibitors

Abacavir is a nucleoside reverse transcriptase inhibitor used in conjunction with other antiretroviral agents in the treatment of HIV infection and it is a popular choice for first-line treatment. Abacavir is generally well tolerated but can cause hypersensitivity in 5% to 8% of patients during the first 6 wk of treatment[1,2]; symptoms include fever, rash, constitutional symptoms, gastrointestinal tract symptoms, and respiratory symptoms (HRS -

Hypersensitivity Reaction Syndrome)[3]. Symptoms worsen with continued usage and can be potentially life threatening if the patient is rechallenged after discontinuation[4].

Hypersensitivity to Abacavir is immunologically mediated, driven by MHC-I antigen presentation and activation of HLA-B*5701. HLA-B*5701 activation restricted to CD8+ T-cells results in the secretion of inflammatory mediators (TNF-α and IFN-γ) and induces the delayed-type hypersensitivity reaction[5,6].

HLA-B*5701 allele occurs at approximately 5% frequency in European populations, 1% in Asian populations, and less than 1% in African populations. In immunologically confirmed hypersensitivity, HLA-B*5701 genotyping is associated with a negative predictive value of nearly 100% and a positive predictive value of approximately 50%: patients without the allele are highly unlikely to develop an immunological hypersensitivity to Abacavir, but only about half of those with the allele will develop HRS.

Thus, although the carriage rate of the HLA-B*5701 allele is low, stratification of patients for Abacavir treatment based on HLA-B*5701 genotyping could virtually eliminate immunologically confirmed hypersensitivity, and appears to be a cost effective healthcare practice[7].

Screening for HLA-B*5701 prior to initiation of Abacavir therapy is widely and strictly recommended for naïve HIV subjects that are going to start antiretroviral treatment; HLA-B*5701 - positive individuals should not be prescribed Abacavir. It should be also remarked that a negative HLA-B*5701 test does not preclude the development of a non-immunologic hypersensitivity reaction to Abacavir or of a clinical hypersensitivity reaction to another antiretroviral agent that may be given along with Abacavir, therefore genotyping should not substitute for clinical vigilance, but can greatly reduce the incidence of Abacavir hypersensitivity by identifying patients at high risk before they are treated[8-14].

Although other nucleoside/nucleotide analogues reverse transcriptase can induce renal damage (in particular, have been reported with Didanosine and Abacavir) there is no doubt that the drug of this class more involved in this effect is Tenofovir (TDF), a nucleotide analogue of adenosine 5 monophosphate administered orally at a dose of 300 mg once daily, in combination with other antiretroviral agents in naive patients' treatment. It is one of the most widely used drugs in patterns of antiretroviral therapy, being placed between the molecules to be preferred in all international guidelines. It can cause damage at the level of the proximal renal tubule[15].

TDF can cause renal toxicity (proximal tubular type), possible acute renal failure, Fanconi syndrome, creatinine dysfunction and hypophosphatemia. Tenofovir renal elimination includes a glomerular step and tubular phase of active secretion, that's why toxicity involves both a reduction in glomerular filtration and tubular function damage.

TDF penetrates, in fact, across the basolateral membrane of the tubular cells mainly through OAT1 and less through OAT3. The extracellular elimination is an active process dependent on MRP2 and MRP4 (members of a superfamily of ATP transporters, involved in the carriage of various molecules and drugs across cell membranes), proteins encoded by *ABCC4* and *ABCC2* genes. The nephrotoxicity mechanism of Tenofovir could be related to a compromised active efflux of TDF through the proximal renal tubule cells by the transporter called MRP2.

According to some studies, the renal proximal tubular epithelium is associated with genetic polymorphism (1249G > A) in the gene encoding the ABCC2-MRP2 transporter, but the positive value of screening in order to identify patients at risk of tubulopathy related to TDF is uncertain, considering this test low sensitivity[16].

Among the NRTIs (Nucleoside Reverse Transcriptase Inhibitors), involved in pharmacogenetic studies, there are Lamivudine (3TC) and Zidovudine (AZT), usually in a fixed dose combination drug, for many years considered a very important however still widely used.

In particular, there are many studies that focus on the relationship between 3TC/AZT pharmacokinetic and pharmacodynamic profile and MDR1, MRP2 and MRP4 polymorphisms.

P-glycoprotein 1 (Permeability glycoprotein, P-gp) is a membrane glycoprotein pump function with the known activity to remove neutral or weakly basic amphipathic substances from the cytoplasm which were penetrated into the cell consuming ATP; it is encoded by *MDR1* gene (multidrug resistance protein 1), also known as ATP-binding cassette sub-family B member 1 (ABCB1). P-gp, MPR2 and MPR4 play a main role in determining the intracellular concentration of nucleoside reverse transcriptase inhibitors. Concerning 3TC/FTC, it was observed in a 33 HIV patients population on antiretroviral regimen that included above formulation, that 3TC concentrations were elevated in 20% of subjects with MRP4 4131T > G variant carriers and that there was a trend of higher AZT concentrations in patients with MRP4 3724G > A variant carriers. However, this study and its subsequent observation are of uncertain clinical significance.

The onset of pancreatitis is related with the use of NRTIs and in particular with Didanosine.

It has been reported in 7% of patients treated with this drug; in a higher percentage of cases showed only increased amylase. In the field of genetic medicine, an increased risk of pancreatitis in the general population has been correlated with mutations in the CFTR (cystic fibrosis transmembrane regulator) responsible for several other clinical conditions such as cystic fibrosis and male infertility, and the mutations of serin protease inhibitor kazal-1 (SPINK-1) encoding a trypsin inhibitor in the cytoplasm of pancreatic acinar cells.

A case-control study conducted in the Swiss court aimed to asses the frequency of mutations in the CFTR and SPINK-1 polymorphisms in HIV-positive patients on antiretroviral regimen containing Didanosine with asymptomatic hyperamylasaemia or symptomatic pancreatitis;

this study suggests that CFTR mutations 1717-1G > A, IV585T and SPINK-1 polymorphism 112C > T are frequent in the studied population and may increase the susceptibility to pancreatitis in patients treated with NRTIs also exposed to additional risk factors, but further studies are needed to confirm these results[17].

Finally, some studies have been conducted to identify a possible correlation between specific mitochondrial polymorphisms and susceptibility to develop peripheral neuropathy (PN) in patients treated with NRTIs. Peripheral neuropathy complicates the clinical picture of HIV patients treated with NRTIs. This adverse event is definitely correlated to drugs belonging to this class because it has been reported also when these ones were taken as monotherapy. In particular, the neuropathy can occur with Didanosine, Zalcitabine and Stavudine. Clinical features of the drug-related PN are similar to the HIV-related neuropathy, but if there is a iatrogenic source PN has an onset and a more rapid progression and it is dose-related[18-20]. Prolonged exposure to NRTIs is associated with skeletal myopathy, lipoatrophy, fatty liver, metabolic acidosis and peripheral neuropathy that occurs with distal symmetrical anesthesia and/or paraesthesia painful structural abnormalities associated with mitochondrial DNA depletion. It has been investigated the association between polymorphisms MTND1 LHON4216C and MTND2 LHON4917G associated with LHON (Leber's Hereditary Optic Neuropathy) and PN in HIV-infected patients treated with NRTIs. The study found that 4917G polymorphism may increase susceptibility to the development of PN in patients treated with NRTIs. However, when subjects with 4917G were excluded from the analysis, the association with 4216C was no longer observed[21].

Considering the association between iron deficiency (essential for mitochondrial function) and some peripheral neuropathies in the general population, some studies have been conducted to examine a possible association between hemochromatosis gene mutations and susceptibility to peripheral neuropathy NRTI-induced, concluding that the iron burden mutations such as C282Y mutation might be associated with a reduced risk of PN in the course of NRTIs[22-25]. Nevertheless this association is particularly controversial.

Non-nucleoside reverse transcriptase inhibitors

NVP is a similar non-nucleoside reverse transcriptase inhibitor (NNRTI) widely prescribed for HIV treatment. Although generally well tolerated and effective, some individuals exposed to NVP show hepatotoxicity and severe cutaneous adverse reactions, including SJS/TEN during the first weeks of therapy (on average 12 d after starting therapy)[26]. This hypersensitivity reaction looks like Abacavir HRS and it is frequent when naive young women with CD4 > 250 cells/μL and naive males with CD4 > 400 cells/μL are treated, these elements suggest that genetic factors may play an important predisposing role[27].

The results of some studies that evaluated the influence of genetic variability in response to NNRTIs treatment, suggest that the development of SJS/TEN is dependent on an immune mechanism. Some studies show a correlation between certain HLA alleles (HLA-B*58:01 and HLA-B*15:02) and the SJS/TEN induced by allopurinol or carbamazepine.

The HLA-DRB1*01 and CYP2B6 gene polymorphisms have been associated with the onset of rash from NVP[28]. Another study has identified the involvement of HLA-B*35:05 in the rash caused by NVP in a Thai population. In addition, an ABCB1 polymorphism (1 member of the ATP-binding cassette subfamily B), also known as MDR1 (encoding the multidrug resistance protein 1) was associated with a lower risk of developing hepatotoxicity. The ABCC10 (encoding the multidrug resistance-associated protein 7) polymorphism rs2125739 has recently been associated with plasma concentrations of NVP. Several studies have finally emphasized the role of NVP hepatotoxicity by CYP2B6 gene polymorphism (516G > T), with the 516TT genotype associated with higher plasma concentrations.

To date, no study, however, assessed the involvement of genetic factors in the SJS/TEN caused by NVP: the gene polymorphisms of cytochromes that metabolize the drug or transporters have been studied only in relation to hepatotoxicity and skin rash. For this reason, a retrospective study was conducted in a population of Mozambique treated with NVP, to test whether the genetic variability of the cytochromes genes metabolizing NVP (CYP2B6, CYP3A4, CYP3A5) and transporters (ABCB1 and ABCC10) could be involved in susceptibility to SJS/TEN. This study describes the relationship between genetic variants of CYP2B6 and the onset of SJS/TEN. In particular, it was found that the 983C allele confers a higher risk of these adverse reactions. It is clear that, since the SJS/TEN is a complex disease, CYP2B6 is just one of many factors involved.

It has been suggested that variants of the MDR1 gene coding for P-gp (the pump transporter efflux of many drugs) can influence Nevirapine toxicity, in particular polymorphism C > T position 3435 of MDR1 was associated with reduced risk of hepatotoxicity[29-33].

Efavirenz is a widely prescribed drug for the HIV infection treatment and in combination with two NRTIs is recommended as a first-line regimen in patients starting antiretroviral therapy. From a pharmacological point of view, Ffavirenz is a non-nucleoside reverse transcriptase inhibitors (NNRTIs) whose metabolism is mediated by Cytochrome P450 2B6 (CYP2B6), which is a genetically polymorphic enzyme. This drug is generally characterized by a good toxicity profile and high efficacy: however, some episodes of viral failure and conditions affecting the central nervous system (CNS) such as nightmares, dizziness, drowsiness, insomnia, inability to concentrate have been reported in some patients with a frequency that can involve approximately half of the patients especially within the first few weeks of treatment[34].

In ACTG 5095 and 5097s it was demonstrated that the presence of a single nucleotide polymorphism (SNP) at position 516 of the CYP2B6 gene correlates with either

the presence of elevated Efavirenz plasma levels and the appearance of CNS adverse events. Subsequent papers also confirmed these findings. In other studies, moreover, similar associations even with the presence of a second polymorphism at position 983 of the CYP2B6 gene have been demonstrated. These two SNPs have a higher frequency in the African population; this phenomenon could therefore explain the particularly higher Efavirenz plasma levels observed in Africans subjects than in individuals of other ethnicities[35-37].

Finally, there is preliminary evidence that the presence of polymorphisms of other genes, such as ABCB1 coding for P-glycoprotein or *CYP3A5* gene, may significantly influence the viral response and/or the daily exposure to the drug in patients treated with Efavirenz. It has been amply demonstrated that the polymorphism increases the predictive value of 516/983 SNPs on the Efavirenz pharmacokinetics; instead, other genetic variants in genes *CYP2B6*, *CYP3A5* and *ABCB1* don't improve the predictive value of the model based on the 516/983 genotype[38].

Finally, this work suggests that the slow metabolizer genotype, according to the polymorphism 516 (G > T) and 983 (T > C), can lead to viral beneficial and that the reduction of the drug dose may increase the risk of viral failure.

The best models to predict the Efavirenz pharmacokinetics are based on the polymorphisms 516 and 983 genotypes: slow metabolizers of white ethnicity are at risk for CNS adverse events, while there is a reduction of the probability of viral failure in black patients.

The presence of a G > T single nucleotide polymorphism (SNP) at position 516 of *CYP2B6* gene results in a Gln-His (Glutamine- Histidine) amino acid change associated with higher plasma EFV concentrations leading to increased drug- related side effects.

The *C3435T* change at a wobble position in exon 26 on chromosome 7 of the human genome has pharmacological consequences, and has been reported in a number of African populations and other ethnic groups in different populations. The frequency of the *C3435T* mutation is significantly influenced by ethnicity with marked differences in genotypes seen between different populations. Several studies have reported a high prevalence of the CC genotype in different African populations, and this prevalence implies overexpression of P-gp. In individuals with CC genotype, access of HIV protease inhibitors to major cellular targets known to express P-gp is restricted and this could have serious implications in the use of protease inhibitors. Patients with the T homozygous genotype have been shown to have low expression of P-gp. The C3435T SNP is also correlated with P-gp expression and function on lymphocytes but not on placenta. Several studies have reported significantly greater CD4 cell count in patients with the MDR1 3435TT genotype and these patients tend towards less pronounced viral infection than those patients with the CT or CC genotype[39,40].

Characterization of MDR1 and CYP2B6 enzymes and utilization of pharmacogenomic testing for identification of different alleles in patients may provide a useful tool for therapy optimization with drugs that are substrates of P-gp and those that are metabolised through the CYP2B6 pathway. CYP2B6 genotyping seems to be a useful tool to predict Efavirenz toxicity and resistance allowing patients to know that as poor metabolizers are at greater risk of increased plasma exposure of the drug and therefore of its adverse effects and probably resistance in case of drug discontinuation[41-47].

Protease inhibitors

Protease inhibitors (PIs) are mainly metabolized by CYP3A4 (the predominant form of Cytochrome P450) of which they also are inhibitors; especially ritonavir is an inhibitor of CYP3A4 and it is used as a booster to increase the plasma exposure of other PI. In view of the PI dual function as substrates and inhibitors, the impact of their polymorphisms is difficult to assess. Finally, PIs are also substrate of Pg-P.

Atazanavir is a widely used PI because of its long-term tolerability, its reduced pill burden and its power. The *UGT1A1* gene codes for the UDP glucuronosyltransferase enzyme (UGT1A1), which mediates bilirubin conjugation with glucuronic acid in the liver, then excreted in the bile. Atazanavir inhibits *UGT1A1*, leading to hyperbilirubinaemia and jaundice in certain subjects. The UGT1A1*28 allele is associated with increased risk of hyperbilirubinaemia during Atazanavir based treatment, and genotyping for UGT1A1*28 before starting antiretroviral treatment containing the aforementioned drug may aid to identify patients at higher risk of hyperbilirubinaemia. Subjects whit two copies of the gene variant (UGT1A1*28 homozygotes) have been reported to have the highest risk and UGT1A1*28 heterozygotes show an intermediate risk of developing hyperbilirubinaemia[48,49].

Atazanavir metabolism is partially due to P-gp efflux pump encoded by the *MDR1* gene, which seems to increase plasma concentrations of Atazanavir in presence of 3435 variable genetic homozygosis C/C, exposing the patient to a risk of hyperbilirubinemia and severe jaundice.

In summary, genotyping for *UGT1A1* and *MDR13435* before starting Atazanavir may help the clinician to identify those subjects at increased risk of exposure to high plasma levels of the drug and the consequent development of side effects[50,51].

In the post-HAART era the occurrence of treatment-related metabolic disorders was observed, among which the most frequent are dyslipidemia, insulin resistance, diabetes mellitus, lipodystrophy, all considered risk factors for cardiovascular events[52]. The genesis of these disorders is multifactorial so the genetic susceptibility of the single patient represents a new field of investigation.

Lipodystrophy is a long-term complication that deeply affects the quality of life of PLWH leading to the need to identify genetic predisposing factors that could optimize the therapeutic management. TNF expression in the adipose tissue plays an important pathogenic role in the abnormal visceral fat distribution; therefore

Table 1 Pharmacogenetics and highly active antiretroviral therapy toxicity

ARVs	Polymorphisms	Effects
NRTIs		
Abacavir (ABC)	HLA-B*5701	Hypersensitivity Reaction Syndrome
Tenofovir (TDF)	ABCC2-MRP2 (1249G > A)	Increased risk of tubulopathy
Lamivudine (3TC)	MRP4 4131T > G	Increased plasma concentrations
Zidovudine (AZT)	MRP4 3724 G > A	Increased plasma concentrations
Didanosine (ddI)	CFTR 1717-1G > A, IV585T,	Higher risk of pancreatitis
Didanosine (ddI), Zalcitabine (ddC), Stavudine (d4T)	SPINK-1 112C > T MTND1 LHON4216C, MTND2 LHON4917G	Leber's Hereditary Optic Neuropathy, Peripheral Neuropathy
NNRTIs		
Nevirapine (NVP)	HLA-B*58:01, *15:02, *35:05	Cutaneous rash, SJS/TEN
	ABCC10rs2125739, CYP2B6 516G > T	Increased plasma concentration, hepatotoxicity Reduced risk of hepatotoxicity
Efavirenz (EFV)	MDR1 3435C > T CYP2B6 G516T, T983C	Higher plasma concentrations, SNC side effects
PIs		
Atazanavir (ATV)	UGT1A1*28, MDR1 3435C/C	Hyperbilirubinemia and jaundice
All PIs	TNF gene 238G > A	Early onset of lypodistrophy
	APOA5 (1131T > C, 64G > C), APOC3 (482C > T, 455C > T, 3238C > G) ABCA1 2962A > G APOE (ε2, ε3 haplotypes)	High risk of dyslipidemia
Others		
Maraviroc (MVC)	SLC01B1 521T > C (rs4149056)	Increased plasma concentrations
Raltegravir (RAL)	UGT1A1*28	Increased plasma concentrations

several studies have been conducted in order to identify the genetic variants involved. It has been shown that the 238 variant was significantly more represented in HIV-infected patients with lipodystrophy than in those without. In particular polymorphism 238G > A appears to be related in some studies but not in others, to an early onset of lypodistrophyc process[53-59].

Regarding dyslipidemia, it is known that there is a correlation in the general population with genetic polymorphisms in apolipoproteins genes; some studies have attempted to reproduce this model even in the HIV population. Several studies showed promising results such as the demonstration of an association between *APOA5* gene polymorphisms (1131T > C and 64G > C) and an increased risk of hyperlipidemia.

Furthermore multiple studies identified some polymorphisms of APOC3 (482C > T, 455C > T, 3238C > G), ABCA1 (2962A > G) and APOE (ε2 and ε3 haplotypes) that are associated with a high risk of dyslipidemia[60-67].

Other antiretrovirals

Maraviroc (MVC), the only coreceptor CCR5 antagonist approved for clinical use, is a therapeutic chance for the treatment of the multiexperienced HIV patients (*i.e.*, with resistance to traditional drugs)[68-70]. A close correlation between MVC plasma levels and therapeutic effectiveness has been described, with the identification of a MEC (Minimum Effective Concentration) of 50 ng/mL. MVC plasma concentrations are the result of absorption, distribution, metabolic and elimination processes mediated by several proteins in different tissues. The hepatic uptake of MVC, and therefore its metabolism, is influenced by the action of a carrier protein, OATP1B1, encoded by *SLCO1B1* gene. It has been shown that the presence, in the heterozygous or homozygous status, C variant allele in the polymorphism 521T > C (rs4149056) SLC01B1 gene is correlated with an increase MVC plasma concentration. Genetic screening before prescribing this drug could be a help for the clinician for a customized therapy.

Raltegravir (RAL) is the first drug of a new antiretroviral class, the Integrase Inhibitors (INI). It is metabolized by UGT1A1 and its variant allele *28 in the homozygous status is associated to a reduction of the enzyme activity resulting in mild higher RAL plasma concentrations, but not statistically significant[71] (Table 1).

PHARMACOGENETICS AND HAART RESPONSE

It is well known that combination antiretroviral therapy has dramatically improved the survival rate and the quality of life of PLWH due to the powerful effect on the viral suppression and immune recovery, that's why the most important surrogate parameters used for the evaluation of the HAART response are represented by the viral load (HIV-RNA) and the CD4+ count.

Several pharmacogenetic studies have been conducted in order to establish a relationship between patients' genetic predisposition and susceptibility to the antiretroviral therapy efficacy, but the obtained data are inconsistent and often conflicting, this is probably due to a partial genetic analysis, different categorization of poor immune recovery or due to small numbers of patients

Table 2 Pharmacogenetics and highly active antiretroviral therapy response

Polymorphisms	Drug response
CYP3A5*1	Increased PIs clearance
CYP2B6 rs3475274, rs28399499	Increased EFV and NVP plasma concentrations
ABCB1 3435C > T	Better viral responses to EFV exposure
ABCB1 rs1045642 (3435T > C)	CT/CC genotype associated with higher CD4 count in EFV, 3TC, NVP containing regimen

PIs: Protease inhibitors; NVP: Nevirapine; EFV: Efavirenz.

evaluated[72].

Drug metabolism through CYP450 system has emerged as an important determinant of several drugs interactions and several efforts are conducted to demonstrate its utility to target an optimal therapeutic regimen in term of drug response.

Among this family of enzymes, the majority of drugs actually used in clinical practice are metabolized by CYP3A4 and CYP3A5 which currently show the most individual variations of gene expression, mainly caused by Single Nucleotide Polymorphisms (SNPs).

As mentioned before there are currently six different classes of antiretrovirals which interferes with the HIV life cycle at a different stage.

CYPs that are primarily involved in the metabolism of NNRTIs and NRTI are CYP2B6 and to a lesser extent CYP3A4. By contrast to the NNRTIs, the large part of PIs are metabolized by the CYP3A enzyme system. CYP enzymes in human liver, in particular CYP3A4, play a pivotal role in PI biotransformation, converting these agents to inactive metabolites[73].

Associations between human CYP3A4 and CYP3A5 genetic variants and predisposition to therapy failure has often been hypothesized and described, mainly in HIV-infected patients treated with Protease Inhibitors whose metabolism is affected by induction or inhibition of CYP3A

Indeed, several recent studies have suggested that the disposition of certain PIs might predicted by CYP3A5*1 genotype. A report published by Mouly *et al*[74] show an association between increased Saquinavir clearance and this genetic variant of the enzyme. The CYP3A5*1 genotype has also been related to 44% faster Indinavir oral clearance in 11 HIV patients[20].

One of the most of robust examples of a pharmacokinetic association is observed with genetic variation in the *CYP2B6* gene and the NNRTI efavirenz and nevirapine. CYP2B6 loss of function alleles (rs3475274 and rs28399499) are associated with pharmacokinetic characteristics of NNRTIs. The metabolizer phenotype predicts Efavirenz and Nevirapine plasma concentrations and clinical response to these drugs[75-77].

Regarding clinical response an association between the metabolizer phenotype and virological failure in African-American has been suggested[78].

The minor allele T at rs3745274 causes a decreased expression and activity of CYP2B6 in the liver. In some studies it has been demonstrated that carriers of the TT genotype compared to GG/GT genotypes experienced an over 3-fold increase in Efavirenz concentrations[79].

Patients with CYP2B6 intermediate and slow metabolizer phenotypes achieve undetectable viral loads after treatment with NNRTIs: the association of the phenotype and response to drugs has important potential for clinical decision-making.

Polymorphisms in ABCB1, which encodes P-glicoprotein, may predict altered pharmacokinetics of some drugs. Two studies suggested that ABCB13435C → T predicted more favourable viral responses to Efavirenz containing regimens[80].

Many studies have assessed the potential association of ABCB1 polymorphisms with changes in drug response, some of these have specifically examined a potential association of genotype with outcome in HIV infected patients; it has been studied the relationship between rs1045642 (3435T > C) genotype with viral load and CD4 count in HIV patients treated with Efavirenz and Nevirapine containing regimens; after 6 mo of these therapies, people having TT genotype showed a significantly higher CD4 count than those having a CT/CC genotype; on the contrary no correlation statistically significant was found with viral load.

Similar results emerged from studies about 3TC and Nevirapine[81] (Table 2).

CONCLUSION

The wide availability of drugs and therapeutic regimens for the HIV infection treatment and the presence of associated adverse effects related to interindividual variability leads the clinician to look for an individualized therapy as much as possible. Pharmacogenetics can provide useful tools for this purpose and can propose models of genetic tests that, however, need to be further studied.

The correlation between genetic variables and the HRS to Abacavir is now recognized as such and therefore its administration is related to the presence of favorable genotypes (negative HLAB5701). The genetic variability related to the adverse effects of Efavirenz and Atazanavir are similarly promising, but not yet present in clinical practice. It is desiderable, considering the need for tailored regimes, pursuing further studies to identify a statically significant correlation between specific genetic profiles and adverse effects related to other antiretroviral drugs (Nevirapine hepatotoxicity, proximal tubulopathy due to Tenofovir, peripheral neuropathy, lipodystrophy, metabolic alterations).

Pharmacogenomics seems to be potentially useful not only in its ability to identify individual susceptibility to drug toxicity, but also in terms of pre-treatment assessment of the patient's individual response to a particular drugs combination. Despite several studies recognize this potential, actually there are no strong

enough data. The analysis of the literature reveals a need for further studies that provide greater sample size, but also a valid model for genetic analysis.

In conclusion, pharmacogenetics represent a way to go toward the goal of personalized medicine in the field of HIV infection, to obtain a therapeutic response optimization of the single patient, a reduction of toxicity HAART related, a lower risk of drug-drug interactions, a right therapeutic dose.

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REVIEW

Non-AIDS definings malignancies among human immunodeficiency virus-positive subjects: Epidemiology and outcome after two decades of HAART era

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Abstract

Highly active antiretroviral therapy (HAART) for human immunodeficiency virus (HIV) infection has been widely available in industrialized countries since 1996; its widespread use determined a dramatic decline in acquired immunodeficiency syndrome (AIDS)-related mortality, and consequently, a significant decrease of AIDS-defining cancers. However the increased mean age of HIV-infected patients, prolonged exposure to environmental and lifestyle cancer risk factors, and coinfection with oncogenic viruses contributed to the emergence of other malignancies that are considered non-AIDS-defining cancers (NADCs) as a relevant fraction of morbidity and mortality among HIV-infected people twenty years after HAART introduction. The role of immunosuppression in the pathogenesis of NADCs is not well defined, and future researches should investigate the etiology of NADCs. In the last years there is a growing evidence that intensive chemotherapy regimens and radiotherapy could be safely administrated to HIV-positive patients while continuing HAART. This requires a multidisciplinary approach and a close co-operation of oncologists and HIV-physicians in order to best manage compliance of patients to treatment and to face drug-related side effects. Here we review the main epidemiological features, risk factors and clinical behavior of the more common NADCs, such as lung cancer, hepatocellular carcinoma, colorectal cancer and anal cancer, Hodgkin's lymphoma and some cutaneous malignancies, focusing also on the current therapeutic approaches and preventive screening strategies.

Key words: Human immunodeficiency virus infection; Malignancy; Highly active antiretroviral therapy; Non-acquired immunodeficiency syndrome-defining cancers

Core tip: Since the introduction of highly active antiretroviral therapy (HAART) the incidence of acquired

immunodeficiency syndrome (AIDS)-defining diseases has declined. This has resulted in a significant improvement in survival of human immunodeficiency virus (HIV)-infected patients. However the incidence of non-AIDS defining cancers (NADCs) did not decrease, and this determines now a relevant burden of mortality among HIV-positive patients. The availability of an even more effective HAART along with chemotherapy and radiotherapy regimens suitable also for HIV-patients could improve the outcome of these patients in the setting of NADCs. Screening interventions to detect precancerous lesions are also of paramount importance in order to decrease mortality of NADCs.

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INTRODUCTION

The early studies among patients receiving transplantation forty years ago showed that Kaposi sarcoma and lymphomas were diagnosed with an high incidence in this immunocompromised population. This findings were confirmed twenty years later when Kaposi sarcoma and some types of lymphoma presented a strong association with an advanced stage of human immunodeficiency virus (HIV)-related acquired immunodeficiency syndrome[1,2]. These malignancies have been classified as acquired immunodeficiency syndrome (AIDS)-defining cancers (ADCs) by Center for Diseases Control and Prevention since 1993[3].

With the introduction of combination antiretroviral therapy there has been a dramatic decrease of the incidence of AIDS-related morbidity and mortality in HIV-positive patients[4-7]. The HAART has also improved the short and medium-term survival in HIV-infected patients with ADCs[8]. As a consequence of the restored immune function, the incidence of AIDS-defining cancers has significantly declined, and the prognosis markedly improved. The HAART showed to modify positively the clinical outcome of Kaposi sarcoma, a typical AIDS-defining cancer, and it represents now a cornerstone for the treatment of all stages of this neoplasm[9]. *In vitro* and *in vivo* studies performed on mice deprived of thymus showed that HAART, and in particular protease inhibitors class, has a direct antitumoral activity. Even the risk of developing non-Hodgking lymphoma was reduced markedly after HAART introduction: Besson *et al*[10] showed in a large French population of HIV-infected patients that the incidence fell sharply between 1993-1994 and 1997-1998 from 86 per 10000 in the 1993-1994 to 42.9 per 10000 person-years. Similarly, another American study among 537 with AIDS-related NHL documented that the annual average incidence of NHL decreased from 29.6 per 1000 person-years in the pre-HAART period (1988-1995) to 6.5 per 1000 person-years in the post-HAART era (1996-2000). The more pronounced changes were observed among the group of diffuse large B-cells lymphomas, with a dramatic decrease of incidence of primary cerebral and of high grade hymmunoblastic lymphomas[11], that are linked to Epstein-Barr virus (EBV) latent co-infection[12].

In contrast with the positive impact of HAART on the incidence of AIDS-defining infectious and malignant diseases, HIV-positive patients remain at increased risk of non-AIDS-related mortality and morbidity, including cardiovascular disease, neuro-behavioral disease and cancers. NADCs have gradually emerged as a major fraction of the overall cancer burden[13,14]. Trends in all-cause mortality emerged from the Data collection on Adverse events of anti-HIV Drugs (D:A:D) study showed a significant decrease from 17.5 per 1000 person-years in 1999-2000 to 9.1 in 2009-11. A similar decrease in the same period was seen for the mortality rate of AIDS-defining conditions (5.9 to 2.0), liver (2.7 to 0.9) and cardiovascular diseases (1.8 to 0.9), whereas NADCs increased from 1.6 per 1000 persons-years in 1999-2000 to 2.1 in 2009-2011[15]. Some large cohort studies, and data derived from linkages among the AIDS and cancer registries, revealed that the risk of developing solid tumors and non-AIDS defining lymphomas was two to three-fold higher than in the general population[16,17]. In the meantime the overall mortality associated with NADCs increased from < 1% in pre-HAART era to 13% after HAART introduction[18]. This changing scenario could be explained by the influence of some demographic features of HIV-positive population such as the advancing age, the role of behavioral risk factors like smoking and alcohol consumption, and chronic coinfection with other viral pathogens (EBV, HCV, HBV and Human Papilloma virus)[19]. There was not demonstrated a clear relationship between immunosuppression and development of NADCs. While some studies showed that a low nadir of CD4 cell count is predictive of a increased risk of developing NADCs[20-22], Engels *et al*[23] did not find a correlation between advanced immunosuppression and the risk of developing NADCs.

Here we focus on the epidemiological and clinical features of the most common NADCs among HIV-positive individuals. We also briefly review their therapeutic approach and the outcome after twenty years of HAART.

LUNG CANCER

Lung cancer was showed to be the most frequent NADCs occurring in HIV-positive people and, it stands as the leading cause of cancer-related deaths among HIV-positive people in a large United States population-based registry[24]. Two meta-analysis estimated that the risk of lung cancer in HIV-infected people was more than two-fold higher than in the general population[25,26], and the risk is relevant for all main lung cancer subtypes (squamous

cell carcinoma, adenocarcinoma and small cell carcinoma). Male sex is more affected, and the mean age when diagnosis of lung cancer occurs is about 15 years lower than in HIV-negative people[27].

Cigarette smoking is the most important risk factor for developing lung cancer and the prevalence of tobacco use among HIV-positive people is higher than in the general population, ranging from 40% to 70% compared to 20% observed among HIV-negative people[28-30]. When considering the role of tobacco in lung carcinogenesis smoking cessation recommendations and interventions represent a critical part in the routine clinical encounter in this high-risk population.

Immunosuppression caused by HIV infection results in chronic activation, disfunction of immune system, and chronic inflammation, all likely promoting carcinogenesis in HIV-infected individuals. Nonetheless the relationship between a low T CD4 cells count, the duration of immunosuppression and the risk of developing lung cancer is not well understood[31]. A large American cohort study of 37294 HIV-infected people showed that HIV infection appears a risk factor for lung cancer even after controlling for other confounding variables, but it did not find an association of lung cancer with low T CD4 cells count[32]. It has been reported that HIV-infected people present more frequently an advanced stage of lung cancer and the outcome is poorer if compared with the general population[33]. However these observations have recently been challenged. One epidemiological study evaluating 322 HIV-positive patients with non-small cell lung cancer showed no difference in stage at cancer diagnosis if compared with 71976 HIV-negative controls, and the median survival was similar between two groups with early stage of disease. In addition the survival of HIV-positive patients with an early stage disease, who underwent surgical resection was similar to that of control group (50 mo *vs* 58 mo; $P = 0.88$)[34].

Non-small cell lung malignancies covers more than 80% of lung cancers among HIV-positive subjects, and the adenocarcinoma is the more frequent histological type, mirroring the current epidemiological trend in the general population[35-37].

Due to the lack of randomized trials and guidelines the choice of appropriate therapy for HIV-infected patients with lung cancer tends to vary based upon patient's clinical conditions and the degree of immunosuppression. Toxicity, poor tolerability and potential of interaction between chemotherapy and HAART are concerns limiting systemic cancer therapy in HIV-positive patients[38,39]. In a retrospective multicenter Italian study of 68 consecutive cases of lung cancer diagnosed in HIV-positive patients, clinical presentation and treatment outcome in the pre-HAART and post-HAART era were compared. The overall median age was 43.5 years and all but one patients (67 out of 68 patients) were heavy smokers. Overall in 58 patients (85.3%) a non-small cell lung cancer was diagnosed, and among these adenocarcinoma was the predominant histological type. Chemotherapy was much more frequent among post-HAART patients, of whom 27 were treated (79.4%) *vs* 16 (48%) in the pre-HAART group ($P = 0.04$). The authors also showed that the overall survival rate was significantly better for the post-HAART group (3.8 mo in the pre-HAART period *vs* 7 mo in the post-HAART period, $P = 0.01$)[40]. Recently The Intergroupe Francophone de Cancerologie Thoracique has initiated a phase II trial of carboplatin plus pemetrexed in HIV-infected patients with advanced NSCLC (NCT01296113). In the United States, an AIDS Malignancy Consortium trial is evaluating the carboplatin/paclitaxel regimen in HIV-infected patients with advanced solid tumors, including lung cancers (AMC-078, NCT01296113). These studies could provide a better knowledge on treatment options and clinical outcome of HIV-positive patients with lung cancer.

COLORECTAL CANCER

Among the NADCs, colorectal cancer (CRC) has been identified as one of the tumors with an increasing in incidence in the HIV population[21]. In a prospective cohort study of 2882 patients with HIV infection the annual incidence of CRC was reported to increase from 0.65 per 1000 patients-years in the pre-HAART era to 2.34 per 1000 patient-years between 1997 and 2002[41]. As a consequence of increased life expectancy of HIV-positive people due to the efficacy of HAART, many people are living long enough to develop CRC. Clinical presentation, treatment and survival of HIV-positive patients affected by CRC were described by the Italian Cooperative Group AIDS and Tumours, where 27 cases of HIV-positive CRC patients were matched with 54 HIV-negative controls retrieved from a national database. HIV-positive patients developed CRC at an earlier age and the disease was more advanced than in the general population. The authors showed also that at the time of diagnosis most of patients had ad advance disease stage and an overall poor outcome, with a probability of survival at 4 years of 15% and 49% for HIV-positive and HIV-negative patients respectively. However it was also noted that chemotherapy was well tolerated in all patients, and in the HAART era there were neither opportunistic infections nor chemotherapy-related deaths[42]. Berretta *et al*[43] also showed that liver metastases due to CRC could be treated with surgical resection, along with chemotherapy, without discontinuing HAART. CRC is a condition that could easily identified at an early stage by screening colonoscopy since many lesions are preceded by premalignant adenomas and could be removed by endoscopy procedures. These observations are supported by the results of a screening colonoscopy study that evaluated the prevalence of neoplastic lesions. Future researches should address the role of screening in the HIV-positive population for CRC in order to improve early diagnosis and survival[44].

HEPATOCELLULAR CARCINOMA

HIV-infected subjects are at greater risk of developing

and dying of hepatocellular carcinoma (HCC). In the HAART era, the incidence of this malignancy was 10 to 36 new cases per 100000 HIV-infected people per year, corresponding to 3-fold to 6-fold excess risk if compared with the general population[13,45].

The high incidence of HCC among HIV-infected patients was also recently documented in a multicenter Italian cohort including 13388 HIV-positive patients enrolled since 1998, where liver cancer ranked as the most frequent NADC[46].

The main risk factors for development of HCC are viral hepatitis and alcohol abuse. The chronic evolution of HBV infection in the liver and the progression to cirrhosis of HCV-related chronic hepatitis are more frequent in HIV-positive individuals than in the HIV-negative people. Moreover HIV-induced immunosuppression may accelerate liver fibrosis and increase the risk to develop HCC[47]. Moreover hepatocytes apoptosis seems to be promoted by upregulation of tumor necrosis factor (TNF) by the HIV surface protein gp120[48].

Another factor that could worsen the liver damage is the antiretroviral therapy which is known to have some direct hepatotoxic effects[49]. These factors could explain the increased incidence of HCC observed in HIV-positive patients, four to seven folds higher than in the general population[50].

An Italian multicenter cohort study comparing 104 HIV-positive patients and 484 uninfected controls with HCC, showed that HIV-infected patients were significantly younger at HCC diagnosis, and they present more commonly HBV or HCV co-infection. The survival was poorer in the HIV-positive patients even though in these patients HCC was more frequently diagnosed at an early stage. However the subgroup of HIV-positive patients receiving HAART and with an undetectable HIV viral load had a better outcome than patients with an higher plasmatic HIV RNA[51]. In this study, even thought the treatment rates were similar between HIV-positive and HIV-negative patients, the overall survival rate was worse in the HIV-positive group, maybe due the fact that in these patients retreatment of an HCC recurrence was considered in a lower number of cases.

Localized therapies such as surgical resection, ethanol injection and radiofrequency ablation should be considered for patients with solitary or small number of HCC lesions[52]. Encouraging data on feasibility of liver transplantation (LT) were showed by Vibert *et al*[53]. Overall survival and relapse rate were not significantly different among HIV-positive patients with HCC compared to HIV-negative control group. This data were recently confirmed by Di Benedetto *et al*[54], who recently compared the outcome of 30 HIV-positive patients who underwent LT with 125 HIV-negative patients: at 1 year and 3 years post LT overall survival (77% at 1 years and 65% at 3 years among HIV-infected *vs* 86.4% and 70% among HIV-negative patients) was similar between the two groups. Therefore HIV-infected patients should be offered the same LT options for HCC treatment that are provided for HIV-uninfected subjects. Prevention of HCC should be addressed to reduce the burden of some risk factors; counselling for alcohol avoidance and promotion of HBV vaccination are important elements of primary prevention. Hepatic ultrasonography and alpha fetoprotein measurement every 6 mo are also essential diagnostic tools for early diagnosis of HCC. Among patients with high risk of developing HCC, such as advanced liver cirrhosis, computed tomography and magnetic resonance imaging are useful to detect hepatic lesions < 3 cm[55]. Recently, with the advent of the new direct-acting antiviral agents, HCV treatment has rapidly changed with a dramatic improvement of cure rates; therefore, eradication of HCV is a more feasible target even in the difficult-to-treat HIV-positive population[56].

HODGKIN'S LYMPHOMA

In immunosuppressed patients, Hodgkin's lymphoma (HD) occurs more frequently than in the general population of the same age, and some epidemiological studies showed that HIV-infected people have a 10-fold higher risk of developing HL than HIV-negative subjects[16,57,58]. HIV-associated HD displays several peculiarities when compared with HD in the general population, such as an unusual aggressive behavior and an overall poor prognosis. More specifically HIV-HL is characterized by the high incidence of more aggressive histological subtypes, mixed cellularity (MC) and lymphocyte depletion (LD), that appears specifically related to advanced immune compromise in HIV-infected patients. A high frequency of EBV association has been shown in HL (80%-100%) tissues from HIV-HL, which indicates that EBV does represent an important factor involved in the pathogenesis of HIV-HL. There are evidences that the EBV-encoded latent membrane protein 1 (LMP1), which is expressed in the majority of HIV-HL, may play a role in the pathogenesis of this lymphoma[59,60]. At the time of HL diagnosis many HIV-positive patients present an advanced stage of disease and systemic "B" symptoms such as fever, night sweats, and/or weight loss > 10% of the normal body weight. Among 290 patients with HIV-HL, an advanced stage of this malignancy was observed in 79% of patients; extranodal involvement was reported in 59% of patients, with bone marrow, spleen and liver involved in 38, 30 and 17 patients respectively. The authors of this study found that the following parameters were associated with a better survival: MC subtype, the absence of extranodal involvement, the absence of "B" symptoms, and prior use of HAART[61]. In a similar study performed in Spain among 104 patients with HIV-HL the complete remission rate was significantly higher in HAART group (91% *vs* 70%, $P = 0.023$)[62]. After the first prospective multi-institutional study performed by AIDS Clinical Trial Group (ACTG), which used the ABVD chemotherapy (doxorubicin, bleomycin, vinblastine, dacarbazine), more intensive chemotherapy regimens including BEACOPP (bleomycine, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, prednisone),

Stanford V (mechlorethamine, doxorubicin, vinblastine, vincristine, bleomycin, etoposide, prednisone), and VEBEP (epirubicine, bleomycin, vinorelbine, cyclophosphamide and prednisone) with radiotherapy have been proposed, and a complete remission (CR) rate > 60% has been obtained[63-66]. Combined administration of HAART and chemotherapy showed to reduce the risk of opportunistic infections, relapses and to improve the CR rate. Moreover the use of high dose chemotherapy and autologous stem cell transplantation (ASCT) seems to be the gold standard as salvage treatment for relapsing or progressing HL in HIV-positive patients[67,68].

ANAL CANCER

Anal carcinoma is an uncommon malignancy in the general population, but it stands as one of the leading NADCs among HIV-positive patients since the HAART introduction[69-71]. In the Swiss HIV Cohort Study a 30-fold higher rate of anal cancer was showed in comparison to the HIV-uninfected subjects[45].

Anal cancer affects primarily men who have sex with men (MSM), with a mean age of 45-50[72]. Squamous cell carcinoma is the most common histological type and it arises from precursor high-grade anal intraepithelial lesions (AIN) within the anal canal[73]. Some high risk types of Human papillomavirus (hr-HPV), especially HPV-16, play a pivotal role in the pathogenesis of anal squamous cell carcinoma (ASCC), and in HIV-positive patients the prevalence of hr-HPV infection was estimated to be three to five fold higher than in the general population[74]. Sexual transmission of HPV through anal intercourse explains the high rate of ASCC diagnosed in HIV-positive MSM subjects[75].

A lower T CD4 cell count has been associated with a reduced clearance of anal HPV infection, and the development of precancerous lesions, such as low grade AIN. The improved survival of at risk HIV-positive patients could also allow the progression of early precancerous lesions to invasive anal cancer. Concurrent chemotherapy and radiotherapy is the first line treatment of anal cancer, and this approach could be safely used for HIV patients. Intensity-modulated radiation therapy has recently proposed to achieve high doses of radiations and reduce dermatological and gastrointestinal toxicity[76,77]. Screening interventions targeted to high risk group, like HIV-infected MSM, are based primarily on anal Pap smear and high-resolution anoscopy. The latter one proved to be cost-effective in the early detection of precancerous anal lesions, which would allow to treat them with minimally invasive localized therapies[78]. Vaccination against hr-HPV has proved to be effective for preventing anal cancer precancerous lesions in women[79]. Further studies are warranted to evaluate if this approach could have similar positive results among high risk HIV-infected patients, such as MSM.

CUTANEOUS MALIGNANCIES

Multiple studies demonstrated that immunosuppressed patients have an increased risk of cutaneous malignancies, and it seems to be most pronounced in solid-organ transplants recipients, who have a 65 to 250 times increased risk as compared to general population[80,81].

Since the early phase of HIV epidemic, Kaposi sarcoma was the most common malignancy with cutaneous involvement[2], whereas the incidence and risk factors associated with cutaneous non-ADCs (NADCs) among HIV-infected persons are less defined. In a large American cohort of 4490 HIV-positive patients retrieved from 1986 to 2006, there were 254 (5.7%) patients who developed skin cancers, and basal cell carcinoma (BCC) was the most frequent non-ADCs, with a ratio of BCC to squamous cell carcinoma (SCC) of 6:1, that differs from transplant recipients who develop SCC in the majority of cases[82]. Similarly in the period between 1985 and 2002 analyzed by an afore-mentioned Swiss study, BCC were more frequent than SCC, and the overall incidence of nonmelanomatous skin cancer was three-fold higher than in the general population (Standardized Incidence Ratio, SIRs = 3.2, 95%CI: 2.2-4.5) in this large national cohort study[83]. More recently the same authors showed that the SIRs of non-melanomatous skin cancers increased between the pre- and early-HAART period, but not between the early- and late-HAART period[45]. In a recent meta-analysis that analyzed 13 studies in the post-HAART and 8 in the pre-HAART era, also the risk of melanoma was showed to be increased among HIV/AIDS population[84]. Even if KS was the most frequent cutaneous cancer, its incidence significantly decreased after 1995, while the age-adjusted incidence rates of cutaneous NADCs remained stable[82]. The factors associated with the development of cutaneous NADCs in this study were aging and the withe/non-Hispanic race, similarly to what has been showed in other HIV-positive cohort and in the general population[85,86]. The development of cutaneous NADCs was also showed to be not related to the CD4+ T lymphocites count and receipt of HAART, but HIV-infected subjects are characterized by an high likelihood of developing subsequent cutaneous malignancies at novel sites. In the afore-mentioned study of Crum-Cianflone *et al*[82], 24% of the participants, who initially presented with a BCC, developed a subsequent BCC, and 8% developed a second type of cutaneous cancer.

These findings were confirmed by another large prospective cohort study which enrolled patients diagnosed with non-melanoma skin cancers, with a median follow-up of 7.3 years. This study showed that the overall 5-years recurrence rates after treatment in HIV-positive patients was 13.8%, and 2.9% in HIV-uninfected patients respectively (HR = 3.1; P = 0.005)[87]. The high rate of recurrences suggests that HIV-infected individuals with an initial cutaneous NADC should be carefully followed up for both recurrent disease and the development of novel cutaneous malignancies. In the last decade some cases of Merkel cell carcinoma (MCC) in HIV-infected people were observed[88], and the risk of acquiring MCC was reported, if compared with the general population, to be 13-fold higher in this population by Engels *et al*[89].

Merkel cell carcinoma (MCC) is an uncommon, highly malignant, primary neuroendocrine tumour of the skin, that usually has its origin in the head, neck or extremities of elderly patients.

In 2008 a polyomavirus (Merkel cell polyomavirus, MCPyV) was reported to be a likely causative agent for the majority of MCCs[90,91]; this has been subsequently well established by multiple international groups[92].

Its clinical behavior is very aggressive and tendency to local recurrence, regional lymph nodes involvement and distant metastases are very high. Thus this tumor has to be regarded not as a localized skin cancer but as a systemic disease. We previously reported on an HIV-infected patient who developed a MCC with the only involvement of inguinal lymph node without evidence of primary skin localization[93]. We decided to administer to the patient, after surgical resection, postoperative radiotherapy and adjuvant combination chemotherapy with carboplatin and etoposide, according to paradigms established for small-cell lung cancer[94]. We did not document significant chemotherapy-related toxicities and the patient did not withdraw concomitant HAART. Even thought immunosuppressed patients with MCC were showed to have a poorer survival as compared to immune competent people[95], our patient did not experience a disease recurrence six years after the time of MCC diagnosis. A good performance status and a stable control of HIV infection with an effective HAART regimen should encourage clinicians to consider, for patients with MCC, systemic chemotherapy and adjuvant radiation in order to avoid regional and distant relapses of this cancer.

OTHERS

Only some retrospective studies and small case series are available to depict the distinct epidemiological and clinical features of other malignancies. In the early HAART period Sutton *et al*[96], showed that the estimated risk of acute myeloid leukaemia was twice if compared with the general population. The authors also showed that intensive chemotherapy proved to be effective to achieve completed remission of acute myeloid leukemia in 11 out of 15 HIV-positive patients. A low T CD4 lymphocytes count, regardless of karyotype, emerged as a predictor of a poor prognosis and short overall survival[97,98].

The incidence of cancers of the mouth and the pharynx, documented among HIV-positive people enrolled in the Swiss cohort from 1985 to 2002, was four-fold higher than in the general population (standardized incidence ratio, SIR = 4.1; 95%CI: 2.1-7.4), and this could be related to the smoking behavior since this was reported in 72% of the overall cohort of HIV-positive patients[83]. On the other hand the prostate cancer incidence rate was showed to be lower in HIV-positive people compared with HIV-uninfected men, even after adjusting for cancer risk factors[99,100].

In a study-linkage performed during 2003-2005 in 12 regions of United States with a population-based cancer ascertainment, Goedert *et al*[101] described the cancer profile of women diagnosed with AIDS. The incidence of breast (SIR = 0.69; 95%CI: 0.62-0.77) and uterine corpus cancers (SIR = 0.57; 95%CI: 0.39-0.81), but not of ovary cancer (SIR = 1.05; 95%CI: 0.75-1.42) was significantly lower than in the general population. The low risk of breast cancer among HIV-infected people could reflect the impairment of endogenous sexual hormone levels, and the ability of HIV to infect, replicate in, and to impair proliferation of breast cells. Breast cancer screening should be performed according to current relevant guidelines for the general population[52].

There is now a general agreement that HIV-positive patients who ensure a good adherence to an effective HAART regimen, and who are not affected by opportunistic infections, should be considered for the same anti-neoplastic treatment protocols for NADCs as in the general population, with a close monitoring of drug toxicity and interactions.

CONCLUSION

There is now a growing evidence that malignancies, whether they are strictly related to advanced stages of HIV infection, or not related to HIV-induced immunosuppression, are one of the main causes of death in the HIV-positive subjects. The effectiveness and tolerability of modern HAART regimens contributed to increase expectancy of life of these patients. Their progressive aging, the role of behavioral risks, such as smoking and alcohol intake, and other viral co-infections could negatively affect NADCs epidemic. In the other hand the availability of HAART and the better mean performance status of HIV-positive patients in the last decade, when compared with these in the pre-HAART era, gave clinicians the opportunity to treat NADCs with more effective chemotherapy regimens and to improve the long term survival. Further studies are needed to evaluate the best therapeutic approaches to NADCs and the impact of targeted cancer screening interventions among HIV-positive individuals.

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REVIEW

Post-transcriptional gene silencing, transcriptional gene silencing and human immunodeficiency virus

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Abstract

While human immunodeficiency virus 1 (HIV-1) infection is controlled through continuous, life-long use of a combination of drugs targeting different steps of the virus cycle, HIV-1 is never completely eradicated from the body. Despite decades of research there is still no effective vaccine to prevent HIV-1 infection. Therefore, the possibility of an RNA interference (RNAi)-based cure has become an increasingly explored approach. Endogenous gene expression is controlled at both, transcriptional and post-transcriptional levels by non-coding RNAs, which act through diverse molecular mechanisms including RNAi. RNAi has the potential to control the turning on/off of specific genes through transcriptional gene silencing (TGS), as well as fine-tuning their expression through post-transcriptional gene silencing (PTGS). In this review we will describe in detail the canonical RNAi pathways for PTGS and TGS, the relationship of TGS with other silencing mechanisms and will discuss a variety of approaches developed to suppress HIV-1 *via* manipulation of RNAi. We will briefly compare RNAi strategies against other approaches developed to target the virus, highlighting their potential to overcome the major obstacle to finding a cure, which is the specific targeting of the HIV-1 reservoir within latently infected cells.

Key words: Human immunodeficiency virus 1; RNA interference; Reservoirs; Epigenetics; Latency; Transcriptional gene silencing; Post-transcriptional gene silencing

Core tip: The lack of progress in developing an effective human immunodeficiency virus 1 (HIV-1) vaccine has motivated the pressing need for alternate therapies to cure HIV. RNAi therapeutics represent an alternate approach to a functional cure by offering specific targeting of the HIV-1 latent reservoir with the significant advantage of allowing cessation of combination antiretroviral therapy.

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INTRODUCTION

Human immunodeficiency virus 1 (HIV-1) infection can be successfully controlled by combination antiretroviral therapy (cART). However, the development of an effective vaccine or an alternative therapy remains the ideal solution since cART has several disadvantages. Adverse effects[1], high costs of therapy, emergence of resistant viruses[2,3] and in particular, the fact that life-long continuous treatment is required[4-6] are just a few examples. Years of research pursuing an HIV-1 vaccine have shown how challenging this task continues to be, with even the most promising trials showing only marginal efficacy[7,8].

Two main obstacles must be overcome to obtain either a vaccine or a cure. First, the high mutation rate of the virus allows extensive accumulation of genetic changes. These genetic changes generate variation with minimal compromise of the virus identity[9-11]; Second, the virus is never eradicated from the body, even after prolonged therapy[6]. While cART has been largely able to deal with the variability of the virus by simultaneously targeting multiple key steps of its replication cycle, it has no direct effect upon latently infected cells[11,12]. The latter, commonly known as latent reservoirs, includes very long-lived resting memory CD4+ T cells[13], macrophages and other cell types[14,15], all of which carry latent proviruses. Provirus refers to the viral form that has been integrated into the cell's genome and is inherited through each cell division. Latent means it is transcriptionally inactive, but is able to re-activate after stimulation[16-19] and is capable of causing substantial viremia when therapy ceases[20,21].

The viral reservoir, a term used to refer to the latently infected cells as a whole, is maintained throughout the life span of an infected individual. During episodes of low-level viremia and/or homeostatic proliferation of T cells the reservoir seems to be replenished, but contribution of each of these processes is still disputed[21-24].

Latently infected cells are considered the major obstacle to a cure for HIV. They remain immunologically and biochemically silent, becoming invisible to the immune system with no expression of viral antigens on their surface. The only known difference between latently infected cells and un-infected cells is a newly integrated "gene": the genome of the HIV provirus.

Considerable effort has been put into understanding the molecular mechanisms of latency in order to develop strategies that specifically target either the latently infected cells or directly target the provirus within them. The establishment of latency results from a variety of molecular mechanisms, mainly transcriptional interference and epigenetic mechanisms. It is believed that there is a repressive epigenetic component in most of the inducible proviruses. This component is facultative heterochromatin, a compact yet dynamic state of chromatin that impedes proviral transcription[25-27]. Opposing approaches, which aim to modify the repressive epigenetic profile established at the HIV promoter, have been developed. These either activate proviral transcription by inducing chromatin relaxation or obstruct transcription through stabilization of heterochromatin.

The first strategy has already been tested in cells from HIV infected (+) patients and is currently being tested in a number of clinical trials (http://aidsinfo.nih.gov/clinical-trials/search/b/0/reservoirs and http://aidsinfo.nih.gov/clinical-trials/search/b/0/vorinostat), using pharmacological drugs or cytokines that directly and/or indirectly induce activation of HIV provirus through a variety of cellular pathways[28-30]. However, while viral transcripts from apparently latently infected cells have been detected, no significant change or reduction in the size of the latent reservoir-proviral integrated DNA-has been observed[30,31]. There is currently a debate as to whether these cell associated viral RNA transcripts represent transcripts driven by the endogenous HIV promoter, the 5'LTR or whether these are so called "read through transcripts" which arise from altered expression from the promoter of the parent gene into which HIV has integrated[32]. The results of further trials of these agents are awaited.

The second strategy is based on RNAi and has the advantage of being specifically directed to viral mRNAs or the provirus regardless of the cell type infected. Aiming to target persistent infection in the first place, most RNAi approaches are designed to directly cleave HIV mRNAs and were first designed in the early 2000s. Significant advances have transpired in the field, beginning from those manipulating PTGS to target viral mRNAs and cellular cofactors that support HIV replication, to those using TGS to induce heterochromatin at the HIV promoter. In this review we will discuss both PTGS and TGS RNAi based approaches for HIV, and provide a brief commentary on other gene therapy alternatives currently under development.

RNAi

RNAi is an evolutionarily conserved mechanism that is present from lower eukaryotes through to mammals. Because it is beyond the scope of this review to discuss each of these, we will mainly focus on the mammalian RNAi pathways. However, we will also include some other species-specific examples to illustrate pertinent points.

The first evidence of RNAi was reported in transgenic tobacco plants expressing antisense or sense RNAs from the coat-protein gene of the tobacco etch virus (TEV)[33,34]. The plants did not show evidence of infection after challenged with TEV, suggesting the presence of a protective nucleic acid-dependent mechanism that was later proved to spread throughout the plant in a systemic way (reviewed in[35]). The precise mechanism was described in the worm *Caenorhabditis elegans (C.*

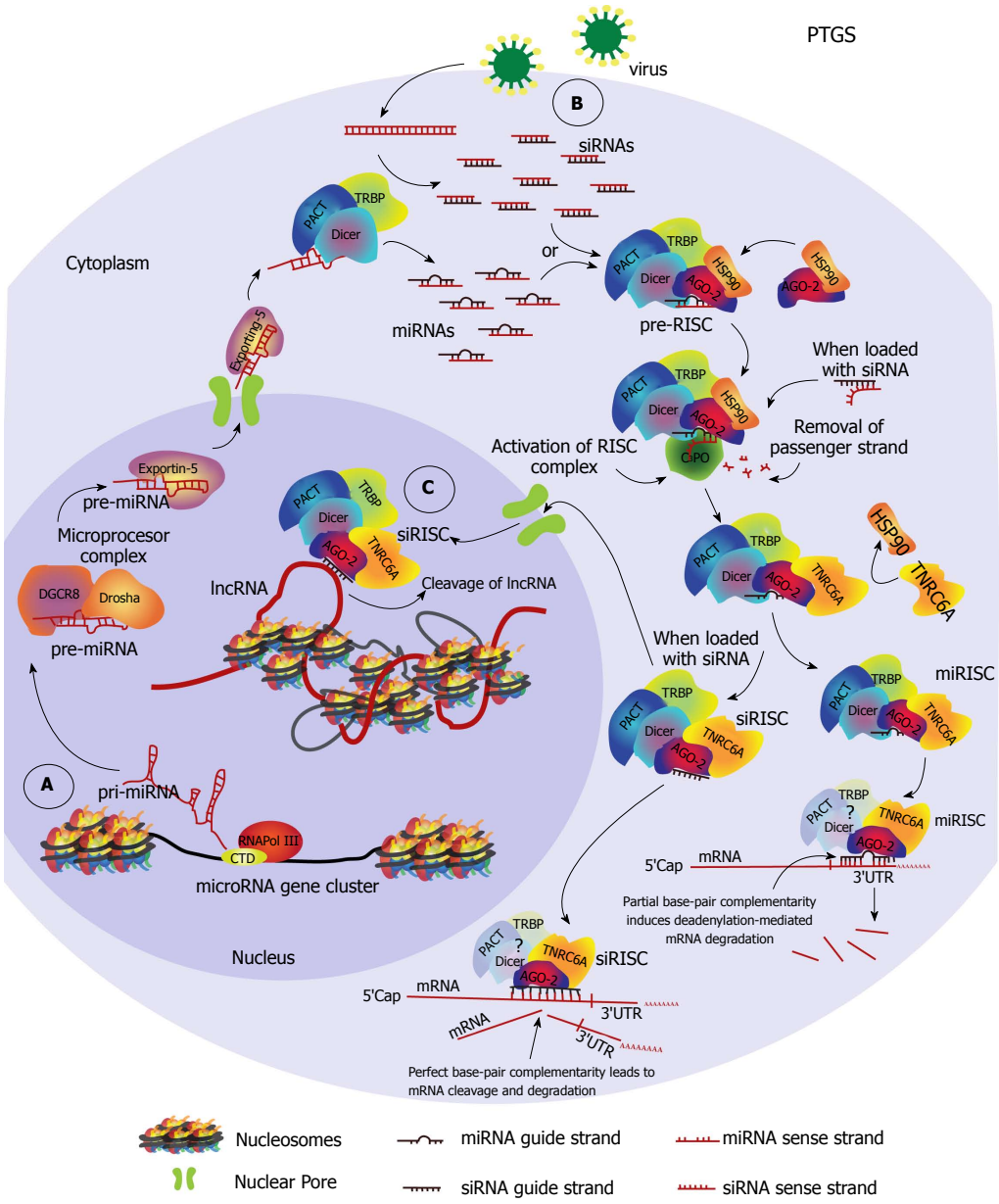

Figure 1 Cytoplasmic and nuclear post-transcriptional gene silencing pathways. A: A primary-microRNA (pri-miRNA) is transcribed by the RNA Polymerase III (RNA Pol III) from a miRNA gene cluster. The pri-miRNA is then processed by the microprocessor complex into the precursor-miRNA (pre-miRNA) which is exported to the cytoplasm by exportin-5. In the cytoplasm, dicer in complex with Tar-RNA-binding protein (TRBP) and protein kinase R activator of transcription (PACT), process the pre-miRNA into miRNA duplexes. MiRNA duplexes are ioaded into argonaute (AGO) proteins 1-4 with help from heat shock protein 90 (HSP90), forming the miRNA pre-RNA-induced silencing complex (pre-miRISC). The pathway is shown for AGO-2. The pre-RISC complex is activated after removal of the passenger strand from the duplex by C3PO, becoming the miRISC. TNRC6A becomes part of the complex. MiRISC finds a target region within the 3'UTR of an mRNA and induces deadenylation-dependent mRNA degradation; B: During viral infections double-strand RNA (dsRNA) intermediates of viral replication are processed by DICER/TRBP/PACT and are loaded into AGO-2 to form the siRISC complex after removal of the passenger strand. Complete complementarity between the guide strand siRNA and the target region induces cleavage of the targeted mRNA. MiRNAs can also induce mRNA cleavage if this condition is satisfied; C: A nuclear post-transcriptional gene silencing pathway can occur when an activated siRISC is imported into the nucleus and identifies a target within a nuclear RNA such as a Long-non-coding-RNA (lncRNA) resulting in cleavage of the RNA molecule.

elegans), in which interference of endogenous gene expression through inoculation of homologous dsRNA molecules was demonstrated and the involvement of a catalytic and an amplification event was suggested[36-38]. It was further demonstrated that RNA interference, as it began to be known, resulted in genetic silencing and co-suppression of the targeted gene[37,38]. Following this discovery, vast exploitation of RNAi for discovery of gene function in reverse genetics of mammalian cells began and soon after was developed as a therapeutic tool, with several clinical trials currently underway for a variety of human diseases (http://www.clinicaltrials.gov/ct2/results?term=RNAi&Search=Search)[39]. This RNAi pathway is known as PTGS (Figure 1). It functions in the cytoplasm and impedes translation of an mRNA into protein, by direct cleavage or by initiating degradation of the targeted mRNA sequence.

It was not until 2004 that the nuclear RNAi pathway

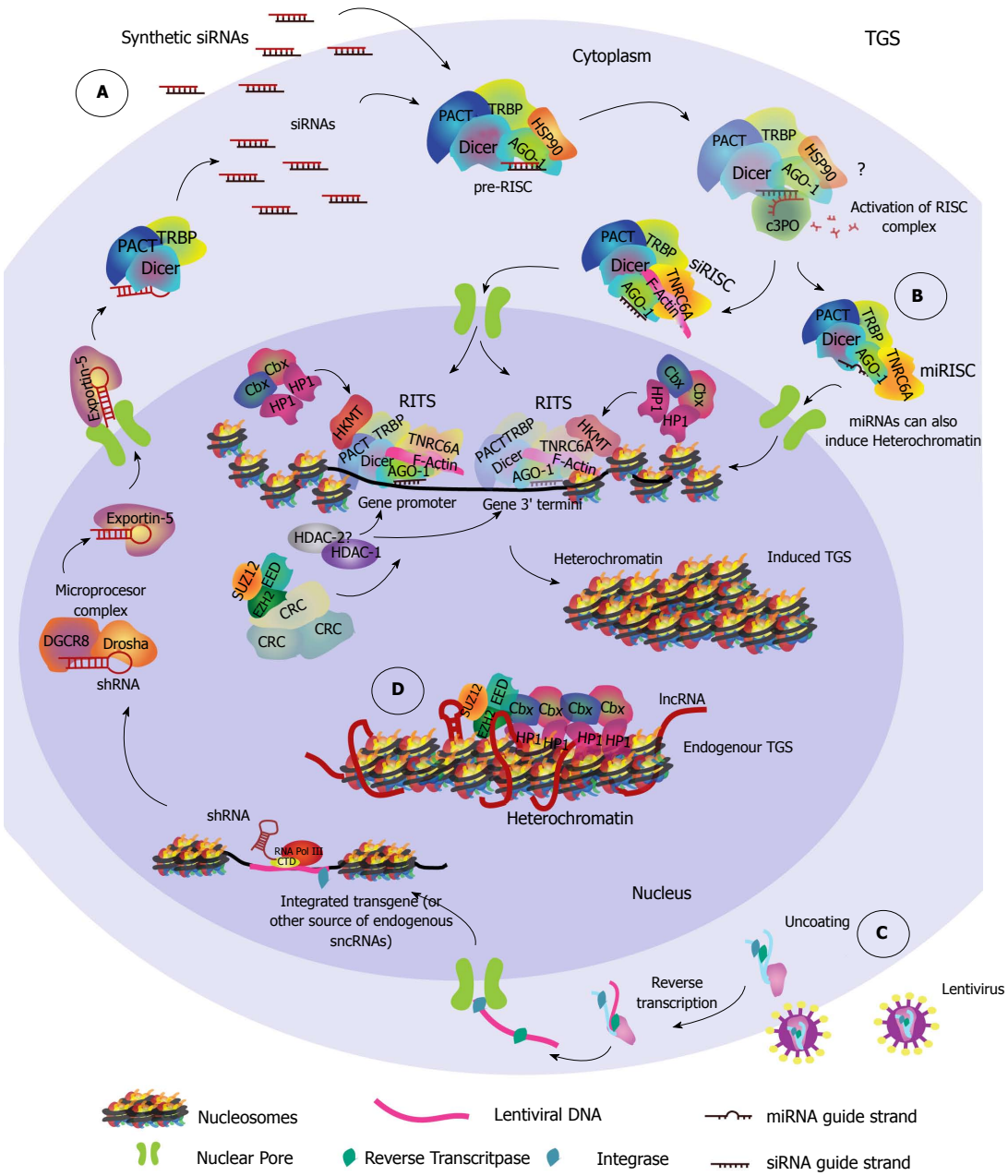

Figure 2 Endogenous and induced transcriptional gene silencing pathways. A: Synthetic siRNAs that have been designed to target the promoter region or the 3' end termini of a gene are loaded into AGO-1, forming the pre-RISC complex. It is currently unknown if removal of passenger strand is required, nonetheless if it occurs it probably takes place in the cytoplasm following the same steps as used in PTGS. F-actin participates in the nuclear import of the RISC complex which, once in the nucleus becomes the RITS complex as histone-lysine-methyltransferases (HKMTs) and other epigenetic related proteins such as Histone-deacetylases (HDAC), DNA methyltransferases (DNMTs), Histone Protein 1 (HP1) and others assemble with it. It is unknown whether RISC related proteins remain in the RITS complex. The RITS complex may vary in its composition depending on the chromatin microenvironment and the small non-coding RNA (sncRNA) target region, therefore only some proteins are shown as an example. Establishment of repressive epigenetic marks (not highlighted for simplicity) and further recruitment of chromatin remodeling complexes (CRCs) results in heterochromatin formation and induces transcriptional gene silencing (TGS). Two independent target regions are pictured together to show the different regions of a gene that can be targeted to induce TGS; B: miRISC complexes whose guide strand targets a promoter region may be exported into the nucleus, form a RITS complex and induce TGS in the same way as was described for siRISC complexes; C: Lentiviruses can be used to drive transgene integration of a DNA cassette designed to express a shRNA that induces TGS. ShRNAs are transcribed by RNA Pol III and are processed through the microRNA pathway. In the cytoplasm they are converted to siRNA duplexes that are loaded into RISC complexes and follow the same import pathway to induce TGS as explained in A; D: Endogenous transcriptional gene silencing is induced by a long-non-coding RNA (lncRNA) whose secondary structure is recognized by members of the Polycomb Group repressive complex 1 (PRC1), such as enhancer of zeste 2 (EZH2), embryonic ectoderm development (EED) and suppressor of zeste 12 (SUZ12). The interaction recruits HP1 and other proteins of PRC1 complexes like the (Chromobox) Cbx family that contain a chromodomain able to induce heterochromatin formation.

TGS, involving chromatin compaction, was identified[40] (Figure 2). Presently, both PTGS and TGS have been found to be functional in the nucleus of mammalian cells, but only TGS seems to repress gene transcription directly through chromatin remodeling.

During RNAi small non-coding RNAs (sncRNAs) are used as guides through sequence homology to target either mRNA transcripts or gene promoters[41-43]. These

sncRNAs are loaded into Argonaute proteins forming the main effector complex; however, other cellular cofactors are required for the process to occur. There are three major kinds of sncRNAs involved in RNAi: small interfering RNAs (endogenous- and exogenous-siRNAs), microRNAs (miRNAs) and piwi-associated RNAs (piRNAs)[44], which we will describe briefly in the next section. The Argonaute proteins are further subdivided into the Argonaute subfamily (AGO-1, AGO-2, AGO-3 and AGO-4, in humans), and the Piwi subfamily (HILI or PIWIL2, $HIWI_1$ or PIWIL1, $HIWI_2$ or PIWIL4 and $HIWI_3$, in humans)[44,45]. There are also species-specific AGO proteins that we will not discuss (recently reviewed in[46]), with the exception of specific examples.

Recently, many novel non-canonical sncRNAs involved in RNAi have been discovered[47-49]. However we will only focus on the three major classes previously mentioned. SncRNAs are generally classified depending on their biogenesis (Dicer/Drosha dependent or independent), their size (about 21-30 nt) and the Argonaute protein they bind (AGO 1-4). They can be endogenous or exogenous depending on their origin. The endogenous sncRNAs are produced from transcription units (Figure 1A), protein coding genes (exons and introns), convergent promoters, long non-coding RNAs (lncRNAs), gene clusters, repetitive elements or retro-elements, such as transposons, while the exogenous sncRNAs are either synthetic or of viral origin (Figure 1B).

SncRNAs

SiRNAs: Exo-siRNAs and endo-siRNAs: SiRNAs are about 21-nt long duplexes generated in the cytoplasm by cleavage of endogenous or exogenous long dsRNA precursors (*e.g.,* lncRNA) by the endonuclease Dicer (Figure 1B). These siRNAs are then loaded onto a specific AGO protein. When exogenous, synthetic siRNAs may be delivered to the cells by transfection/nucleofection protocols or may originate from expression of artificial integrated constructs (lentivirus transduction), such as short-hairpin RNAs (shRNAs). Naturally occurring exo-siRNAs in mammalian cells were not discovered until very recently, and were found to originate from dsRNA intermediates of viral replication in mouse embryonic stem cells[50]. On the other hand, mammalian endo-siRNAs were identified in somatic tissue and found to be processed through a non-canonical Drosha-independent mechanism, from Dicer cleavage of a long nuclear hairpin RNA expressed from short interspersed nuclear elements (SINEs)[51].

Generally, siRNAs direct the cleavage of their cognate mRNA through PTGS when they mutually base pair with perfect complementarity[52-54]. Mutations in the siRNA or in the target region within the mRNA sequence usually reduce or abolish silencing, which is why RNAi is considered a very specific mechanism[55,56]. In addition, siRNAs can also induce TGS, a nuclear RNAi pathway, whenever they target a complementary sequence within the promoter or the 3′ end of a gene[57,58]. Additionally, siRNA-directed transcriptional gene activation (TGA) has also been reported for several genes[57,59].

miRNAs: In their canonical pathway miRNAs are first transcribed as primary-miRNAs (pri-RNA) by RNA Pol II, and are then processed by the nuclear RNAse III protein Drosha - an RNAse type III enzyme (Figure 1A). Drosha and co-factor DiGeorge syndrome critical region gene 8 (DGCR8) form the Microprocessor complex. This complex generates precursor-miRNAs (pre-miRNA) that are further processed, exported to the cytoplasm by Exportin 5, and cleaved by Dicer. Dicer generates 22-nt miRNA duplexes that are analogous to siRNA duplexes. Non-canonical pathways exist which are Drosha, Dicer or DGCR8 independent. Importantly, unlike siRNA-duplexes, miRNA-duplexes frequently contain mismatches and about the first 2-7 nts at the 5′ end of the guide strand, known as the seed region, may target the 3′ untranslated (3′ UTR) region of multiple mRNAs. Based on this multiple targeting ability, other biochemical characteristics and evolutionary conservation, microRNAs are clustered into families (http://www.mirbase.org/index.shtml). In miRNAs, complete base pair complementarity with target mRNA is found within this seed region, which allows for mismatches towards the 3′ end. MiRNAs predominantly direct deadenylation-dependent mRNA-decay that results in translational repression, but they are also able to induce sequestration. While common in plants, in mammals on rare occasions when miRNAs show complete complementarity to the target region they can induce cleavage of the mRNA[60-62]. Deadenylation and other ways of translational repression and sequestration result from partial complementarity between miRNA and the target mRNA[44,47,63,64]. In a similar way to siRNAs, when mature miRNAs show complete homology to a promoter region, they are able to induce TGS[65-67] (Figure 1C). However, this miRNA pathway is not well described due to the few cases that have been reported.

piRNAs: PiRNAs are longer (about 25-31 nt) than siRNAs or mature miRNAs and have fundamental roles in maintenance of stemness, transgenerational inheritance and genome instability through targeting of repetitive sequences (*e.g.,* endogenous retroviruses and transposon elements), among other functions (reviewed in[68]). In mammals, piRNAs are expressed in germ cells and somatic germ cells (SGC), but their role in somatic stem cells, such as hematopoietic stem cells, remains controversial[69,70]. Even though there is expression of piwi-pathway-specific AGO proteins in human CD34+ stem cells[71], further evidence is still required to confirm a functional piRNA pathway in somatic stem cells different to SGCs.

Interestingly, piRNAs direct specific genome rearrangements in ciliates and this precise genome editing results in either somatic elimination[72] or retention[73], indicating the versatility of this particular RNAi pathway. PiRNAs can target mRNAs through a PTGS-like mechanism, however they may also induce TGS by directing heterochromatin formation at the target regions[74,75]. Intriguingly, members of the piRNA pathway are highly expressed in certain human cancer cells (reviewed in[76]), though it is still

unknown whether they are the cause or the effect. To our knowledge there are no reports regarding the use of synthetic piRNAs and since they have not been manipulated for human therapy we will not explore these further. However, the ability of piRNAs to establish a permanent, stable and inheritable silencing through directed epigenetic chromatin modifications and other mechanisms makes them of great interest for future study, especially since silencing mediated through their activities is inherited to every single cell of a multicellular organism.

RNAi pathways: PTGS and TGS

PTGS: In humans, RNAi induced mRNA cleavage is directed only by AGO-2[54,77]. Loading of the sncRNA duplex onto the AGO proteins is well described for AGO-2 and involves the heat-shock protein 90 (HSP90) (Figure 1). HSP90 aids in the recruitment[78] and stabilization[79] of unloaded AGO within processing bodies (P-bodies). Inhibition of HSP90 results in unpaired siRNA- and/or miRNA-dependent silencing, respectively. These P-bodies are cytoplasmic structures that contain mRNA decay factors, untranslated mRNA, translational repressors and RNAi related factors[80]. The active silencing complex is named RISC or miRISC, depending on the type of sncRNA (siRNAs or miRNAs, respectively) that is loaded onto the AGO protein. We will refer to both as RISC, unless specified.

The sncRNA-duplex/AGO-2 complex is called pre-RISC (pre-RNA-induced silencing complex) and requires the removal of one of the strands of the RNA, the passenger strand, in order to become an active RISC complex[81,82] (Figure 1). The strand that remains in RISC is known as the guide strand. Passenger/guide strand selection depends on the individual thermodynamic properties of each sncRNA molecule within the duplex; these properties generally create energetic asymmetry between the duplex ends, allowing differentiation and selection of the guide strand[83]. Asymmetry means that the duplex is energetically less stable at one 5′ end and causes unwinding to begin at this site. As a result, the strand whose 5′ end lies in the less stable end of the duplex will be loaded onto the AGO protein, becoming the guide strand. Whenever the energetic difference between the duplex ends is small or negligible, both strands may be randomly loaded[83,84].

Dicer seems to play a role in sensing and positioning the guide strand, facilitating removal of the passenger strand. This ability appears to be activated through its interaction with Transactivation Response (TAR) RNA-Binding Protein (TRBP) and Protein Kinase RNA (PKR) Activator (PACT), both double-stranded RNA binding proteins (dsRBP)[85] (Figure 1). However, there is contradictory evidence regarding this role for Dicer[85,86], and further research may be needed to clarify these observations. Nonetheless, it is important to mention that proper selection of the guide ensures specificity towards silencing the intended target.

Pre-RISC activation requires the slicer activity of AGO-2, specifically the nicking of the passenger strand[87-89]. After nicking, the endonuclease component 3 promoter of RISC complex (C3PO), composed of Trax and Translin proteins in humans, is able to cleave and remove the passenger strand[87,90,91]. This results in activation of pre-RISC into the RISC complex. Within RISC, the guide strand is used to scan mRNAs for a region with full or partial base pair complementarity. Once the region is found the mRNA is either cleaved, deadenylated or stored during translational repression[63,92,93] (Figure 1). Storage and repression of translation may have a role in gene regulation of processes that require a quick response, as translation can be initiated from stored transcripts rather than relying on *de novo* transcription[94].

While AGO-2 can direct either cleavage or translational repression of mRNAs, non-catalytic AGO proteins like AGO-1 seem mostly involved in translational repression, since they are unable to cleave mRNA transcripts[46]. Furthermore, it is currently unknown how RISC activation occurs for non-catalytic AGO proteins (AGO-1, 3 and 4). However, owing to their inability to cleave mRNAs, activation of the silencing complex must either be different or rely on help from additional cofactors.

For silencing to occur, human AGO-2 requires direct binding with TNRC6A, also known as GW182, a mRNA binding protein rich in glycine/tryptophan repeats[95,96]. Interaction between GW182 and AGO-2 proteins is crucial for miRNA-mediated silencing and appears to take place directly after the passenger strand is removed by C3PO[96,97]. Both AGO-2 and GW182/TNRC6A have been shown to co-localize with siRNA and miRNAs within GW bodies (GWB)[98], another term for P-bodies. Based on these observations, it has been proposed that silencing by miRNAs requires an effector complex formed of at least one AGO and one GW182/TNRC6A protein[99] (Figure 1).

Interestingly, human GW182/TNRC6A was found to transport AGO-2 proteins to the nucleus during miRNA-induced silencing of a nuclear non-coding RNA[100]. The latter constitutes evidence of a nuclear PTGS pathway (Figure 1C). Indeed, increasing evidence supports a functional nuclear PTGS pathway, with a recent study demonstrating not only the presence of PTGS related proteins in the nucleus of mammalian cells, but an active AGO-2-RISC complex able to efficiently cleave two nuclear lncRNAs, Malat1 and Neat1[101]. These studies add to previous evidence indicating the existence of a nuclear RNAi pathway and suggest that PTGS and TGS may be closely related.

We have aimed to provide a detailed overview of the molecular mechanism of PTGS in order to understand the unknowns of TGS and further compare the manipulation of PTGS or TGS for HIV gene therapy. PTGS pathways have been exploited for HIV therapeutics, and several clinical trials are currently testing PTGS-based gene therapy approaches directed to cellular and viral transcripts. A major disadvantage of using PTGS to treat HIV is that PTGS requires viral transcription because it acts on mRNAs. First, this gives the virus the chance

to evolve resistance mutations and escape silencing; Second, latent proviruses will not be targeted since they are not undergoing active transcription. Improvements in siRNA/miRNA design and expression have been developed aimed at overcoming these caveats and will be discussed in the HIV-1 section.

TGS: TGS is a conserved mechanism of gene regulation across species and has been extensively studied in the plant model *Arabidopsis thaliana (A. thaliana)*, the worm model *Caenorhabditis elegans (C. elegans),* and the fission yeast *Schizosaccharomyceae pombe* (*S.pombe*). The first evidence for TGS was observed in plants, and it was found to require siRNA-induced DNA methylation for heterochromatin formation (recently reviewed in[102]). However, the mechanism in *S.pombe* shed insight on the identification of TGS in mammals. In this microorganism, siRNAs generated from centromeric repeats are first processed by Dicer, then loaded onto AGO-1, and together with the proteins Chp1 and Tas3 form the silencing complex, namely the RNA-induced initiator of transcriptional gene silencing (RITS) complex. This RITS complex is analogous to the RISC complex from PTGS. RITS is then directed through siRNA base pair complementarity to a specific locus at which it induces recruitment of Clr4 (histone methyltransferase) and Swi6 (chromo domain binding protein) in order to establish and spread heterochromatin domains[103-105].

Human sncRNA-directed TGS is mainly, but not exclusively directed by AGO-1 rather than AGO-2, and is generally triggered by promoter-targeted sncRNAs (Figure 2). Recent evidence suggests that it may be also triggered by sncRNAs that target the 3' termini of genes[58,106]. While increasing evidence suggests a role for AGO-2 in nuclear gene silencing[100], it seems to be predominantly through a nuclear PTGS that involves RNA cleavage[101], with only few described exceptions[107]. There is also evidence of RNA-induced nuclear silencing without heterochromatin formation, involving both AGO1 and AGO2[108], and there seems to be various RNA-directed nuclear-pathways that control transcription at different stages[109]. However, it is generally accepted that heterochromatin and its associated markers (*i.e.,* histone methylation and deacetylation) is a characteristic feature of TGS. Therefore, for this review we will focus on the different endogenous TGS mechanisms that involve heterochromatin formation induced by sncRNAs loaded into an AGO protein.

Heterochromatin is considered a hallmark of repressive silent chromatin, ubiquitous in eukaryotic organisms. In mammals, its establishment at a particular locus is a result of protein interactions and cross talk with multiple silencing mechanisms such as DNA methylation, genomic imprinting and Polycomb group of proteins (PcG)[65,103,110]. The epigenetic profiles across mammalian genomes are very heterogeneous and show a wide range of silencing dynamics. Silencing extends from permanent and inheritable to inducible, dynamic silencing. The former is mainly but not restricted to, constitutive heterochromatin and is found in centromeres and telomeres[111]; while the latter, predominantly within facultative heterochromatin, controls specific gene expression during differentiation and development[112].

SncRNA-directed TGS in mammalian cells has been a controversial topic since its discovery, nearly a decade ago, with some still doubting its existence. These doubts have relied on the inability to explain in detail the molecular mechanisms driving TGS. In particular, the much awaited identification and characterization of a functional nuclear mammalian RITS complex, because there are apparently no RNAi proteins with homology to Tas3 and Chp1 present in the nucleus and AGO-1 is non-catalytic. At present, most of the evidence of mammalian sncRNA-AGO-1 directed TGS relies on synthetic siRNAs or shRNAs driving TGS to control infectious agents, such as HIV-1[113], or cellular genes that support viral replication[114]. Nonetheless, the relatively slow accumulation of evidence has supported the existence of this functional pathway, with evidence for miRNA-induced TGS in senescence[107] and in differentiation[65]. We will explain the basis for the doubts and show the recent evidence supporting mammalian sncRNA-directed TGS.

The breakthrough proving the existence of a TGS mechanism in mammalian cells came with the identification of the human ortholog for Clr4, known as Suppressor of variegation (Su(var)3-9) in *D. melanogaster* and Su(var)39H in humans; and then with the ortholog for Swi6, known as Histone Protein 1 - alpha (HP1-α) (in both *D. melanogaster* and humans)[115-117]. However, no human orthologs for Chp1 and Tas3 proteins from fission yeast RITS complex have been yet identified. At present, there are more questions than answers about the series of events in humans that result in siRNA-AGO-1 mediated heterochromatin formation and activation of the RITS complex. It is possible that both PTGS and TGS share a core multi-protein complex, which may differ in accessory subcellular or pathway-specific co-factors, because the initial steps of TGS may potentially resemble those of PTGS.

There is also controversy regarding the activation of the RITS complex during TGS. It is assumed that removal of the passenger strand occurs during TGS to allow the RITS complex to scan for the target sequence that is complementary to the guide strand. However, since AGO-1 lacks the catalytic amino acid tetrad DEDH responsible for the slicing function, it is not clear how this process occurs[118]. AGO-1 needs to nick the passenger strand from the siRNA duplex, so C3PO or a similar complex would be able to remove the passenger strand.

On one side, it was shown *in vitro* from bacterially expressed human AGO proteins, that AGO-1 is able to cleave the passenger strand, but requires assistance for removal of the cleaved fragments[119]. This has been interpreted as non-catalytic AGO proteins being very inefficient catalysts and having an extremely low nickase activity.

This is in agreement with findings in mouse embryonic stem cells, in which the absence of the four mammalian AGO proteins resulted in apoptosis, but the expression of any one of the other AGO proteins in isolation, was

enough to rescue the cells and restore a functional RNAi pathway, showing evidence for functional redundancy[120]. In addition, another study showed that non-catalytic AGO proteins are loaded within the duplex but removal of passenger strand takes place approximately 2 to 3 d[121]. The process of passenger strand removal is currently unknown.

In contrast, the crystallographic structures of human AGO-1 in association with endogenous RNA (1.75 Å) and in association with Let-7 miRNA (2.5 Å) were used to show that while highly similar to hAGO-2-RNA structures, there was an absolute requirement for the introduction of the catalytic tetrad by introduction of a single point mutation as well as the reconstitution of a loop called PL3, in order to restore the slicer functionality of AGO-1[122]. These observations argue against a catalytic role for AGO-1.

It seems more likely that other proteins aid non-catalytic AGOs during this step. These cofactors would be present in the AGO knockout mice study and in the cells used to show removal of passenger strand after a few days, but not in the bacterial system, in which cleaved fragments remained loaded to the AGO proteins. Comprehensive studies are required to address this question definitively.

An increasing number of studies have found PTGS-related proteins in the nucleus of mammalian cells, such as GW182/TRNC6A and the endonucleases hC3PO and Dicer[100,123-125]. These proteins appear to have functions related to both to the mechanisms underpinning PTGS in the nucleus and to the regulation of chromatin and transcription.

For example, human Dicer has been shown to interact with NU153, a non-canonical nuclear transport nucleoporin, as demonstrated by co-localization within the nucleus[125]. In addition, human Dicer has been shown to associate with the chromatin structures of ribosomal DNA[124]. It also has a role in termination of transcription[126], in regulation of intergenic transcription in the human β-globin gene cluster[127] and in regulation of nuclear receptor (NR) signaling, as evidenced by direct binding of Dicer to NR promoter regions[128]. Further, Dicer has been reported to be required in heterochromatin formation in fission yeast[129] and in vertebrates[130], suggesting its presence in the nucleus of human cells could be due to an as yet unidentified role in mammalian TGS (Figure 2B).

We previously mentioned that GW182/TNRC6A shuffles AGO-2 proteins between the nucleus and cytoplasm through a non-canonical nuclear localization signal[100]. Additionally, GW182/TNRC6 associates with all four RNA loaded-AGO proteins during PTGS. Therefore, it is a possibility that Dicer is contained within a loaded AGO-1-TRNC6A complex during the nuclear shuffling that occurs during TGS[131] (Figure 2). Furthermore, the interaction between GW182/TNRC6 and AGO-1 occurs through binding of the GW repeats of GW182/TNRC6 to the Piwi domain of AGO-1[77]. This is intriguing because the fission yeast RITS member protein, Tas3, has a GW-repeat-containing motif and interacts with AGO-1 to promote TGS[132]. It is therefore possible that, the Tas3/AGO-1 interaction in fission yeast could be analogous, not homologous, to the AGO-1 and GW182/TNRC6 interaction in humans. Consistent with this hypothesis, the plant specific PTGS-related GW protein NERD was found to be involved in TGS in *A. thaliana*[133]**.** Thus, there is evolutionary evidence supporting the likelihood of a link between the two mammalian pathways in the nucleus.

Protein complexes containing AGO-2, TNRC6A, Dicer and TRBP have been immunoprecipitated from human isolated cell nuclei. These protein complexes were able to induce PTGS, with the specific cleavage of four different nuclear lncRNAs mediated by corresponding siRNAs[101]. Similar complexes were immunoprecipitated with nuclear AGO-1 and found to harbor the same PTGS proteins, supporting the notion of a core complex for both pathways. However, this study did not identify proteins that have been implicated in the loading of sncRNA onto AGO proteins, such as C3PO and HSP90 within mammalian cell nuclei. In previous studies the identification of these proteins could have been the result of contamination from cytoplasmic remnants. This study highlighted the importance of ensuring that isolated nuclei are free from endoplasmic reticulum (ER) to avoid contamination with cytoplasmic AGO-containing complexes. Recently, a comprehensive protocol was developed to ensure that purified nuclei are free from ER contamination[134].

It is important to note that the majority of studies aimed at understanding the mechanisms of loading and activation of silencing complexes incorporating non-catalytic AGO proteins have done it in the context of PTGS, either in the cytoplasm or in the nucleus. These studies have not specifically targeted genes embedded in chromatin. Therefore, a possibility remains that siRNAs or miRNAs that are only homologous to specific regions such as promoter regions, can be identified and differentially processed. In this way, complexes could share a common core, but would vary in accessory proteins that modify their function to induce either TGS or PTGS.

Consistent with this model, a recent study unveiled a sorting mechanism in humans, which directs differential loading of AGO-1 proteins for unique sncRNAs in the setting of a viral infection[135]. However the determinants of this selection remain unknown. Nonetheless, most sncRNAs were loaded in equivalent ratios to AGO-1 and AGO-2 proteins and thus these unsorted sncRNAs may be used to scan targets in both cellular compartments. Therefore we hypothesize that when there are targets in both compartments, both pathways are likely to occur, depending how efficient each of these sncRNAs is for the pathway.

While the understanding of the molecular mechanisms of PTGS is reasonably complete, and there is some evidence of commonalities with TGS, there are far many more uncertainties in the TGS mechanism. Several important early steps in the TGS mechanism remain to be fully deciphered, including the precise mechanism that determines RITS recognition of target, the characteristics or type of target and the determinants of induction of different epigenetic heterochromatin profiles. In addition,

while human TGS can be thought of at a single cell level, its implication needs to be considered within the context of a multicellular organism. Many changes or epigenetic check points occur early during embryogenesis and development or during cell differentiation. While some changes are dynamic allowing differentiation of cells down different pathways, once certain check points are reached epigenetic profiles are more stable and are inherited to daughter cells through multiple cell divisions.

At present, there is evidence supporting two main models describing target recognition. The first is a siRNA/DNA-binding model[65,136], during which the RITS complex binds directly to chromatin. This binding seems to be dependent on the interaction between the siRNA and its DNA-target sequence. Once the interaction has taken place it triggers the *in situ* recruitment of chromatin remodeling factors that induce heterochromatin and establish silencing (Figure 3A). We previously introduced the unresolved question of how the passenger strand is removed. In the TGS model however, each strand of the duplex will find a target on DNA, in the same location but on different DNA strands. Therefore, for the sake of identifying the target region, both strands are potentially useful. In HIV-1, a siRNA guide-strand targeting a promoter region will find two target sites. One on the 5′ LTR of the sense strand, and the other in the antisense strand in the region that is complementary to the 3′LTR of the sense strand (Figure 3B).

In the second model the RITS complex binds to an RNA intermediate, finding its target in either an antisense transcript or in a sense nascent transcript (recently reviewed in[59] and in[137]). In this model, only one strand of the duplex acts as the guide strand (Figure 3C). Presently, there is more experimental evidence supporting the RNA model given that, owing to its similarity with lncRNAs silencing mechanisms, more studies have tested this hypothesis. Though, there are still critical gaps in the data and more evidence is required to further evaluate the DNA model. It is possible that each of the models occur under particular conditions and potentially a variety of mechanisms control the diverse and precise regulation of gene expression in humans.

Establishment of heterochromatin is a progressive process. Once the RITS complex has found its target region a series of events follow, which generally initiate with removal and or replacement of specific histone-tail post-translational modifications to alter the biochemistry and structure of the associated chromatin (Table 1). Numerous histone modifications important for histone structure and gene regulation have been described[138], however we will only be discussing canonical acetylation and methylation marks that have been related to TGS and HIV-1. The different histone tail modifications are generated and recognized by histone deacetylases (HDACs), histone and DNA methyltransferases (HMTs and DNMTs, respectively), and chromatin modifying complexes. Ultimately, the combination of histone tail modifications and the recruitment of protein complexes make up a pattern that relates to the specific transcription state of a gene (a recent review can be found in[139]).

HDACs are required early in heterochromatin formation and remove acetylation (Ac) marks that are frequently found in actively transcribing chromatin. HDACs appear to be continuously recruited to epigenetically repressed loci[140], however, in very robust silencing, HDACs may not be continuously recruited. HDACs are recruited to chromatin by different mechanisms that are in some cases dependent on DNA methylation in CpG islands (discussed below). This differential recruitment is attributed to HDACs being able to form higher order complexes that may or may not include methyl-CpG-binding domain (MBD)-containing proteins[141].

The removal of Ac marks is necessary for the establishment of methylation repressive marks and chromatin compaction[142]. Several lysine residues from histone tails can be methylated by specific histone lysine methyltransferases (HKMTs) in order to repress chromatin (Table 1). Methylated residues are recognized by HP1 and HKMTs, both of which bind to chromatin and dimerize to induce chromatin compaction[143]. Nucleosome compaction exposes hidden lysine residues that become accessible to further methylation by HKMTs. Progressive methylation recruits more HP1-α and chromatin remodeling complexes. Chromatin remodeling complexes promote the establishment and spread of heterochromatin through a positive feedback loop with HP1[144] (Figure 3A).

Heterochromatin is also the final outcome of DNA methylation, genomic imprinting[145] and Polycomb (PcG) mediated silencing[65,146]. Therefore, RNAi-induced TGS has the potential to induce a variety of epigenetic profiles.

CpG islands (CGIs) are genomic regions that are unusually high in their CG or CpG content when compared to the genomic average of these nucleotides. CGIs are predominantly found in promoter regions and are demethylated during active gene transcription[40]. Conversely, methylation of promoter CGIs is associated with epigenetic gene repression. Thus, DNA methylation accounts for another layer of control of gene expression. It is well known that DNA-nucleotide-methyl-transferases (DNMT) methylate CpG residues[147] and seem to catalyse the reverse reaction[148]. However, the Ten-Eleven-Translocation enzymes (TET) are considered the main CpG DNA demethylases[149] while proteins containing DNA-methyl-CpG-binding domain (MBD) recognize the methylated status[150] in order to induce heterochromatin. However, it is not known how methylation is selectively established at precise promoters.

Genomic DNA methylation of CpG islands is fundamental for the programmed repression of genes during embryogenesis in mammalian cells. The methylation pattern is erased in the early embryo in order to establish the totipotent state, but is re-established during implantation with pluripotency genes being methylated and thus repressed[151,152]. Methylation of CGIs is then recognized by HKMTs that contain a methyl-binding domain (MBD) domain, in this case G9a. G9a establishes H3K9me3 and recruits HDACs, inducing HP1-α binding and local heterochromatinization. Heterochromatinization of HP1 promotes *de novo* DNA methylation by DNMT3 and

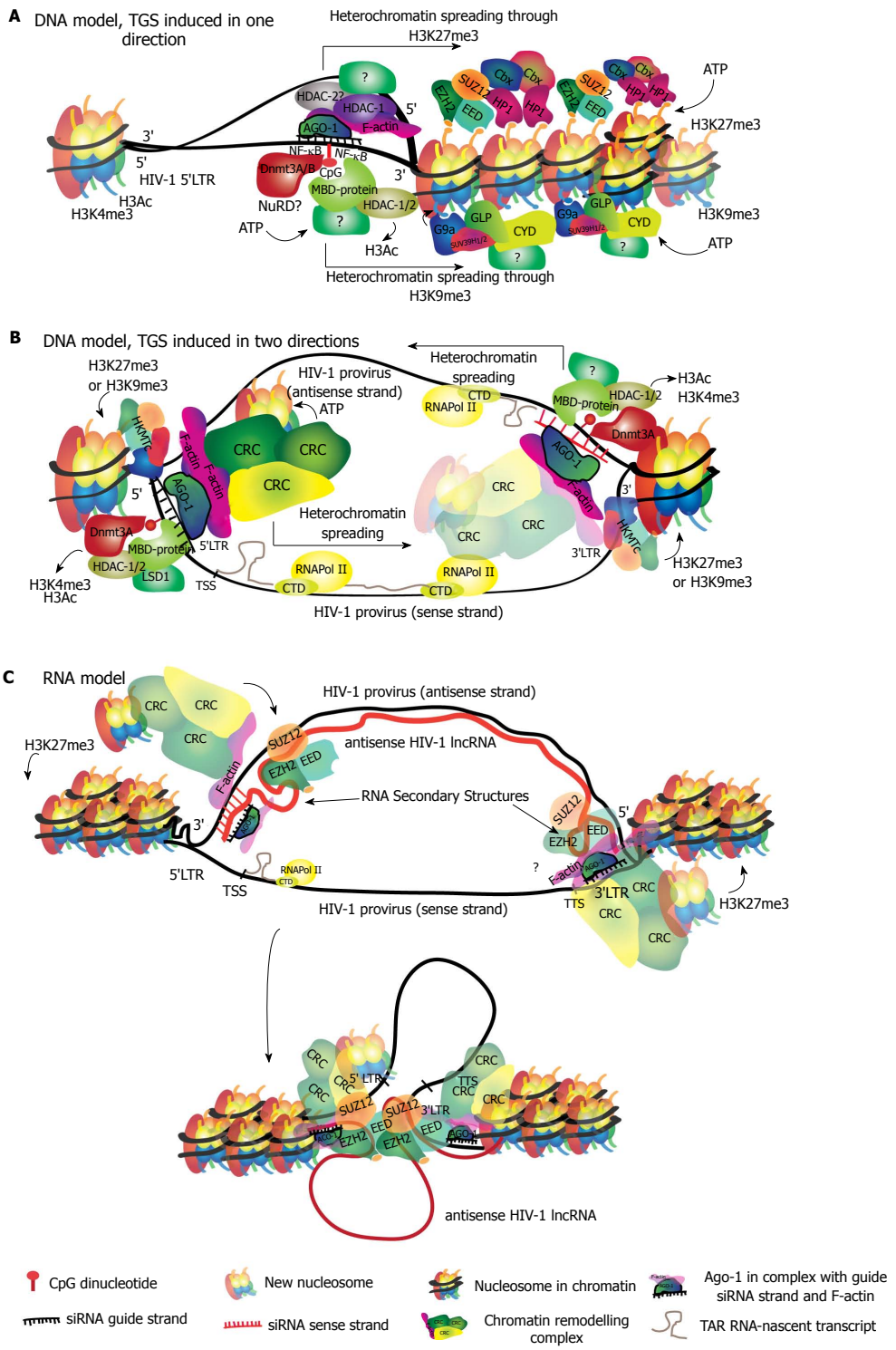

Figure 3 Models describing possible molecular mechanisms of siRNA-induced transcriptional gene silencing in human immunodeficiency virus 1. A: DNA model in which the siRNA guide-strand finds its target in the 5'LTR promoter of the HIV-1 genome binding directly to the DNA. This binding triggers the recruitment of HDACs and HKMTs, which further recruit CRCs to induce chromatin compaction. Two mutually exclusive pathways are shown simultaneously, for simplicity. While both pathways may be initiated with the DNA methylation of CpG dinucleotides, they differ in the proteins that are recruited to the locus. The pathway characterized by H3K27me3 is shown above and involves initial recruitment of PCR2 (EZH2-SUZ12-EED) followed by the specific CRCs readers of H3K27me3. The H3K9me3 recruits G9a or SUV39H1/2 followed by specific CRCs as well. In this model heterochromatin is likely to spread in only one direction; B: In this DNA model, both strands of the siRNA duplex find a target on opposite DNA strands given that both, the 5'LTR and 3'LTR from the HIV-1 genome, have the same sequence. Regardless of the epigenetic pathway that is induced, heterochromatin will spread in the 5' to 3' direction from each end of the HIV-1 genome; C: In the RNA binding model, antisense transcription generates a HIV-1 specific lncRNA that covers all the HIV-1 genome. The siRNA guide-strand will bind the 3'UTR of this transcript, which corresponds to the 5'LTR sequence. The binding recruits PCR2, which establishes H3K27me3 and may also interact with the secondary structures of the lncRNA. Higher order interactions may bring together the 3'end of the HIV-1 genome, recruiting CRCs and inducing heterochromatin. A binding site for the same siRNA strand remains in the DNA sense strand at the 3'LTR, which could potentially contribute to heterochromatin formation. HIV-1: Human immunodeficiency virus 1; DNMT3 A/B: DNA methyl-transferase A/B; HDAC: Histone deacetylase; MBD-protein: Methy-CpG-binding protein; Cbx: Chromobox family; HKMT: Histone lysine (K) methyl-transferase; CRC: Chromatin remodelling complex.

Table 1 Canonical histone modifications implicated in TGS and TGA

Histone residue	Modification	Function	Writers	Erasers	Readers	Reviewed in
H3K4	Ac	Transcription activation				[228]
	me1 (enhancer sequences)	Transcription activation	SET1 (tri)[229], SET7 (mono)[230], MLL[231], SMYD2[232]	LSD1 (mono and di)[233], JARID1A/ KDM5A JARID1B/ KDM5B (di and tri)[234]	CHD1[235], RAG2[236], TAF3[237], BPTF[238], BHC80[239], ING FAMILY[240], PYGO2[241]	[166,242]
	me2/me3 (regulatory elements at the 5' end of active genes, and in poised genes)	Transcription activation, resolution of bivalency from poised genes				
H3T6	Phosphorylation	Transcription activation	PKC B		LSD1	[243]
H3K9	Ac	Transcription activation, histone deposition	GCN5/PCAF[244]	SIRT6[245]	BRD4[246]	[247]
	me1/me2 me3 (non-genic regions, centromeric heterochromatin, satellite sequences, long terminal repeats)	Transcritional silencing, heterochromatin	SUV39H1/2[143], G9a[248], SETDB1[249]	JMJD1A/KDM3A[250], JMJD1B/KDM3B[251], JMJD1C/TRIP8, JMJD2A/KDM4A (B/C/D)[252]	HP1[253], EED 17406994), TDRD7[254], MPP8[255], UHRF1/2[256], GLP[248], CDY FAMILY[257]	
H3K27	me1/me2/me3, heterochromatin and facultative heterochromatin	Transcritional silencing, heterochromatin, poised genes	EZH2, EZH1[258]	JMJD1A/KDM3A, JMJD1B/KDM3B, KDM6A/UTX, JMJD3/KDM68, JMJD3/KDM6B[259]	Cbx proteins[165], EED[260]	[166,261]
H3K36	Ac	Transcription activation	GCN5, PCAF[244]			[262]
	me1/me2 (in the body and 3' end of genes)	Transcription elongation	NSD1, NSD2[263], SET2[264], SMYD2[232], MMSET[265]	ASH1[266], JHDM1[267], JHDM1A/KDM2A, JHDM1B/KDM2B[268]	ISW1B[269]	
	me2/me3 (gene bodies)					
H4K20	me1	Transcritional silencing, heterochromatin, repression of proinflammatory genes	PR-SET7/SET8[270]	PHF8[271]	L3MBTL1[272]	[273]
	me2		SUV420H1, SUV420H2[274]	PHF2[275]	PHF20[276], L3MBTL1[277]	
	me3 (non-genic regions, centromeric heterochromatin, satellite sequences, long terminal repeats		SUV420H2[274], SMYD5[275]	PHF2[275]	NcoR[275]	

H: Histone; K: Lysine residue; T: Threonine residue; me1: Monomethlyation; me2: Di-methylation; m3: Tri-methylation; SET1: Su(var)3-9, Enhacer of Zeste, Trithorax protein 1; SMYD2: SET and MYND domain containing protein 2; MLL: Mixed lienage leukemia protein; PKC-β: Protein kinase C beta; GCN5: general control nonderepressible-5; PCAF: P300/CBP associated factor; SUV39H1/2: Suppressor of variegation 3-9 homolog 1 or 2; SETDB1: SET domain bifurcated 1; NSD1: Nuclear receptor binding SET domain protein 1; MMSET: Multiple myeloma SET domain; PR-SET7/SET8: SET domain containing lysine methyltransferase 8; LSD1: Lysine (K) specific demethylase 1; JARID1A/KMT5: Jumonji/ARID 1 domain containing protein 1A; SIRT6: Sirtuin 6; JMJD1A: Jumonji domain containing protein 1A; ASH1: Absent small or homeotic like 1; JHMD1: Jmjc domain containing histone demethylase 1; PHF8: PHD finger protein 8; CHD1: Chromodomain-helicase-DNA binding protein 1; RAG2: Recombination activating gene 2; TAF3: TATA box binding protein (TBP)-associated factor; BPTF: Bromodomain and PHD finger containing transcription factor; ING Family: Inhibitor of growth; PYGO2: Pygopus family PHD finger 2; BRD4: Bromodomain-containing protein 4; TDRD7: Tudor domain-containing protein 7; MPP8: Methyl-H3K9-binding protein 8; UHRF1/2: Ubiquitin-like containing PHD and RING finger domains 1 or 2; CDY Family: Chromodomain Y chromosome family; GLP: G9a-like protein; ISWI1B: Imitation switch protein 1B; L3MBTL1: Lethal (3) malignant brain tumor-like protein 1; NcoR: Nuclear receptor coRepresor.

further spreads silencing by repeating the loop[151] (Figure 3A). In humans, DNMT3 establishes *de novo* methylation and is responsible for tissue-specific DNA methylation patterns[153,154].

In the case of TGS, recent studies have shown that DNA methylation of CGIs is not required for siRNA-guided heterochromatin formation in fission yeast, as was initially described[155]. Similarly, the signatures of TGS in mammals appear to be somewhat diverse and may require DNA methylation in some cases. Interestingly, there is an RNAi-directed DNA methylation process that triggers TGS in plants[156], which is reminiscence of a mechanism in mammalian cells: piRNAs are known to direct DNA methylation in the male germ line in order to repress expression of transposable elements, but a similar mechanism has not been described on somatic cells[157]. However, there is some indirect evidence of a similar mechanism in mammalian somatic cells when transduced with lentiviral vectors. In fact, reduced expression of the introduced transgene was observed during differentiation in a murine model and silencing was found to be the result of DNA methylation of the promoter of the lentivirus driven

gene[158]. Furthermore, it is well known that a considerable amount of integrated vectors become silent[159], and this effect seems to be dependent on the promoter chosen to drive the ectopic expression of the gene[160,161]. These observations could be related to ubiquitous RNA guided-DNA methylation pathway mechanism in mammalian cells aimed at controlling endogenous retroviruses. It is clear though, that *de novo DNA* methylation can provide stability for the inheritance of gene repression patterns through generations[151]. In this instance, TGS involving DNA methylation is likely to characterize robust silencing of a gene.

The PcG defines a group of genes that play a fundamental role in development and whose deletion results in early embryonic lethality in mice[162]. The PcG perform an antagonistic role to the trithorax group (TrxG) of proteins by inducing epigenetic gene repression. Both, PcG and TrxG, ensure the maintenance of proper expression patterns throughout the life span of a multicellular organism. There are two main repressive multi-subunit complexes formed by PcG: Polycomb-repressive complexes 1 and 2 (PCR1 and PCR2)[163,164].

PCR1 efficiently compacts chromatin through a variety of subunits that either identify and bind to H3K27me3, or mono-ubiquitilate Lys119 of histone 2 variant 2 (H2A), both of which promote nucleosome compaction. PCR1 is actually a group of functionally related but diverse protein complexes made up of different subunits that vary its function[165]. In addition to its role in development, roles in senescence, self-renewal, cancer and even gene activation have been recently identified for PCR1[165]. Interestingly, both complexes appear related, with PCR1 eventually acting downstream of PCR2 on certain loci.

PCR2 establishes the repressive epigenetic signature, H3K27me2/3 through its enhancer of zeste 1 and 2 subunits (EZH1, EZH2)[164] and induces chromatin compaction. In addition to H3K27me3, the activation mark H3K4me3 is also established by PCR2. Characteristically, genes co-expressing both, K3K37me3 and H3K4me3 epigenetic marks, are poised for transcription in undifferentiated cells. This state of epigenetic bivalency is resolved by the exclusive expression of H3K4me3 in transcriptionally active loci or H3K27me3 in transcriptionally repressed loci[166].

A direct link between PCR2 and TGS during regulation of granulopoyesis was elegantly demonstrated. Further this process was shown to be fundamental in driving progenitor lineage decisions at checkpoints of differentiation, in particular at the *NF1-A* gene. In this study, miRNA-223 directly bound to the NFI-A promoter region through its seed region and induced TGS of this gene through recruitment of the PcG proteins, YY1 and SUZ12, along with AGO-1 and Dicer[65]. This evidence supports previous findings of a siRNA-directed TGS, involving AGO-1, recruitment of EZH2, induction of H3K9me2 and the PTGS protein TRBP2[114]. Furthermore, the primary miRNA-208b has recently been shown to interact with EZH2, a Polycomb-group protein associated with gene silencing through chromatin remodeling[146]. Together, these studies clearly show that not only siRNAs, but also endogenous promoter-targeted miRNAs are able to trigger TGS in mammalian cells through recruitment of PcG proteins.

Interestingly, genes that are repressed by PcG express short-RNAs (about 50-200 nts) that interact with PCR2 to promote silencing[167]. However, no AGO proteins are involved in this case and the mechanism resembles that of X-chromosome inactivation (*Xi)* (explained in the next section), with SUZ12 subunit of PCR2 binding to a short RNA-stem loop from the BSN gene that mimics Xist A-Repeat (RepA) stem-loop. The important concept to highlight is that short RNAs can be transcribed from repressed loci and are used to guide repressor complexes to maintain these loci in a silent state.

Genomic imprinting is the mechanism by which parental-origin specific expression of imprinted genes is controlled in somatic cells (reviewed in[168]). It requires the DNA methylation of a region within the imprinting control region (ICR) that lies in the cluster of imprinted genes. This ICR is only demethylated in the germ cells but is then specifically re-methylated during fertilization depending on whether the maternal or the paternal allele is to be expressed in the somatic cells[169]. It is considered to be a very strong and stable silencing.

A well-studied case, that would be an example for the second TGS model, is *Xi*. During *Xi,* expression of the lncRNA *Xist* represses transcription from the paternal chromosome[110]. However, *Xist* is further regulated by the antisense lncRNA *Tsix*. After transcription, lncRNA *Tsix* induces silencing of *Xist* by recruiting PCR2, establishing H3K27me3 marks and enhancing *de novo* hyper methylation by DNMT3A[170]. The crucial link between RNAi and genomic imprinting in *Xist* regulation seems to be in the cleavage of the *Xist-Tsix* duplex by Dicer, which generates siRNAs targeting *Xist* leading to heterochromatin formation. These siRNAs in turn silence *Xist* and in this system deletion of Dicer appears to abolish silencing[145]. Currently, there is a dispute regarding the role of Dicer in this process and thus of RNAi in *Xi*, because Dicer knockout embryonic stem cells have shown contrasting results with either a defect in *Xi* (arguing in favor) or no defect at all (arguing against). A very detailed discussion about these contrasting results can be read in[171]. It is worth noting that other nuclear endonucleases could potentially induce cleavage in the absence of Dicer. However, recent findings showed that depletion of Dicer in human female cells has no effect in the epigenetic silencing of *Xi*, but results in up-regulation of X-linked genes, indicating that Dicer may be important for dosage compensation of those genes in differentiated cells[172].

Xi is just one of several examples of genomic imprinting during which specific DNA methylation and a lncRNA drive long-range epigenetic heterochromatic silencing through recruitment of PcG (Figure 2D). Because genomic imprinting involves recruitment of PcG proteins to an RNA intermediate, establishment of epigenetic repressive marks and short RNAs derived from the targeted genes, it supports the model of an RNA intermediate in sncRNA-directed TGS.

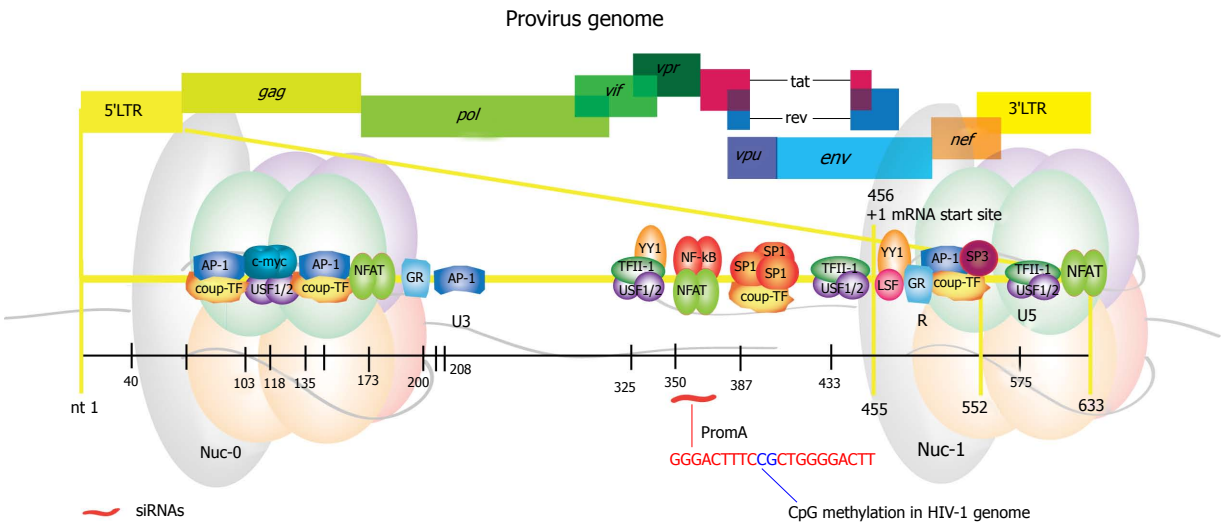

Figure 4 Map of the human immunodeficiency virus 1 genome showing in magnification the 5'LTR region with the location of transcription factor binding sites. The specific coordinates within the HIV-1 genome for each of the shown DNA regulatory elements is listed on Table 1. 5'LTR: 5' long terminal repeat; gag: Group specific antigen; pol: Polymerase; vif: Viral infectivity factor; vpr: Viral protein R; vpu: Viral protein unique; tat: Trans-activator of transcription; rev: RNA export element; env: Envelope; nef: Negative factor; 3'LTR: 3' long terminal repeat; Ap-1: Activator protein 1; COUP-TF: Chicken ovalbumin upstream transcription factor; c-myc: V-myc avian myelocytomatosis viral oncogene homolog; USF 1/2: Upstream stimulatory factor 1 or 2; NFAT: Nuclear factor activated T cells; GR: Glucocorticoid receptor responsive element; YY1: Ying-yang 1; TFII-I: Transcription factor II-I; NF-κB: Nuclear factor κ beta; SP: Specificity protein; LSF: Late SV40 factor; U3: Untranslated region 3; R: R region; U5: Untranslated region 5; Nuc-0: Nucleosome 0; Nuc-1: Nucleosome 1.

All these endogenous silencing mechanisms are an example of the different possibilities that may result when inducing TGS through sncRNAs (Figures 2 and 3). TGS is part of an enormous gene regulation network that involves a wide variety of mechanisms and protein interactions, whose combination yield diverse specific gene silencing outcomes. While we do not know yet how to induce each of these different epigenetic profiles, this mechanism has the power to silence the HIV-1 promoter in an inheritable, stable and permanent fashion, which we have reported through siRNA-induced TGS.

VIRUS: HIV

HIV establishes a long-term infection in dividing and non-dividing cells. The integrated proviral form is flanked by two long terminal repeats (LTRs) that originate from reverse transcription and are fundamental for viral replication[173]. HIV provirus behaves like a cellular gene; it has its own promoter located in the 5′ LTR and is rich in responsive elements for binding of several cellular transcription factors (Figure 4). It also has a 3′ LTR, which ensures the viral mRNAs are polyadenylated and capped mimicking cellular transcripts[174]. Of note, both LTRs have the same sequence and the 3′LTR is transcribed into the 3′ UTR of the viral transcripts.

Upon integration, the provirus goes through an initial phase of abortive transcription. This phase is characterized by the presence of a non-processive RNA Pol II at the promoter region that is overcome upon expression of the viral trans-activating protein (Tat). Tat is imported back to the nucleus and binds the trans-activator response element (TAR), an RNA hairpin structure coded by the HIV promoter, greatly enhancing transcription[175]. Although most integrated proviruses are able to overcome abortive transcription, some become latent[27].

HIV latency

HIV latency is an interesting model to study because it is likely to be the result of various endogenous TGS mechanisms. Studies have described a variety of epigenetic profiles at the HIV promoter some of which are associated with extremely robust silencing such that reactivation of HIV is resistant in the face of substantial cell activation.

Generally, H3K9me3 is considered to be mutually exclusive with H3K27me3, and are found in different loci. More specifically, H3K9me3 is associated with silencing of endogenous retroviruses and retro-transposons, and is also enriched in constitutive heterochromatin regions and pericentromeric heterochromatin[176]. On the other hand, H3K27me3 is associated with a more dynamic silencing of varying strengths, which may depend on the presence of the H3K4me3 activation mark, as well as other undefined factors.

In HIV-1 infection, H3K27me3 has been found enriched in the 5′LTR promoter in cell line models of latent infection in which the virus reactivates upon stimulation[177]. This is consistent with H3K27me3 being generally a more flexible epigenetic repressive mark and with the likelihood that most of the inducible latent provirus is silenced through pathways involving H3K27me3, rather than H3K9me3. H3K9me3 has only been found in a few HIV-1 latency studies and re-activation of latent provirus carrying this mark has either not been observed after strong stimulation (with Phorbol-Myristate-Acetate treatment) or has required

Table 2 Coordinates of transcription factor binding sites in the HIV-1 5'LTR

Name	Position[1]	Function	Cell type	Notes	Ref.
Nuc-0	About 40-200	Structural	Consistent across different cell types	Stable. Stability seems independent of transcription	[278]
AP-1/COUP-TF	About 103	Activation/Repression			[279]
c-myc/RBF-2 (USF1/2)	118-124	Repression/Activation	HeLa-CAT-CD4 and J-Lat J89 (Jurkat)	Binds the sequence CACTGAC in HIV promoter, but the canonical sequence is CACGTGAC Recruited by Sp1, can bind directly to the promoter to recruit HDAC1 RBF-2 can potentially bind to the CTGAC of this motif.	[280,281]
AP-1/COUP-TF	About 135	Repression/Activation	Cell type variation	COUP-TF binds to the nuclear responsive element	[180,279]
NFAT	173	Activation	Consistent across different cell lines	NFAT consensus sequence TGGAAA maps on antisense strand	[282]
GRE-I	192-197	Repression/Activation	Cell type variation	GRE-like element AGAACA	[283-285]
AP-1	About 208			AP-1 recently found to be crucial for latency	[286]
YY1/RBF-2	About 336	Repression/Activation	Jurkat, HeLa	Putative E-box element RBEIII. Sequence overlaps YY1, RBF-2/TFII-I and AP-1 binding sites	[281,287,288]
NFAT/NF-kB	350	Activation/Repression	Consistent across different cell types	Two shared in-tandem binding sites for each transcription factor. NF-kB in the sense strand, NFAT in the antisense	[289-291]
COUP-TF/Sp1/CTIP-2	About 388	Activation/Repression	Microglial, Oligodendrocytes, T lymphocytes	COUP-TF synergises and interacts with SP1 to activate, while CTIP2 directly binds to SP1 and represses transcription	[279,292,293]
Nuc-1	450-610	Structural	Consistent across different cell types	This nucleosome is remodelled to induce HIV latency or transcriptional gene silencing	[278]
RBF-2/AP-4	435-440	Activation/Repression	HEK293T, Jurkat	Both bind the E-box element CAGCTG, which has been named RBEI	[288,294-296]
GRE-II	450-455	Activation/Repression	Cell type variation	GRE-like element TGTACT	[283-285]
LSF/YY1	about 440-483	Repression	HeLa	LSF recruits YY1. This interaction recruits HDCA1 to initiate repression	[281,297,298]
GRE-III	471-476	Repression/Activation	Cell type variation	GRE-like element AGACCA	[283-285]
COUP-TF/AP-1/SP3	About 485	Repression/Activation	Microglial	Synergises and interacts with SP3	[180,279]
RBF-2	About 576	Activation/Repression	Jurkat	Binds an atypical RBEIII element: ACTGCTGA	[288,294]
NFAT	618	Activation	Consistent across different cell lines	NFAT consensus sequence TGGAAA maps on sense strand	[291]

[1]Genomic coordinates are based on the HBX2 numbering system. Nuc-0: Nucleosome 0; AP-1: Activator protein 1; COUP-TF: Chicken ovalbumin upstream transcription factor; c-myc: V-myc avian myelocytomatosis viral oncogene homolog; RBF-2: Ras-responsive binding factor 2; USF1/2: Upstream stimulatory factor 1 or 2; NFAT: Nuclear factor of activated T cells; GRE-I: Glucocorticoid responsive element I; YY1: Ying-yang 1; NF-κB: Nuclear factor kappa beta; Sp1: Specificity protein 1; CTIP-2: COUP-TF interacting protein 2; Nuc-1: Nucleosome 1; AP-4: Activator protein 4; GRE-II: Glucocorticoid responsive element II; Sp3: Specificity protein 3.

silencing of HP1-γ or other factors through RNAi[178,179]. This supports H3K9me3 as a more robust repressive epigenetic mark.

Similarly, Suv39H1, another HKMT responsible for H3K9me3, has been found to be recruited to latent HIV promoter in microglial cells[180], while in a different T-cell latency model, G9a, another HKMT responsible for H3K9 methylation), was found to be a determinant of proviral latency[181]. Moreover, the HKMT LSD1 is also recruited to the HIV promoter by the cofactor CTIP2 and establishes H3K9me3 to promote latency, rather than activation[178]. Additionally, EZH2, one of the PCR2 subunits that establish H3K27me3, has been found to be present at the LTR of latent provirus. Knockdown of EZH2 resulted in higher transcriptional activation of the HIV promoter than when knocking down Suv39H1[177], indicating that the former is associated with a more responsive epigenetic silencing.

Recently, a nuclear lncRNA expressed as an antisense transcript initiated from the viral 3'LTR, was found to modulate HIV-1 replication[182]. This lncRNA was further shown to exert epigenetic modulation of the 5'LTR HIV promoter by recruiting both DNMT3 and EZH2, resembling a genomic imprinting mechanism[183]. These observations are consistent with HIV CpG islands being methylated in a latency model[184]. It has been described that transcriptional silencing by *Xist* requires RepA, which is a short RNA transcript containing the A-repeat that forms an RNA secondary structure to which EZH2 and other PcG members bind, and whose deletion prevents silencing[185]. Given the similarity of the HIV antisense lncRNA mechanism to that of *Xist,* the TAR RNA-loop secondary structure fundamental for HIV transcription could potentially be involved in an interaction with EZH2. While the latter statement is hypothetical, the evidence thus far points towards a robust silencing of HIV by this lncRNA. The scope of this discovery may be extrapolated to the barriers to achieving reactivation of latent provirus as a therapeutic approach. Reactivation strategies to purge the latent reservoir, such as the use of histone deacetylase inhibitors (HDACis) have not been successful, despite using a variety of agents like Vorinostat and Panabinostat, with

different potencies and specificities in inducing HIV specific chromatin relaxation[32]. The mechanism by which this HIV antisense lncRNA maintains latency might explain in part this difficulty, because a very robust and deep silencing may be established in a great deal of latent proviruses that make up the reservoir. Moreover, it could be potentially harmful to aim at disrupting this HIV lncRNA silencing because strategies directed to it could have an impact on other genomic regions strongly repressed by similar mechanisms.

Pan-HDACis have been developed that target more than one class of HDACs and the development of HDACis with isozyme specificity are on the scope[186]. However, HDACis will not specifically target only HIV, instead these drugs induce general chromatin relaxation on cellular genes and so have effects that are no HIV-specific. In addition, given the evident epigenetic complexity of HIV latency, more than one type of enzyme involved in epigenetic silencing will be needed to fully disrupt the latent provirus.

Collectively, the characteristic heterogeneity observed in the studies describing either HIV latency or on those aimed at re-activation of the latent provirus may be explained by the considerable density of binding sites for cellular transcription factors within the 5′LTR (Figure 4 and Table 2), in conjunction with the modulation executed by the HIV antisense lncRNA. Thus, it is possible that inducing TGS through siRNAs/shRNAs that target different regions within these DNA binding elements could result in the establishment of varied epigenetic profiles.

RNAI FOR HIV

PTGS for HIV

Initial applications of RNAi to HIV were designed to target viral mRNA transcripts through the PTGS pathway[187]. These first attempts used transfection of one siRNA directed against important viral transcripts such as *gag*[187], *env*[188] and *rev*[189], and also cellular genes important for HIV-1 replication cycle, such as CD4[190] and CCR5 or CXCR4 chemokine receptors[191]. Suppression was not sustained whenever only viral mRNAs were targeted due to the emergence of resistant variants[192-194]. It became clear that a combinatorial RNAi against HIV would provide better protection and this correlated with delayed viral escape[195]. Further analysis of resistant viruses was useful to guide the design of more effective shRNAs[194]. Indeed, escape-proof shRNAs were identified that exerted potent and prolonged HIV suppression[196]. However, this approach was not completely robust as escape was observed from combinatorial shRNAs despite these being specifically designed to target previously characterized resistant viral variants[197]. Since then, multiple design approaches have been developed using a variety of strategies in search of the best combination of siRNA/shRNAs molecules that might prevent viral escape[198,199].

Following these findings, shRNAs targeting both conserved viral genes and host cellular genes required for viral replication became the preferred way to overcome this problem. Indeed, targeting only cellular genes such as CD4[190] and CXCR4 and particularly the CCR5 chemokine receptor dramatically reduced the emergence of resistant viruses[200]. Currently, PTGS is not envisaged as a stand-alone strategy for treating HIV. Rather its putative use is in combination with other types of gene therapy technologies, which we will discuss in the section for alternative gene therapy approaches.

TGS for HIV

The field of sncRNA-induced TGS for HIV therapeutics is less developed and has been hampered by the doubts regarding the existence of the pathway in mammalian nuclei. Nonetheless, siRNA and shRNA approaches have been efficiently developed that achieve long-term *in vitro* suppression of HIV replication, accompanied by epigenetic profiles which resemble those described in studies of the latent form of HIV-1.

We designed a siRNA, designated PromA, directed to the tandem repeat of NF-κB binding sites found in the HIV promoter (Figure 4). It can induce prolonged suppression of active HIV-1 infection *in vitro* and induces methylation of the CpG dinucleotide that maps to the sequence linking NF-κB tandem sites[201]. This HIV suppression was associated with recruitment of AGO-1 and HDAC1, and increased presence of H3K9me2 at the HIV promoter and involved nucleosome remodeling[202]. Later, long-term suppression (about 90 d) in conjunction with enrichment of H3K27me3 was observed when using stable expression of a shRNA targeting the same region[203]. H3K9me2 and H3K9me3 were also enriched but at much lower levels (H3K27me3 >>> H3K9me2 > H3K9me3). Suppression was then proved to be specific, as mutations in the shRNA sequence impaired virus suppression[204]. Interestingly, we identified F-actin as a key player in nuclear transportation of promoter-targeted siRNAs in mammalian cells, using the same siRNA constructs[205]. Results from this study are consistent with selective transport of promoter-targeted sncRNAs, which has also been shown for AGO-1 by other groups[135], as mentioned earlier.

Using a TGS-based gene therapy for treating HIV infection has several advantages over other therapies. First, TGS acts directly at the HIV promoter giving the virus virtually no opportunity to develop resistance; Second, it is likely able to act on latent provirus, whereby it potentially locks the virus in the latent state impeding future re-activation; Third, small doses of the effector molecules are sufficient to induce silencing since integrated provirus in a clinical setting is limited to less than 2 to 3 copies per cell[206]; And fourth, the silencing could potentially be inherited, though this remains to be definitely demonstrated.

Furthermore, an interesting point to note is that since the 5′LTR promoter contains the same sequence as the 3′LTR, a siRNA/shRNA designed to target the promoter region will also have a second target in the proviral 3′LTR. This could be potentially beneficial, as

heterochromatin could be induced from both ends of the provirus (Figure 3B). Other potential targets are the 3'UTRs of viral mRNAs, whose targeting mainly depends on the efficiency of a siRNA to induce PTGS, or both PTGS and TGS simultaneously. In the latter case, PTGS would function until TGS is established, impeding transcription of viral mRNAs. However, an efficient siRNA/shRNA targeting both PTGS and TGS pathways has not yet been identified. Indeed, our siRNA PromA targeting the NF-κB did not show a significant PTGS effect on viral mRNAs[202] when we measured the effect in a setting mimicking an active HIV transcription owing to its clinical relevance, rather than using a weak promoter. In addition, the 1-LTR and 2-LTR circle intermediates of abortive HIV integration, which reside within the nucleus, may be targeted as well. While transcription and translation of viral genes from these unintegrated DNA forms has been observed, the contribution of these to actual infection is not clear[207]. And lastly, the linear DNA intermediate, that is synthesized in the cytoplasm by the RT enzyme and will become integrated as provirus, also contains the two viral LTRs, and several host proteins are known to interact with it[207]. While PTGS acts only post-HIV integration on viral mRNAs, rather than on incoming viral RNA genomes[208], the effect of promoter-targeted siRNAs in the incoming reverse-transcribed HIV genome and other unintegrated DNA forms has not been investigated.

Essentially, if sequence complementarity and/or sequence features of the promoter-targeted siRNA are the main determinant for target binding, then an activated RITS complex could potentially bind to any type of molecule containing the target sequence.

OTHER GENE THERAPY STRATEGIES FOR HIV

Hope for an HIV cure re-emerged after the successful bone marrow transplantation of Timothy Ray Brown-the leukemia patient known as the Berlin patient - with stem cells homozygous for the Δ32 deletion in the CCR5 gene (CCR5Δ32)[209]. This gene encodes an important co-receptor used by the virus to enter the host cells and individuals carrying the homozygous mutation have proven resistant to HIV infection by CCR5-tropic viruses[210]. Timothy was cured from both leukemia and HIV. Years after the transplant, he remains virus-free even when no longer under cART[211]. Since then, researchers have been developing various strategies to transform hematopoietic stem cells (CD34+) into HIV resistant cells, with the aim of reproducing this outcome.

Consequently, CCR5 has become the favorite cellular factor to target, especially since HIV CCR5-tropic strains are predominantly present during early stages of the disease and often persist into later stages[212,213]. Moreover, individuals with this mutation appear to be otherwise healthy apart from an as yet unconfirmed increase in susceptibility to West Nile infection[214] and hepatitis B virus infection[215]. These statements have raised the concern of whether CCR5 is implicated in immune system-related diseases[216]. An interesting discussion in this topic can be read in[217]. Thus, the effect of knocking down CCR5 could results in unpredicted effects.

Presently, different genetic therapy technologies are being tested for their *in vivo* ability to generate HIV resistant cells. From combined PTGS approaches, to genome editing with Zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALENs) or clustered regularly interspaced short palindromic repeats elements (CRISPR) associated caspase 9 (Cas9).

The most recent strategies involving PTGS use triple combination vectors. For example, a viral vector expressing shRNA against CCR5, an shRNA against TRIM5α isoform and a TAR-decoy against HIV[218] was successfully tested in a humanized NOD-RAG$^{-/-}$IL2r$\gamma^{-/-}$ knockout mouse model. Similarly, a strategy using a viral vector expressing an shRNA against HIV *tat/rev*, a TAR-decoy element and ribozyme against CCR5[219] was initially tested using modified autologous CD4+ T cells in HIV positive patients who had failed therapy (NTC01153646), and is now been tested as an adjunct therapy using modified CD34+ T cells in patients with acquired-immune deficiency syndrome (AIDS)-related non-Hodgkin Lymphoma (NHL) (NCT01961063) and in patients with AIDS-related NHL requiring stem cell transplantation (NCT00569985). Importantly, long-term expression of the effector molecules from this construct has been detected in multiple cell lineages from treated patients, in which a combination of transduced and untransduced CD34+ cells were used[220].

ZFN strategies predominantly target CCR5. Recently, a phase I clinical trial (NTC00842634) testing the transfusion of CCR5 ZFN-modified autologous CD4+ T cells into HIV positive patients[221] showed that the procedure was feasible and safe. During an anti viral therapy treatment interruption the modified cells had a higher survival over non-modified cells. Also, patients showed decreased HIV DNA levels in blood. Currently, the effect of repeated doses of the ZFN-modified CD4+ T cells is being tested (NCT02225665). Although, these clinical trials use modified CD4+ T cells rather than CD34+ cells, recent studies in a humanized mice model showed low engraftment, but proper multi-lineage differentiation of the CCR5-ZFN CD34+ cells[222].

TALENs and CRISPR have not yet been trialed in humans. However, the results from *in vitro* studies are very promising[223], with CRISPR editing able to excise the provirus from infected cells, and thus able to target latent proviruses[224]. ZFNs have also been used to target the provirus, using lentivirus to achieve stable expression of the nucleases[225]. However, the above-mentioned ZFN-related clinical trials used adenovirus vectors. Generally, genome-editing approaches use non-integrative adenoviral vectors. Adenoviral vectors are diluted after each cell division and direct transient expression of the editing nuclease. Transient expression has been the choice for genome-editing approaches on

the grounds that a continuous expression of a selected editing nuclease could be potentially risky as it may result in off-target genome editing. To date, it remains to be addressed if ZFN/TALEN/CRISPR genetically modified CD34+ are safe to use in humans and whether they are feasible approaches towards a functional cure.

CONCLUSION

Presently, a variety of strategies are being tested in order to breakthrough this highly challenging treatment barrier. There are still several large hurdles to be surmounted. Currently there is a lack of adequate delivery systems for targeting cells with HIV infection and the latent reservoir. Further TGS/PTGS approaches require stable expression from vectors, such as lentiviral vectors but this must be combined with high transduction and engraftment rates, for therapy to be effective. In the same way, genome-editing approaches rely on vectors that drive transient expression of the editing enzyme, but get diluted after each cell division. Thus, achieving high genome editing efficiency is one of the limitations.

Importantly, TGS and CRISPR genome editing have the potential to target proviruses directly, and therefore could be effective in targeting latent provirus. Yet this strength may also be an inherent weakness and thus a careful selection of the targeted sequences of HIV-1 is fundamental. Unfortunately, 5'LTR sequences from proven replication competent proviruses are the least represented in curated databases in comparison to other HIV genomic regions. Nonetheless, combinatorial strategies are also an option within these therapies, and may be designed to target an additional host factor as well.

Gene therapy technologies that target only CCR5 may be unable to target latent provirus that is already present. In addition, they may select HIV-1 viruses with tropism for the CXCR4 co-receptor, allowing escape and potentially more rapid disease progression. This evolution is more likely if latent provirus remains in untargeted compartments.

The combinatorial strategies from PTGS, which target the virus and a host factor such as CCR5, provide an additional mechanism that directly restricts the virus and could possibly delay or imped viral evolution. In this regard, it could potentially provide some protection from CXCR4-tropic emerging viruses or re-activating from latent proviruses.

Basically, with present technologies none of the effector molecules for these therapies can be directly administered to an infected patient. Rather, autologous cells are obtained, genetically modified, and then transferred back to the patient. Generally, these therapies aim at modifying CD34+ cells in order to develop multi-lineage HIV resistance and thus long-term protection to the infection. Indeed, the limitation of most of these therapies relies on the efficiency of several steps throughout the complete intervention process. For instance, the efficiency or success to which the autologous cells are first, modified *ex vivo*; Second, re-mobilized or transplanted; third, engrafted within the bone marrow; and fourth, either achieve a sustained and prolonged multi-lineage expression of the modified trait/gene or achieve a certain percentage of modified cells from all the lineages enough to provide protection. Furthermore, the engrafted modified cells will share a niche with the wild-type cells, unless ablation of the immune system is performed before. Therefore, understanding the interactions and signaling between these two populations sharing a niche could give us a better prediction of the long-term success of these therapies. Factors such as symmetric and asymmetric cell division[226], unidentified endogenous mechanisms of genomic mosaicism detection in stem cells[227] and other cellular and molecular pathways may play an important role. For instance, if it is confirmed that Piwi proteins are expressed in hematopoietic stem cells, this could potentially have an impact in those therapies that rely on integrative gene therapy vectors.

Finally, other concerns remain such as the worldwide implementation of these gene-therapy strategies and their cost, particularly in developing countries. Consequently, the development of delivery methods that facilitate the clinical application of these therapies is an important quest.

The various RNAi strategies to target HIV reviewed here provide a potential alternate approach to combating HIV infection and the latent reservoir, with the results of current and future RNAi therapeutic trials poised to reveal whether this approach represents a possible pathway towards a functional HIV cure.

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REVIEW

Women's willingness to be tested for human immunodeficiency virus during pregnancy: A review

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Abstract

Mother-to-child-transmission of human immunodeficiency virus (HIV) is a primary cause of pediatric infections with HIV. Many of these infections involve women who were not tested early enough in pregnancy, or who did not receive prevention services. HIV testing of pregnant women is considered to be one of the key strategies for preventing mother-to-child-transmission of HIV, but HIV testing rates among pregnant women in various countries remain suboptimal. Understanding the factors relating to women's willingness to be tested for HIV during pregnancy is critical for developing strategies to increase HIV testing rates among pregnant women. Extensive research points to various factors relating to women's willingness to be tested for HIV during pregnancy, and various recommendations aimed at improving testing rates among pregnant women have been suggested based on the research. In light of the goals set by the United Nations to reduce the rate of infants infected with HIV, it is necessary to summarize what is currently known regarding factors related to women's willingness to be tested for HIV during pregnancy. The purpose of this review is therefore to examine factors related to women's willingness to be tested for HIV during pregnancy, and to summarize recommendations for practice and further research.

Key words: Female; Human immunodeficiency virus infection; Pregnancy; Testing/screening; Patient acceptance of health care; Research

Core tip: The willingness of women to be tested for human immunodeficiency virus (HIV) during pregnancy is a complex phenomenon. There is frequent inconsistence in research results; however, studies have shown that certain major factors are steadily identified over time as associated with the phenomenon. Numerous factors related to pregnant women's willingness to be tested suggest multiple possible interventions to maximize HIV testing efficiency and increase testing rates. There is a need in further research of the phenomenon, as the majority of the research literature focuses on sub-Saharan Africa.

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INTRODUCTION

In 2012, there were more than 210000 new cases of human immunodeficiency virus (HIV) in children. This figure is in addition to the existing 3.4 million children already living with the virus worldwide[1]. More than 90% of these infections were the result of Mother-To-Child Transmission (MTCT). Many new MTCT cases of HIV occurred in women who did not receive testing for HIV early in their pregnancy, or who did not have access to preventative prenatal care [2].

Over 90% of MTCT of HIV occurs in sub-Saharan Africa, where women in their reproductive years represent 50% of the HIV-infected population[3]. MTCT of HIV is not limited to low-income countries: according to one source, each year in the United States there are between 100 to 200 new cases[2].

HIV testing of pregnant women has been advocated by UNAIDS as one of the key strategies for preventing MTCT[4]. In 2004, the World Health Organization (WHO) and UNAIDS issued recommendations for routine HIV testing of pregnant women in resource-limited countries[5]. At present, routine prenatal HIV testing is considered to be standard care in the United States and other developed nations. Nearly half of African countries have also adopted routine prenatal HIV testing policies, with 42.7% of them adopting these policies in 2006[6]. Despite these positive developments, HIV testing rates of pregnant women in many countries remain suboptimal[7].

Testing for HIV is voluntary and dependent on the willingness of women to receive testing. Understanding factors related to women's willingness to receive HIV testing during pregnancy is critical to developing strategies to increase HIV testing rates for pregnant women. Extensive research elucidates various factors as being related to women's willingness to be tested for HIV during pregnancy. Considering the goals set by the United Nations to reduce the rate of infants infected with HIV[8,9], the purpose of this review is to summarize the current research on factors related to the willingness of women to receive testing during pregnancy, and to formulate recommendations for practice and further research. Moreover, this review summarizes the advantages of testing for HIV during pregnancy, and provides recent statistics of testing rates for HIV during pregnancy in various countries.

ADVANTAGES OF TESTING FOR HIV DURING PREGNANCY

Testing for HIV detects HIV infections that would otherwise be missed. Timely HIV detection provides opportunity for interventions to alter the course of disease and prolong life[10]. Testing for HIV during pregnancy has additional advantages. First, results of a systematic review and meta-analysis showed a high incidence of HIV infection in pregnant and postpartum women in African countries as compared to non-African countries (cumulative HIV incidence being 3.6% and 0.3%, respectively), thus making testing during pregnancy especially important[11]; Second, most women in countries with low- to mid-level incomes will visit prenatal health clinics at least once during the course of their pregnancies[12], making the prenatal visit a valuable opportunity to test women for HIV[13].

Without appropriate health care management of HIV positive pregnant women, there is a high risk of MTCT. The risk of HIV MTCT in low- and mid-income countries is 15% to 40%. Of these, 5%-10% of MTCT will occur during the pregnancy itself, another 10%-20% of MCTC will occur during labor and delivery, and breastfeeding will account for 5%-20% of MTCT cases. Proper therapy for HIV in pregnancy is crucial to prevent MTCT[14]. The most important advantage of testing women for HIV during pregnancy is that timely identification of a pregnant woman infected with HIV allows planning and initiation of care, which may significantly lower the risks of MTCT of HIV, and, consequently, lead to healthier populations[15].

In high-income countries, MTCT rates have decreased dramatically following the introduction of recommendations for routine HIV testing for all pregnant women[16-18]. Programs promoting Prevention of Mother-To-Child Transmission (PMTCT) of HIV, including routine testing for HIV, have also led to a substantial decrease in MTCT rates in Sub-Saharan Africa, by around 50% since 2009[9].

Identification of the HIV-status of a pregnant woman allows establishment of three interventions for PMTCT of HIV: (1) administration of antiretroviral prophylaxis to mothers during pregnancy and delivery, and to newborn infants following delivery; (2) delivery of infants by scheduled cesarean section; and (3) avoidance of breastfeeding in favor of appropriate replacement feeding[15]. These interventions have been shown to be both effective and cost-effective in lowering MTCT of HIV[19-21]. Maternal treatment with antiretroviral therapy reduces cases of MTCT of HIV to < 2% of deliveries by women with HIV. The appropriate (scheduled cesarean) mode of delivery[22] and avoidance of breastfeeding[23,24] have also been shown to support reduction of MTCT of HIV. At present, delivery by scheduled cesarean section is recommended for women with a viral load of over 1000 copies/mL. PMTCT programs can lower MTCT rates to about 5% even in low-income countries with limited availability of combination antiretroviral regimens, and those without the ability to provide delivery by cesarean section and replacement feeding. However, this is dependent on women being tested for HIV during pregnancy as well as their enrolling in and completing a PMTCT program[25].

Early identification of HIV infected pregnant women enables health care providers to test infants for HIV infection following delivery, as well as for the early administration of prophylaxis to protect HIV-infected infants and those whose HIV status remains unknown from opportunistic infections. It also allows an opportunity for counseling women on the risks of infant infection *via* breastfeeding and proper initiation of appropriate replacement feeding[15,26].

Testing early in the pregnancy has several advantages over testing in late stages of pregnancy. Testing and identification of HIV status during pregnancy allows health care providers to use the three known effective interventions for prevention of MTCT of HIV (maternal antiretroviral treatment during pregnancy and delivery and postpartum infant prophylaxis; delivery by scheduled cesarean section; and avoidance of breastfeeding)[15].

When HIV infection is not detected during pregnancy, it is not possible to administer antiretroviral treatment during the course of the pregnancy itself. In such cases, the only remaining strategy for health care providers is to administer antiretroviral therapy during delivery and to the infant immediately following birth, and to instruct the mother to avoid breastfeeding and to begin replacement feeding. However, it is known that antiretroviral prophylaxis is more effective in preventing MCTC when begun during pregnancy[15]. In a study by Wade *et al*[27], maternal prophylaxis treatment during delivery with intravenous zidovudine, together with a six-week course of zidovudine administered to the newborn following delivery reduced the rate of MCTC by 60%.

HIV TESTING RATES AMONG PREGNANT WOMEN

Worldwide efforts to increase testing rates for HIV among pregnant women, such as implementation of routine HIV testing of all pregnant women, have led to a general increase in testing rates in various countries[7]. However, reported testing rates remain suboptimal. It is not always clear whether suboptimal testing rates are due to pregnant women's refusal to be tested or due to the fact that they have not been offered or have not had access to testing services.

The CDC reported that the percentage of pregnant women tested for HIV in the Unites States remained stable overall during the time period from 2000 to 2010, at around 50%-60%. The percentage of pregnant women tested remained stable among non-Hispanic whites, non-Hispanic blacks, and all age groups, although the rate did increase significantly among Hispanics[28]. Remis *et al*[29] reported a dramatic increase in the percentage of women in Ontario undergoing prenatal HIV testing, from 33% in 1999 to 96% in 2010. It has been suggested that measures undertaken to increase HIV testing, such as sending reminders to health care providers who did not order prenatal HIV testing, at least partially contributed to such success.

According to a report by UNICEF, in 2012 an estimated 40% of pregnant women in low- and middle-income countries received HIV testing, which represented an increase from 30% in 2010 and 8% in 2005[30]. Interestingly, other recently available sources usually point to higher testing rates in several low- and middle-income countries. For example, the Indian Health Service has reported a 22% increase in HIV testing rates over a 4-year period[2]. Another source cited that the national health services in South Africa in 2007/2008 tested 80% of pregnant women for HIV[31]. It has been reported that in Uganda in 2010, 63% of pregnant women were tested for HIV[32].

Several other relatively recent studies of prenatal testing for HIV in low- and middle-income countries also point to higher testing rates. In a study by Kizito *et al*[33], of a total of 20738 women who received prenatal services at Entebbe Hospital in Uganda from May 2002 to January 2006, 62.8% accepted testing for HIV. In contrast, in a study by Chandisarewa *et al*[34], following the initiation of routine HIV testing in urban Zimbabwe, 99.9% were tested for HIV. These results may not have been representative of the total population of pregnant women in these countries[33,34].

Despite the high reported testing rates, as long as HIV testing coverage of pregnant women is not 100%, every pregnant woman with unknown HIV-status potentially endangers the health of her future child and the health of future generations. This is particularly true in countries with high HIV infection rates[33].

FACTORS RELATED TO WOMEN'S WILLINGNESS TO BE TESTED FOR HIV DURING PREGNANCY

The term "HIV testing" is often used to describe both testing and counseling. Several voluntary testing approaches have been applied. In "opt-in testing", health care providers ask patients if they would like to receive HIV testing, while in "opt-out testing" patients are notified that, unless they decline, HIV testing is included in routine prenatal care. The WHO recently proposed a formulation that distinguishes between two types of HIV testing: client-initiated testing and provider-initiated testing. Client-initiated testing corresponds to what is usually referred to as voluntary counseling and testing (VCT) or "opt-in testing", while provider-initiated testing corresponds to "opt-out testing"[35].

Various terminology has been used to describe services of HIV testing ("opt-in testing", "opt-out testing", "client-initiated testing", "VCT", "provider-initiated testing"). The literature also uses varying terminology to describe the target variable: willingness, readiness, HIV test acceptance, HIV test uptake, *etc*. To simplify matters, the original terms from the studies mentioned in this literature review were used.

Studies report varying willingness of women to be tested for HIV during pregnancy. For example, around

50% of respondents expressed willingness to be tested in a Chinese study by Li *et al*[36] and in an Ethiopian study by Moges and Amberbir[37]; in contrast, other studies reported higher willingness to be tested (more than 75% of respondents expressed willingness to be tested)[38-40]. Some African studies demonstrated significant gaps between the willingness of pregnant women to receive HIV testing and their actual testing rates, as in studies from Sudan[41] and Tanzania[42]. Similarly, a South African study showed that pregnant women had a good level of knowledge and understanding about HIV testing in pregnancy, and their perceptions of HIV testing were positive, but they were not consistent with their behavior. That is, the women's positive attitudes towards HIV testing were not reflected in their actual behavior[43]. The difference between women's willingness to receive HIV testing and actual testing rates implies that willingness to be tested for HIV during pregnancy is a complex phenomenon influenced by an interplay of factors[42,44].

Based on the classification used by Deblonde *et al*[45] in their literature review on impediments to HIV testing in Europe, in the present review factors related to the willingness of women to be tested for HIV during pregnancy have been classified as policy-related factors, woman-related factors, and health care provider-related factors.

POLICY RELATED FACTORS

HIV testing rates among pregnant women depend on the prenatal HIV-testing approaches used at a particular location. The CDC reviewed HIV testing rates among pregnant women and found that opt-out testing resulted in higher rates of testing (71%-98%) than the opt-in approach (25%-83%). The opt-out approach has been shown to be more successful in terms of testing rates than the opt-in approach in sub-Saharan Africa[6,34,46-48]. It has been suggested that the opt-out approach destigmatizes the test, which might explain higher testing rates when the opt-out approach is applied[34]. It is also possible that the opt-out approach merely requires less effort on the part of the woman to be tested.

It should be noted that there is no uniformity in testing approaches, as various countries use different testing approaches[44]. Testing approaches may frequently vary within a single country. For example, at present both the opt-in and the opt-out approaches are used in the United States[49]. In addition, there are countries where prenatal HIV testing is still performed only in women who are in risk groups for HIV infection, such as Israel[50], although the Israeli Ministry of Health has recently recommended universal testing for all pregnant women[51].

WOMAN RELATED FACTORS

Based on this literature review, major woman related factors may be summarized as referring to social factors, fear of the HIV test results, knowledge (of HIV/AIDS and MTCT of HIV), perceived susceptibility to HIV, perceived benefits of the test, prior HIV testing, and sociodemographic characteristics (age, marital status, education, and economic factors).

Social factors

A considerable amount of literature on the willingness of women to be tested for HIV during pregnancy has focused on social factors. Women infected with HIV/AIDS often describe stigma as a major factor influencing their health behaviors[52]. Women's fear of receiving stigma and discrimination at the hands of their community, spouses, family, and health care providers have been shown to be major impediments to HIV testing during pregnancy in various countries[36,37,53-58]. Even in settings where prenatal HIV testing is normative, women's expectation that they will experience stigma as a result of HIV testing can impede their willingness to be tested[59]. Conversely, intensive family support[60] and support from significant others[61,62] have been recognized to be facilitating factors.

An important role in the willingness of women to be tested for HIV during pregnancy has been attributed to the male partner, who can be either a barrier or a source of support[31,37,40,42,55,63-65]. Women feel that their spouses' support and approval for HIV testing is a necessary condition for them to agree to receive an HIV test during pregnancy[37,63,66-68]. Bajunirwe and Muzoora[63] found that rural Ugandan women had a higher tendency than did urban women to believe that they need their spouses' approval to receive testing.

Fears of negative reactions from the male partner as a factor influencing the willingness of women to receive HIV testing have been discussed in several studies[59]. In light of societal expectations of women's sexual monogamy to their spouse[69], a male partner may blame an HIV-infected woman for unfaithfulness. As a consequence, women may face negative repercussions due to their identification as being infected with HIV, such as domestic violence[59].

In their study of pregnant women in rural Kenya, Turan *et al*[59] found that fear of their spouses' reaction and possible repercussions were a more powerful influence on the willingness of women to be tested for HIV during pregnancy than were their concerns regarding any other significant others. Turan *et al*[59] suggested that because community members are not easily able to identify if a woman is infected with HIV, women have less fear of receiving negative consequences from the whole community.

It should be noted that male partner factors also play a role in whether women return for results, as demonstrated by Msuya *et al*[70]. In their study, when women's spouses did not undergo testing, the women themselves were less likely to return to the clinic to receive their own test results.

Fear of the test results

Fear of the test results has been shown to be a major barrier to being tested for HIV during pregnancy, both in earlier and in more recent studies[31,37,55,56,58]. Dube and Nkosi[43] found that half of the women in their study felt

that getting tested for HIV was emotionally stressful. Similarly, Moges and Amberbir[37] found that pregnant women resist HIV testing because they are afraid to receive a positive result. A similar finding was also seen in Tanzania[42]. In contrast, an Ethiopian study by Maedot *et al*[71] found that pregnant women who felt that they were capable of coping with a positive HIV test result were identified as being more likely to accept VCT.

Knowledge, perceived susceptibility to HIV, and perceived benefits of the test

Most reviewed studies demonstrate that women's willingness to be tested for HIV during pregnancy was influenced by their knowledge about HIV/AIDS and MTCT[36,37,39,41,58,60,72-74]. Other studies found that knowledge was not related to willingness to be tested. It has been suggested that results need to be interpreted within the context of a particular society[75].

Turan *et al*[59] found that knowing someone who was HIV-positive was associated with willingness to receive HIV testing during pregnancy. They suggested that knowledge of MTCT and knowing someone who was HIV-positive might increase women's awareness of the possibility of MTCT and the advantages of receiving HIV testing. Indeed, many studies found that high perceived susceptibility to HIV was associated with willingness to receive HIV testing during pregnancy[36,37,42,65,76-78]. Research from multiple countries has shown that many pregnant women did not believe that they were at risk for contracting HIV because they are in monogamous relationships and trust their male partner[37,40,42,79].

Many studies also identified an association between women's perception of the benefits of the test, either for their infants' or for their own health, and the willingness of women to be tested[37,38,42,62,63]. However, in a study by Baiden *et al*[80], willingness to be tested for HIV was not associated with women's view on the usefulness of the test.

Several studies have found that HIV testing participation was related to the number of prenatal care visits a pregnant woman had already received[58,60,68]. Women who have less access to prenatal health care are less likely to know about PMTCT and other preventative care[58]. It has been shown that improving women's access to prenatal care improves PMTCT uptake[81].

Several studies have identified certain obstetric factors, such as bad obstetric history, or being multi gravida *vs* primigravida, as associated with uptake of VCT[41,63,65,67,82]. It is possible that multigravida women had more contact with prenatal care services and therefore had prior experience of HIV testing, or that they were more aware of the MTCT of HIV.

Prior HIV testing

Several studies found that prior HIV testing was related to experience with HIV counseling and testing (HCT)[66,83]. In contrast, in a study in Ghana by Holmes *et al*[84] it was found that 95% of women who had been previously tested for HIV declined to receive additional testing. Similarly, Peltzer *et al*[31] found that women declined to receive HCT because they already had been tested for HIV previously.

It is possible that the inconsistency in results may reflect women's varying underlying beliefs. For example, Matovu *et al*[83] suggested that women may seek repeat testing in order to be certain that they have not been infected. In contrast, it has also been suggested that women who have received a negative test result in the past, and who do not feel that they are at any new risk, will not consider repeat testing to be useful to them[84]. Similarly, focus group discussions in a study by Matovu *et al*[83] suggested that women who have received repeat negative HIV testing results feel that they may not be susceptible to HIV, or that they have been lucky. Such beliefs raise concerns, as a previous negative result does not absolutely guarantee a negative result during a subsequent pregnancy.

Sociodemographic characteristics

Age: In a Sudanese study, women older than 26 years had higher acceptance of VCT[41]. Similarly, in a study conducted in Burkina Faso, the uptake rate of VCT increased linearly with age, being particularly low among adolescents (15-19 years)[67]. Enosolease and Offor[85] have shown that older Nigerian women had higher rates of acceptance of HIV testing. It has been suggested that older women may be more aware of a higher cumulative risk of infection and are more likely to take autonomous decisions[67]. Other studies have found that older age was actually associated with test refusal[58,66]. There were also studies which found no correlation at all, as in a UK study[77]. These findings suggest that age is a confounding factor.

Marital status: Fabiani *et al*[86] found that being married was associated with lower VCT uptake. Conversely, in a study by Matovu *et al*[83], VCT acceptance was actually higher among married women. Perez *et al*[58] also found that women living with a partner were more likely to accept HIV testing. It is possible that inconsistence in results reflects variation in women's perception of susceptibility to HIV. Some married women may perceive themselves as less susceptible to HIV because they trust their husbands[37], while other married women may actually feel that they are more at risk[58]. Perez *et al*[58] also suggested that married women are more accepting of HIV testing because they feel that they can depend on their spouse to support them in the event of a positive test result.

Education: Previously conducted studies in different countries have demonstrated that higher educational status was associated with higher willingness to accept VCT[37,54,58,63,74,79,84,86]. Perez *et al*[58] suggested that women with less education also have less knowledge about and access to prenatal healthcare.

Conversely, it has also been found that lower education sometimes leads to higher rates of test acceptance[87,88].

Barragán *et al*[87] suggested that when HIV testing is encouraged by healthcare workers, women's low level of knowledge may not be an impediment to their acceptance of testing.

Economic factors: Moges and Amberbir[37] found that the occupational status of women in Northwestern Ethiopia was an important factor in their readiness to utilize VCT. Employed pregnant women accepted VCT at higher rates than unemployed married women. Similar results were obtained in a Vietnamese study[54] and in a Sudanese study[41]. The researchers suggested that when women leave the home and are employed, they have greater access to information about VCT compared to unemployed married women who spent most of their time at home[37]. Perez *et al*[58] also found that rural Zimbabwean women with unemployed partners and lower incomes were less likely to be tested because these women are less economically independent and less able to make decisions for themselves.

HEALTH CARE PROVIDER RELATED FACTORS

Based on the literature review, health care provider-related factors may be classified as relating to access to antiretroviral therapy, site characteristics, woman-provider dynamics, and belief in the HIV test's reliability. Perceived ability to get continuous medical care following a positive HIV test result [71], knowledge about the availability of the antiretroviral therapy[89], and lack of access to antiretroviral therapy[66] are often listed among factors associated with the willingness of women to be tested during pregnancy.

In a study from Kenya, Anand *et al*[90] found that site factors were the most significant element in PMTCT program acceptance. In a government hospital in Uganda, administrative problems, lack of resources and lack of staff were cited as significant causes for failure to counsel women about routine HIV testing during pregnancy[91]. Dahl *et al*[66] found that the longer that testing sites were in operation, the higher their rates of HIV test acceptance. Larsson *et al*[25] examined the willingness of women in rural Uganda to receive HIV testing. They found that for women of all income levels, those who lived further than three kilometers from an HIV testing site were less likely to be tested. Long waiting times were identified as another major reason for refusing the test[31].

Several studies have demonstrated the positive effects of combining PMTCT programs with prenatal care[92-94]. There are several possible explanations for this: it may be that combining these services helps women by reducing the cost, time, and travel required to receive care. Additionally, women seeking care at a site that offers combined services are less likely to be identified as seeking HIV testing, and therefore may be less fearful that they will be stigmatized or discriminated against by their communities[92]. In addition, it has been previously documented that perceived unreliability of test results and distrust of HIV testing technologies can discourage uptake of HIV testing[66,95,96]. Combining services may improve and increase technology available at testing sites, which in turn can improve women's confidence in both the technology and their health care providers.

Site characteristics seem to play an important role in acceptance of testing for HIV and also in women's intention to come back for results, which is crucial in prevention of MTCT. In a study by Sarker *et al*[68], operational factors were the most significant reason why women failed to return to a testing site to get their results. These operational factors included poor scheduling of the post-test counseling sessions and a lack of doctors. Msuya *et al*[70] also found that the site of recruitment was associated with women's motivation to return to receive their HIV test results.

Varga *et al*[97] examined impediments to willingness to be tested for HIV of 15 to 19-year-old mothers in rural and urban Limpopo Province, South Africa. The study found that the relationship and communication between the pregnant women and the HCT counselor was a significant factor influencing the rate of acceptance of HIV testing, as were the counselors' profiles, which impacted the interaction between pregnant women and clinic staff. Peltzer *et al*[31] found that the more trust pregnant women had in their HCT counselor, the higher their rate of acceptance of pre- and post- test counseling. A study in Alberta, Canada, found that counselors' gender and education were the most significant influence on women's willingness to participate in routine opt-out prenatal HIV testing[98].

Studies have also found that lack of confidentiality was associated with less participation in HCT[31,42,56,97]. Negative experiences with medical personnel[55,82], as well as low quality of pre-test counseling, were also associated with less participation in HCT[66,95,96]. In addition, women's failure to understand the HIV testing procedure as explained during group counseling, as well as dislike for group counseling, were listed among the major reasons for refusing the test[31]. Perez *et al*[58] showed that group counseling had a negative effect on acceptance of HIV testing. The researchers suggested that this demonstrates a need for revision of counseling methods.

RECOMMENDATIONS

The extensive literature on this topic provides recommendations aimed at increasing HIV testing rates among pregnant women in different countries. Numerous factors related to pregnant women's willingness to be tested suggest multiple possible interventions to maximize HIV testing efficiency and increase testing rates. In general, major recommendations suggested in various studies aimed at increasing HIV testing rates among pregnant women can be conceptually mapped as falling into one of three primary domains: male partner involvement, education, and improvement of site-level factors.

Male partner involvement

Male partner involvement in the process of prenatal HIV testing is necessary. This involvement includes HIV counseling for couples and testing with facilitated disclosure. There is a need to advance strategies to address women's fear of negative repercussions from their spouses in response to a positive HIV test result. One potential strategy is teaching prenatal healthcare providers to facilitate discussions between women and their spouses about HIV testing in order to improve their acceptance of testing. However, women should be also equipped with tools to help them safely and effectively communicate with their spouses about HIV testing. Prenatal healthcare workers should be aware of signs of domestic violence and include domestic violence reduction programs in their prenatal care.

Education

Women's education in general should be promoted, including knowledge of HIV testing, MTCT of HIV, and PMTCT programs. Emphasis should be put on HIV susceptibility and benefits of the HIV test, while various misperceptions should be corrected through proper counseling. Moreover, education should include support and empowerment of women to reduce fear of the test and provide tools to cope with the results.

Improvement of site-level factors

Improving women's willingness to participate in HIV testing and PMTCT programs should include addressing deficiencies at the site level as well as focusing on participant factors. Positive site-level factors to encourage HIV testing in pregnant women include improving staff availability and knowledge, improving scheduling and patient management, developing better counseling methods, and increasing health care providers' access to test kits and on-site laboratory capabilities. Mistrust towards HCT providers should be reduced. These factors should be periodically evaluated, including comparison of sites with high and low rates of HIV testing and PMTCT acceptance.

CONCLUSION

There are several factors that are usually identified as associated with the willingness of women to be tested for HIV during pregnancy. Studies have shown that certain major factors remain stable over time. However, frequent inconsistent results concerning certain factors suggest that there is no magic formula for understanding and predicting women's willingness to be tested. The inconsistencies in results may reflect the complexity of the phenomenon of women's willingness to be tested. Factors may be interrelated and influenced by cultural and social characteristics of a society, requiring further research and meta-analyses of the phenomenon.

It should be noted that the majority of the research literature focuses on sub-Saharan Africa. There is dearth of research on factors related to the willingness of women in countries with middle and high income levels to be tested for HIV during pregnancy. Research on the willingness of women to be tested in certain countries is completely lacking.

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REVIEW

Insights into human immunodeficiency virus-hepatitis B virus co-infection in India

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Abstract

Shared routes of transmission lead to frequent human immunodeficiency virus (HIV)-hepatitis B virus (HBV) co-infection in a host which results in about 10% of HIV positive individuals to have chronic hepatitis B infection worldwide. In post-antiretroviral therapy era, liver diseases have emerged as the leading cause of morbidity and mortality in HIV-infected individuals and HBV co-infection have become the major health issue among this population particularly from the regions with endemic HBV infection. In setting of HIV-HBV co-infection, HIV significantly impacts the natural history of HBV infection, its disease profile and the treatment outcome in negative manner. Moreover, the epidemiological pattern of HBV infection and the diversity in HBV genome (genotypic and phenotypic) are also varied in HIV co-infected subjects as compared to HBV mono-infected individuals. Several reports on the abovementioned issues are available from developed parts of the world as well as from sub-Saharan African countries. In contrast, most of these research areas remained unexplored in India despite having considerable burden of HIV and HBV infections. This review discusses present knowledge from the studies on HIV-HBV co-infection in India and relevant reports from different parts of the world. Issues needed for the future research relevant to HIV-HBV co-infection in India are also highlighted here, including a call for further investigations on this field of study.

Key words: Human immunodeficiency virus-hepatitis B virus co-infection; India; Genetic diversity; Liver diseases

Core tip: Various parameters of hepatitis B virus (HBV) infection including molecular epidemiology, disease profile and treatment outcome remains unexplored in human immunodeficiency virus (HIV)-positive individuals from India, a major reservoir for HBV and HIV infection of the globe. Only few reports particularly from eastern Indian HIV-HBV co-infected cohort represented some interesting findings in context to the global reports on this co-infection. Comparing with the available worldwide studies, issues that should be addressed for research in India are identified and a call for further investigations on HIV-HBV co-infection in India is highlighted through

this article. This is needed for proper management of HIV-HBV co-infected Indian population.

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INTRODUCTION

Human immunodeficiency virus (HIV) and hepatitis B virus (HBV), the two important blood-borne human pathogens, are major public health concerns in the current era. Since the introduction of combination antiretroviral therapy (cART) in 1996, acquired immunodeficiency syndrome (AIDS) related deaths among HIV-infected individuals have been reduced significantly worldwide[1]. In this situation of improved life expectancy due to ART, liver disease associated mortality has emerged as the leading cause of deaths in HIV-infected global population. Of all the possible causes of liver-related deaths, HBV co-infection has become one of the important burdens among HIV-positive individuals in post-ART era. Moreover, HBV shares its routes of transmission (sexual contact, percutaneous route and perinatal route) with HIV[2] and thus the incidence of HIV-HBV co-infection becomes a frequent phenomenon in a host[3]. As a consequence, in an estimated 40 million people living with HIV worldwide, approximately 10% (2-4 million) have chronic HBV co-infection [defined by the presence of serum hepatitis B surface antigen (HBsAg) for more than 6 mo][2]. In addition, the biological signs of prior HBV infection [defined by the presence of serum anti-hepatitis B core antibody (HBcAb)] could be observed in 90% of HIV-positive individuals in the regions with high endemic HBV infection such as south-east Asia and sub-Saharan Africa.

During the setting of co-infection, HIV and HBV simultaneously interact in a host complicating pathogenesis and disease progression of these two infections, immune-responses to both the virus and treatment outcome against them. Till date, several studies have been conducted worldwide which have shown significant negative impact of HIV on the natural history of HBV infection[4-8] whereas such confirmed evidences are missing that state the effect of HBV on HIV infection[9]. Co-infection with HIV modifies the natural history of HBV infection by increasing the rate of HBV chronic infection, lowering the rate of HBsAg, hepatitis B e antigen (HBeAg) seroconversion and increasing HBV replication[5,6]. The deleterious effect of HIV leads to more rapid progression towards end-stage liver diseases (liver cirrhosis and hepatocellular carcinoma) and higher risk for liver-disease related mortality in HIV-HBV co-infected individuals as compared to those infected with HBV only[10,11]. Simultaneously in presence of HIV, management of HBV becomes complicated greatly[12].

Most of the developed parts of the world (for, *e.g.*, western Europe, Australia and United States) where HBV is less endemic (HBsAg prevalence < 2%) and some of the developing countries (mostly sub-Saharan African countries) with endemic HBV infection (HBsAg prevalence ⩾ 8%) have largely contributed to the studies regarding HIV-HBV co-infection in these parts of the globe. Harbouring the third largest population of HIV infection and the second largest pool of chronic HBV infection (HBsAg prevalence 2%-7%) of the world, reports on HIV-HBV co-infection in India are scarce. Interestingly, few reports available from India indicate that studies on HIV-HBV co-infection are required from national as well as global perspectives. Therefore here we have highlighted HIV-HBV co-infection in India in comparison to the reports from different parts of the world to understand the present scenario of this co-infection in this subcontinent.

EPIDEMIOLOGICAL SCENARIO OF HIV-HBV CO-INFECTION

HIV-HBV co-infection showed global heterogeneity in epidemiological pattern. Two major determinants of this variation are geographical origin and risk groups of infected patients[2]. In regions of low endemicity of HBV infection (prevalence of HBsAg < 2%) such as United States, Western Europe and Australia, prevalence of chronic hepatitis B was reported to be 5%-14% among HIV-positive individuals[7,13-17]. In the countries of developed parts of the world, acute HBV infection occurs in adolescents and young adults through primarily sexual transmission (both heterosexual and homosexual), followed by percutaneous transmission. HIV-infected men who have sex with men (MSM) showed the highest frequency of chronic HBV infection (9%-17%)[3]. In contrast, perinatal transmission (south and south-east Asia) and horizontal transmission (Africa) are the major threats in the intermediate (HBsAg prevalence 2%-7%) and high endemicity (HBsAg prevalence ⩾ 8%) zones of HBV infection where persons obtain HBV infection in childhood[18]. Adults could acquire HIV-HBV co-infection through sexual contact and unsafe blood transfusion process in the resource limited settings of low-income countries[18,19]. Most studies reported 10%-20% prevalence of HIV-HBV co-infection in these countries[2]. Moreover reports from different parts of sub-Saharan Africa suggests that HBsAg prevalence could vary considerably (from Kenya; 6% to Nigeria; 16.7%)[20,21]. Evidences of variations in the prevalence of HBsAg among different risk groups were also found across the countries of this continent[22]. Regarding the epidemiological scenario several studies have been performed worldwide on multi-centre cohort of HIV-HBV co-infection which revealed the overview of prevalence, clinical and virological profile of these patients from a country[7,8,10,15-17,23-25]. Recently, a study including a multi-national cohort from 11 countries showed the concordant prevalence of HIV-HBV co-

infection in Africa, America and Asia similar to the previous reports[25].

In contrast, sporadic reports from India[26-37] has addressed the issue of prevalence of HBsAg among HIV-infected patients majority being from the northern part of the country[32-37]. Taking together these reports, HBsAg prevalence among HIV-infected Indian population could be estimated as 2%-14%. These reports mostly included HIV-positive patients either from one ART centre[31,37] or from single risk group for, *e.g.*, injecting drug users[27], female sex workers[30]. However in another two studies quite high frequency of HBsAg were found - approximately 22% (6/27)[32] and approximately 30% (34/110)[26]. These variations in results were observed possibly due to small sample size data, lack of multi-centre studies and unavailability of multi-risk group data. Thus, overall epidemiological trend of HIV-HBV co-infection in India still remains obscure (Figure 1). Nevertheless, two findings from these sporadic studies are concordant with the worldwide reports[21,22,25], *i.e.*, (1) the male gender is predominant over the females; and (2) sexual contact is the chief transmission route of HIV-HBV co-infection in India[26-31].

INFLUENCE OF HIV ON NATURAL HISTORY OF HBV INFECTION

To date, significant adverse effects of HIV co-infection on the natural history of HBV infection have been demonstrated in several studies from the perspectives of increased chronicity, accelerated rate of advance liver disease development and heightened mortality rates[4-6,10,11]. In a retrospective study, Bodsworth *et al*[4] showed increased rate of HBV chronicity development in HIV seropositive homosexual men than those without HIV (23% *vs* 4%)[4]. In several studies, HIV-HBV co-infected individuals showed decreased rate of HBeAg seroclearance along with increased HBe antigenemia[5,6,8]. Incidence rate of HBeAg seroclearance was decreased five times in HIV-positive patients compared to HIV-negative ones during a mean follow up for 5 years in a study from France[8]. Moreover in accordance to high HBeAg positivity, high serum HBV DNA load is associated with HIV-HBV co-infected patients[5,6]. HBeAg positive HIV-infected patients mostly had higher HBV viraemia as compared to HBeAg negative individuals. Thio *et al*[25] showed that 66% of HBeAg negative subjects had HBV DNA < 2000 IU/mL in a multi-national treatment-naïve cohort suggesting HBeAg as a predictive factor for HBV treatment in absence of HBV DNA quantification data[25]. Remarkably, studies describing the impact of HIV on HBV related mortality showed that HIV-HBV co-infected individuals had increased rate of liver associated deaths as compared to those with HBV mono-infection[7,10]. HIV-HBV co-infected men had 17 times higher incidence of liver-disease related deaths than HBV mono-infected ones[10].

Besides the worldwide reports, studies regarding the abovementioned parameters are lacking in India. However in one study from eastern India showed high HBV DNA load among HBeAg negative HIV infected individuals where 61% had HBV DNA ⩾ 2000 IU/mL and required HBV treatment[31]. According to the study by Thio *et al*[25], detection of HBeAg may be useful to assess the need for treatment in a setting where HBV DNA quantification facility is unavailable. Saha *et al*[31] demonstrated that HBeAg could be helpful to indicate the need for treatment among HBeAg positive HIV-HBV co-infected patients from eastern India however DNA quantification is necessary to consider HBeAg negative patients for treatment or not. Thus eastern Indian HIV-HBV co-infected individuals need serious attention for their clinical management and the conformation of this finding from the different parts of the country is an urgent necessity.

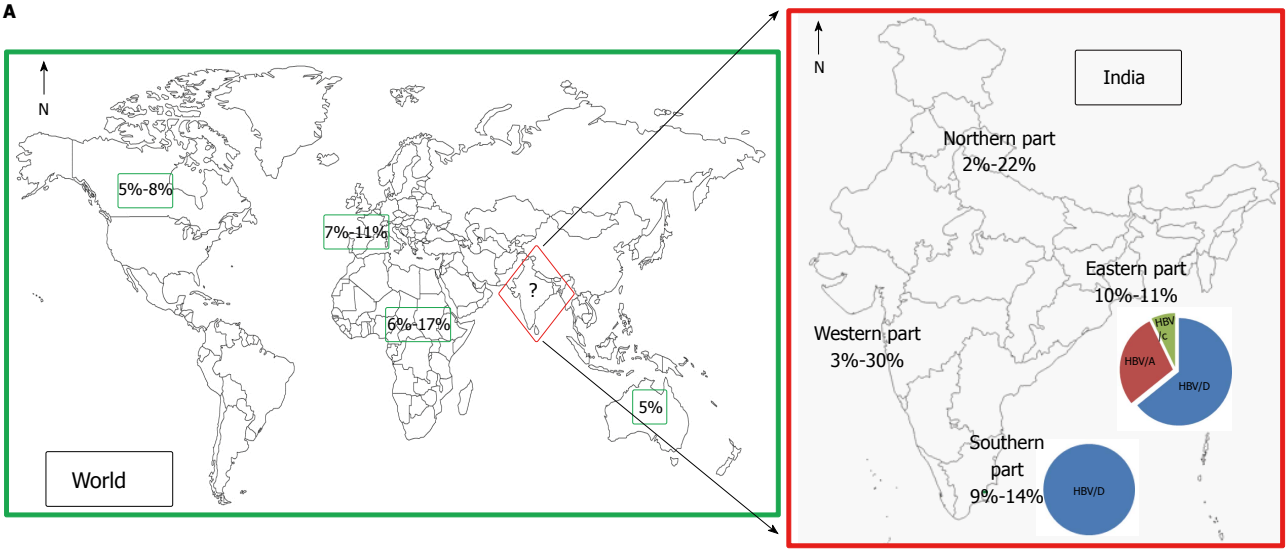
The aforementioned negative impacts on natural history of HBV might be the consequences of influence of HIV on the diversity of HBV genome, modification of host immune response and ART related complications. Some reports could be found to address the genetic diversity of HBV among HIV co-infected individuals[23,38-43]. HBV genome diversity can be described from two aspects - genotypic and phenotypic.

INFLUENCE OF HIV ON GENOTYPIC DIVERSITY OF HBV

Genotypic diversity is related to the natural history and the genotypes of HBV infection occurring during the gradual evolution of HBV in a host without selective pressures. Having a high mutation rate (10^{-5}/replication cycle), HBV results in the generation of different genotypes and each genotype can further be divided into several sub-genotypes. So far ten HBV genotypes (A-J) have been described depending upon their > 8% nucleotide divergence in complete genome sequences, whereas subgenotypes have that divergence of > 4% - < 8%[44]. HBV genotypes and subgenotypes showed varied distribution according to the geographical regions. Moreover, HBV genotypes/sub-genotypes differ considerably in the mutational patterns, ethnicity and their clinical as well as treatment outcomes[44].

In HIV-HBV co-infection, distribution of HBV genotypes was found to vary with geographical origin which is similar to HBV mono-infection[45]. In a recent collaborative study from 19 French university hospitals, 223 HIV-HBV co-infected patients were evaluated[24], where primarily prevalence of HBV/A were found in European and HBV/D in African patients. While, HBV/E was found mainly in patients with sub-saharan African origin, as this genotype is reported to be confined mostly to that region. Interestingly, a report from Mexico observed differential predominance of genotype between HBV mono-infected (HBV/H) and HIV-HBV co-infected patients (HBV/G)[46]. Moreover in a recent report on a multi-national HIV infected cohort (n = 113), Thio

A



B

Research areas in HIV-HBV co-infection	Worldwide reports excluding India (References)	Nationwide reports from India (References)
Multi-centre study on prevalence and characterization	√ [5-8,16,17,23-25]	–
Effect of HIV on natural history of HBV infection	√ [4-6]	–
Genetic diversity (Genotypic and phenotypic) of HBV among HIV co-infected individuals	√ [23-25,38-41,46,48-50]	√ [31,42,43,47]
Altered immune response in HIV-HBV co-infection and effect of HIV on genetic diversity of HBV	√ [25,58,59,90,91]	√ [42]
Increased liver injuries and end stage liver disease development	√ [11,38,92,93]	–
Increased liver mortality	√ [7,10,11]	–
Detection of Lamivudine resistance	√ [23,52-59]	√ [31,42,60]
Reports on other anti-HBV drugs	√ [62-64]	-

C

	pol ORF (amino acid)	pre-S2/pre-S1/S ORF (amino acid)	BCP + pre-C/C ORF (nucleotide)	X ORF
Mutations reported in HBV among HIV-co-infected individuals in India	3TC-resistant double (L180M + M204V) and triple mutation (V173L+ L180M + M204V)[31,42,60]	Pre-S2 deletion, immune-escape mutations(M133I/T, Y134N/H, Q129R), vaccine-escape mutation (G145R), premature stop codon (C69Stop)[42,43,47]	A1762T/G1764A, G1896A, frameshift mutation (-1G)[43]	-

Figure 1 Human immunodeficiency virus-hepatitis B virus co-infection: World *vs* Indian scenario. A: Prevalence of this co-infection is elucidated from most of the developed parts of the world as well as from sub-Saharan African countries as multi-centre and multi-cohort study. The few regional reports available from various regions of India are also represented, highlighting the need for multicentric study; B: Most of the research areas remain unexplored in India as compared to the worldwide reports; C: Mutations in hepatitis B virus (HBV) genome (four open-reading frames) reported among human immunodeficiency virus (HIV)-HBV co-infected Indian patients. BCP: Basal core promoter.

et al[25] reported the predominance of HBV/A (72%) and HBV/D (16%) in HIV-HBV co-infection worldwide and the divergence of HBV genotype with geographical regions[25]. Till date only one study could be found to report an association of HBV genotype (HBV/G) with liver severity, *i.e.*, the degree of liver fiborsis in HIV-HBV co-infected patients[38].

In comparison to worldwide data, only four studies could be found from India that analyzed the genetic diversity of HBV among HIV co-infected patients; three from eastern India[31,42,43] and one from north-eastern India[47]. Reports from other parts of the country are still lacking. Interestingly, a recent multi-national study[25] that included patients from India (n = 13), showed

100% prevalence of HBV/D. In contrast, three studies from eastern India, that included a larger number of patients (n = 73[42], 119[31] and 85[43]), reported predominance of HBV/D, followed by HBV/A and found a few HBV/C infected patients. Pal *et al*[42], showed that the HBV genotypes/subgenotypes found among co-infected patients from eastern India are consistent with the previous data on HBV mono-infection, but the proportion differs between the HIV-HBV co-infected and the HBV mono-infected patients. Significantly higher prevalence of HBV genotype D (HBV/D - 67%) and HBV sub-genotype D2 (HBV/D2 - 68%) was observed among HBsAg positive HIV co-infected patients from this region[42]. Moreover, the predominance of HBV/C among the HBsAg negative HIV co-infected IDUs from Manipur, a state of northeastern India, has also been reported[47]. The presence of HBV/C has been thought to be correlated with drug-trafficking routes and epidemic use of injection drug in that geographical region. Additionally in this study, HBV recombinant strains (HBVA/D, HBVA/C) were found from two IDUs. Few studies could be found worldwide to report recombination in HBV DNA among HIV co-infected patients[48-50]. However, clinical consequences of these recombinants are unknown.

EFFECT OF HIV ON PHENOTYPIC DIVERSITY OF HBV

Phenotypic diversity results from the attempts to escape from host immune pressure or selective pressure of drugs. In HIV-infected individuals known HBV phenotypic diversity as well as novel viral variants have been reported which arise from several mutations in the four open-reading frames (ORFs) of HBV genome (pol, pre-S1/pre-S2/S, pre-C/C and X).

The basal core promoter (BCP) mutations reported to be associated with fulminant hepatitis in HBV-mono-infection namely T1753C, A1762T, G1764A occur in X ORF leading to down-regulation of HBeAg by decreasing its mRNA synthesis. The BCP double mutations (A1762T/G1764A) and triple mutation (T1753C/A1762T/G1764A) could also be found in HIV infected patients from United States, Australia and Thailand[23,41]. But the frequency of A1762T and G1764A was lower in HIV-HBV co-infected individuals as compared to those with HBV mono-infection (39.8% *vs* 59.3% and 39.8% *vs* 61% respectively)[41]. Moreover, presence of HBV precore stop codon mutation was reported in the co-infected cohort from different parts of the world though prevalence of G1896A (W28Stop) varied among these studies[23,38,39]. Among HIV-HBV co-infected patients, a novel -1G deletion mutation in precore/core region of HBV was reported[40]. This mutation was found to be associated with genotype A and high HBV load. This mutation was suggested to be associated with altered pathogenesis in this population by two mechanisms - firstly, development of a premature stop codon and truncated pre-core/core protein might be responsible for increased viral load and secondly, stop codon in the MHC-class II restricted epitope might lead to immune escape. In the same study, in addition to -1G mutation, substitutions in *x* gene, polymerase gene, precore/core gene and regulatory regions were also found[40]. Study by Audsley *et al*[41] supported the earlier result showing -1G frameshift mutation to be unique for HIV-HBV co-infected patients (10.8%). The only report[43] demonstrating the molecular epidemiology of HIV-HBV co-infected individuals from eastern India also found these BCP, precore/core mutations however prevalence of these mutations varies from the worldwide reports. In this Indian cohort lower frequency of A1762T/G1764A (13.6%) and-1G mutation (1.75%) were found, but the prevalence of G1896A is high (22%) as compared to the reports available from different parts of the globe[43]. This discrepancy could be explained by the high prevalence of HBV/D than HBV/A and HBV/C in India.

In a recent study analyzing complete HBV genome from HIV co-infected patients, pre-S2 deletion was more frequently found in pre-S1/pre-S2/S ORF among HIV-HBV co-infected individuals as compared to those infected with HBV only (14.6% *vs* 3.3%)[41].The majority of Pre-S2 deletions were located close to the N-terminus of the Pre-S2 protein. In contrast this deletion mutation is uncommon in eastern Indian cohort with HIV-HBV co-infection (5.41%)[43]. Some immune escape mutations (P120T/S and G145R/K/A) could also be found in context to HIV-HBV co-infection[38]. In the study from eastern India, low frequency of some immune escape mutations (Q129R, M133I/T, Y134N/H and G145RS) has been reported from the surface gene region of HBV genome[42,43]. Interestingly, in the upstream of "a" determinant region, a stop codon at C69 was found mainly in HBV/D2 isolates from these HBsAg positive HIV co-infected patients. This mutation was previously reported in Iranian HBV mono-infected patients with cirrhosis[51]. Though, the effect of this nonsense mutation remains unknown among HIV-HBV co-infected patients.

Besides spontaneous genetic variability, several diversities could be found in HBV genome, mainly in polymerase gene, under the selective pressure of nucles(t)ide analogues having anti-HBV activity. As a first line ART, lamivudine has been extensively used among HIV co-infected individuals. Benhamou *et al*[52] first estimated that after 4 years of lamivudine (3TC) therapy, 90% of a HIV-HBV co-infected cohort developed drug-resistant HBV which was higher compared to HBV mono-infected patients (67%). Furthermore, a later study showed increased frequency of double mutation (rt L180M + rt M204V) and triple mutation (rtV173L + rt L180M + rt M204V) during longer duration of 3TC therapy and they found 3TC resistance in 94% of the HIV-HBV co-infected patients experiencing 3TC for > 4 years[23]. The high frequency of lamivudine-resistance associated with HIV-HBV co-infected patients could also be supported in several studies from different parts of

the world so far[23,39,53-59]. Another adverse consequence of the 3TC-resistant triple mutation is that it generates vaccine escape mutation (E164D + I195M) in the overlapping surface gene region. Therefore, possibility to infect the unvaccinated as well as the HBV vaccinated persons makes this a serious health issue. In the earlier study of Pal *et al*[42], the presence of 3TC-resistant triple mutation was observed among the HIV-HBV co-infected patients from eastern India. In a recent study from same part of the India demonstrated the high incidence of 3TC-resistant double and triple mutation among HIV-HBV co-infected patients who had exposure of 3TC as a sole HBV-active agent during prolonged ART[60]. Studies on 3TC-resistance among HIV-HBV co-infected patients from different parts of the world found higher frequency of 3TC-resistant double mutation compared to triple mutation[23,39,53-57]. It is noteworthy to mention in this context that HIV-HBV co-infected patients from eastern India receiving long-term ART showed predominance of 3TC-resistant triple mutation over double mutation and the former prevailed in significantly higher frequency among HBV viraemic patients experiencing 3TC for ⩾ 4 years [frequency of 3TC-resistant triple mutation (vaccine escape mutation): 60% *vs* double mutation: 10%][60]. Moreover these 3TC-resistance associated vaccine-escape HBV mutants showed the presence of liver damages in these HIV-HBV co-infected patients. This finding by Pal *et al*[60] underscored the urgent need to study the overall burden of 3TC-resistant mutations in HIV-HBV co-infected Indian pool for proper management of 3TC-resistant mutants of HBV from clinical and public health perspectives in this country. Considering the adverse effects of 3TC-monotherapy, use of combination therapy using tenofovir has been introduced by World health organisation worldwide and National AIDS Control Organization in India for the management of HBV among HIV co-infected individuals[61]. Among other drugs used for treatment of HBV, development of drug resistance have been reported for adefovir and entacavir among HIV-HBV co-infected patients[62,63], however tenofovir resistance could not be detected among this population. So far, Tenofovir has been reported to suppress HBV DNA even in presence of lamivudine resistance and thus is recommended for the treatment of HBV infection in setting of HIV co-infection[64]. Use of tenofovir has been started in India among HIV-HBV co-infected patients from 2012[61], however the evaluation on treatment response for HBV during tenofovir treatment is still missing.

HIV ASSOCIATED IMMUNOLOGIC STATUS AND GENETIC DIVERSITY, SEROLOGICAL OUTCOME OF HBV INFECTION

Besides the genotypic and phenotypic diversity, modulation in HIV-associated immune status could not be overlooked. HIV-HBV co-infected subjects were mostly associated with lower CD4+ T-cell count as compared to HIV mono-infected ones[25]. This observation indicates towards the potential effect of HIV related immune dysfunction on HBV diversity as well as in the clinical outcome of HBV infection among HIV-positive patients. Few available reports showed interesting findings. The study by Pal *et al*[42] highlighted the influence of HIV induced immune modulation on the genetic heterogeneity of HBV among HIV-HBV co-infected patients from eastern India. Here a trend of negative association between the frequency of the HBV/D2, the predominant HBV subgenotype, isolates and CD4+ T cell counts was found. The HBV/D2 isolates showed decreased genetic diversity in low CD4+ T cell count group which in turn was attributed to increased HBV viremia and favourable selection of HBV/D2 isolates in HIV induced low immune pressure. Moreover, increased non-synonymous substitutions with increase in CD4+ T cell count in this study underscored the possibility that ART induced immune reconstitution might lead to the development of vaccine/immune escape and lamivudine resistant mutations among HBV/D2 infected patients. In contrast to HBV/D2, interestingly in HBV/A1 genetic variability was modified differently in presence of HIV. This contrasting substitution pattern with varying immune suppression between HBV/A1 and HBV/D2 was proposed to be related to the differences in host immune response against these two subgenotypes. An earlier study from Argentina showed that as a consequence of lower CD4+ T cell count, HBV subjects from HIV co-infected patients had low quasispecies diversity as well as evolutionary rate when compared to that from HBV mono-infected patients[65].

Another study reported the association of HBV serological outcome with CD4+ T-cell count. Landrum *et al*[66] showed increased proportion of chronic HBV infection in patients with CD4+ T-cell count < 200 cells/mm^3 (19%) compared to those with ⩾ 500 cells/mm^3 (11%) and 200-499 cells/mm^3 (16%). Individuals with HBV infection occurring after HIV diagnosis had high risk of chronic HBV infection and also this risk reduced after initiation of highly active antiretroviral therapy. However, conformation of these findings is missing due to limited studies in the respective fields.

HIV-HBV CO-INFECTION IN INDIA- A CONCERN

In India, HIV and HBV mono-infection have been studied thoroughly from different parts of the country[67-89]. These reports represent the subcontinent as a region epidemic of HIV infection and intermediately endemic to HBV infection. But, "HIV-HBV co-infection in India" remains unexplored even after knowing the epidemiological trends of these virus and adverse effects of HIV on outcome of HBV infection in setting of co-infection. In comparison to the global scenario of researches on HIV-HBV co-infection, information from India is scanty and thus need investigations in this field (Figure 1). Few

studies from eastern India have shown some interesting findings highlighting the need for the studies from the different parts of this country to get the national scenario on the whole. The foremost requirement is to elucidate the overall burden of HIV-HBV co-infection in India, not only the prevalence of chronic HBV infection but the rate of prior infection should be studied to know its threat level among HIV infected population of India. To fulfill this aim, multi-centre study with different risk groups across the country should be included. Besides epidemiological studies, characterization of virological parameters (HBV genotype/subgenotype distribution, their association with degrees of immunosuppression, HBeAg status) and clinical aspects (ALT, fibrosis stage) also needs attention. Moreover, full genome sequencing of HBV from HIV co-infected Indian population is required to get results whether genetic diversity from this cohort shows concordance with worldwide reports and to screen for the possible presence of any unusual mutations. Data obtained from HIV-HBV co-infected patients from eastern India, *i.e.*, effect of HIV on genetic heterogeneity of HBV also needs conformation from the other parts of this country. Another important aspect for future research includes the study on response of anti-HBV treatment among HIV-HBV co-infected patients of India in this ART era. This requires the evaluation on the incidence of drug resistant mutations, follow-up studies to elucidate its clinical and treatment outcome on combination therapy. Taken together, research on HIV-HBV co-infection in India could lead to better understanding of this global health problem which would explore the scenario to the rest of the world. Finally this will help to develop the strategy for proper management of HIV-HBV co-infection in Indian population.

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REVIEW

Next-generation sequencing in clinical virology: Discovery of new viruses

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Abstract

Viruses are a cause of significant health problem worldwide, especially in the developing nations. Due to different anthropological activities, human populations are exposed to different viral pathogens, many of which emerge as outbreaks. In such situations, discovery of novel viruses is utmost important for deciding prevention and treatment strategies. Since last century, a number of different virus discovery methods, based on cell culture inoculation, sequence-independent PCR have been used for identification of a variety of viruses. However, the recent emergence and commercial availability of next-generation sequencers (NGS) has entirely changed the field of virus discovery. These massively parallel sequencing platforms can sequence a mixture of genetic materials from a very heterogeneous mix, with high sensitivity. Moreover, these platforms work in a sequence-independent manner, making them ideal tools for virus discovery. However, for their application in clinics, sample preparation or enrichment is necessary to detect low abundance virus populations. A number of techniques have also been developed for enrichment or viral nucleic acids. In this manuscript, we review the evolution of sequencing; NGS technologies available today as well as widely used virus enrichment technologies. We also discuss the challenges associated with their applications in the clinical virus discovery.

Key words: PCR; Next-generation sequencers; Virus discovery; Sequence-independent single-primer amplification; Virus discovery based on cDNA-AFLP; Rolling circle amplification; Metagenomics

Core tip: Rapid development and commercial availability of next-generation sequencers (NGS) systems have dramatically changed almost every field of biological research, especially microbiology and metagenomics. Different NGS systems have been adapted and used for numerous applications in virology too. These systems are capable of rapidly sequencing and analyzing a complex mixture of nucleic acid templates, in a massively parallel

fashion, making them ideal tools for viral metagenomics and discovery. This manuscript reviews the prevailing NGS technologies, their application in virus discovery to serve as a guide for the readers, working in the field of virology, public health and in biothreat mitigation programs.

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INTRODUCTION

Viral infections are a cause of significant health burden globally, particularly in the less developed countries. During the 20th century, methods for virus detection, characterization and taxonomical classification were established, that helped in the discovery of a number of important viruses, in prevention of viral infections and treatment. By the late-1950s, it was generally believed that most of the human pathogenic viruses had been discovered, but the emergence of a number of previously unknown viruses [Hepatitis viruses, Hantavirus, human immunodeficiency virus, Marburg virus, severe acute respiratory syndrome (SARS), Coronavirus Ebola virus] during the later part of the century strongly challenged this belief[1].

It has now become obvious that due to different anthropological activities, such as extensive globalization of travel and business, rapid unplanned urbanization, deforestation, *etc.*, epidemiology of viral diseases have changed significantly[1]. This change has led to the increased exposure of different human populations to newer pathogens, including viruses, mostly zoonotic in nature[2,3]. The emergence of Ebola virus, Nipah virus, Sin Nombre Hantavirus, SARS, Influenza viruses (H1N1, H7N9), and MERS viruses in the recent past[4], clearly signify the onset of many others in the near future. According to a recent statistical estimate, there are at least 320,000 mammalian viruses that are waiting to be discovered[5]. The World Health Organization (WHO) has correctly cautioned that, "It would be extremely naïve and complacent to assume that there will be no other disease like AIDS, Ebola, or SARS, sooner or later"[6].

Apart from natural outbreaks, the risk of pathogens, especially deadly viruses, to be used as biological weapons and agents of bioterrorism have also increased in the recent years[7]. Being exceptionally diverse, in term of etiology, morphology, nucleic acid type and sequence information, clinical manifestations, *etc.*, rapid detection and identification of viruses pose great challenge to clinical investigators. Nevertheless, during natural or deliberate outbreaks, identification and characterization of viruses in clinical samples is extremely essential to facilitate prevention and quarantine strategies, implement specific diagnostic tools, and also to determine explicit treatment strategy.

This article will review the gradual evolution and recent advances in the field of virus discovery, with special reference to the next-generation sequencing (NGS) technologies and related molecular biology methodologies.

EVOLUTION OF VIRUS DISCOVERY TECHNIQUES

Classical approaches to virus discovery

Classically, virus discovery from clinical samples was based on filtration (to remove host cells and other microbes), inoculation of the cell free filtrate in suitable cell cultures followed by purification of the viruses from cultures and their characterization[8-10]. Morphological changes in the cultured cells, collectively known as cytopathic effect, such as formation of syncytia, cell rounding, lysis, detachment, or inclusion bodies, *etc.*, indicate the presence and successful infection of the virus(es) in the cells[11]. Virus isolate(s) are purified from the cultured cells or culture supernatant using density gradient and other high speed centrifugal techniques. This is followed by structural characterization of viral particles, antigens, nucleic acids, through different biophysical and biochemical methods[4]. Although classical methods are sometimes considered as time-consuming, tedious and need significant experimental basis, but the cell inoculation method still remains an exceptional source of enriched viral particles required for serological, molecular characterization and other purposes. Nonetheless, in many cases, viruses are not readily infective to cell cultures, which severely hamper their characterization. Additionally, repeated passaging of the virus to obtain high titer could change the population of virus being sought[12].

Nucleic acid sequence-dependent amplification approaches to virus discovery

Subsequently, with the development of nucleic acid sequence-dependent techniques, such as PCR-sequencing and microarrays, the requirement of cell culture based traditional methods became obsolete to a large extent[13-16]. These techniques were comparatively much faster as compared to classical techniques and led to the discovery of several new genotypes of known viruses. Among PCR and microarray based methods, the former gained enormous popularity due to its ability to rapidly amplify very small amounts of viral sequences from clinical samples. Even though, the prior requirement of sequence information (to design primers and hybridization probe), made this technique suitable for discovery of new genotypes of known viruses, but not appropriate for absolutely novel viruses. This limitation was later addressed by the development of consensus or degenerate PCR[17,18]. Although, this PCR method was tolerant to considerable sequence variation, but it lacked its original sensitivity and was still critically dependent on prior sequence information of the virus genera/family being investigated.

Moreover, this method could only amplify small fractions of viral genome, which were sometimes not enough for further analysis.

Nucleic acid sequence-independent amplification approaches to virus discovery

The limitations of sequence dependent techniques prompted the investigators to resort to "metagenomics", a technique that does not presume any knowledge about the organisms being investigated[19]. Metagenomics is the study of total genetic material present in a given sample, without culturing the organisms present in it. Conventional metagenomics analyses involved direct amplification of the nucleic acids through PCR, cloning and sequencing, *etc.*[15,20]. At the outset, this technique was intensely used for assessing the bacterial diversity within highly diverse samples ranging from soil, oceans, and lakes to human gut and stool, which demonstrated the power of this technique to discover genetic materials of unknown origin[21]. Subsequently, early virus discovery investigators developed a number of random amplification techniques for viral metagenomics, such as sequence-independent single-primer amplification (SISPA), virus discovery based on cDNA-AFLP (VIDISCA), rolling circle amplification (RCA), *etc.*, to amplify viral genetic materials for cloning and sequencing[15,20,22]. Extensive use of these viral metagenomic techniques, led to the discovery of different viruses, including human T-cell lymphotropic virus type-1, Torque Teno virus, different Parvoviruses, Coronaviruses, Polyomaviruses, Hepatitis C virus, Sin Nombre virus, Human Herpesviruses 6 and 8, and West Nile virus *etc.* in clinical samples[23-26].

NGS-based metagenomic approaches to virus discovery

In all the above nucleic acid sequence based virus discovery approaches, the Sanger sequencing method played a very significant role. However, with the commercial availability of high throughput sequencing technologies in 2005, a gradual shift in generation of sequencing technologies became evident. These massively parallel sequencing technologies evolved rapidly and entirely transformed almost every field of biological research including clinical research laboratories[27]. NGS is presently the most attractive approach towards metagenomics, including viral metagenomics, due to its independence from the requirement of prior sequence information. Furthermore, being highly sensitive, NGS can rapidly recuperate nearly full genome sequences of viruses, with relatively less amount of starting material as compared to conventional cloning based approaches[28,29]. Moreover, the large dynamic detection range of the NGS has established it as the most powerful technology available till date, which has catalyzed the rate of virus discovery[30-33]. In combination with conventional methods such as SISPA, VIDISCA, RCA, *etc.*, NGS can dramatically augment turnaround time and sensitivity of virus discovery[23]. Additionally, NGS has enormous, exciting applications in virology, including analysis of viral evolution and quasispecies analysis, antiviral resistance, vaccine, *etc.*[23,33-35].

A comparison of different virus discovery approaches, their advantages and limitations, applicability in different scenarios, *etc.*, is presented in Table 1.

EVOLUTION OF SEQUENCING TECHNOLOGIES

First-generation sequencers

Originally, two different DNA sequencing methods were described almost simultaneously, the Sanger's method, and the Maxam-Gilbert's method[36,37], both considered as the first-generation of sequencing methods. Sanger's method was based on DNA sequencing with chain-terminating inhibitors, while Maxam-Gilbert's method was based on base-specific chemical modification and cleavage of the DNA backbone[38]. Due to its ease and possibility of automation, Sanger's method became instantly popular and was successfully commercialized into DNA sequencing machines. As a result, for almost last 3 decades, the Sanger's method dominated as the gold standard for DNA sequencing[39]. This sequencing method was primarily accomplished by amplification of templates with fluorescently labeled chain-terminating nucleotides, followed by capillary electrophoresis of the amplicons and reading the fluorescence signals, which can provide consistent sequence information of templates up to 1000 bp. Despite its wide use for sequencing pure templates, this sequence method was constrained by its low throughput, higher cost, time and labor involved in sequencing larger genomes. Furthermore, complete dependence on specific primers, inability to sequence the genetic material from a mix of diverse organisms severely restricted its use for direct metagenomic applications.

Second-generation sequencers

To overcome the technological constraints of the Sanger sequencers, second-generation or the NGS technologies were developed, based on a large number of innovations in the amplification technology, sequencing chemistry, microfluidics, imaging technologies, and Bioinformatics, *etc.*[40]. These novel sequencing technologies, initially commercialized by two companies, namely Roche and Illumina, and later by Life Technologies have spectacularly high throughput and high sensitivity, making them more appropriate for direct application in metagenomic studies. As compared to Sanger sequencers, currently available 2nd-generation NGS platforms are capable of generating only short sequence reads, but the true magnificence of NGS lies in their capability to sequence and analyze complex mixes of DNA in a massively parallel manner, generating millions to billions of sequence reads in a single run. Consequently, these technologies are often referred to as "short read" technologies and are distinguished by "third generation" sequencing technologies (or "long read") that provide significantly longer reads (kilobases). However, at present, these long read technologies have, on the whole, lower throughput and accuracy[41-43].

Table 1 A comparative evaluation of the different virus discovery approaches showing advantages and disadvantages associated with them

	Classical approaches (Cell culture and infection based)	Nucleic acid sequence-dependent amplification approaches	Nucleic acid sequence-independent amplification approaches	Next-generation sequencers-based metagenomic approaches
Requirement of cell culture systems	Yes, required for virus particle enrichment	Not required	Not required	Not required
Information about the cytopathic effects of the virus	Yes, could be achieved through cell changes	No information could be achieved	No information could be achieved	No information could be achieved
Requirement of special equipments for purification	Yes, Ultracentrifuge/high speed centrifuges, density gradient is required for preparing pure virus	Not necessary, semi pure preparations obtained through low speed centrifuges are suitable	Not necessary, semi pure preparations obtained through low speed centrifuges are suitable	Not necessary, semi pure preparations obtained through low speed centrifuges are suitable
Information about detailed morphological/structural features of the virus	Yes, could be achieved through Electron/Atomic Force microscopy	No information on virus morphology/structure could be achieved directly	No information on virus morphology/structure could be achieved directly	No information on virus morphology/structure could be achieved directly
Time required for virus identification	Long time is required for identification, ranging from days to weeks	Comparatively faster, days required if cloning and sequencing is involved. Faster with microarray based approaches	Comparatively faster, virus could be identified within few days	Fastest available approach, identification could be done within days and even some times within hours
Requirement of prior knowledge about the virus	Not required	Some information is required regarding genus/family to design primers/probes	Being sequence independent technique, no information is required	Being sequence independent technique, no information is required
Dynamic detection range	Very narrow	Narrow	Wide	Extremely wide
Tolerance to non-viral materials	Vulnerable to other pathogens capable of infecting cell	Being sequence dependent, less vulnerable to other sequences from host and other pathogens	Being sequence in-dependent, more vulnerable to other sequences from host and other pathogens. Virus enrichment techniques required before analysis	Being sequence in-dependent, more vulnerable to other sequences from host and other pathogens. Virus enrichment techniques required before analysis
Suitability for discovery of new viruses	Yes	Less suitable, good at discovery of genotypes/ variants of known viruses	Yes	Yes
Suitability during outbreaks	Not suitable due to requirement of long time	Not suitable due to requirement of prior sequence information	Yes, but still considerable time is required during outbreaks	Being fast, very much suitable in detecting pathogens in an outbreak scenario

Even though, widely distinct in their sequencing chemistry and detection technology, NGS platforms are common in terms of massively parallel sequencing of clonally amplified or single DNA molecules. On these platforms, sequencing is executed by repetitive cycles of polymerase-mediated nucleotide extension (Roche-454, Illumina GA) or oligonucleotide ligation (SOLiD). Using a "wash-and-scan" technique, sequence data is acquired as large sets of fluorescence or luminescence images of the flow-cell surface, subsequent to each repetitive sequencing cycle step[44]. This data is later compiled by using a computer-intensive pipeline for image integration, quality assessment, storage, processing and analysis. A typical NGS run generates several hundred megabases (Mb) to gigabases (Gb) of nucleotide sequence data, depending on the platform.

Although NGS platforms commercially available today, provide massive parallel sequencing, but due to their technological features and data output capabilities, every platform is suitable for certain specific applications. Hence, as per explicit requirements, NGS platform needs to be carefully selected. In cases of virus discovery, which is the scope of this review, NGS platforms capable of generating longer sequence reads are preferable over the others. Long reads are extremely useful for *de novo* read assembly and generation of longer contigs, which endow with improved statistical power of finding related sequences in nucleotide database searches[45]. Conversely, for characterization and analysis of virus variants and quasispecies, platforms providing high quality reads, *i.e.*, less error and increased depth became the choice, over longer read lengths. In this review, we will discuss briefly the most popular NGS technologies (Illumina and Roche 454), widely used in virology. The details of the technologies, sequencing chemistries and other applications have been reviewed elsewhere in details[10,31,34].

The most widely used NGS is the Illumina sequencing technology, where clonal amplification of the template is attained to form DNA clusters, using primers attached to solid surface and sequencing is achieved *via* reversible dye-terminator technology. Although Illumina sequencing has higher sequence yield at a relatively low cost per base, this platform has a characteristic systematic base calling bias, exhibit differences in sequence quality, a higher sequencing error rate and increased single-base errors associated with GGC motifs[46-49].

On the other hand, 454 sequencing platforms are based on parallel pyrosequencing, utilizing sequencing-by-synthesis chemistry and chemiluminescence is detected to achieve nucleotide sequence. This method amplifies DNA through an emulsion PCR, generating

clones of DNA using a single template. The main benefit of this technology is its ability to produce long reads, while restricted by its high error rate in homopolymers containing regions, and a high rate of artificial amplification[50-52]. The error rates of NGS are higher relative to the Sanger sequencers, and also require advanced computational tools and statistical calculations before further data processing and assembly[53]. Due to the NGS platform specific errors, presently, use of barcoding strategies, simultaneous sequencing of the samples by two different NGS platforms or high coverage sequencing have been recommended to counteract the effects of errors[54-56]. Nevertheless, these issues are being continually addressed and resolved in the newer versions of these platforms to make them more robust, both in terms of quality and quantity.

With the advancement in instrumentations, NGS platforms are now available as benchtop sequencing instruments in the form of the 454 GS Junior (Roche) and MiSeq (Illumina) which, despite having a small footprint, offer exciting NGS capabilities for clinical settings, at modest running costs[45]. MiSeq includes the Nextera, TruSeq, and reversible terminator-based sequencing by synthesis chemistry and has highest data integrity with broader range of application, including amplicon sequencing, clone checking, small genome sequencing etc. The MiSeq provides maximum throughput per run with lowest error rates, while the 454 GS Junior generates longer reads (approximately 600 bases) with better assemblies, but is limited by lower throughput and homopolymer-associated errors.

Apart from the two most widely used NGS technologies, another technology known as the SOLiD technology (by Life Technologies) is commercially available, but its representation in the scientific literature is limited compared to Roche 454 and Illumina, which might be attributable to its recent availability or complexity of data processing and assembly[57]. Nevertheless, SOLiD is slowly but gradually being accepted as a very reliable platform and has recently been used for *de novo* sequencing of a large mammalian genome[58].

Technical details of the NGS technologies have been extensively reviewed earlier[23,45,59]. A comparison of the currently available NSG systems is also available at the Genohub website (https://genohub.com/ngs-instrument-guide/).

Third-generation sequencers

The third generation of the sequencers has evolved lately, that include the Ion Torrent (Life Technologies), Single-Molecule Real-Time technology SMRT (Pacific Biosciences), and the Nanopore sequencing technology (Oxford Nanopore Technologies). Third-generation sequencers are distinct from their predecessors in two primary features: (1) template amplification is not needed prior to sequencing, which cuts down template preparation time; and cost (2) the signal is registered in real time, directly, during the enzymatic reaction. Apart from the Ion Torrent, rest of the third-generation sequencing technologies is quite recent, and still in the evaluation stages. Moreover, data on their application in the field of virus discovery is extremely scanty. Hence, all these will be discussed only briefly in this review.

The Ion Torrent Personal Genome Machine is based on a semiconductor based sequencing technology and does not require a fluorescence or chemiluminescence based image scanning, resulting in high speed, low cost sequencing system within small size equipment. Cyclically, the semiconductor microfluidic chip is flooded with each nucleotide, and a voltage is generated if it is incorporated, and no voltage is generated when not incorporated. This is based on the fact that every time a nucleotide is incorporated into the DNA molecules, a proton is released, causing a change in voltage, which is subsequently detected and registered by the chip[45,60].

Using the SMRT, single large DNA molecules can be sequenced with high processivity of up to 7 kb, with average read lengths of 3-4 kb[23,61]. On a SMRT cell, numerous Zero-Mode Waveguides are embedded with single set of enzymes and DNA template. During the reaction, enzyme incorporates a nucleotide into the complementary strand, cleaving off fluorescent dye linked with the nucleotide, and this fluorescent signal is captured[61].

Nanopore sequencing is another recently developed method of the third-generation sequencing[62,63]. Nanopore is a tiny biopore with diameter in nanoscale, and involves a heptameric transmembrane channel α-haemolysin (αHL) from *Staphylococcus aureus.* This protein has the ability to tolerate extraordinary voltage and current conditions (up to 100 mV, 100 pA). Under a standard condition of ionic flow, when a DNA molecule is passed through the channel of, *etc*., HL, current is modulated according to the size difference between every deoxyribonucleoside monophosphate (dNMP). This current modulation is detected by standard electrophysiological techniques and the dNMP is identified[62]. Nanopore sequencers are extremely small (size of a USB drive), can sequence long read faster (> 5 kb at a rate of 1 bp/ns), free of fluorescence/chemiluminescence and other enzymes, less sensitive to temperature and other conditions. These benefits make it fit as an extremely rapid sequencing device for field conditions, but the requirement of highly purified DNA needs to be addressed for their wide application in virus discovery.

Among the different NGS platforms available today, choosing the right one for correct application is extremely essential before embarking on a metagenomic project. In case of absence of a reference genome, or where highly divergent sequences are expected, such as in case of virus discovery, *de novo* sequencing and assembly is necessary. Such an assembly requires extensive computational power and datasets containing longer reads with higher coverage are preferable[64-66]. When reference genomes for assembly are available, technologies that generate short reads could also be used to have a high coverage of the metagenomes[53].

When compared in terms of publications, Illumina technology is the most widely used platform, irrespective of application. Earlier the use of this platform was not suited for virus discovery or *de novo* sequencing projects due to its short reads. However, regular augmentation in read length for Illumina platforms has made it suitable for *de novo* assembly of genomes, at a sensitivity, comparable to specific PCR[53,67,68]. However, according to the number of publications, specifically for metagenomic studies, pyrosequencing technology (Roche 454) is preferred over the other NGS approaches producing shorter reads. Of late, Roche has announced the discontinuation of its 454 technology by the mid-2016, which leaves the new investigators with alternative NGS platforms available today.

SAMPLE PREPARATION FOR VIRAL METAGENOMICS AND DISCOVERY

NGS has emerged as the most promising tool for the detection and discovery of novel infectious agents in clinical specimens[23]. However, being unbiased method of sequencing, NGS is greatly affected by very low virus-to-host genome ratios in clinical samples[69-71]. Hence, enrichment of pathogen genetic material or depletion of host genetic materials is essential to maximize sensitivity for discovery of novel pathogens, including viruses in clinical samples[23,72,73]. A schematic representation of the different steps involved in NGS based virus metagenomics and discovery is depicted in Figure 1.

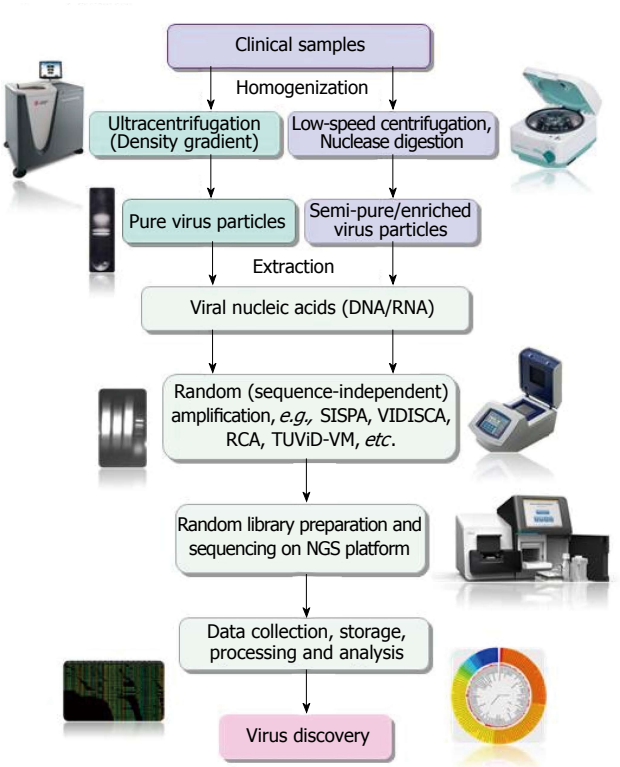

Figure 1 Diagrammatic representation of main steps of clinical virus discovery by next-generation sequencer based technologies.

Physical enrichment of virus particles

A number of virus enrichment protocols involving physical and enzymatic techniques have been successfully applied for clinical samples. These include virus capsid purification through freeze/thaw cycles of cell disruption, filtration through appropriate pore membranes (0.45 μm and 0.22 μm), centrifugation, prior nuclease digestion of host genome, *etc.*, followed by extraction of capsid-protected viral nucleic acids, their conversion to cDNA (in case of RNA virus) and non-specific PCR amplification[15]. The efficiency of enrichment in NGS-mediated virus discovery, especially the prior nuclease digestion has been clearly demonstrated by different studies[12,72,74]. Recently Hall *et al*[74] reviewed literatures available on methods for enrichment of viral nucleic acids from clinical samples for NGS-based studies. They found that both ultracentrifugation-mediated enrichment and low-speed centrifugation together with filtration and a nuclease digestion step is widely used for enrichment of viral nucleic acids.

Alternatively, approaches to deplete host genetic materials include use of methylation-specific DNase activity, host ribosomal RNA removal, duplex-specific nuclease normalization methods[75-77]. Such techniques on one hand increase the detection sensitivity of the NGS platform, circumventing the cost and time involved in generating and analyzing huge amounts of background data on the other hand. Ideally, in a clinical setting virus enrichment methods are required to be rapid, standardized and undemanding in terms of cost, manpower or instrumentation facility.

Enrichment of viral nucleic acids through non-specific amplification techniques

A number of virus enrichment methods have been applied successfully for NGS studies of different clinical samples. Of them, the sequence-independent single primer amplification (SISPA), developed by Reyes and Kim[78], was modified for successful amplification of viral sequences from serum by Allander *et al*[79] and later by others for identification of novel viruses through Sanger sequencing[80-83]. Recently, SISPA was used in combination with NGS and shown to be successful in detection of Hepatitis B and C viruses (HBV, HCV) in solid tissue samples[72]. In a recent study, SISPA-NGS strategy was found to be helpful in detection of Schmallenberg virus (SBV) in veterinary samples[84], suggesting the utility of this technique in screening of field animals that are intermediate hosts to many human viruses. In some of the recent studies no specific physical enrichment of virus particles was applied, but NGS was done on SISPA generated random PCR products, that also resulted in rapid detection of hemorrhagic fever-associated Yellow Fever Virus (YFV), Lujo virus (LUJV), and a new Arenavirus (related to lymphocytic choriomeningitis virus, LCMV) in diverse clinical samples[85-87].

Likewise, another well-established sequence-independent amplification technique is the virus discovery

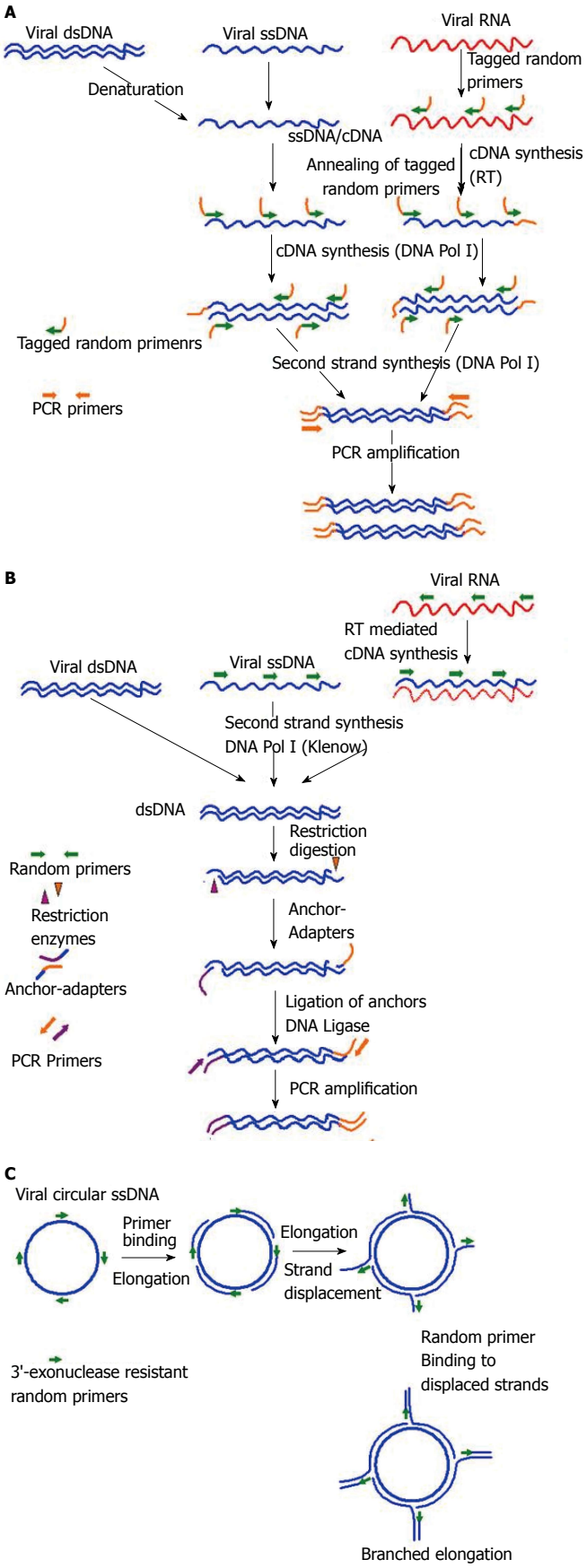

Figure 2 Different virus nucleic acid enrichment techniques. A: Sequence-independent single-primer amplification. Initially viral RNA and ssDNA is transcribed into complementary DNA (cDNA) using reverse transcriptase (RT) and DNA Pol I respectively, with the help of tagged-primers having defined sequence at the 5' end while random nucleotides at the 3' end. Subsequently, second strand synthesis is performed using DNA Pol I (Klenow) to make the cDNA double stranded (dsDNA). Now all the nuceic acids present in the reaction are dsDNA fragments have tagged sequence at their ends. Finally, anchored dsDNA is amplified with primers annealing to the adapter specific sequences, PCR product are checked and ready for analysis through cloning-sequencing or direct sequencing through next-generation sequencers (NGS); B: Virus discovery based on cDNA-AFLP. Initially viral RNA is reverse transcribed into complementary DNA (cDNA) using RT and random primers. Subsequently, second strand synthesis is performed using DNA Pol I (Klenow) to make the cDNA double stranded (dsDNA). In this step, other viral single stranded DNA (ssDNA) viral is also converted to dsDNA. Now all the nuceic acids present in the reaction are dsDNA. In the next step dsDNA are digested with a set of frequent cutter restriction endonucleases, which produce asymmetric cuts. Now specially designed matching anchor-adapters are ligated ends of the restriction fragments using DNA Ligase. Finally, anchored dsDNA is amplified with primers annealing to the adapter specific sequences, PCR product are checked and ready for analysis through cloning-sequencing or direct sequencing through NGS; C: Rolling circle amplification. Amplification of multiply primed single stranded circular viral genomes. 3'-exonuclease resistant primers randomly bind the genome and are elongated by the Phi29 polymerase. The growing strand subsequently displaces the preceding strand of the DNA, making the strand available for binding of random primers and further elongation. This cyclic displacement and elongation leads to a highly branched structure of growing DNA, which is linear in topology. Rolling circle amplification has the capability to specifically enrich the circular ssDNA genomes in an environment of other genetic materials, and could then be characterized by NGS.

cDNA-amplified fragment length polymorphism (VIDISCA), used for discovery of a novel human SARS-associated coronavirus, HCoV-NL63[88,89]. Later this technique was successfully used in combination with Sanger sequencing to discover other novel viruses in clinical samples[90,91]. To late, the utility of this technique in combination with NGS for virus discovery has been demonstrated in veterinary samples, as well as in clinical samples[92,93]. Additionally, Shaukat *et al*[92] modified the VIDISCA method at the reverse transcription step by using specially designed mix of random hexamers that do not anneal to ribosomal RNA, further increasing the specificity of the assay. Apart from SISPA and VIDISCA, multiply-primed RCA has also been demonstrated to enrich circular viral genomes, suitable for sequencing through NSG platforms[94-96]. A diagrammatic representation of SISPA, VIDISCA and RCA is depicted in Figure 2 respectively. Recently, Kohl *et al*[97] reported an ultra-centrifugation and DNA digestion based enrichment protocol followed by SISPA for detection of known and new viruses in human tissue samples. This technique, termed as tissue-based universal virus detection for viral metagenomics was demonstrated to complete within 28 h, making it suitable for discovery of zoonotic and biothreat agents of viral origin during outbreaks[97].

Alternatively, in another study, the authors used a barcoding strategy to carry out unbiased deep sequencing in multiple clinical samples and removed human and other low-quality sequences through bioinformatic filtering pipeline and identified viruses belonging to the Herpesviridae, Flaviviridae, Circoviridae, Anelloviridae, Asfarviridae, and Parvoviridae families in serum samples from tropical febrile illness[2].

Apart from virus discovery and detection in clinical samples, analysis of quasispecies, drug-resistant viral

Table 2 Important bioinformatics challenges associated with application of next-generation sequencers in viral diagnostics action taken or proposed to overcome challenges

Bioinformatics challenges associated with application of NGS in viral diagnostics	Action taken or proposed to overcome challenges
Generation of huge volumes of data by NGS platforms-"data deluge"	Advancement in storage and computation facilities, availability of computer with greater storage and highly powerful processors, cluster/grid computing and cloud computing. Computation facilities needs to be updated with emergence of newer platforms delivering larger datasets
Challenges in uploading data for submission to databases and supercomputing servers for analysis	Requirement of uninterrupted and extremely fast networks
Challenges in storage, public archival and ease of access	Creation of specialized data archive such as the Sequence Read Archive by NIH and ENA (European nucleotide Archive) by EBI. Sharing of data within the three major databases (NIH, EBI and DDBJ) for public accessibility
Challenges in analysis and visualization of large volumes of data, beyond the scope of computation facilities available in molecular biology laboratories	Creation of metagenomic or NGS data analysis pipelines and integrated tool kits, such as those available at NIH-NCBI, EMBL-EBI, MGRAST, CASAVA, MetaVir, Megan, UCSC Genome Browser, BioLinux, *etc*., availability of cloud computing based servers such as Galaxy
Challenges in alignment, *de novo* assembly, gene prediction and phylogenetic analyses NGS datasets, especially short read datasets	Availability of alignment algorithms/programs such as ABySS, ELAND, SOAP, Bowtie, Cloudburst, Zoom, BWA, SHRiMP, MOM, SeqMap, Metagene, Velvet, QSRA, ALLPATHS, EDENA, VCAKE, FragGeneScan, BLAST, GLIMMER, EULER-SR, Avadis, Eagle View, *etc.*
Interpretation of huge amount of data generated in metagenomic analyses by NGS platforms	Proper interpretation of analyzed data is of utmost importance to identify newer pathogens as well as their clinical significance

NGS: Next-generation sequencers.

variants and monitoring of genetic consistency of live viral vaccines there are numerous applications of NGS, which are directly associated with human viral diseases. NGS-based virus detection technique has also been shown to be useful in surveillance of vector-borne and zoonotic viruses[23]. This possibility of detecting arthropod-borne viruses was demonstrated using Dengue virus-infected mosquito pools (Aedes aegypti), where, use of NGS resulted in highly sensitive detection of mosquito pools containing infected vectors[98]. Similarly, in a surveillance study focused on the discovery of bat-transmitted pathogens, using coronavirus consensus PCR and unbiased NGS, a new coronavirus related to SARS-CoV was documented[99].

BIOINFORMATICS CHALLENGES ASSOCIATED WITH NGS

Regardless of the field of applications and platforms used, ever-increasing capacities of NGS platforms and their wide usage have resulted in extremely unprecedented volumes of data. This is commonly referred to as "data deluge", and is represented by huge NGS datasets deposited in specialized data archive such as the SRA, a primary archive of NIH, dedicated for submission and storage of raw data and alignment information, generated by all major NGS platforms. Being part of the International Nucleotide Sequence Database Collaboration at the National Center for Biotechnology Information, data submitted to either of the databases SRA, ENA (European nucleotide Archive of European Bioinformatics Institute, EBI) and the DDBJ (DNA Database of Japan) are shared amongst them. SRA serves as an initial point for downstream analysis of NGS data and also provide access to data from human clinical samples to authorized users. According to a recent comparison of GenBank statistics (Release 197, 8/2013 *vs* Release 203, 8/2014), total nucleotide entries to the GenBank represent an annual growth of more than 43%, and annual growth exclusively for virus sequence entries is 21%[100]. This data deluge has posed significant hardware, software and bioinformatics challenges towards storing, transfer, analysis and interpretation of the data[101].

All NGS platforms are advancing towards the capability to sequence longer DNA fragments, and to generate even larger volume of data sets[53]. To analyze such gigantic volumes of data, exceptionally massive computational facilities are also required, which has entirely revolutionized the field of Bioinformatics[60,102]. Once NGS sequence has been generated, the biggest of the challenges comes, *i.e.*, computational requirements for storage and analysis of the massive data sets. Although a detailed description of bioinformatic processes involved in metagenomics data analysis is beyond the scale of this review, the key processes involved in the NGS data analysis are quality assessment, sequence assembly and annotation of the dataset against a database of nucleotide or protein sequences[34]. Quality assessment and data cleaning involves filtering out of low-quality sequences from the dataset, followed by alignment and error correction to separate true variance from the experimental noise[23]. After sequencing and quality assessment, there are two approaches for assembly of the reads. The sequence reads are then mapped to the available reference genome, or individual sequencing reads are assembled *de novo,* using different assembly servers[34,103]. The *de novo* approach is generally followed for discovery of viruses, considering the fact that reference genomes or related sequences may not be available in the databases. To determine the affinity of the assembled reads or the contigs, Basic Local Alignment Search Tool (BLAST) is used, that computes regions of similarity and statistical significance of possible

matches between a query sequence and GenBank submissions[104]. Despite the availability of the BLAST, analyzing a viral metagenome may still be a challenging task in case of highly divergent or novel viral families, which are not represented in the database.

In the Table 2, we have summarized the challenges associated with handling and analysis of NGS generated data, their solutions presently available or suggested.

CONCLUSION

During the last decade, numerous innovations in virus enrichment techniques, sequencing chemistry and signal detection technologies, availability of high end dedicated bioinformatic servers for analysis of the NGS data has greatly accelerated the discovery of viral pathogens in clinical samples. Apart from its increasing applications in virus discovery, NGS has been successfully used in monitoring of antiviral drug resistance, investigation of viral evolution, diversity and quasispecies, and evaluation of the human virome. The supreme advantage of the NGS platforms is their ability to characterize hundreds of different pathogens simultaneously that are not otherwise cultivable using conventional approaches. Nevertheless, there are a number of challenges that need to be overcome for these technologies to become routine in clinical settings. The initial cost of set-up, turnaround time, requirement of powerful computational facilities along with the requirement of a highly skilled group of people are the major barriers to their wide application in resource-limited countries, where the cases of emerging viruses are the highest.

Despite the broad utility of NGS in virus discovery, extremely high sensitivity of this technique also makes it prone to unintentional contamination. The use of random primers for enrichment and the deep sequencing may result in significant potential for carryover contamination from laboratory reagents. Simultaneous analyses of blinded controls may be one approach towards excluding such possibilities, but it will also double the cost of sequencing. Another outcome of the NGS data is the rapid rate of discovery of viruses. However, the absence of appropriate cell culture systems or animal models limit the possibility of experimental studies on these new viruses, thereby the clinical significance of these new viruses remains to be properly understood.

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MINIREVIEWS

Perinatally infected adolescents living with human immunodeficiency virus (perinatally human immunodeficiency virus)

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Abstract

The availability of highly potent antiretroviral treatment during the last decades has transformed human immunodeficiency virus (HIV) infection into a chronic disease. Children that were diagnosed during the first months or years of life and received treatment, are living longer and better and are presently reaching adolescence and adulthood. Perinatally HIV-infected adolescents (PHIV) and young adults may present specific clinical, behavior and social characteristics and demands. We have performed a literature review about different aspects that have to be considered in the care and follow-up of PHIV. The search included papers in the MEDLINE database *via* PubMed, located using the keywords "perinatally HIV-infected" AND "adolescents". Only articles published in English or Portuguese from 2003 to 2014 were selected. The types of articles included original research, systematic reviews, and quantitative or qualitative studies; case reports and case series were excluded. Results are presented in the following topics: "Puberal development and sexual maturation", "Growth in weight and height", "Bone metabolism during adolescence", "Metabolic complications", "Brain development, cognition and mental health", "Reproductive health", "Viral drug resistance" and "Transition to adult outpatient care". We hope that this review will support the work of pediatricians, clinicians and infectious diseases specialists that are receiving these subjects to continue treatment.

Key words: Adolescents; Human immunodeficiency virus-infection; Antiretroviral therapy; Puberty; Growth; Complications

Core tip: We have performed a literature review about different aspects that have to be considered in the care and follow-up of perinatally human immunodeficiency virus-infected adolescents and young adults. Articles reporting original research, systematic reviews, quantitative

or qualitative studies and published from 2003 to 2014 were selected. Results are presented in the following topics: "Puberal development and sexual maturation", "Growth in weight and height", "Bone metabolism during adolescence", "Metabolic complications", "Brain development, cognition and mental health", "Reproductive health", "Viral drug resistance" and "Transition to adult outpatient care".

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INTRODUCTION

The World Health Organization defines adolescence as the period in life from ages 10 to 19, *i.e.*, the second decade of life[1]. Puberty is the main biological component of adolescence and results in physical and mental changes due to the reactivation of the neurohormonal mechanisms of the hypothalamic-pituitary-adrenal/gonadal axis, as well as those determined by the historical context and sociocultural conditions of each individual.

The body image of adolescents is affected by the changes in their body attributes (hair, breasts) and functioning (ability to have sexual intercourse, menarche, voice change); similarity to the adult body; significance of recognizing the other; and interaction with others with bodies that can now awaken desire, which now become desirable and desiring[2]. This process also includes parallel losses that must be properly assimilated: loss of the childhood body, childhood parents, and childhood identity[3].

Normal adolescence syndrome is the name given to the set of characteristics proper to this developmental period, which include the following: search for oneself and one's identity, group tendency, the need to intellectualize and fantasize, religious crisis, temporal displacement, development of sexuality, assertive social attitude, successive contradictions, progressive detachment from parents, and continuous mood swings[4].

As a result of the aforementioned features, adolescents are liable to increased exposure to alcohol and drug use, vulnerability to traffic accidents, fights, misdemeanors, and difficulty in maintaining appropriate self-care activities, such as the use of condoms, adoption of harm reduction measures, and proper use of medications. These characteristics, together with issues related to the social vulnerability of youth, contribute to the significant number of infections by the human immunodeficiency virus (HIV) that occur during this stage of life[5].

There is no stereotype universally representative of adolescents perinatally infected by HIV (PHIV). Some HIV-infected children reach adolescence fully aware of their condition, while others do not. In some cases, they are the only family member with an HIV infection, or they belong to families with good adherence to treatment and, thus, go through childhood having benefited from the full effects of combination antiretroviral therapy (cART): viral suppression, adequate growth, and good quality of life. In other cases, treatment is irregularly performed, and the affected youths exhibit advanced forms of the disease in adolescence, eventually requiring new drugs to ensure their survival. Other youths mature in institutions, where antiretroviral therapy (ART) may or may not be properly performed. In short, the living conditions and treatment history should be thoroughly investigated in the case of PHIV adolescents, as non-adherence to treatment is associated with the emergence of viral drug resistance, with the consequent need to change the antiretroviral regimen, and it is also associated with situations and characteristics typical of adolescence[6].

We should bear in mind that, independently of their personal history, we are interacting with individuals who tend to have a defiant attitude and who exhibit some degree of emotional instability. This situation is the context within which we must investigate adolescents' awareness of their condition and treatment. In many cases, concepts such as HIV, CD4, or viral load are too abstract to be easily understood, and adolescents tend to be more concerned with their transforming bodies, their losses and gains[7].

Thus, the professionals who provide care to PHIV youths should feel an affinity with adolescents. Although pediatricians are trained to handle adolescents, the ideal situation is that of a multidisciplinary staff that is available to meet the peculiar demands posed by adolescent care in an integrated manner[8].

Adolescents' caregivers should participate in all aspects of treatment, including the moment when diagnosis is communicated, therapeutic decision-making, and adherence to treatment. Healthcare professionals should be receptive to the caregivers' insecurities, doubts, fears, and anguish, which might appear at different times during follow-up.

The staff should assume that the adolescents' families have the skills and conditions to help them cope with their problems and, thus, should help the relatives to become aware of their resources and possibilities and emphasize their positive aspects, helping them to feel increasingly more self-assured and competent. To establish a partnership with the patients' relatives is the best strategy in the terms of health or education actions or prevention; it is an efficient, positive, productive, and inclusive approach that increases the opportunities to promote changes.

STRATEGY FOR ARTICLE SEARCH

This study consisted of a literature review of articles included in the MEDLINE database *via* PubMed, located using the keywords "perinatally HIV-infected" AND "adolescents". Only articles published in English or Portuguese from 2003 to 2014 were selected. The types of articles included original research, systematic reviews,

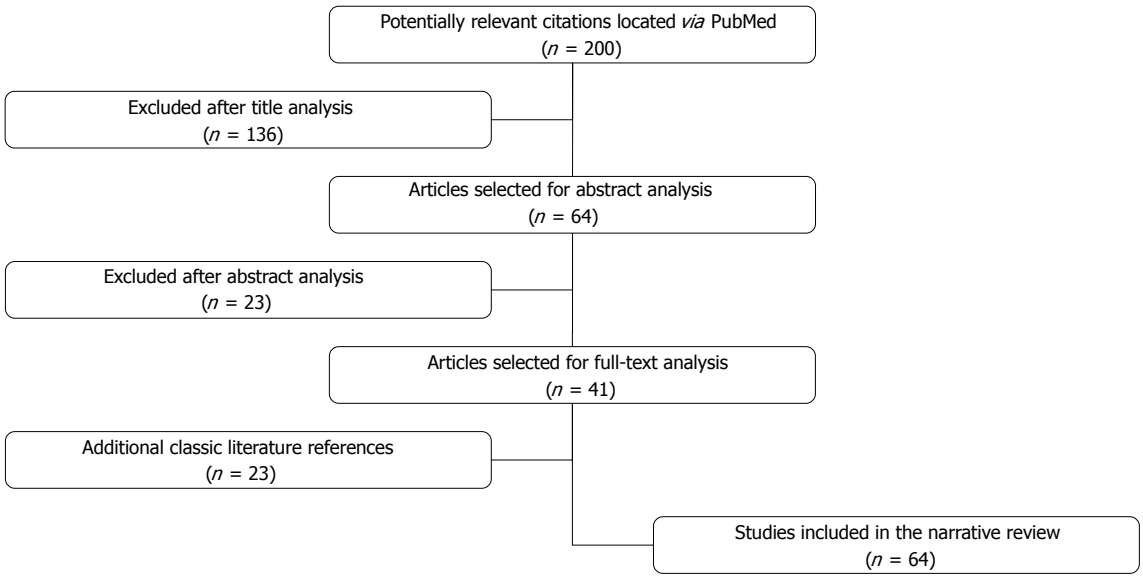

Figure 1 Flow chart representing the process of article selection.

and quantitative or qualitative studies; case reports and case series were excluded.

The application of the aforementioned criteria located 200 articles based on their titles. The abstracts of 64 of such articles were analyzed, which resulted in 41 articles selected for full-text analysis and data extraction. In addition, classic literature references considered relevant for the subject of interest were included. As a result, a total of 64 articles were included in this review. Figure 1 shows the flow chart of article selection.

PUBERTAL DEVELOPMENT AND SEXUAL MATURATION

The body changes that are characteristic of puberty include remarkable physical growth and sexual maturation. According to Marshall and Tanner, puberty is characterized by acceleration followed by cessation of growth, changes in the amount and distribution of fat, and development of the gonads and secondary sex characteristics[9,10].

The sequence of body changes that constitute sexual maturation comprises the development of the gonads, reproductive organs, and sex characteristics. Thelarche is the beginning of breast development in girls; gynecomastia is the enlargement of the breast tissue in boys; pubarche refers to the first appearance of pubic hair, menarche to the first menstrual cycle, semenarche to the first ejaculation, and sexarche to the first sexual intercourse.

Just as in other chronic diseases, HIV infection acquired in the perinatal period also affects sexual maturation. This interference might result from direct virus action, secondary infections, nutritional disorders, and the action of cytokines. The delay in sexual maturation seems to be greater in the later pubertal stages[11,12].

One observational study conducted in the United States found a significant delay of pubertal onset in a group of 2086 adolescents with vertically transmitted HIV infection compared to uninfected youths born from HIV-infected mothers[13].

GROWTH IN WEIGHT AND HEIGHT

Slow weight gain and growth deficits are common among children with vertically transmitted HIV infection. These children exhibit early and progressive reductions of linear growth and body mass index, in addition to sustained deficit in anthropometric indexes compared to non-infected individuals[14,15]. These disorders, starting in childhood, might continue into adolescence. Weight loss is common among HIV-infected individuals, independent of the use of highly active antiretroviral therapy (HAART), and it seems to have a multifactorial etiology.

Weight loss occurs early in the course of infection, preceding the manifestation of significant compromise of the immune system[16]. Growth failure is a well-known indicator of disease progression among HIV-infected children and adolescents and usually precedes the decrease of CD4+ cells. Improvement of growth parameters might be used as a measure of HAART efficacy; control of viral replication exerts a positive effect on the weight and height[17].

Deficits in growth precede and may contribute to the onset of immunodeficiency and opportunistic infections in HIV-infected individuals[18].

The differences in growth patterns are probably due to differences in the manifestations of the disease in HIV-infected children and adolescents; growth delay is greater in patients with viral loads above 100000 copies/mL[19].

The final height of individuals with vertically transmitted HIV infection is usually shorter than the target height. This fact suggests that the height loss accumulated

throughout childhood and adolescence might influence the final height in that population[20].

BONE METABOLISM DURING ADOLESCENCE

Puberty is a significant period vis-à-vis the acquisition of adequate bone mass. Some of the factors that influence normal bone mineralization are as follows: calcium intake, vitamin D levels, physical activity, hormones, genetic factors, and nutritional status[21]. The adolescent growth spurt is characterized by large bone mass accumulation; the incidence of fractures due to relative bone fragility is high as a result of the dissociation between bone expansion and mineralization[22]. The peak of bone mineralization corresponds to the accumulation of calcium in this tissue. The bone mineral density (BMD) decreases before the adolescent growth spurt, and it increases over the subsequent four years. The peak calcium accretion rate was found to occur at a median age of 12.5 years old in girls and 14 years old in boys[23].

In the current scenario of HIV-infection in growing and developing children, characterized by increased survival and prolonged ART use, the long-term impact on the children's bone metabolism is not well known[24]. The BMD is lower in HIV-infected children and adolescents compared to the non-infected population[25]. Children using HAART exhibit complications resulting from low BMD[26,27]. These complications are potentially more severe in adolescents than adults due to the adolescent growth spurt and puberty[27].

The etiology of low BMD is multifactorial and might be directly related to the virus, ART, comorbidities, or factors unrelated to HIV infection. Periodic BMD testing is indicated during adolescence, and youths found to have low BMD should be instructed to perform high-impact exercises and use calcium and vitamin D supplements[28].

METABOLIC COMPLICATIONS

The long-term benefits of HAART are widely known. Increasing numbers of children with vertically transmitted HIV infection are reaching adulthood and, thus, becoming chronically ill adults[27]. In addition to its impact on the survival of this population, prolonged HAART also seems to have cardioprotective effects in HIV-infected children and adolescents[29]. However, as part of this scenario of improved survival, many youths develop severe metabolic complications, including lipodystrophy, dyslipidemia, insulin resistance, lactic acidosis, and bone mass loss. Dyslipidemia, which is mainly associated with the use of protease inhibitors, may increase the risk of cardiovascular disease in adulthood[27].

Chokephaibulkit *et al*[30] found that the levels of parathyroid hormone were significantly higher among adolescents with vitamin D deficiency. Insulin resistance has also been reported in children and adolescents with vertically transmitted HIV infection in association with higher body mass index values[31,32].

HIV-associated lipodystrophy is a particular cause of concern in adolescence, as the disordered distribution of the body fat - loss of fat in the face and lower limbs and enlarged dorsocervical fat pad and chest fat - might have significant repercussions in this stage of life, when the individual develops the adult body that serves to present oneself to the world. Multidisciplinary healthcare staff should be aware of the possibility that lipodystrophy may act as a hindrance to ART adherence[8].

In addition to the aforementioned body changes, ART is also associated with increased cholesterol and triglyceride levels[33], which make dietary and exercise advice indispensable in the clinical management of these patients[8]. Adolescents at high risk for atherosclerotic disease might benefit from early changes in their lifestyle, as well as from clinical interventions that aim to improve their long-term prognosis[34].

Routine and systematic cardiac evaluation has paramount importance in the follow-up of HIV-infected children and adolescents, as cardiovascular disease has become a part of care for long-term survivors. Accelerated atherosclerosis has also been found in young adults without traditional coronary risk factors[35].

BRAIN DEVELOPMENT, COGNITION, AND MENTAL HEALTH

Neuroimaging data collected from healthy children and adolescents show that the brain volume attains its peak by 10.5 years of age among girls and 14.5 years among boys; the grey matter decreases and the white matter increases during adolescence[36]. This developmental stage is known as "synaptic pruning". The increase in white matter reflects greater axon myelination, with increased neural transmission speed and better quality of brain connectivity.

Some evidence indicates that structural and functional changes in different brain areas are associated with greater rational and emotional planning skills (prefrontal cortex), higher memory capacity (temporal lobe), language skills (frontal lobe), higher intelligence quotient (frontal and occipital lobes), and better reading skills (temporal and parietal lobes). The central executive function processes in this developmental stage include working memory, processing speed, and cognitive flexibility.

One study assessed 16 PHIV adolescents undergoing ART using neuroimaging methods and found increased grey matter and decreased white matter relative to healthy controls[37]. Those findings agree with well-documented alterations in subcortical structures among HIV-infected adults, such as neural loss across the entire prefrontal cortex, cerebral atrophy, and white matter demyelination affecting periventricular areas, the corpus callosum, internal capsule, anterior commissure, and optical tract in particular. The cognitive domains most affected among HIV-infected adults are motor skills,

expressive language, episodic memory (encoding and retrieval), and executive function (processing speed, attention, and working memory), the latter of which seems to contribute substantially to learning, particularly during childhood[38-40]. Prospective memory, which is related to "remembering to remember", is also impaired; this impairment has a close relationship with the action of taking medicine at the right time and, thus, with adherence to treatment. Therefore, the brain and cognitive development of adolescents living with HIV may be impaired in different ways, resulting in lower intelligence and poorer academic performance, executive deficits (abstraction, problem-solving, cognitive flexibility, and cognitive deficits in social skills and planning), limited memory skills, language deficits (in cases with encephalopathy), reduced information processing speed, attention deficit, and impaired motor coordination[41,42].

The results of a literature review on the neurodevelopment of PHIV children and adolescents suggest that such youths do not perform as well as controls in evaluations of cognition, processing speed and visual-spatial tasks and are at higher risk of mental health problems[43]. One study used a neuropsychological battery to assess the cognitive domains of attention/processing speed, psychomotor ability, and problem-solving skills in 16 PHIV adolescents. The results showed that the performance of the PHIV youths was poorer compared to the control group, which consisted of age-matched HIV-uninfected volunteers[44].

In regard to mental health, the incidence of psychiatric disorders is higher among PHIV adolescents and uninfected youths belonging to HIV-infected families compared to the general population[45-47]. Several studies found that up to 70% of such adolescents meet psychiatric diagnostic criteria. Some authors found correlations between diagnosis in adolescents and psychiatric disorders among their caretakers[45-47].

REPRODUCTIVE HEALTH

PHIV adolescents start their sexual life at approximately the same age as the HIV-uninfected population[48,49]. Studies on pregnancy showed that its progression and outcomes are similar among PHIV women and women with sexually transmitted HIV, except for the proportion of women with undetectable viral load close to labor and delivery, which is lower among PHIV women[50-52]. The difficulty of attaining viral suppression in that population of pregnant women is probably due to their long previous exposure to various ART regimens, with the consequent emergence of resistance-related mutations in HIV; that fact also accounts for the high rate of cesarean deliveries in that group.

VIRAL DRUG RESISTANCE

Currently, few ART-naive PHIV adolescents are admitted for treatment. The ongoing strategy of diagnosing women living with HIV during pregnancy allows the early diagnosis of perinatal infection among the exposed infants, while global guidelines emphasize the relevance of starting treatment within the first months of life[53,54]. However, difficulties in adherence to treatment throughout childhood account for the emergence of resistance-associated mutations in the virus, as well as successive drug changes.

When the hindrances to adequate adherence to treatment during childhood are not removed, PHIV adolescents might not achieve appropriate viral suppression and often exhibit multidrug resistance-associated mutations[55]. Several studies have shown that the proportion of PHIV adolescents with viral suppression is approximately 50%[56-59].

The possible transmission of virus strains with resistance-associated mutations by this population to their sexual partners is a significant cause of concern. A longitudinal study that followed up 330 PHIV adolescents who responded to audio computer-assisted self-interviews (ACASI) in the United States found that 62% of the sexually active youths reported engaging in unprotected sexual intercourse. The viral load was over 5000 copies/mL in 42% of the sexually active PHIV adolescents, and in almost all, the virus exhibited resistance-associated mutations[49].

TRANSITION TO ADULT OUTPATIENT CARE

The advances made in AIDS treatment have significantly improved the survival of children and adolescents with vertically transmitted HIV infections[60]. The demand for transfer to adult outpatient care increased concordantly with the extent to which such youths now reach adulthood. More than 25000 HIV-infected individuals aged 13-24 years old are currently undergoing the transition to adult outpatient care in the United States[61].

For this transition to be successful, the focus must fall on comprehensive care, including the patients' mental and reproductive health, gender identity, sexuality, stigmas, social issues, cognitive development, adherence to ART, detachment from pediatric outpatient care, and communication with the staff in charge of patient care[61-63]. Integral care poses a major challenge; however, it is crucial for the management of this population of patients to reduce the impact of the transition and improve their long-term follow-up. Integral care is necessary to ensure that the therapeutic success achieved in childhood will continue during adulthood[60,62,64].

CONCLUSION

The aim of this review is to provide information to the pediatricians and infectious disease specialists in charge of continuing the treatment given to PHIV adolescents since childhood. The clinical and laboratory monitoring of these youths should be able to detect problems such as delayed growth and physical and neuropsychological development, metabolic and bone disorders, and issues related to their reproductive health. Possible therapeutic

failures should be addressed, considering the individual and family history relative to ART. The follow up of adults perinatally infected with HIV will pose new challenges vis-à-vis the benefits and complications of ART.

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MINIREVIEWS

Purinergic signaling and human immunodeficiency virus/acquired immune deficiency syndrome: From viral entry to therapy

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Abstract

Human immunodeficiency virus (HIV) infection is a serious condition associated to severe immune dysfunction and immunodeficiency. Mechanisms involved in HIV-associated immune activation, inflammation and loss of CD4+ T cells have been extensively studied, including those concerning purinergic signaling pathways. Purinergic signaling components are involved in viral entry and replication and disease progression. Research involving the participation of purinergic signaling in HIV infection has been not only important to elucidate disease mechanisms but also to introduce new approaches to therapy. The involvement of purinergic signaling in the pathogenesis of HIV infection and its implications in the control of the HIV infection are reviewed in this paper.

Key words: Human immunodeficiency virus; Purinergic signaling; Immune activation; Inflammation

Core tip: This paper reviews the latest findings regarding the involvement of the purinergic signaling system and human immunodeficiency virus (HIV) infection. On the last 10 years, several studies have been published on the participation of purinergic signaling in HIV infection. The findings helped to elucidate disease mechanisms and proposed new targets and approaches to therapy. We have found that basic and clinical research on this field are very promising and must be further pursued.

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INTRODUCTION

Acquired immune deficiency syndrome (AIDS) is a pandemic disorder caused by the human immunodeficiency virus (HIV). HIV infection is characterized by persistent immune activation, inflammation and loss of CD4+ T cells, which altogether lead to immunodeficiency[1-3]. The pathological mechanisms involving these dysfunctions along with disease progression markers and prospective ways of halting disease progression are targets of extensive research. Although a great number of pathological mechanisms have been proposed, a lot remain unclear.

The connection between purinergic signaling and HIV infection is not consistently in favour of the host or the pathogen. Purinergic receptors may favour viral entry[4,5] whilst its enzymes may help to halt disease progression[6] and boost immune response against the virus[7]. However, the increasing knowledge of purinergic signaling components and their connection to HIV infection has been remarkably valuable in the understanding of the disease mechanisms. Furthermore, this knowledge raises the possibility of using purinergic receptors antagonists, purinergic signaling mediators and their analogs in HIV therapy[8-10].

The aim of this paper is to review purinergic signaling and its involvement, through its components (enzymes, receptors) and mediators [adenosine triphosphate (ATP) and adenosine), in HIV virus entry and replication, disease progression, and potential therapeutic strategies.

BACKGROUND ON PURINERGIC SIGNALING

Following the identification of ATP, along with purinergic co-transmission and the P1 and P2 receptors in the 70's, the purinergic signaling system has been intensely studied[11,12]. The receptors were characterized and the ATP mechanisms of release and breakdown have been described. Consequently, the involvement of the purinergic system in the pathophysiology of several human disorders has been uncovered and the possibility of using these pathways as targets for therapy has been raised[12].

ATP, adenosine diphosphate (ADP), Adenosine monophosphate (AMP) and adenosine are extracellular purines that mediate a series of physiological and pathological processes[12]. Receptors to purines are specific cell surface molecules called purinergic receptors. Two distinct purinergic receptor families have been indentified: P1 and P2 receptors. P1 are specific to adenosine and comprise 4 subfamilies, while P2 receptors are selective to ATP and AMP and contain two subfamilies, P2X and P2Y, based on their chemical properties. Additionally, P2X is subdivided into 7 subtypes and P2Y into 8[13,14]. Purinergic receptors families, subfamilies and subtypes are shown on Table 1.

Extracellular nucleotide concentrations are regulated by ectoenzymes that hydrolyse these nucleotides; the NTPDase1 (CD39) cleaves ATP to AMP, NTPDase2 (CD39L1) cleaves ATP to ADP, 5'-ectonucleotidase (CD73) produces adenosine from AMP, ectonucleotide pyrophosphatase/phosphodiesterase (E-NPP) breaks down ATP into AMP, and alkaline phosphatases (AP) which dephosphorylates nucleotides[15]. Adenosine, which has its physiological effects mediated by P1 receptors, can be either transported into the cell or inactivated by adenosine deaminase (ADA) and purine nucleoside phosphorylase (PNP)[15]. Figure 1 shows a schematic representation of the purinergic pathway. Although each purinergic signalling component has its own function they do not operate independently. In physiological conditions they act in cooperation interfering with the function of other elements. When considering pathogenic conditions these cross-talks and networks must be taken into consideration[16].

In viral infection, purinergic extracellular nucleotides and receptors not only participate in the innate and adaptive responses but also modulate the immune responses[17]. The release of ATP by damaged cells generates a danger signal acting as a DAMP, it also stimulates the NOD-like receptor mediated inflammasome and the activation of the capase-1 pathway[18]. Extracellular ATP also acts as a costimulatory signal to T cells and drives the differentiation of gut T helper 17 (Th17) cells[19].

The purinergic signaling system is involved in a series of processes including, neurotransmission, neuro and immune modulation, secretion, cell proliferation, differentiation, apoptosis, cell death, phagocytosis, chemotaxis and embryonic development[11,17,20,21]. Purinergic signaling has been linked to a series of acute and chronic inflammatory diseases[22] including inflammatory bowel disease[23], cancer[13], ischemia[24] and acute lung injury[25]. Therapeutic approaches using the components of the purinergic system are being developed for a number of diseases[12] such as cancer[20,26,27], diabetes[28], osteoporosis[29], and neurodegenerative diseases[30] as well as HIV[8-10]. Consequently, the involvement of the purinergic system in the pathophysiology of several human disorders has been uncovered and the possibility of using these pathways as targets for therapy has been raised[12].

The first study linking the purinergic signaling system and HIV infection was published in 2005, Leal *et al*[6] identified an increased NTPDase activity in CD39-positive lymphocytes of HIV-infected patients. Further studies associating CD39 and HIV disease progression were published in 2011 and 2013[31-33]. In 2007, a study highlighted the protective effect of adenosine receptors against neuronal damage in primary murine cultured brain cells[34], followed by later studies correlating dementia and other HIV-associated neurocognitive disorders to ATP release and P2X in primary neuron-glia co-cultures from mouse striatum[35] and adenosine receptors in HIV-infected macrophages[36]. Studies on pannexin hemichannels and purinergic receptors and HIV infection have been

Table 1 Purinergic receptors families, subfamilies and subtypes, subdivisions

Family	Subfamily	Subtype	Ligand	Cell type expression
P1	A1	NA	Adenosine	Neutrophils, monocytes, macrophages, and DCs
	A2A	NA	Adenosine	Neutrophils, monocytes, macrophages, DCs, T, B and NK cells
	A2B	NA	Adenosine	Neutrophils, monocytes, macrophages, DCs, T and NK cells
	A3	NA	Adenosine	Neutrophils, monocytes, macrophages, DCs, T and NK cells
P2	P2Y	P2Y1	ADP	Neutrophils, monocytes, macrophages, DCs, T, B and NK cells
		P2Y2	ATP, UTP	Neutrophils, monocytes, macrophages, DCs, T, B and NK cells
		P2Y4	UTP (ATP, UDP)	Monocytes, macrophages, DCs, T and B cells
		P2Y6	UDP, UTP	Neutrophils, monocytes, macrophages, DCs, T and B cells
		P2Y11P2Y8	ATP	Monocytes, macrophages, DCs, T and B cells
		P2Y12	ADP	Neutrophils, monocytes, macrophages, T and B cells
		P2Y13	ADP, ATP	Neutrophils, monocytes, DCs, T and B cells
		P2Y14	UDP, glucose	Neutrophils, DCs, T, B and NK cells
	P2X	P2X1	ATP	Neutrophils, monocytes, macrophages, DCs, T, B and NK cells
		P2X2	ATP	B cells
		P2X3	ATP	B and NK cells
		P2X4	ATP	Neutrophils, monocytes, macrophages, DCs, T, B and NK cells
		P2X5	ATP	Neutrophils, monocytes, macrophages, DCs, T and B cells
		P2X6	ATP	B and NK cells
		P2X7	ATP	Neutrophils, monocytes, macrophages, DCs, T, B and NK cells

NA: Not applicable; DC: Dendritic cells; NK: Natural killer cells. Adapted from Junger WC, 2011[56].

published in the last few years[4,5]. ADA also has been the subject of study in the context of HIV infection, proving to be a immune response booster[7] and a biomarker for disease progression[37] and accelerated aging associated with HIV infection[38].

HIV INFECTION AND THE IMMUNE SYSTEM

Host defence against HIV depends on a combination of adaptive and innate responses[39]. Despite the ability of these responses to briefly control disease progression, they are not capable of eliminating the virus. The complex interaction between the host response and HIV virus has been extensively studied and the HIV has been shown to take advantage of host metabolic pathways and proteins, known as host permissive factors, allowing the virus to thrive and persist in the host organism[40-42].

As with other viruses, the first line of defence against HIV is the innate response. Germ line-encoded Pattern-Recognition Receptors (PRRs) are essential players in the innate response. PRRs include toll-like receptors (TLRs), membrane-bound C-type lectin receptors (CLRs), NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs), and unidentified proteins capable of recognizing DNA or RNA[43]. These PPRS recognize pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs). Although the knowledge of HIV-specific PPRs and PAMPs remains scarce, RIG-I was recently described as being involved in recognizing cytosolic HIV genomic RNA[44]. The recognition of PAMPS and DAMPS by the PRRs triggers a cascade of signaling pathways on the surface of antigen-presenting cells (APCs) and dendritic cells (DCs), which initiate host inflammatory and immune responses. When the APCs and the DCs are stimulated, the CD4+ T cells and the natural killer (NK) cells are activated in the lymph nodes and the adaptive response is initiated[45,46].

DCs are not only important APCs along with macrophages and monocytes in both innate and adaptive responses[3], but they also modulate the adaptive response together with NK cells[46]. Once the DCs are activated, they produce cytokines and induce T helper 1 (Th 1) responses and consequently cytotoxic T lymphocyte (CTL) responses[46]. However, HIV specifically targets CD4+ T cells along with macrophages and DCs which are essential for the antiviral response[2].

PURINERGIC SIGNALING AND THE IMMUNE SYSTEM

In physiological conditions, the cells are able to maintain a balance in the levels of ATP[47]. In pathological conditions, on the other hand, injured, necrotic and activated cells release ATP into the extracellular environment, where it interacts with P2 receptors or is degraded by ectoenzymes. Purinergic signaling seems to be an important regulator of the activation of NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome. P2 receptors control the potassium efflux contributing to the activation of the inflammasomes[48,49]. The release of ATP from necrotic cells activates the NLRP3 inflammasome *via* the P2X7 receptor[18,50]. HIV-1 acts as primary signal to activate the NLRP3 inflammasome[51], since local release of ATP is stimulated by the binding of HIV gp120 to its receptor which results in activation of purinergic receptors[5,52]. The activation of the inflammasome is important in the antiviral response since it may lead to the elimination of the infected cells by pyroptosis[53-55]. Figure 2 illustrates the activation of NLRP3 inflammasomes in a HIV infected CD4 T cell. In the specific case of HIV infection pyroptosis might not be a protective method since it does not

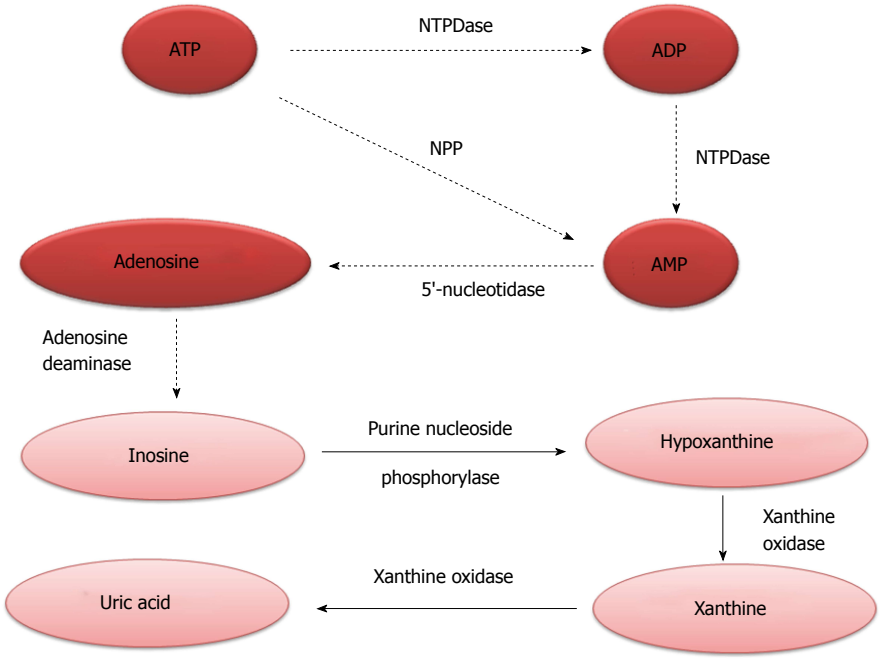

Figure 1 Schematic representation of the purinergic pathway. ATP is broken down to ADP and AMP by NTPDase or directly to AMP by pyrophosphatase/phosphodiesterase (NPP). AMP is converted to adenosine by 5′-ectonucleotidase (CD73). Adenosine deaminase (ADA) transforms adenosine into inosine which is converted in hypoxanthine by purine nucleoside phosphorylase (PNP). Xantine oxidase catalyzes the oxidation of hypoxanthine to xanthine and xanthine to uric acid.

eliminate the infection, instead it creates a pathogenic cycle in which the death of CD4+ T cells results in the release of inflammatory signals that attract more CD4+ T cells which subsequently die creating a state of chronic inflammation[54].

Recruitment of inflammatory cells to the sites of infection as part of an antiviral response involves the release of nucleotides and an autocrine purinergic signaling pathway[42,56]. The release of nucleotides triggers the polarization of purinergic proteins and receptors contributing to migration of phagocytes to the sites of inflammation and infection[42]. P2 purinergic receptors are also involved in chemotaxis[42]. Purinergic receptors are not only involved in chemotaxis but also in the modulation of immune responses[56].

The multiple and complex processes leading to inflammation and immune activation are not fully understood. Appay and Sauce[2] (2008) proposed a simplified model for immune activation and inflammation in HIV infection in which three well-known major events, depletion of CD4+ T cells, immune activation and exhaustion of regenerative capacity, all contribute to inefficient immune response and loss of T cell homeostatic regulation[2]. During acute infection, the depletion of gut mucosal CD4+ T cells triggers the loss of protective mechanisms such as the epithelial barrier and immune cells that otherwise would block translocation of microbial products from the gut into the circulation[57-59]. These microbial products such as bacterial DNA and lipopolysaccharides activate the innate response receptors and a signaling cascade, consequently boosting the production of inflammatory cytokines[60]. These events prompt a systemic immune activation that characterizes the chronic phase of HIV infection and consequent loss of peripheral CD4+ T cells[60]. Chronic immune activation and inflammation increases cell turnover and causes the accelerated aging of the immune system driving HIV-specific CD8+ T cells to exhaustion[2].

DCs represent an important link between the innate and adaptive responses. ATP enhances the antiviral response by activating DCs which then migrate to the lymph nodes. Extracellular ATP was found to interfere with the transfer of HIV-1 from immature DCs to CD4+ T cells thereby controlling the spread of the virus by halting viral replication[61].

ATP RELEASE AND PURINERGIC RECEPTORS IN HIV-ASSOCIATED NEUROCOGNITIVE DISORDERS

ATP release and purinergic receptors are involved in pathologies of the central nervous system (CNS) in neurodegenerative, neuropsychiatric and neurocognitive diseases[62]. Regarding the toxic effects of extracellular purines in HIV infection, a connection between HIV-associated neurocognitive disorders (HAND) and the pathological release of purines by HIV-infected macrophages was found. High concentrations of ATP, ADP, AMP and small amounts of adenosine were found in HIV-infected macrophages along with glutamate, suggesting that ATP release from these cells might be involved in neuronal damage in HIV-infected patients[53]. These purinergic molecules are thought to mediate calcium influxes through activation of calcium receptors, causing damage

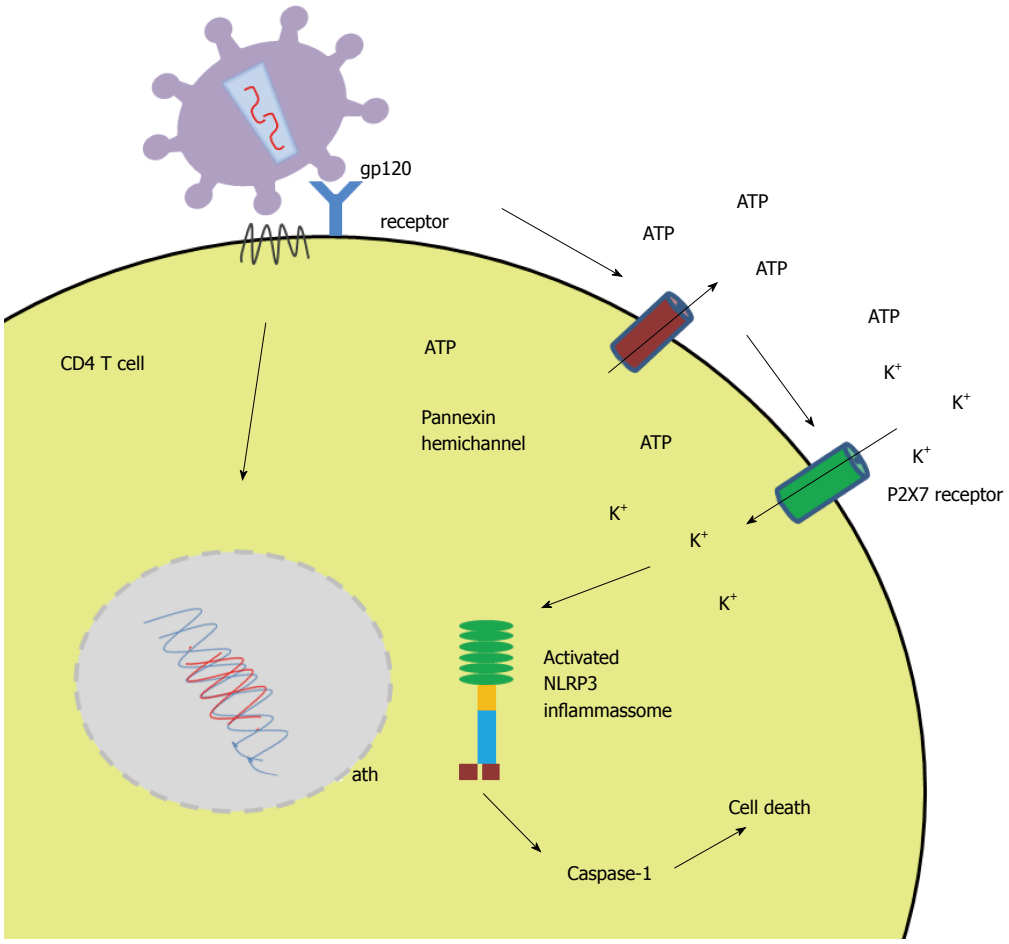

Figure 2 Schematic illustration of ATP release and activation of NOD-like receptor family, pyrin domain containing 3 inflamassome in a human immunodeficiency virus infected CD4 T cell. Once human immunodeficiency virus gp120 binds to its receptor ATP release is stimulated through pannexin hemichannels with consequent activation of P2X7 receptor. The influx of potassium causes the activation of NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammassome leading to cell death *via* Caspase-1.

or death of neurons[35].

Several mechanisms are involved in the neurocognitive impairment that affects HIV infected patients. The HIV transactivator of transcription Tat induces the release of cytokines and chemokines from microglia, microglia, macrophages, neurons, and astrocytes in the CNS and causes disruption of the blood-brain barrier with resulting neurotoxicity[63]. Recently it was suggested the P2X receptors are involved in HIV and opioid neuropathogenesis[36]. The use of TNP-ATP, a non-selective P2X antagonist, prevented the neurotoxic damage caused by exposure to morphine and/or HIV Tat or ATP in striatal neurons[36]. P2X4 receptors are expressed by subpopulations of striatal neurons and glia and are activated by excess levels of extracellular ATP caused by morphine and/or HIV-1 Tat[36]. The involvement of P2X4 in HIV and opioid neuropathogenesis was confirmed by the fact that selective blockade of P2X1, P2X3, or P2X7 receptors, not P2X4, were not able to prevent Tat or morphine induced neurotoxicity, suggesting this particular receptor might be a potential new target for prevention of HAND[36].

HIV-associated dementia is linked to inflammation and consequent production of proinflammatory cytokines. The adenosine receptor A1AR was shown to inhibit Tat-mediated proapoptosis by attenuating intracellular Ca^{2+} and production of nitric oxide[34]. More recently, it was demonstrated that the activation of the A2A adenosine receptor inhibits Tat-induced TNF-α production in monocytes thereby suggesting that adenosine is an important regulator of cytokine production and its pathway a possible therapeutic target for CNS inflammatory disorders[64].

PANNEXIN HEMICHANNELS AND PURINERGIC RECEPTORS ARE INVOLVED IN THE PROCESS OF HIV VIRAL ENTRY AND IMMUNE ACTIVATION

ATP is transported mainly through a combination of vesicular release, connexin and pannexin hemichannels[12], but also includes P2X7 receptors and maxi-ion channels[65]. The role of pannexin hemichannels have been studied in a number of pathophysiological events including calcium signaling, cellular differentiation, cellular migration, inflammation, cell death, innate and

adaptive immune responses and HIV viral entry[66,67]. To ensure an efficient entry into the cell, the HIV-1 gp120 protein binds to CD4+ T cell receptor and chemokine coreceptors CXCR4 or CCR5. It has been demonstrated that this binding increases the intracellular free calcium, induces the opening of Panx-1 hemichannels in response to ATP release and activation of purinergic receptors[68]. Once opened, Panx-1 hemichannels facilitate virus entry by changing ionic gradients with further release of further signals such as ATP that activate extracellular purines and receptors[68]. Orellana *et al*[68] (2013) suggested that Panx-1 hemichannels are part of the apparatus of host proteins of which the virus takes advantage to enter the cells and replicate, and that the opening of these hemichannels might be involved in other events such as viral fusion.

In addition to Panx-1 hemichannels, other purinergic signaling pathways components may favor virus entry and subsequent events in viral infection. Purinergic receptors are also implicated in virus entry. A recent study demonstrated that the HIV-encoded Env complex triggers the release of ATP and subsequently activates the purinergic receptors initiating a cascade of events[4]. The extracellular ATP released through the Panx-1 hemichannels act on purinergic receptors, including P2Y2 which along with pannexin-1, ATP and Pyk2 were shown to be essential for HIV-1 replication[4], since purinergic receptor inhibitors and antagonists blocked HIV-1 replication[4]. P2Y2 activates the proline-rich tyrosine kinase, an important step in the cascade, which is also a critical effector of HIV-1 infection[4]. Overexpression of P2Y2 in peripheral blood mononuclear cells (PBMCs) increased the depolarization of the plasma membrane, an event required for Env-dependent HIV-1 fusion[4].

P2X1 is also involved in viral entry, while P2X7 and P2Y1 might be involved in later events of viral infection; antagonists for all these receptors blocked viral replication but only antagonists for P2X1 blocked viral entry[5]. The binding of gp120 to host CD4 and co-receptors induces ATP release and subsequent activation of P2X1 receptors required for viral entry. P2X7 and P2Y1 may require greater amounts of accumulated ATP to be activated for involvement in later steps of the viral cycle[5]. Hazleton *et al*[5] (2012) also suggests that other products of ATP are also involved in the process of entry and replication of HIV. Further studies are necessary to elucidate the mechanisms and explore the therapeutic potential of purinergic receptors antagonists.

As mentioned previously, P2X7 is involved in HIV-1 replication and much higher concentrations of ATP are needed to activate this receptor. ATP release though Panx-1 hemichannels are thought to achieve a sufficiently high local concentration of ATP to induce P2X7 activation, which in turn activates the opening of Panx-1 hemichannels, creating a cycle that leads to persistent immune activation[69]. This positive feedback loop might be one of the multiple mechanisms involved in persistent activation during HIV infection[52]. The role of purinergic receptors in HIV entry, fusion and replication is summarized in Figure 3.

ECTOENZYMES AND HIV DISEASE PROGRESSION

Whilst ATP may promote inflammation, adenosine is considered a mostly anti-inflammatory molecule and a crucial regulator in innate and adaptive immune responses[70]. Ectoenzymes CD39 (NTPDase-1) and CD73 (5′-ecto-nucleotidase) are known to dephosphorylate extracellular ATP to adenosine, playing an important role in immune modulation[33,71,72]. The role of the ectoenzymes in physiological processes and diseases has been extensively studied, specially the NTPDase and 5′-ecto-nucleotidase activities[73]. Co-expression of CD39 and CD73 plays an important part in keeping the balance between activation and regulation of the immune response against HIV and, subsequently, in the halting of disease progression[51,74].

CD39 cleaves ATP into AMP while CD73 converts AMP into adenosine, increasing the expression of A2AR agonist and cyclic AMP (cAMP). CD39 is a marker of lymphoid activation which requires ATP as an energy source. This enzyme hydrolyses ATP into AMP to maintain the ATP levels, preserve cellular integrity and modulate the immune response[6]. Our group has found that the NTPDase-1 activity is increased in lymphocytes of HIV-positive patients suggesting that it might be due to apoptosis and the need to reduce the toxic effects of ATP release[6]. In the last few years a series of studies have investigated the role of T regulatory (Treg) cells expressing CD39 in HIV infection[31-33]. Nikolova *et al*[33] (2011) have established that the expansion of Treg CD39+ cells in HIV-infected individuals might contribute to disease progression, since they inhibit T cell proliferation. Another study also describes the increased expression of CD39 in Treg cells and its association with disease progression and immune activation[32]. In addition, CD39 was found to be involved in the inefficiency of the CD8+ T cell response during chronic HIV infection by inhibition of important cytokine production[33]. In support of this finding, Jenabian *et al*[31] (2013) show that the expansion of Treg CD39+ cells inhibits IL-2 production thereby suppressing the function of CD4+ T cells; this occurs through demethylation of an essential CpG site in the *IL-2* gene promoter. CD39+ Treg cells were shown to inhibit HIV replication mediated by cAMP in conventional T cells, suggesting they have a protective effect against HIV infection[75].

Unlike CD39, that is overexpressed in HIV-infected individuals, CD73 has shown to be depleted, which leads to decreased adenosine suppression and failure to control persistent cell activation contributing to disease progression[76,77]. Another interesting finding is that once the pool of CD4+CD73+ cells is depleted they can no longer be expanded, even if the CD4+ cell levels are recovered[76].

ADA is a purinergic signaling enzyme responsible for catalyzing the deamination of adenosine and also involved in modulating the T cell response against HIV[7,78-80]. ADA boosts both the CD4+ and CD8+ memory cell response

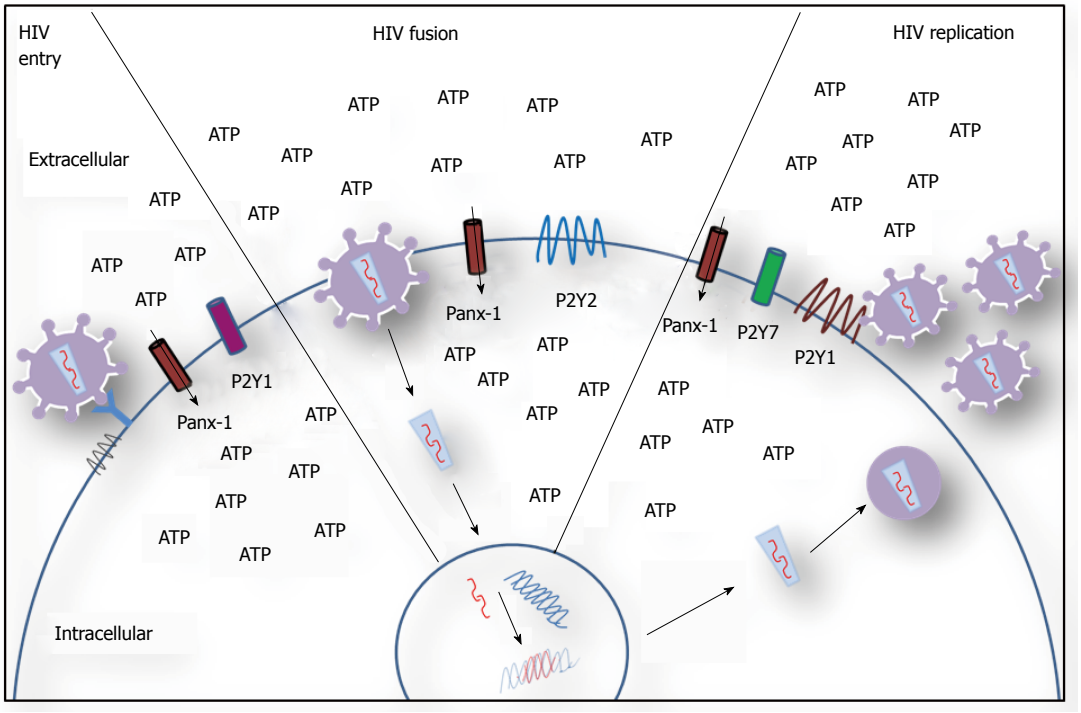

Figure 3 Schematic representation of purinergic receptors involvement in human immunodeficiency virus infection. Pannexin hemichannels (Panx-1) are open in response to ATP release and activation of purinergic receptors, facilitating viral entry, fusion and replication. The blockage of viral entry by P2X1 antagonists suggests it is involved in this stage of infection. P2Y2 increases cell membrane depolarization facilitating fusion. P2X7 and P2Y1 are involved in later steps of viral cycle.

to HIV and also reduces the suppression mediated by Treg cells[7,78-80]. In DCs, ADA has been shown to be capable of boosting immunogenicity and increasing the secretion of pro-inflammatory cytokines[7,78,80]. All this data taken together suggests that ADA would be a strong candidate target for therapeutic and vaccine approaches[7,78,79].

ADA AS DISEASE PROGRESSION AND SENESCENCE BIOMARKER

HIV infected individuals are subject to accelerated aging, this might be due not only to the chronic inflammation and immune activation inherent of HIV infection[81], but also as a consequence of highly active antiretroviral therapy (HAART)[82]. In fact, immune activation has been a major cause of morbidity and mortality from AIDS-defining and non-AIDS defining diseases in patients undergoing antiretroviral treatment[83]. The increased production of inflammatory cytokines arising from the chronic inflammatory status enhances a process called "inflammaging"[84]. The term "inflammaids" was employed to define the extensive activation of innate and adaptive immunity during HIV infection, which resembles the process of inflammaging[85,86]. The HIV infection *per se* predisposes infected patients to premature aging, however the interplay between these pathological changes and HAART makes the situation even more complex[82]. The age-associated disorders that affect HIV infected patients include neurological and metabolic diseases and immunosenescence; the premature aging also has an impact on disease progression markers such as CD4+ and CD8+ T cell counts[87]. Since immune activation is a strong predictor of disease progression and consequent immunosenescence and premature aging, several biomarkers have been identified[37,38,88]. ADA has been shown to be not only a suitable marker of immune activation and disease progression[37] but also a suitable biomarker of senescent human CD8+ T cells[38].

PURINERGIC RECEPTORS, ATP/ADENOSINE AND ATP ANALOGS AS POTENTIAL THERAPEUTIC APPROACHES

The study of the implications of ATP and purinergic signaling in HIV infection has highlighted its potential use in therapeutic approaches[4,8,9,89].

As discussed earlier in this paper, studies have shown that purinergic receptors are essential for viral entry and replication[4,5]. A recent study has demonstrated that the inhibition of purinergic signaling blocks HIV-1 membrane fusion[10]. This study reveals that PPADS, a nonselective purinergic antagonist, is capable of inhibiting cell-to-cell and cell-free HIV-1 infection in both X4- and R5-tropic virus infections[10]. This finding highlights the potential use of P2X-selective purinergic antagonists to inhibit HIV-1 fusion.

Wagner[8] (2011) proposes the use of ATP combined with HAART to eliminate infected cells, exploiting the ability of ATP to modulate the immune response[17].

Recently, the use of ATP analogs was proposed to inhibit HIV-1 transcription by preventing cyclin-dependent kinases (Cdks) binding to Tat thus inhibiting Tat-dependent transcription[9].

CONCLUSION

HIV infection is a serious condition with a huge impact on global health. Despite all efforts to contain the spread of the virus, prolonging the life span and quality of life of infected individuals, prevalence[90], morbidity and mortality[91] rates are still high. Much is known about HIV infection but there are still areas in critical need of both basic and clinical research[92,93].

The understanding of pathogenic mechanisms involved in HIV infection and progression to AIDS, in which metabolic pathways such as purinergic signaling are involved, is crucial to achieve important objectives in controlling this condition.

Metabolic pathways have been the subject of investigation in the search for new therapies[52] and even a cure for HIV[94]. Even though the involvement of the purinergic signaling pathway in viral infections, including HIV, are multiple and ambiguous, it deserves further attention[22]. Studies on the subject have helped to elucidate disease mechanisms and propose new targets and approaches to therapy.The identification of prognostic biomarkers and the recognition of candidate target genes or pathways to improve therapy are high priority areas where HIV infection is concerned. Purinergic signaling system components are relevant topics of study both for development of biomarkers as well as potential therapeutic targets.

The data reviewed in this paper reveals that this research field must be encouraged. We believe further studies targeting purinergic signaling enzymes and receptors must be particularly pursued due to its relevance in the process of understanding of the pathogenic mechanisms and the promise of new therapies in the future.

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MINIREVIEWS

Diagnostic assays developed for the control of foot-and-mouth disease in India

Gaurav Kumar Sharma, Sonalika Mahajan, Rakesh Matura, Saravanan Subramaniam, Rajeev Ranjan, Jitendra Biswal, Manoranjan Rout, Jajati Keshari Mohapatra, Bana Bihari Dash, Aniket Sanyal, Bramhadev Pattnaik

Gaurav Kumar Sharma, Sonalika Mahajan, Rakesh Matura, Saravanan Subramaniam, Rajeev Ranjan, Jitendra Biswal, Manoranjan Rout, Jajati Keshari Mohapatra, Bana Bihari Dash, Aniket Sanyal, Bramhadev Pattnaik, Project Directorate on Foot and Mouth Disease, Indian Council of Agricultural Research, Mukteswar, Uttarakhand 263138, India

Author contributions: Sharma GK designed the scope of the minireview and wrote the paper; Mahajan S and Matura R reviewed the literature and compiled the data; Saravanan S, Ranjan R and Biswal J analyzed the FMD virus diagnosis data; Sharma GK, Mahajan S and Dash BB analyzed the herd immunity data; Rout M and Mohapatra JK analyzed the FMD sero-surveillance data; Sharma GK and Pattnaik B compiled and revised the manuscript; Sanyal A compiled the molecular diagnosis of FMD data and revised the manuscript.

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Abstract

Foot-and-mouth disease (FMD) is a highly contagious and economically devastating disease of livestock, primarily affecting cattle, buffalo and pigs. FMD virus serotypes O, A and Asia1 are prevalent in India and systematic efforts are on to control and eventually eradicate the disease from the country. FMD epidemiology is complex due to factors like co-circulation, extinction, emergence and re-emergence of genotypes/lineages within the three serotypes, animal movement, diverse farm practices and large number of susceptible livestock in the country. Systematic vaccination, prompt diagnosis, strict biosecurity measures, and regular monitoring of vaccinal immunity and surveillance of virus circulation are indispensible features for the effective implementation of the control measures. Availability of suitable companion diagnostic tests is very important in this endeavour. In this review, the diagnostic assays developed and validated in India and their contribution in FMD control programme is presented.

Key words: Foot-and-mouth disease; Diagnosis; Sero-surveillance; Sero-monitoring; Multiplex polymerase chain reaction; Real-time polymerase chain reaction; Lineage differentiating polymerase chain reaction

Core tip: To inform scientific community, this short review summarizes existing foot-and-mouth disease diagnostics developed in the recent past and used in India. Immediate and future requirements in the diagnostics are highlighted.

Sharma GK, Mahajan S, Matura R, Subramaniam S, Ranjan R, Biswal J, Rout M, Mohapatra JK, Dash BB, Sanyal A, Pattnaik B. Diagnostic assays developed for the control of foot-and-mouth

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INTRODUCTION

Foot and mouth disease (FMD) continues to pose threat to the livestock sector in the world. The annual direct loss due to FMD in India is estimated at United States dollar4.45 billion[1]. Economic losses due to trade barrier imposed by FMD free countries could be much more. FMD is caused by a single stranded positive sense RNA virus, belonging to the genus *Aphthovirus* of family *Picornaviridae*[2]. Seven serotypes (O, A, C, Asia1, SAT-1, SAT-2 and SAT-3) and multiple antigenic variants within the serotypes of FMD virus (FMDV) exist because of the variable antigenic nature of its structural proteins.The FMDV genome of approximately 8.5 kb is polyadenylated at 3′ terminus and carries a small protein VPg at its 5′ end[3,4]. It encodes four structural proteins (SPs) (VP1-4) and at least 8 non-structural proteins (NSPs). Structural proteins, VP1, VP2, VP3 and VP4 are formed by post-translation cleavage of a precursor coded by 1D, 1B, 1C and 1A genes, respectively[3]. Non-structural proteins of FMDV consist of L, 2A, 2B, 2C, 3A, 3B1, 3B2, 3B3, 3C, and 3D. The L gene which encodes L protein is situated at the extreme 5′ end of the coding region while all other NSPs are encoded by the P2 and P3 regions, which are situated towards the 3′ end of the viral RNA (Figure 1). The P2 region codes for 2A, 2B and 2C while P3 codes for 3A, 3B1, 3B2, 3B3, 3C and 3D.

Most of the developed countries are free from FMD, whereas the disease is present in many developing countries including India. Epidemiology of FMD in India is complex due to prevalence of many variants of FMDV serotypes (O, A, Asia1), mixed farming system, diverse landscape, animal husbandry practices and very large population (about 500 million) of susceptible livestock[5].

The disease in cattle and buffalo is characterized by high fever, depression, excessive salivation, formation of vesicles on the tongue and oral cavity, epidermis of the coronary band and inter digital space, udder and teats. Formation of vesicles in the oral cavity results in reduced food consumption, weight loss and emaciation. Vesicles may also develop in the epithelium of the pharynx, larynx, trachea, oesophagus and rumen. In young animals, it may lead to death due to myocarditis. Many times it leads to secondary bacterial infection in affected animals. While mortality is generally less than 3%, morbidity is very high and economic losses become unbearable for the farmers on account of decreased productivity and protracted convalescence in affected animals. Though, mortality is notably high in young pigs. Incubation period ranges from 2 to 14 d, but is generally shorter than a week.

FMDV can be transmitted by direct contact, aerosols, mechanical carriage by men or fomites and through animal products such as meat, offal, milk, semen or embryos. Infected pigs shed large quantities of virus in aerosols[6] and spread the virus down wind. Under favourable conditions of low temperature, high humidity and moderate winds, virus in aerosols may spread up to 250 km over sea[7] and 60 km over land[7]. Virus can remain infective on soil for 3 d in summer and for up to 28 d in winter[8].

FMD symptoms could be confused with other vesicular diseases like Swine Vesicular Disease (cattle and sheep are resistant), Vesicular Exanthema (cattle and sheep are resistant), Vesicular Stomatitis virus (sheep/goats are resistant). Availability of rapid and sensitive FMD diagnostic assays is essential in order to confirm the initial cases and prevent further spread of the disease. Infected animals may secrete the virus before clinical symptoms develop and the virus could spread rapidly in the susceptible population; hence rapid and early identification of the infected/carrier animals is critical.

Timely identification of serotype of the virus involved in the outbreak is of the utmost importance for disease control. Besides, apparently healthy animal population in endemic settings are to be regularly screened for the presence of antibodies against SPs and NSPs of FMDV and for the presence of the virus in the oro-pharynx to confirm the carrier status. Many diagnostic assays have been developed throughout the world for rapid and specific detection of FMDV and the antibodies against the FMDV proteins. Most of these assays are developed and validated considering the local requirements and prevailing virus pool, whereas some assays have been developed for use in the broad geographical areas. Now a day, molecular methods for FMD diagnosis are playing important role when compared to the conventional methods.

The episodes of FMD outbreaks are to be actively monitored, recorded and investigated in order to support the vaccination based control programme in the country. For all these activities, availability of rapid, sensitive, specific and economical diagnostic assays representing the FMDV pool in circulation is of prime importance and necessity.

A systematic vaccination based control is in operation for control and eventual eradication of FMD from India since 2003-2004 by Government of India (Department of Animal Husbandry, Dairying, and Fisheries)[5]. The total FMD susceptible livestock population in the country is about 500 million comprising of more than 300 million cattle and buffalo, 71.5 million sheep, 140.5 million goats, and 11 million pigs[9]. Availability of indigenously developed diagnostic assays is crucial and indispensible to support such a huge control programme. In this review, the role of diagnostic assays developed, validated and used over the last decade in the country (Table 1) along with their contribution in control of FMD in India is being discussed.

FMDV DETECTION IN CLINICAL MATERIALS

FMD is primarily diagnosed by demonstrating FMDV

Table 1 Diagnostic assays for foot-and-mouth disease virus diagnosis with their associated advantages and disadvantage

FMD diagnostic assay	Specimen materials	Target region	Sensitivity	Specificity	Advantages	Disadvantages
Sandwich ELISA	RNA from TE, FL, TE,	VP1 protein	80%	100%	Easy to perform Suitable for handling large number of samples	Less sensitive, not suitable for certain type of clinical samples
Multiplex PCR	RNA from TE, FL, TE, Semen, Milk	1D region	Minimum detection limit of 1×10^{-1} $TCID_{50}$/mL	100% specific for cross serotype detection	Rapid and sensitive Suitable for samples like semen and milk	High risk of generating false positives
Taqman real-time PCR	RNA from TE, FL, TE, Semen, Milk	1D region	Minimum detection limit of $10^{1.0}$ $TCID_{50}$/mL	100% specific for cross serotype detection	More sensitive and specific than gel based assay	high risk of generating false positives
Virus isolation and neutralization	Triturated material of TE, FL, TE,	--			Gold standard assay for FMD diagnosis	Slow takes 1-4 d for confirmatory results
RNA transfection	RNA from TE, FL, TE, Semen, Milk	--	--	--	FMDV can be isolated from deteriorated clinical materials	--
LAMP	RNA from TE, FL, TE, Semen, Milk	3D region	Minimum detection limit up to 1.1×10^{-4} $TCID_{50}$/mL	--	Require no specialized instruments, can be used as point-of-care diagnosis	High risk of generating false positives
3AB3 I-ELISA	Serum	3AB3 region	96%	99.1% -96.4%	Sensitive and Specific	Only for bovine species
3ABC C-ELISA	Serum	3ABC region			Specific assay Universal for all species	Less sensitive than I-ELISA
2Ct I-ELISA	Serum	2C region			Sensitive and Specific	Only for bovine species

FMD: Foot-and-mouth disease; ELISA: Enzyme-linked immunosorbent assay; PCR: Polymerase chain reaction; LAMP: Loop-mediated isothermal amplification.

particles or viral genome in the clinical materials *viz*. tongue epithelium, foot epithelium, saliva, milk and semen, *etc*. Detection of intact virus particles by sandwich enzyme-linked immunosorbent assay (ELISA) and virus neutralization test provides confirmatory diagnosis, whereas detection of the viral genome by polymerase chain reaction (PCR) or loop-mediated isothermal amplification (LAMP) assay is more sensitive method of diagnosis. Samples collected from the FMD suspected animals are processed and routinely analyzed by these assays. The details of the suspected clinical samples tested during the last seven years are presented in the Table 2.

Table 2 Details of the clinical materials suspected for Foot-and-mouth disease tested by sandwich enzyme-linked immunosorbent assay and multiplex polymerase chain reaction during the last five years

Year	Sample tested	Serotype O	Serotype A	Serotype Asia1	Total FMD diagnosed
2009-2010	1155	423	15	7	445
2010-2011	345	83	10	10	171
2011-2012	567	265	4	40	309
2012-2013	701	218	15	52	285
2013-2014	3130	1295	24	10	1329
Total	5898	2284	68	119	2539

FMD: Foot-and-mouth disease.

Sandwich ELISA

FMD antigen detection ELISA was shown to be rapid and simpler to perform[10]. The assay is generally regarded as the primary test for FMD diagnosis especially at the regionally located FMD diagnostic laboratories in the country[11]. The suspected clinical materials are first submitted to the regionally located FMD diagnostic laboratories in the country working under ICAR-PDFMD, Mukteswar where samples are processed and tested by an in-house sandwich ELISA for identification of FMDV serotype(s). The assay is based on the detection of FMDV structural proteins (Figure 1) and utilizes the serotype specific polyclonal antibodies generated in guinea pig and rabbits[12]. This antigen-capture sandwich ELISA has 100% specificity for heterologous FMDV and 80% sensitivity for detection of complete virus particles in clinical samples[12]. The assay is easy to perform at regional FMD diagnostic laboratories and large number of samples can be processed without risk of laboratory cross contamination. The assay is being used countrywide since two decades at 23 regionally located laboratories in the country[11]. As the assay specifically detects the intact virion particles in clinical materials in a serotype specific manner, the lower sensitivity could be attributed to the improper storage and transportation of samples that leads breakdown of the virus particles. The clinical materials are then submitted to the Central Laboratory, Mukteswar for detailed virological and genome analysis. Since 2009-2010, more than 5000 clinical materials have been tested by the sandwich ELISA, both at the regional FMD diagnostic laboratories and the central laboratory.

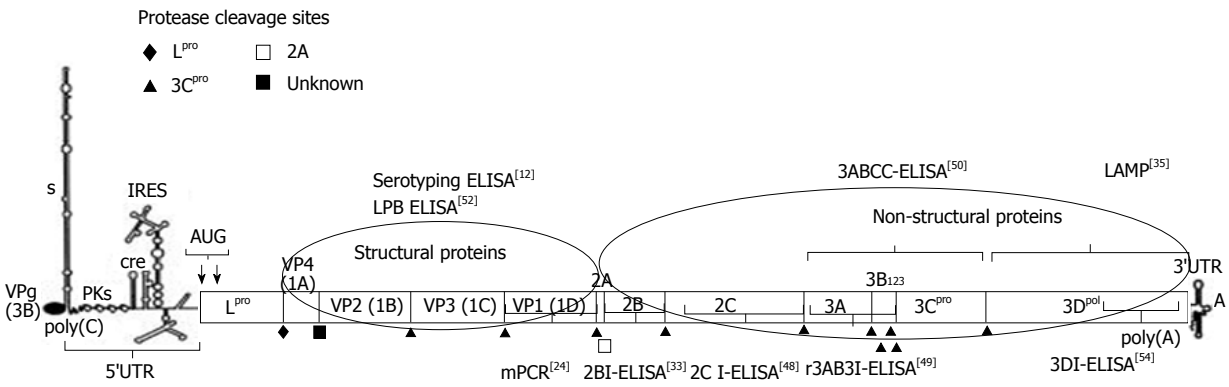

Figure 1 Genome structure of the foot-and-mouth disease virus. Describes in details the different regions of foot-and-mouth disease virus (FMDV) which codes four structural and 8 non-structural proteins. The regions of genome encoding structural proteins are targeted for FMDV serotype determination by various assays, whereas the genome regions coding for non-structural proteins are targeted for serotype independent diagnosis. The SP of FMDV is targeted for serotyping by antigen trapping enzyme-linked immunosorbent assay (ELISA) or for measurement of antibody response by liquid phase blocking ELISA. Antibodies against various non-structural proteins are targeted for differentiation of FMD infected from vaccinated animals.

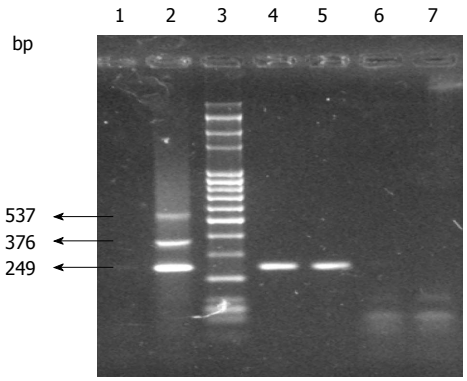

Figure 2 Depicting the foot-and-mouth disease virus serotyping by gel based multiplex polymerase chain reaction assay. Foot-and-mouth disease virus serotype determined by gel based ready-to-use lyophilized one-step realtime-polymerase chain reaction. Lane 1; negative control; Lane 2: Positive control of serotypes O (249 bp), A (376 bp) and serotype Asia1 (537 bp); Lane 3: 100 bp DNA ladder; Lane 4 and 5: Positive sample of serotype O; Lane 6 and 7: Negative samples for FMD.

Virus isolation

Of the established diagnostic approaches, virus isolation (VI) in cell culture is considered as the "gold standard" as described in OIE Terrestrial Manual 2012[13]. This method can be highly sensitive (depending upon the cell culture system used), although it can be slow, taking between 1 and 4 d to generate the results and require a containment laboratory facility. However, virus isolation from clinical materials is indispensible for antigenic profiling of the virus and vaccine matching. Primary cells, such as bovine thyroid (BTY), are highly susceptible to a wide range of FMDV serotypes[14], but they are difficult and costly to prepare and lose FMDV susceptibility after multiple passages[15]. Primary lamb kidney (LK) cells are also very sensitive to FMDV, and unlike BTY cells, LK cells maintain their sensitivity to FMDV infection after cryopreservation[16]. Immortalized cell lines [*e.g.*, baby hamster kidney (BHK-21) fibroblasts and porcine kidney epithelial cells], are much easier to maintain but are less susceptible to specific animal-derived FMDV serotypes[17-20]. Recently LFBK-$\alpha_v\beta_6$stable cell line has been established and was observed to be an excellent cell line for FMDV diagnostic- and research-based cell applications[21].

In India, all the clinical samples collected/submitted for FMD diagnosis are subjected to virus isolation using the cell lines (BHK21, IBRS, and LFBK cells). Virus isolates after characterization are archived in the National FMDV repository. Currently the repository contains more than 1850FMDV isolates comprising of serotype O (*n* = 1180), A (*n* = 298), Asia1 (358) and C (*n* = 15). The oldest isolate available in the repository is of the year 1962 (Serotype O). Such a vast pool of virus isolates aids in selection and identification of suitable vaccine candidates through vaccine matching exercise from time to time.

RNA transfection method for FMDV rescue

Isolation of virus from the clinical materials is not always possible due to several factors[22]. Under such scenarios, the transfection based virus-rescue method as described by Belsham *et al*[23], has been optimized in India[22]. Success rate of RNA transfection for virus isolation was observed to be 62% against 16% in conventional cell culture method that enhances the number and diversity of virus isolates being usedin vaccine matching exercise. Till date, 88 serotype O, 24 serotype Asia1 and 09 serotype A viruses have been rescued using RNA transfection method from the samples where conventional method of cell culture passage failed to isolate the virus[11].

Multiplex PCR

In vitro amplification based detection of genome is more rapid and sensitive than conventional VI[24]. Initially, assays were developed targeting the conserved 3D region[25,26] and 5′ UTR region[27]. Subsequently, multiplex PCR for (mPCR) targeting VP1 region were developed for detecting FMDV and differentiating amongst the serotypes[24,28,29].

On the similar lines, mPCR was also developed in India[24] and the success rate of FMD diagnosis and serotype detection increased by 8%[30]. In this assay, the serotype-specific primers targeting 1D region and common reverse primer (NK61) targeting 2B region were used for multiplexing (Figure 1). Figure 2 indicates the mPCR based serotype identification describes the identification of serotype involved by multiplex PCR where product size of 249, 376 and 537 bp are specific for serotypes O, A, and Asia1, respectively. The minimum detection limit of the mPCR has been estimated as 1 x 10^{-1} $TCID_{50}$/mL for serotypes O, A, and Asia1[24].

Although, the mPCR suffered from the disadvantage of generating false positives due to carry-over of PCR amplicons and thus, not considered as an ideal assay for routine testing of large numbers of samples especially at regionally located FMD diagnostic laboratories[31]. To overcome the chances of cross-contamination and make it more feasible for regional FMD laboratories, a ready-to-use thermo-stable RT-PCR mixture was developed[30]. All the components of the reaction mixture were mixed together in a vial and lyophilized (Lyodryer, United States). The lyophilized vials are to be reconstituted with nuclease free water before use and supplemented with the extracted RNA from the suspected materials followed by *in-vitro* amplification in a thermal-cycler. This thermostable RT-PCR mix made the assay more user friendly and clinical samples can be now diagnosed by PCR at the field level FMD diagnostic laboratories with uniformity in the results. In addition, the requirement of keeping live FMDV for positive control became obsolete. Since 2005, more than 2037 suspected clinical materials have been successfully tested by the mPCR in the country[11].

Reverse transcription-LAMP assay

Reverse transcription-LAMP (RT-LAMP) assay is an autocycling and strand displacement DNA synthesis method[32] which has recently been employed in FMD diagnosis as point-of-care test. The RT-LAMP based targeting 3D and IRES region for detection of FMDV have been reported earlier[33,34]. In the recent past, LAMP based assay for FMDV detection and serotype differentiation (O, A and Asia 1) has been developed[35]. RT-LAMP based assay targeting 3D region has also been developed in India and is being used routinely for rapid detection of FMDV (Figure 1)[36]. LAMP assay requires only a water bath instead of a thermal-cycler as in PCR. In addition, gel documentation system is also non-essential as hydroxynaphthol blue (HNB), an azo dye is used as the indicator. The sensitivity and specificity of the RT-LAMP assay developed were estimated as 4.2 x 10^{-4}, 2 x 10^{-4} and 1.1 x 10^{-4} $TCID_{50}$/mL for FMDV serotypes O, A and Asia1 respectively. LAMP assay for FMD diagnosis was validated by simultaneous testing of the clinical samples (n = 139) by mPCR and LAMP and the results revealed higher sensitivity in case of LAMP.

Real time PCR assay

Reverse-transcription real time PCR (RT-qPCR) assays have been developed and evaluated for the identification of FMDV in different parts of the world using fluorogenic dyes. Both SYBR Green and TaqMan chemistries have been widely utilised in qPCR assays for FMD, however TaqMan provide an additional advantage of multiplexing. In India, a qPCR assay targeting 1D region of FMDV was developed in multiplex format for simultaneous detection and identification of FMDV serotypes in the suspected clinical materials[37]. The sensitivity of the TaqMan based multiplex qPCR was found to be $10^{1.7}$ $TCID_{50}$/mL, $10^{1.0}$ $TCID_{50}$/mL, $10^{1.7}$ $TCID_{50}$/mL for serotype O, Asia1 and A respectively[37]. The qPCR assay was found to be more sensitive than gel based assay and provides an estimate through standard curve of viral load in the samples. With high sensitivity and specificity, the qPCR assay has been used as the primary tool for the detection of FMDV in persistently infected carriers among exposed ruminants which is of great importance in disease control[38].

FMD diagnosis in semen and milk

FMDV can be actively secreted in semen of FMD infected bull before onset of clinical symptoms and up to 5-8 mo post infection[39]. It has also been reported that FMDV can survive in frozen semen straw, thus artificial insemination can possibly serve as the source of FMDV transmission to wider and farther areas. The extenders used during the production of semen straws provide the conditions conducive to survival of the virus for more than 320 d when stored at -50 ℃[40]. Routinely used FMD diagnostic methods such as VI and antigen ELISA require modifications for detecting FMDV in semen samples[41,42]. Even mPCR assay was found to be far less sensitive for semen samples. The major reason behind PCR failure was the presence of PCR inhibitors in semen[28,39]. Hence, existing mPCR assay was improvised for the detection of FMDV genome in semen samples[43]. The RNA from suspected semen samples (neat or extended) was extracted by a modified method to remove the PCR inhibitors[43]. This modified mPCR has been used for screening of 980 animals for presence of FMDV genome in semen till now. It was also established that, FMDV could be detected in semen of the infected cattle bull for about 5 mo but not more than 8 mo[43].

LINEAGE DIFFERENTIATING PCR

There is co-circulation, extinction, and emergence and re-emergence of genotypes/lineages within the serotypes from time to time in India. The emergence or re-emergence of any new lineage warrants rapid and accurate detection to facilitate early warning[44,45]. Detailed nucleotide sequence of these viruses are analysed to detect emergence of any new group. A rapid multiplex PCR assay was developed for detection of the dominating $VP3^{59}$-deletion group of serotype A

Table 3 Details of the total number of serum samples screened for reactivity to foot-and-mouth disease virus NSP 3AB3 during the last five years in India

Year	Total samples tested	Total positive	% animals 3AB3 reactors in India
2009-2010	29763	8303	27.90
2010-2011	31042	8341	26.87
2011-2012	37467	10410	26.09
2012-2013	40934	10811	26.41
2013-2014	52224	15268	29.20
Total	191430	53133	27.70

virus with 100% sensitivity and specificity[44]. Genotype differentiating RT-PCR was developed as a fast, cost-effective and user-friendly alternative to 1D region based phylogeny for detection and differentiation of genotypes VI and VII of serotype A[45]. Similarly, a simple, fast and multi-primer RT-PCR assay has been developed and validated to differentiate genetic lineages of serotype Asia1 viruses[46]. These assays have been proven as useful tools in preliminary molecular epidemiological investigation of FMD in the country.

SERO-SURVEILLANCE OF FMD IN INDIA

Sero-surveillance is of prime importance in India where FMD control programme is in operation for last 10 years. As per the OIE guidelines, in regions adopting vaccination to control FMD, sero-surveillance should be performed by an assay capable of differentiating infected from vaccinated animals (DIVA)[47]. Detection of antibodies against various non-structural proteins (NSPs) of FMDV has been successfully utilized for DIVA[48,49]. Considering the complex epidemiology of the disease in the country, assays for DIVA were developed and validated taking into account the factors such as vaccine quality (in terms of level of NSP contamination in the formulation) and coverage in India. A tool box of one competitive and four indirect ELISAs utilizing 3AB3, 3ABC, and truncated 2C ($2C_t$) NSPs of FMDV (Figure 1) was developed in India[50-52]. The performance of these in-house DIVA assays was compared with the two commercially available kits (PrioCheck® FMDV-NS and Svanovir FMDV 3ABC-Ab ELISA kit) and indigenously developed assays were found to be equally capable in detecting infected animals among the vaccinated population[53]. However, the in-house assays performed better than the commercial kits in case of intensively vaccinated samples[53]. The r3AB3 indirect ELISA is routinely used for countrywide screening of bovines[51] and results obtained for the serum samples collected at random from the country are presented in the Table 3. The diagnostic sensitivity of this assay is 96% while the diagnostic specificity varied between the naïve and vaccinates as 99.1% and 96.4%, respectively. This assay detects antibodies to 3AB (3AB-Ab) from 10 to as late as 900 d post-infection in experimentally infected cattle. Recently 3B[54], 2B[55] and 3D[56] NSP based assays have also been developed in India and are under validation.

SERO-MONITORING OF FMD

Post vaccination sero-monitoring is critical to monitor protective antibody level in animals before and after every round of vaccination. Under the Government of India initiated vaccination based FMD control programme (FMDCP) 120 million cattle and buffaloes are routinely vaccinated at 6 mo interval to progressively build herd immunity[5]. However, vaccines against FMD only protect the animal from clinical disease and not from the super infection by other serotypes of FMDV. Additionally, the vaccine induced protection remains only for about 4-6 mo[57] and with the decline in herd immunity risk of clinical disease increases due to the creation of infection window. Therefore, quantitative estimation of protective antibody response (titer) in vaccinated animals through sero-monitoring is indispensable for devising appropriate vaccination regime and successful implementation and monitoring of the control programme[58,59]. With the current sampling policy in the country, village is considered as a herd and from each district covered under FMDCP, 10 villages are randomly selected for sampling, and from each village 20 serum samples (10 cattle and 10 buffalo) are collected at random before (0 d) and 28 d post vaccination (dpv) to have un-biased estimate of vaccination performance and the resulting level of herd immunity. Antibody titers against the serotypes O, A and Asia1 are determined by four fold dilution liquid phase blocking ELISA (LPBE)[60,61]. With the expansion of FMDCP, there is a considerable rise in the number of serum samples to be tested. Thus, a high throughput LPBE assay was developed recently to fasten the process and save time and labour (Manuscript communicated). This high throughput assay utilizes the linear regression method for extrapolation of titers of test serum samples from the known internal controls[60]. In addition, the reagents used in the assay are thermo-stable facilitating the transportation to the regional laboratories under high ambient temperature.

CONCLUSION

Considering the fact that India has a large livestock population (about 500 million) susceptible to FMD, the country requires economical companion diagnostic tests tailor-made for the suitability under Indian scenarios to run the progressive control programme for FMD. Though India is now self-sufficient to produce most of the diagnostic kits, but still a lot of improvisation is needed in the current assays. The polyclonal antibodies used in several assays could be replaced with the recombinant antibodies. Some success has been achieved in development of single-chain variable fragment (scFv)[62] but work is being continued to develop scFv and nanobodies against highly immunogenic epitopes of FMDV and assess their applicability in diagnostics.

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SYSTEMATIC REVIEWS

Associations among depression, suicidal behavior, and quality of life in patients with human immunodeficiency virus

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Abstract

AIM: To investigate the potential associations among major depression, quality of life, and suicidal behavior in human immunodeficiency virus (HIV) patients.

METHODS: A detailed MEDLINE search was carried out to identify all articles and book chapters in English published from January 1995 to January 2015.

RESULTS: Based on the main findings, the prevalence of major depressive disorder (MDD) ranged from 14.0% to 27.2%. Furthermore, the prevalence of suicidal ideation varied from 13.6% to 31.0% whereas, attempted suicides were reported to range from 3.9% to 32.7%. Interestingly, various associated risk factors for both depression and suicide were identified in HIV patients. Finally, consistent associations were reported among MDD, suicidal ideation, and poor quality of life in individuals living with HIV.

CONCLUSION: Although additional studies are needed to elucidate this complex association, our results suggest the importance of early detection of both MDD and suicidality in patients living with HIV.

Key words: Major depression; Suicidal behavior; Quality of life; Human immunodeficiency virus infection

Core tip: Among patients with human immunodeficiency virus (HIV) the prevalence of major depressive disorder (MDD), suicidal ideation, and attempted suicides ranged from 14.0% to 27.2%, from 13.6% to 31.0%, and from 3.9% to 32.7%, respectively. Multiple risk factors for both depression and suicide were identified in HIV patients. Importantly, a consistent association has been reported between MDD, suicidal ideation, and poor quality of life in individuals living with HIV. The early detection and adequate treatment of depressive symptoms and suicidality should be considered fundamental tasks when managing HIV infected patients, particularly in those individuals who are severely medically ill.

Serafini G, Montebovi F, Lamis DA, Erbuto D, Girardi P, Amore M, Pompili M. Associations among depression, suicidal behavior, and quality of life in patients with human immunodeficiency virus. *World J Virol* 2015; 4(3): 303-312 Available from: URL: http://www.wjgnet.com/2220-3249/full/v4/i3/303.htm DOI: http://dx.doi.org/10.5501/wjv.v4.i3.303

INTRODUCTION

Chronic medical conditions such as human immunodeficiency virus (HIV) infection have been found to be associated with elevated stigma and discrimination, psychological distress, and poor social support[1]. Almost half of individuals diagnosed with HIV suffer from one or more comorbid psychiatric disorders[2] and experience a poorer health-related quality of life compared to individuals without comorbidities[3].

Major depressive disorder (MDD) is a highly comorbid psychiatric condition in patients with HIV, and the presence of MDD is associated with poor adherence to treatment, disease progression, and lower quality of life[4]. It has been well established that depressive symptoms may contribute to both HIV progression and mortality[5]. For example, in a 7-year longitudinal study, Ickovics *et al*[6] found that women with HIV and chronic depressive symptoms had twice the risk of dying by suicide compared to those with few or no depressive symptoms. Specifically, the mortality rates were 54% among patients with chronic depressive symptoms and 48% among those with intermittent depressive symptoms as compared to 21% for women having few or no depressive symptoms and low CD4 cell counts[6]. MDD is also associated with premature drop-out and poorer outcomes after treatment[7,8], as well as persistent drug use in heroin users[9].

In addition to depression, suicidality, which includes both suicide ideation and attempts, is considered to be another major psychiatric problem associated with HIV/acquired immune deficiency syndrome (AIDS)[10]. Similar to MDD, an association between suicidality, poor quality of life, poor adherence to antiretroviral therapy, and non-disclosure of HIV status to significant others has been also reported[10,11]. Overall, socio-demographic variables (*e.g.*, female gender, younger age); psychiatric conditions such as substance abuse, MDD, and a history of prior suicide attempts; neuropsychiatric side-effects of antiretroviral therapy and psychotropic medication; psychosocial factors including heterosexual orientation, poor social support, loss of employment, maltreatment, and sexual abuse; and clinical factors (*e.g.*, stress reactions, the perception of pain, physical impairment, psychological/physical symptoms, and AIDS diagnosis) have been found to contribute to suicidality among HIV patients[12-19].

Thus, individuals with HIV infection may have a higher risk of suicide than those individuals without HIV[20-22]. In addition, many individuals living with HIV are reluctant to disclose their HIV serostatus to friends and/or family due to the fear of stigmatization[23,24]. Moreover, individuals who experience this type of fear may have disadvantages with regards to seeking HIV testing, education, or treatment[25].

Interestingly, the introduction of antiretroviral medications significantly improved both HIV health-outcomes and life expectancy of HIV infected patients, which led to a significant reduction of suicide rates[26]. However, patients living with HIV/AIDS are still dying in large numbers and, therefore, examining quality-of-life issues remains an important area of research[27]. In particular, individuals with HIV reported profound alterations in day-to-day activities, significant relationships, and health status[28]. Accordingly, several psychometric instruments and health questionnaires have been specifically developed to evaluate quality of life among individuals with HIV.

Based on the current literature, it remains unclear whether or not individuals with HIV and poor quality of life are at a higher risk of depression/suicidality than those with HIV and a higher quality of life. Thus, the present review aimed to investigate the nature of the associations between MDD, quality of life, and suicidal behavior in HIV patients.

MATERIALS AND METHODS

In order to provide a critical review of the associations among depression, suicidality, and quality of life among patients with HIV infection, we performed a detailed search using the largest existing databases (PubMed/MEDLINE, Scopus, Web of Science, and Psychinfo) to identify all articles and book chapters in English published between January 1995 and January 2015. Specifically, the following search terms were used: "Major depression" OR "Major Depressive Disorder" OR "MDD" AND "Suicidal Behavior" OR "Suicide attempts" OR "Suicide ideation" OR "suicidality" AND "Quality of life" AND "HIV infection." Full-text articles were evaluated for relevance when a title or abstract appeared to describe a study eligible for inclusion. Abstracts that did not explicitly mention the association between depression, suicidality, and quality of life among individuals living with HIV were excluded. We also excluded meta-analytic studies and reviews. Overall, we identified 36 articles; however, only 12 full-text articles included in our review.

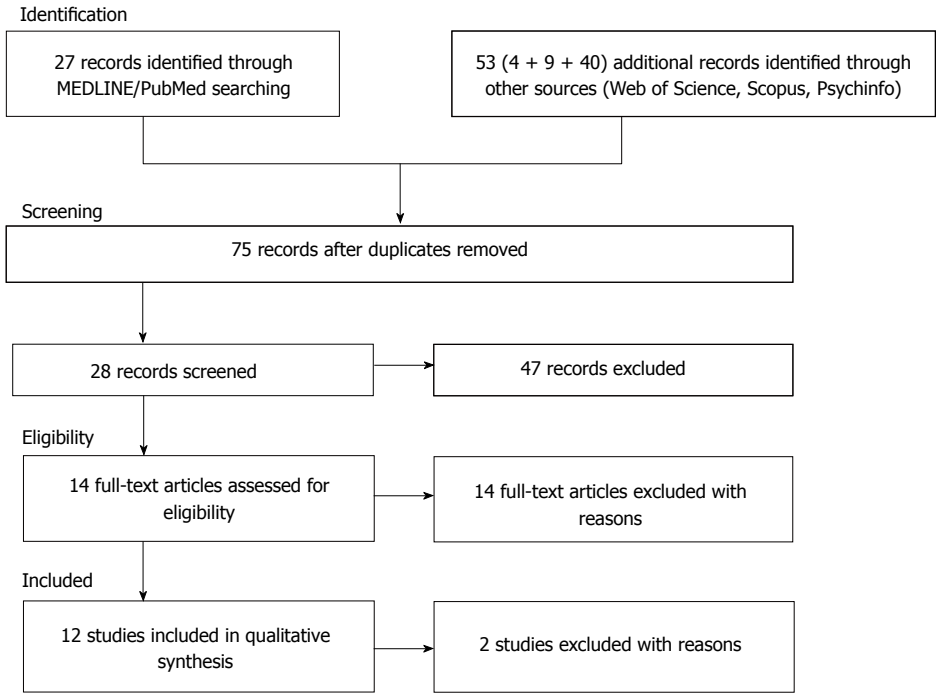

Figure 1 Flowchart of the search and selection process.

Study design and eligibility criteria

To achieve a high standard of reporting, we have adopted Preferred Reporting Items for Systematic Reviews and Meta-Analyses' (PRISMA) guidelines[29]. The PRISMA Statement consists of a 27-item checklist and a four-phase flow diagram for reporting in systematic reviews. PRISMA includes the broader effort to improve the reporting of different types of health research as well as to improve the quality of research used in decision-making in healthcare.

RESULTS

Number of studies selected

The combined search strategy yielded a total of eighty articles of which, after a complete analysis, twenty-eight full-text articles were screened and fifty-two were excluded. We excluded articles that were not published in peer reviewed journals, those that were not in English language, articles without abstracts, abstracts that did not explicitly refer to the main topic, and those with unclear data regarding materials and methods and subjects which were analyzed. We assessed fourteen articles for eligibility but two full-text articles were excluded due to low-relevance to the main theme. Therefore, twelve articles fulfilled our inclusion criteria and were included in the present review (Figure 1).

Type of studies selected

Studies that investigated the prevalence of depression, suicide ideation, suicide thoughts, in patients living with HIV: Nine studies investigated the prevalence of suicide ideation/thoughts and associated risk factors in HIV infected patients. Ogundipe *et al*[30] found that 13.6% of 295 people living with HIV/AIDS (PLWHA) reported suicidal ideation.

Moreover, Kinyanda *et al*[31] demonstrated that the prevalence of moderate to high risk for suicidality (MHS) and lifetime suicide attempts of 618 recruited patients was 7.8% and 3.9%, respectively.

In a sample of 62 randomly selected HIV+ women, Lewis *et al*[32] investigated the existence and impact of major depression. The researchers also found that activities of daily living (ADL) and the subjective questionnaire of cognitive functioning were useful instruments to assess depression.

In another study, the clinical/behavioral characteristics of 71 patients in northern Taiwan as related to their HIV status have been explored by Lee *et al*[33]. Based on the main findings, anxiety was reported in 21.0% of patients, depression in 27.2%, memory alterations in 32.7%, and suicide attempts in 32.7%. Atkinson *et al*[34] also reported that HIV+ individuals (N = 203) reported a significantly higher rate of lifetime MDD than HIV- participants (14% *vs* 5%). However, both HIV+ and HIV- reported similar rates of current MDD.

Furthermore, Sherr *et al*[11] demonstrated that the prevalence of suicidal ideation was 31% in a sample of 778 patients living with HIV. Interestingly, heterosexual men and black respondents were twice as likely to experience suicidal ideation when compared to gay men or women and White/Asian respondents. In addition, individuals who did not disclose their HIV status were twice as likely to report suicidal ideation as compared to other subjects.

In another study conducted in a sample of 28 Chinese HIV+ participants and 23 matched HIV- controls, nearly 79% of HIV infected individuals had a significantly higher

lifetime rate of major depression relative to 4% of the comparison group[35]. Moreover, 18% of these patients reported active suicidal thoughts.

Haller and Miles[36] also examined suicidality among 190 HIV+ participants and reported that 26% had suicidal thoughts within 30 d of hospitalization, 49% revealed that they had a suicide plan, and 48% indicated they had suicide intent. Further, individuals with suicidal ideation had predominantly MDD (64%).

Finally, Kalichman *et al*[37] found that in a sample of individuals aged 45 or older and living with HIV/AIDS (N = 113), 27% reported suicidal thoughts.

Studies that investigated the associated risk factors in patients living with HIV: Six studies investigated the relevance of associated risk factors in HIV infected patients. Ogundipe *et al*[30] identified unemployment, emotional distress, religion, HIV status non-disclosure, and previous suicidal attempt as significant predictors of suicidal ideation in their sample. In another study, Kinyanda *et al*[31] reported that significant unique predictors of MHS included female gender, increasing negative life events, a previous psychiatric history, and MDD.

Other articles focused on the importance of associated risk factors in patients diagnosed with HIV. For example, sexual intercourse without condoms during the six previous months were found more frequently in HIV-negative heroin users compared to HIV-positive heroin users[33] whereas higher levels of physical and psychological symptoms independently predicted suicidal ideation[11].

Furthermore, Haller and Miles[36] suggested that individuals with MDD, dysthymia, substance abuse, thought disorders, post-traumatic stress disorder (PTSD), or borderline/avoidant personality disorders were more likely to report suicidality.

Interestingly, subjects with suicidal thoughts more frequently used escape and avoidance whereas positive-reappraisal coping strategies were used less frequently[37]. The authors[37] demonstrated the existence of associations among suicidal thoughts and perceived poor social support from friends/family even after controlling for depression.

Studies that analyzed the association between depression, suicidality and quality of life in HIV patients: Eight of the included studies examined the associations between depression, suicidality and quality of life in samples of HIV patients. First, a significant association between suicidal ideation and being unmarried, poor medication and quality of life was found by Ogundipe *et al*[30] in a sample of 295 PLWHA.

Furthermore, Pompili *et al*[38] reported that HIV patients with a poorer health-related quality of life (HRQoL) had higher hopelessness levels (these subjects were at high suicide risk) and were more likely to have depression than those with a higher HRQoL. In addition, higher scores on all dimensions of the Temperament Evaluation of Memphis, Pisa, Paris and San Diego-self administered version (TEMPS-A) were reported in patients with a poorer HRQoL relative to those with higher HRQoL. Furthermore, Atkinson *et al*[34] reported that 203 HIV+ were more likely to have a lifetime substance use diagnoses than HIV- participants (14% *vs* 6%). They also found that worse daily functioning and life quality as well as unemployment were independently predicted by both depression and HIV status.

Palliative care and quality-of-life issues are still two relevant areas of research in patients with advanced AIDS. Breitbart *et al*[39] investigated the impact of treatment for depression on the desire for hastened death in a sample of 372 patients with advanced AIDS. They reported a significant association between desire for death and depression; also, desire for death was reduced in those patients who responded to antidepressants medications. However, nearly half of those individuals who received antidepressant medications and/or supportive psychotherapy or counseling showed little or no improvement in depressive symptoms. In another study, Jin *et al*[35] found that worse daily functioning was independently predicted by both major depression and HIV+ status in a sample of 28 Chinese HIV+ participants and 23 matched HIV- controls.

Furthermore, in a sample of HIV-infected women directly after diagnosis, Krabbendam *et al*[40] found that the women experienced strong emotions and quality of life impairment, suggesting that high emotional distress may be occurred in specific phases of HIV illness. In addition, Haller and Miles[36] demonstrated a significant association between quality of life variables and suicide ideation in 190 HIV patients. Specifically, leisure/social and family/friends were strongly associated with suicidal ideation in their sample. Finally, higher emotional distress together with poorer HRQoL were found in a sample of individuals living with HIV-AIDS (N = 113) who reported suicide thoughts as compared to those who had not considered suicide[37] (Tables 1 and 2).

DISCUSSION

The present mini-review aimed to investigate the associations among MDD, quality of life, and suicidal behavior.

First, our findings indicated that the prevalence of a current MDD diagnosis varied from 14.0% to 27.2% according to the selected samples[33,34]; however, as high as 79% of HIV patients reported a lifetime diagnosis of MDD[35]; Second, the prevalence of suicidal ideation ranged from 13.6% to 31.0%; whereas, the prevalence of attempted suicide ranged from 3.9% to 32.7%[11,30,31,33]; Third, various associated risk factors for depression and suicide were found to be important in HIV patients[11,30,31,33,36,37].

Other recent studies[41-43] confirmed this high but more variable prevalence of MDD in HIV patients ranging between 18% to 81% at some stage of the illness according to the different populations which were investigated, the different study designs as well as the different diagnostic

Table 1 Studies that investigated using specific psychometric instruments the association between depression/suicidality and quality of life in human immunodeficiency virus patients

Ref.	Study design	Sample size	Follow-up	Psychometric instruments assessing MDD, suicidality, and quality of life	General findings	Limitations	Conclusion
Ogundipe *et al*[30]	Cross-sectional study	295 PLWHA (102 males and 153 females; mean age 37.3 ± 8.7 yr)	No	GSQ-28, BDI, and WHOQOL-BREF	Overall, 13.6% of PLWHA reported suicidal ideation. A significant association between suicidal ideation and being unmarried, poor medication adherence and altered quality of life has been reported. Unemployment, emotional distress, religion, HIV status non-disclosure and previous suicidal attempts were significant predictors of suicidal ideation among PLWHA	(1) The cross-sectional nature of the study; (2) Subjects have been not assessed for the presence of prior suicide attempts; (3) Participants have been not evaluated during a follow-up period	Suicide should be considered a major health issue in subjects with HIV infection. Specific psychosocial and clinical factors may be useful to identify PLWHA who are at-risk for suicide
Pompili *et al*[38]	Cross-sectional study	88 outpatients (71 men and 17 women; mean age 42.9 ± 10.3 yr)	No	GMDS, BHS, SHSS, TEMPS-A, and SF-36	More severe depression and hopelessness have been found between patients with a poorer HRQoL when compared to those with a higher HRQoL. Higher scores on all dimensions of the TEMPS-A were also reported in those with a poorer HRQoL relative to subjects with a higher HRQoL	(1) The small sample size; (2) The cross-sectional nature of the study; (3) Data on HIV severity, illness duration, or age of symptom onset were not collected; (4) Data were collected *via* self-report and not validated by psychiatric examinations	Patients with a poorer HRQoL were more likely to have depressive affective temperaments, depression and suicide risk than patients with higher HRQoL
Kinyanda *et al*[31]	Cross-sectional study	618 HIV outpatients (169 male, 449 female; mean age in the 25-44 age band)	No	M.I.N.I., coping style index derived by variables of the MAC, and International HIV Dementia Scale	Prevalence of MHS and life-time attempted suicides resulted 7.8% and 3.9%, respectively. After univariate analyses, female gender, food insecurity, increasing negative life events, high stress score, negative coping style, past psychiatric history, psychosocial impairment, diagnoses of PTSD, GAD, and MDD resulted associated with MHS. After multivariate analyses, only female gender, increasing negative life events, a previous psychiatric history, and MDD were independently associated with MHS	(1) The cross-sectional nature of the study; (2) the small number of subjects with some of the diagnosed psychiatric disorders; (3) the threshold as a cut-off point for MHS has been not validated in the African socio-cultural context; (4) the use of the "risk for suicidality" measure instead of "suicidality"	Both social and psychological stressors may act on previous and current psychiatric morbidities triggering suicidality
Lewis *et al*[32]	Cross-sectional study	62 HIV-positive women (mean age 35.7 ± 6.6 yr)	No	BDI-FS, MM of the Primary Care Evaluation of Mental Disorders, ADL, and SCQ	ADL and subjective questionnaire of cognitive functioning were useful instruments to measure depression in HIV-positive women	(1) The cross-sectional nature of the present data; (2) The small sample size which may limit the generalization of findings; (3) Participants have been not evaluated during a follow-up period; (4) the sample includes only women	Diagnosis of depression is of great importance, not only clinically, but also to ensure the judicious allocation of scarce medical resources in the regions worst affected by HIV
Lee *et al*[33]	Cross-sectional study	576 patients (503 male, 73 female; mean age 40.6 ± 9.3 yr) of which 71 were HIV positive, and 514 had hepatitis C	No	A semi-structured questionnaire assessing demographics, quality of life, HIV risk behavior, and psychiatric symptoms,and WHOQOL-BREF	Overall, 21.0% of the subjects reported anxiety, 27.2% depression, 32.7% memory loss, and 32.7% attempted suicide. Based on the main findings, HIV-negative heroin users were more likely to have sexual intercourse without condoms during the six previous months	(1) The sample may be not representative of the Taiwanese heroin users population; (2) It was not possible to validate whether patients replied the questions truthfully	No significant differences were found between the HIV-positive and HIV-negative patients on psychiatric symptoms or quality of life

Atkinson *et al*[34]	Cross-sectional study	203 HIV-infected former plasma donors and 198 HIV-negative donor controls (122 male, 279 female; mean age 40.2 ± 6.4 yr)	No	WMH-CIDI, BDI-II, MOS-HIV, Modified HIV Stressor Scale, ADL, and Social Support Scale	HIV+ subjects reported a significantly higher rate of lifetime MDD (14% *vs* 5%) than HIV- participants. Both HIV+ and HIV- reported similar rates of current MDD. HIV+ were more likely to have lifetime substance use diagnoses than HIV- (14% *vs* 6%). Importantly, worse daily functioning and life quality as well as unemployment were independently predicted by both depression and AIDS	(1) Rates of depression may be underestimated by the used psychometric measures; (2) Recurrence of MDD episodes and bipolar disorder cases have not been examined; (3) The sample is derived by an agrarian setting; (4) The preliminary nature of the findings	High lifetime rates of MDD and suicidality were found in this HIV-infected agrarian cohort presumably due to the existence of a pre-HIV mood disorder, direct effects of HIV, social stigma, negative impact of HIV/AIDS on employment together with the perception that HIV is a terminal condition
Sherr *et al*[11]	Cross-sectional study	778 HIV-positive clinic attenders (183 heterosexual women, 76 heterosexual men, 496 gay/bisexual; mean age 40.5 yr)	No	Suicidal ideation reported using a self-report item based on feelings in the preceding week, levels of optimism in relation to treatment and infectiousness, MSAS short-form, and EuroQol-5D	Suicidal ideation was reported by 31% of patients. Heterosexual men and black respondents were twice more likely to have suicidal ideation relative to gay men or women and White/Asian respondents, respectively. Also, those with lack of disclosure were twice more likely to have suicidal ideation than those without. Higher physical and psychological symptoms independently predicted suicidal ideation	(1) The cross-sectional study design; (2) Subjects have been not evaluated for the presence of previous suicide attempts; (3) Participants have been not tested during a follow-up period	Suicidal ideation rates among HIV-positive clinic attenders were high
Jin *et al*[35]	Cross-sectional study	28 HIV+ participants and 23 matched HIV- controls (38 male, 13 female; mean age 35.4 ± 6.7 yr)	No	CIDI Depression Module, BDI-I, Module E of the CIDI assessing lifetime suicidality, ADL	Overall, 79%of HIV-infected subjects had a lifetime rate of major depression relative to 4% of the comparison group. 9% of patients received treatment for depression, but 18% showed active suicidal thoughts. Worse daily functioning was independently predicted by both depression and HIV+ status	(1) The small sample size that may limit the generalization of the present findings; (2) The effects of gender could be not separated; (3) The sample was selected for feasibility purposes	High rates of major depression and suicidality have been found in HIV-infected Chinese subjects

ADL: Activities of daily living; BDI: Beck Depression Inventory; BHS: Beck Hopelessness Scale; BDI-FS: Beck Depression Inventory-Fast Screen for Medical Patients; CIDI Version 2.1: Composite International Diagnostic Interview Depression module; DDRS: Desire for Death Rating Scale; GAD: Generalised anxiety disorder; GSQ-28: General Health Questionnaire; GMDS: Gotland Male Depression Scale; HRQoL: Health-related quality of life; MDD: Major depressive disorder; M-QOL: McGill Quality of Life Questionnaire; MSAS: Memprial Symptom Assessment Schedule; MAC: Mental Adjustment to Cancer Scale; MM: Mood Module; MHS: Moderate to high risk for suicidality; MOS-HIV: Medical Outcomes Study-HIV; PLWHA: People living with HIV/AIDS; PTSD: Post-traumatic stress disorder; SAHD: Schedule of Attitudes toward Hastened Death; SF-36: Short-Form 36-Item Health Survey; SCQ: Subjective Complaints Questionnaire; SHSS: Suicidal History Self-Rating Screening Scale; TEMPS-A: Temperament Evaluation of Memphis, Pisa, Paris and San Diego-auto questionnaire version; M.I.N.I.: The Mini-International Neuropsychiatric Interview; WHOQOL-BREF: World Health Organization Quality of Life Assessment-Brief Version; WMH-CIDI, version 3.0: World Mental Health Composite International Diagnostic Interview; HIV: Human immunodeficiency virus.

criteria which were used.

As reported by Hirsch Allen *et al*[44], depression may be evaluated both dimensionally as well as categorically and this is the first source of variability.

In addition, MDD in HIV patients may vary according to several variables such as the population of interest, main research hypotheses as well as comparisons with other studies/populations. Importantly, depressive symptoms that do not meet diagnostic criteria may be also associated with significant psychosocial impairment and disability[44]. Moreover, the role of somatic symptoms related to depression may be frequently neglected in HIV infected patients due to their frequent overlapping with somatic complaints directly related to the disease.

Overall, clinicians should carefully consider that screening, diagnosing, and quantifying depressive symptoms represent three different but equally critical/challenging tasks when managing depressed HIV infected patients.

MDD often contributes to the negative psychological effects of HIV, increases emotional distress, and exerts a critical impact on adherence to treatment over time in HIV-infected individuals[45]. Clinicians encounter a challenging task in diagnosing MDD in HIV patients given the complex nature of this association. One of the most debated issues is whether or not MDD is a manifestation of HIV brain disorder or, conversely, MDD should be considered the primary disorder that may be exacerbated by the presence of

Table 2 Studies that investigated without using specific psychometric instruments the association between depression/suicidality and quality of life in human immunodeficiency virus patients

Ref.	Study design	Sample size	Follow-up	Quality of life instruments	General findings	Limitations	Conclusion
Breitbart *et al*[39]	Follow-up study	372 patients with advanced AIDS, of which 42 were re-assessed at the follow-up (280 men, 92 female; mean age 44.4 ± 9.4 yr)	2-mo follow-up	Depression module of the SCID, HIV version, Ham-D, SAHD, DDRS, no specific psychometric instruments were used to measure quality of life	A significant association between desire for death and depression was found but desire for death was reduced in those patients who responded to antidepressants medications. However, approximately half of subjects who received antidepressant medications and/or supportive psychotherapy or counseling demonstrated little or no improvement in depressive symptoms	(1) The study was not a controlled clinical trial of antidepressant therapy; (2) Systematic bias (*e.g.*, with more refractory patients being less likely to remain in the study) may be not excluded; (3) The failure to find significant differences about the proportion of patients with a high desire for hastened death may reflect the limited power of these analyses	Depressed patients who were successfully treated with antidepressant medications reported a significant reduction of desire for death
Haller *et al*[36]	Cross-sectional study	190 HIV patients (129 male, 61 female; mean age 37.3 ± 7.4 yr)	No	UM-CIDI, MCMI-III, Suicide Screener (seven-item structured interview), quality of life derived by HIV-PARSE	Overall, 26% of subjects reported suicide thoughts within 30 d of admission, 49% a suicide plan, and 48% a suicide intent. Individuals with suicidal ideation had predominantly MDD (64%), drug dependence (52%), and depressive personality disorder (50%). After regression analyses, those with MDD, dysthymia, substance abuse, thought disorder, PTSD, and borderline/avoidant personality disorders were more likely to have suicidality. Concerning the quality of life variables which were measured, leisure/social and family/friends were strongly associated with suicidal ideation	(1) The cross-sectional nature of the findings; (2) No specific psychometric instruments were used.	Subjects with substance use disorders, unstable interpersonal relations, and a restricted social environment may be considered at-risk individuals and need to be regularly screened for suicidality
Kalichman *et al*[37]	Cross-sectional study	113 HIV-AIDS subjects (mean age 53, age range 47-69)	No	Beck Depression Index, and WOC	Subjects who reported suicide thoughts (27%) have also higher emotional distress and poorer health-related quality of life relative to those who had not considered suicide. Furthermore, escape and avoidance were more frequently used whereas positive-reappraisal coping strategies were less frequently used by those with suicide thoughts. An association between suicide thoughts and the perception of reduced social support from friends and family was also reported. The mentioned differences remained even after controlling for symptoms of depression	(1) The small sample size; (2) The cross-sectional nature of the findings. These factors may limit the generalization of the findings	Relevant emotional distress and suicide thoughts were experienced by subjects in midlife and older individuals with HIV-AIDS
Krabbendam *et al*[40]	Cross-sectional study	24 HIV women (mean age 32 yr with a range of 20-49 yr)	No	In depth interviews using a qualitative semi-structured approach providing insights into feelings, perceptions, beliefs	Strong emotions and quality of life impairment were experienced by HIV-infected women directly after diagnosis. It has been suggested that one counseling session was not effective	(1) The small sample size and the cross-sectional nature of the findings may seriously limit the generalization of the present findings; (2) Counseling given once was reported to be not effective	Continuous counseling may be provided by support groups. Importantly, the counselors may be used as examples

DDRS: Desire for Death Rating Scale; SCID: Depression module of the Structured Clinical Interview for DSM-IV, HIV version; Ham-D: Hamilton Rating Scale for Depression; MDD: Major depressive disorder; MCMI: Millon Clinical Multiaxial Inventory; PTSD: Post-traumatic stress disorder; SAHD: Schedule of Attitudes toward Hastened Death; UM-CIDI: University of Michigan Composite International Diagnostic Interview; WOC: Ways of Coping Questionnaire; HIV: Human immunodeficiency virus.

HIV. Interestingly, some authors hypothesized that MDD and its clinical presentation should be considered an adjustment reaction to the diagnosis of HIV infection[41].

Moreover, the presence of depression may significantly impair the number and activity of lymphocytes in HIV-positive patients dramatically reducing the role of natural killer cells, which increases the mortality in this population[41,42,46]. Del Guerra *et al*[43] have suggested that HIV may predispose patients to the onset of MDD through the interaction between the following neurobiological mechanisms: (1) Chronic increase of inflammatory cytokines and abnormal activation of microglia and astrocytes; (2) Consistent reduction of monoamine levels; (3) Neurotoxicity; and (4) Reduction of neurotrophic factors and subsequent impaired neuroplasticity processes, and psychosocial factors.

Our findings indicate a significant association between suicide ideation/thoughts and poor quality of life in HIV patients[30,36,37,40]. Moreover, results also suggest that depression was significantly associated with[38,39] or predicted a poor quality of life in HIV patients[34,35]. According to population-based studies[47], an higher prevalence of suicide in subjects with HIV may be found relative to the general population, and comorbid mood disorders may be identified in more than half of subjects. Among HIV infected individuals, those with AIDS were more likely to report current suicidal ideation, lifetime suicidal thoughts, and suicide plans compared to those without AIDS. This may be explained by the fact that individuals diagnosed with AIDS usually have a poorer HRQoL than those with HIV infection. Also, the presence of severe depressive symptoms in these patients was a significant predictor of daily functioning together with unemployment, and life quality[34,48]. Moreover, depressive symptoms may be independent predictors of significant impairment in daily functioning and quality of life regardless of the effects of HIV, as suggested by Jin *et al*[35]. The negative consequences of MDD on daily functioning and employment have also been found in previous studies[49]. Taken together, these findings suggest the clinical relevance of early detection and adequate MDD treatment, particularly in those individuals who are severely medically ill.

The present review should be considered in the light of limitations. First, some included studies may reflect the authors' choice according to their expertise and may include small sample sizes, which limit the generalization of the findings; Second, most studies were cross-sectional in nature and did not allow for causal interpretations of the associations between depression, quality of life, and suicidality in HIV patients; Third, some studies predominantly investigated the presence of suicide ideation instead of considering the impact of prior suicide attempts on the quality of life of HIV patients; Fourth, some studies were limited by the possible underestimation of MDD and suicidal behavior rates given the use of self-reported psychometric instruments and the limited power of some analyses to find significant differences regarding the proportion of patients with depression and suicidality.

The prevalence of depression and suicidal ideation are consistent in patients with HIV, suggesting the importance of early detection for both these conditions in this population. Also, significant associations have been reported among MDD, suicidal ideation, and poor quality of life in HIV populations. However, further studies are necessary to elucidate this complex association.

COMMENTS

Background

It has been reported that individuals living with human immunodeficiency virus (HIV) are at risk for both depression and suicidality. Most of individuals diagnosed with HIV suffer from one or more comorbid psychiatric disorders and experience a poorer health-related quality of life compared to individuals without comorbidities.

Research frontiers

It is quite unclear whether or not subjects with HIV and poor quality of life are at higher risk of depression/suicidality compared with HIV and higher quality of life.

Innovations and breakthroughs

Among patients with HIV the prevalence of major depressive disorder (MDD), suicidal ideation, and attempted suicides ranged from 14.0% to 27.2%, from 13.6% to 31.0%, and from 3.9% to 32.7%, respectively. A significant association has been reported among MDD, suicidal ideation, and poor quality of life in HIV populations.

Applications

Further additional studies are needed to elucidate the exact nature of the association between MDD, suicidality, and quality of life in HIV patients. The early detection and adequate treatment of these conditions is absolutely recommended in clinical practice, in particular in those individuals who are severely medically ill.

Terminology

PRISMA: The PRISMA Statement consists of a 27-item checklist and a four-phase flow diagram for reporting in systematic reviews in the effort to improve the reporting of different types of health research and the quality of research used in decision-making in healthcare. Hopelessness: Hopelessness may be defined as a negative perspective concerning the future, loss of motivation, and expectations. Hopelessness predisposes patients with psychiatric disorders to suicidal behavior and has been identified as an important risk factor for suicide; TEMPS-A: (Temperament Evaluation of Memphis, Pisa, Paris and San Diego) The TEMPS-A is a self-rating questionnaire consisting of 109 items for men and 110 for women assessing subaffective trait expressions as they were conceptualized in Greek medicine and in German psychiatry.

Peer-review

The review has great clinical implication as well.

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Contents

Quarterly Volume 4 Number 4 November 12, 2015

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Volume 4 Number 4 November 12, 2015

Contents

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REVIEW

Hepatitis delta virus: A fascinating and neglected pathogen

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Abstract

Hepatitis delta virus (HDV) is the etiologic agent of the most severe form of virus hepatitis in humans. Sharing some structural and functional properties with plant viroids, the HDV RNA contains a single open reading frame coding for the only virus protein, the Delta antigen. A number of unique features, including ribozyme activity, RNA editing, rolling-circle RNA replication, and redirection for a RNA template of host DNA-dependent RNA polymerase Ⅱ, make this small pathogen an excellent model to study virus-cell interactions and RNA biology. Treatment options for chronic hepatitis Delta are scarce and ineffective. The disease burden is perhaps largely underestimated making the search for new, specific drugs, targets, and treatment strategies an important public health challenge. In this review we address the main features of virus structure, replication, and interaction with the host. Virus pathogenicity and current treatment options are discussed in the light of recent developments.

Key words: Hepatitis delta virus; Hepatitis B virus; RNA replication; Pathogenesis; Treatment

Core tip: Hepatitis delta virus (HDV) is the etiologic agent of probably the most severe form of virus hepatitis. HDV replication and spread depends on the presence of hepatitis B virus which provides the envelope proteins coded exclusively by its own genome. About 20 million people are currently chronically infected with HDV and no specific therapy is still available. Here, we review the current knowledge on HDV biology, epidemiology, pathogenesis, and treatment. Future trends and perspectives are discussed in the light of recent developments on HDV biology and its interaction with the host.

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INTRODUCTION

Over 35 years have passed since Rizzetto *et al*[1] reported the discovery of what has been called Delta antigen in a patient with diagnosis of severe hepatitis B infection. Subsequent research on the nature of this antigen led to the identification, in 1980, of a new hepatotropic virus, hepatitis delta virus (HDV)[2,3]. This new infectious agent was later found to be a sub-viral agent dependent on the presence, in infected cells, of hepatitis B virus (HBV) to accomplish the replication cycle[3,4]. In nature, both viruses, HBV and HDV, share the same envelope proteins coded exclusively by the HBV genome[5,6].

Today, the World Health Organization estimates that about 400 million people are chronically infected with HBV worldwide[7,8], of which approximately 20 million are co-infected with HDV[9,10]. The Amazon basin and some central African and east European countries are among the regions with higher prevalence. However, there is still a considerable lack of information concerning a significant number of countries mostly situated in Africa, Asia, and Latin America (Figure 1). The geographic distribution of the so far identified eight HDV clades is also far from being uniform. Clade 1 may be found worldwide, in contrast with clade 3 which seems to be confined to the Amazon region (Figure 1). The most frequent outcome of the acute co-infection with HDV is virus clearance and patient's recovery. However, in up to 5% of the infected individuals a chronic form of HDV infection will develop[11]. In the case of super-infection, when a chronic HBV carrier gets super-infected with HDV, the outcome is distinct. About 70%-90% of super-infected individuals will become chronic carriers for both viruses, HBV and HDV[12].

As compared to the individuals that are chronic carriers of HBV alone, HDV additionally increases the risk of hepatocellular carcinoma (HCC) and mortality threefold and twofold, respectively, in HDV/HBV carriers[13,14]. Currently, in clinical practice, there are no drugs used that directly and specifically target HDV. None of the currently approved anti-HBV drugs efficiently blocks HDV infection[7,9,14-17].

All of the above, given additional HDV-inflicted liver pathogenesis, and inability to efficiently circumvent HDV infection by anti-HBV drugs, makes HDV a very serious pathogen, and it does call for additional attention to HDV and development of specific anti-HDV interventions.

HDV is mostly endemic in low income countries in which the budget for new, potentially expensive drugs is, of course, not the first priority. Accordingly, development of new treatment options based on specific drugs has not only proved to be difficult (the virus apparently does not code for any specific enzymatic activity that could be targeted) but may also represent an uninteresting option for pharmaceutical companies, speaking from a strictly financial point of view.

Nevertheless, this small human pathogen bears a set of features that make it a formidable model to study fundamental aspects of host-pathogen interactions and RNA biology including mechanisms of transcription, replication, and genome evolution[18,19]. The small size and structure of the genome bearing only one open reading frame (ORF), which is edited by host enzymes, its ribozyme activity and still largely undeciphered mechanism of RNA-directed RNA replication, are prominent examples of the uniqueness of this human pathogen[19].

In this review, we will address the specific features of HDV structure and replication, its interaction with host cells and HBV. Future perspectives of research based on recent important developments will be discussed.

The virus and its replication

The virus: HDV is an enveloped spherical subviral agent about 36 nm in diameter[19]. The virus particle contains a ribonucleoprotein (RNP) core consisting of one copy of the RNA genome and approximately 200 copies of the only virus encoded protein, the Delta antigen (HDAg)[20]. The HDV envelope contains hepatitis B virus surface antigens (HBsAg), provided solely by HBV. In accordance, the two viruses share virtually indistinguishable envelopes[6].

The virus genome is a circular single-stranded RNA molecule of around 1.7 kb and negative polarity[21,22]. A significant degree of internal base-pairing (about 70% of all nucleotides) is an important feature, with potential not yet unveiled functional implications, observed in this molecule[23,24]. This structure is similar to that described for plant viroids, albeit the latters have a smaller size and do not code for any protein (Table 1). On the contrary, the HDV genome displays one ORF which codes for the only viral protein, the Delta antigen[25-27]. This protein can be found in virions under two distinct forms: Small (S-HDAg, 195 aa) and large (L-HDAg; 213 or 214 aa, depending on the genotype). L-HDAg is synthesized mainly later in the replication cycle[28,29] as a consequence of an editing mechanism that takes place in the so-called anti-genome, an exact copy of the genome that arises as a replicative intermediate during RNA replication. The editing reaction is catalyzed by cellular adenosine deaminase 1 which converts an amber stop codon into a tryptophan codon (UGG) allowing a 57 nucleotide and consequently 19 aa extension of the ORF[30,31].

Both L-HDAg and S-HDAg share the same functional domains with the exception of the L-HDAg-specific C-terminal extension, which bears an isoprenylation signal present in cysteine residue 211[32]. Farnesylation of this residue is reported to be crucial albeit not sufficient for interaction with HBsAg and subsequent virion packaging and release from the cells[33,34]. The common functional motifs are a nuclear localization signal (NLS; aa 66-75), a coiled-coil domain (aa 12-60), and a

Table 1 Similarities and differences between hepatitis delta virus and plant viroids

HDV (1700 nt)	Pospiviroidae (200-400 nt)	Avsunviroidae (200-400 nt)
Circular ssRNA	Circular ssRNA	Circular ssRNA
Extensive intramolecular base pairing	Extensive intramolecular base pairing	Extensive intramolecular base pairing
A DNA-directed RNA polymerase makes both plus and minus strands	A DNA-directed RNA polymerase makes both plus and minus strands	A DNA-directed RNA polymerase makes both plus and minus strands
Encodes for protein	No proteins encoded	No proteins encoded
Virion maturation depends on a helper virus	Replication does not depend on the presence of a helper virus	Replication does not depend on the presence of a helper virus
Symmetric rolling circle RNA replication	Asymmetric rolling circle RNA replication	Symmetric rolling circle RNA replication
Replicates in the nucleus	Replicates in the nucleus	Replicates in chloroplasts
Ribozyme activity	No ribozyme activity	Ribozyme activity

HDV: Hepatitis delta virus.

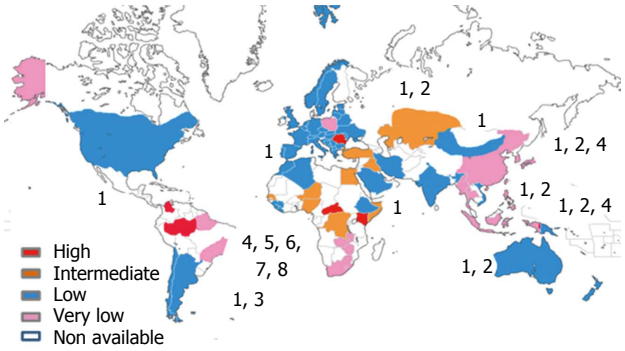

Figure 1 Prevalence and geographical distribution of eight hepatitis delta virus clades in the world.

bipartite arginine-rich RNA binding domain (aa 97-107 and 136-146; ARM1 and ARM2, respectively)[35-37]. More recently, however, it was shown that mutation in the core arginines of both ARM1 and ARM2 did not impair the RNA-binding ability of a C-terminal HDAg-160 truncated form of HDAg[38]. The authors suggested that HDAg establishes numerous contacts with HDV RNA to assemble ribonucleoprotein complexes.

Delta antigens: Several properties have been assigned to S-HDAg but none related to any known enzymatic activity. Among the reported putative and observed functions are the promotion of nuclear import of HDV RNPs[39], regulation of HDV RNA editing[40], facilitation of ribozyme cleavage (chaperone)[41,42], and facilitation of accumulation of processed RNA transcripts[43,44]. Both Delta antigens are post-translationally modified by host enzymes. Several post-translational modifications (PTM) have been described in HDAg and these include phosphorylation, methylation, acetylation, and sumoylation[45-48]. Phosphorylation occurs at multiple sites and can be mediated by different host kinases, dsRNA-activated protein kinase R, protein kinase C, and ERK1/2[49-51]. All these modifications may have distinct functional significance but it seems consensual that they are all involved in promoting virus RNA replication[52].

Methylation of Arg 13 on S-HDAg by arginine methyltransferase Ⅰ was reported and proposed to be important to enhance both genomic RNA and mRNA synthesis[46]. Additionally, cellular p300 acetyltransferase was found to acetylate Lys72 on the NLS of S-HDAg[53]. Although speculative, this modification may have impact on the efficiency of nuclear import.

Finally, sumoylation was the most recent PTM to be reported on S-HDAg. It occurs at multiple lysine residues and is catalyzed by host small ubiquitin-related modifier isoform 1. Sumoylation was proposed to be important to promote genomic RNA and mRNA synthesis[48].

Undoubtedly, these observations represent only a tiny part of the whole picture drawn by HDAgs inside the cell. In fact, Delta antigens can also be found as peptides of different smaller sizes in the nucleus of HDV replicating cells[54]. Do these additional smaller forms correspond to distinct functional features? The answer is still far from being clear as no evidence supporting this point of view have been reported. In addition, it has been shown that S-HDAg can form multimers in HDV replicating cells[20,55,56]. These multimers may play an important role in virus replication by facilitating the accumulation of virus RNAs. Moreover, it is known that HDAgs are basic proteins with an estimated overall + 12 charge[57]. Thus, it is not surprising that, at least *in vitro*, the protein can bind nonspecifically to several types of nucleic acids including dsDNA and several distinct RNAs[58].

Furthermore, S-HDAg may also be involved in sequestering and manipulating host cell components to facilitate HDV replication. In this context, it is not surprising that the search for S-HDAg interacting proteins unveiled a considerable number of potential partners. First Cao *et al*[59] used an immunoprecipitation followed by mass spectrometry approach being able to identify more than 100 host proteins in the assay. Later, Gowans *et al*[60] performed a yeast two-hybrid screen using a human liver cDNA library and identified 30 host candidate proteins capable of specifically interacting with S-HDAg. Making use of RNA silencing strategies some of these candidate interactions were found to be of potential functional significance. However, the above mentioned strong positive charge of HDAgs compels one to be careful when analyzing the specificity and role of these interactions in the HDV replication cycle.

S-HDAg is predicted to be an intrinsically disordered protein, a property already assigned to several other virus and cellular proteins[61]. This feature may be responsible for the lack of success in all, to our knowledge at least in three different laboratories, attempts to crystalize and solve the 3D structure of the Delta antigen. These properties of the Delta antigen make the study of HDV biology much more complex than perhaps initially believed. However, as we shall discuss below, they are not the only most important ones.

HDV replication: HDV replication takes place in the nucleus of infected cells[60,62,63]. The study of the HDV replication has long been difficult due to the lack of an appropriate cell culture system capable of supporting all steps of the virus life cycle, from attachment to release from the cells. Primary human hepatocytes have been long the only cells known to support the complete life cycle of HDV[64]. These are expensive and not easy to cultivate. Thus, other approaches needed to be developed and a number of alternatives arose with time. Among them are the Hepa RG cell line and the stably transfected HEK-293 cells expressing S-HDAg under the control of a tetracycline inducible promoter[65,66]. Although not representing ideal models, they became important tools for HDV research. The recent identification of the sodium-taurocholate co-transporting polypeptide (NTCP, encoded by SLC10A1) as the bona fide receptor for HBV and HDV culminated a long run that included a number of tested hypothesis and putative isolations[67,68]. It represented an important breakthrough since it allowed engineering cell lines overexpressing it and consequently also supporting the initial steps of virus attachment and entry. So far, these human NTCP-expressing cell lines include human HepG2 and Huh7 as well as mouse Hepa1-6, AML-12, and primary mouse hepatocytes[69].

After the uncoating of virus particles, HDV RNPs are transported to the nucleus, where RNA replication takes place[70]. The existing data indicates replication of the virus genome involves a double rolling-circle mechanism with formation of multimeric anti-genomic and genomic molecules[71]. These RNA multimers are cleaved at precise monomeric intervals by a rybozime activity present in both genomic and antigenomic molecules[72,73]. The presence of ribozymes in HDV RNAs is a feature shared with the viroid family of Avsunviroidae[74] (Table 1).

Although it is well established that the presence of S-HDAg stimulates virus RNA accumulation, the precise role of this virus antigen in the mechanism of HDV RNA replication remains elusive. Controversy on which host polymerase or polymerases are involved in synthesis of genomes and antigenomes lasted, for a long time. Some groups claimed that both RNA pol Ⅰ and pol Ⅱ are involved in genome and antigenome synthesis, respectively[75]. Mainly, these evidences were obtained in *in vitro* assays using different inhibitory concentrations of α-amanitin and on reports showing the presence of virus RNA in the nucleolus[75,76]. By contrast, other groups, using different types of transcription inhibitors, actinomycin D, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole, α-amanitin, provided data suggesting the involvement of solely RNA pol Ⅱ[77]. Furthermore, the presence of virus RNA in the nucleolus could not be observed in the absence of Delta antigen suggesting that this presence lacks functional relevance[62,63]. In recent years, the use of immunoprecipitation and proteomic approaches, among others, led to the identification of several pol Ⅰ, pol Ⅱ, and pol Ⅲ subunits as binding partners for HDV RNA[59]. These results need to be interpreted with care since the observed binding to HDV RNA could be a result of indirect interaction through other non-identified partners. However, independently of the host polymerase(s) involved in replication of virus RNAs a striking question is still hanging in the air: How does the virus redirect a host DNA-dependent RNA polymerase to use an RNA template? Here, the eventual participation of the S-HDAg, which as mentioned before displays a net positive charge and intrinsic disorder, may play a crucial role allowing the virus to overcome obstacles posed by the host environment for its replication.

The search for promoter sequences in virus RNA has also been followed by a few groups with inconclusive results. Yet, there is evidence from *in vivo* models supporting that mRNA synthesis initiates at nt 1630[78,79]. It may additionally be possible that multiple binding sequences for host RNA polymerases are present both in the virus genome and antigenome. This "nonspecific" binding could be a consequence of the RNA secondary structure bearing an extensive base-pairing with a number of predicted internal loops. Additionally, S-HDAg could also play an important role since it can bind nonspecifically to several nucleic acids, from dsDNA to ssRNA. It could be possible that S-HDAg plays a role as mediator between the virus RNA and a host RNA polymerase promoting its binding to several sequences in the genome and antigenome. Alternatively, S-HDAg could simply act as a chaperone, stabilizing RNA molecules and making them available for transcription. Assembly of HDV virions takes place in the cytoplasm. In this cellular compartment HBV-derived HBsAgs interact with HDV RNPs that are exported from the nucleus[80,81]. This interaction was shown to be mediated by L-HDAgs[82,83]. Tavanez *et al*[81] used heterokaryon assays to show that HDV RNPs shuttle between the nucleus and the cytoplasm. The authors claimed that nuclear import is mediated by an NLS located in Delta antigens (aa 66-75) and provided evidence that export to the cytoplasm is mediated by a cis-acting sequence in virus RNA[35]. However, Lee *et al*[84] (2001) have shown a year before that aa 198-210 in L-HDAg were able to promote the export of a reporter protein. More recently, Freitas and Cunha used a well-established CAT reporter system to investigate a possible presence of nuclear export elements (NEEs) in HDV RNAs[85]. The authors showed that NEEs may be present in both genomic and antigenomic molecules and that nuclear export is, at

least in part, sensitive to leptomycin B, an inhibitor of the host CRM1-mediated export pathway. Whether a NES present in L-HDAg or a NEE in virus RNA are responsible for promoting HDV RNP export may be considered still controversial. Consequently, further research is mandatory to unequivocally answer this question.

Clinical manifestations and therapy: It is widely and for a longtime known that HDV infection is associated with a broad range of clinical manifestations, from asymptomatic to fulminant hepatitis. In the latter cases, mortality often reaches 80% of the affected individuals[86,87].

Concomitant infection of HBV and HDV usually displays more severe symptoms when compared with a single HBV infection. Nevertheless, the most frequent outcome is virus clearance, a situation reported in about 95% of the cases[88]. In contrast, HDV super-infection of chronic HBV patients results in progression to chronicity in up to 80% of patients. Moreover, about 60%-70% of these patients will develop cirrhosis[89]. These patients usually progress more rapidly to cirrhosis, show increased liver decompensation, and eventually death when compared with those chronically infected with HBV alone[90,91].

The factors influencing the distinct clinical course in coinfected and superinfected patients are still poorly understood. In both cases the organism produces a strong anti-HDAg antibody response which is, unfortunately, unable to modulate the course of infection[92-94]. The majority of superinfected patients progresses to chronic disease independent of the presence of high titers of anti-HDV antibodies. Despite the limited number of studies there are evidences supporting a role of cytotoxic T cells in HDV infection including the destruction of infected hepatocytes[95]. In any case, immunology of HDV infection is perhaps one of the most poorly understood aspects of the disease.

From the histologic point of view there are no detectable differences between anomalies observed in the liver of HDV-infected patients and patients with other acute or chronic virus liver disease[96,97]. These anomalies mostly consist of hepatocellular necrosis and inflammation and may represent, at least in part, a consequence of the immune response of the host. Proteomic and systems biology approaches have more recently been used to investigate changes in protein expression patterns and metabolic pathways altered during HDV replication. Although the model systems used can hardly be considered ideal, the obtained results provided consistent evidence that HDV replication results in significant alterations in pyruvate and glycolysis metabolism[98-100]. Of note, these studies have shown that cancer was the most likely disease associated with HDV replication and provided evidence that the G2/M cell cycle checkpoint is altered as a consequence of the presence of the virus[100]. Definitely, these observations, of which a significant number of arise from proteomic experiments and analysis, need to be interpreted and handled with care. In any case, it seems uncontroversial that further research on liver biopsies of infected patients may possibly help confirming these findings.

There is no efficient therapy for chronic HBV/HDV infection. Pegylated interferon-α (PEG-IFN-α) is perhaps the most popular therapy and the one that has shown some antiviral activity against HDV[15,101]. However, the efficacy is limited - a temporary reduction in virus titers is usually observed in 15%-40% patients - and the need for prolonged administration often results in severe adverse effects[101,102]. These effects include fatigue, weight loss, and psychiatric disturbances. Ribavirine, lamivudine and other nucleotide analogues have also been tested but have shown a very limited, if any, efficacy[103-106]. The Hep-Net International hepatitis D intervention trial included 77 patients from Germany, Greece, and Turkey. In this study a PEG-IFN-α2a therapy was compared with adefovir and a combination of PEG-IFN-α2a and adefovir[107]. Adefovir showed a very limited efficacy and the combination therapy based on PEG-IFN-α2a and adefovir was only superior in reducing HBsAg levels but not in HDV RNA[17]. In any case, HDV RNA relapses were often observed in a long-term follow-up (median time 4.5 years). The nucleoside analog entecavir, which showed antiviral efficacy in the woodchuck model of hepatitis B, was assayed in thirteen chronic hepatitis D patients for one year also proving to be ineffective[17]. It thus seems evident that current anti-HBV drugs are unable to efficiently circumvent HDV infection.

Today, it is usually recommended to treat chronic hepatitis D with PEG-IFN-α for at least one year if the patient tolerates the eventual adverse effects. However, in patients with advanced liver disease, liver transplantation may represent the only available option[108]. It is thus clear that current therapeutic options are unsatisfactory and there is an urgent need for more effective and specific anti-HDV drugs that will directly target HDV. Prenylation inhibitors may become an interesting and effective option and have been shown to be safe when used to treat neoplasias[109,110]. As discussed before, prenylation of L-HDAg is essential for interaction with HBsAg and virion assembly, and thus may be regarded as a potential target for therapeutic intervention.

Most recently, and as a consequence of the identification of NCTP as the host cell HDV receptor, inhibitors of viral entry have been tested and proposed as potential anti-viral drugs. Namely, Myrcludex B, a synthetic N-acylated preS1 lipopeptide and cyclosporine A were shown to inhibit virus entry by interfering with the receptor functions of NCTP, however, currently there is no data available regarding the performance of this drug in actual HDV-infected individuals[111,112].

However, it is clear that a higher investment in research of fundamental aspects of HDV biology as well as of anti-HDV specific compounds is crucial in order to improve the quality of life and life expectancy of chronic HBV/HDV carriers.

Recent trends in HDV research

In the past few years a number of interesting developments have occurred in the field of HDV research and its interaction with HBV.

Using super-infection with WHV-enveloped HDV of the woodchucks that were chronic carriers of WHV and already developed HCCs, it was found that HDV was able to infect fractions of the cells of WHV-induced HCCs. These results suggest that at least a certain percentage of HCC cells *in vivo* express functional WHV receptors and support the attachment, entry, trafficking, and complete replication cycle of HDV[113]. The data also opens new avenues of research that will further address the mechanisms of the relationship between established HCCs and ongoing virus infection.

A second study compared several types of HDV that differed only by the envelope proteins of HBV that coated the virions[114]. Twenty five different types of HBV envelope proteins that belonged to twenty five different HBV variants of nine genotypes A-I were analyzed. It was found that all nine HBV genotypes tested were able to support the production of infectious HDV virions that contained HDV genome of genotype I. Significant differences in infectivity were found for the envelope proteins of different HBV variants. The data generated strongly suggest that HBV envelope proteins facilitate not only attachment and entry, but also at least one additional immediate post-entry step of the HDV life cycle. In addition, testing of infectivity suggested that it cannot be concluded that the envelope proteins of HBV produced during chronic stage of HBV infection are mainly responsible for assembly of the virions with diminished infectivity. The study also suggested that correctly regulated disassembly of HDV RNP from the HBV envelope proteins after entry is critical for the overall infectivity of HDV particles[114].

Finally, a third recent study demonstrated that infectious HDV virions can be assembled by the envelope proteins derived from the naturally integrated HBV DNA in the absence of ongoing HBV replication[115]. These findings suggest that HDV can possibly persist *in vivo* in the absence of HBV replication (or when HBV replication is suppressed by a drug), when functional HBV envelope proteins are supplied from integrated HBV DNA. Such a mechanism of HDV persistence was not explored previously. The results obtained explain, at least in part, inability of anti-HBV drugs to efficiently block HDV infection *in vivo*. Additionally, they also suggest that HDV can be actually a more independent and more significant pathogen than it is currently assumed[116].

Origin of the virus

As discussed earlier, HDV bears a number of characteristics similar to those found in plant viroids (Table 1).

These similarities may allow speculation on a possible HDV origin from the plant world. According to this hypothesis, HDV could have evolved to encode the Delta antigen thus providing an explanation for its larger genome when compared with viroids[117]. However, a deeper analysis of this homology was evaluated as non-significant and this hypothesis seems to be, at least for the time being, ruled out.

One of the key features of HDV genomic and antigenomic RNA molecules is their ribozyme activity. Ribozymes are considered to be characteristic of viroids. However, the two HDV ribozymes are not only structurally different from those of Avsunviroidae but also display similarities to several HDV-like ribozymes found in eukaryotes[74,116]. This finding rather supports the hypothesis of a human transcriptome origin of HDV.

We can thus conclude that the plant or animal origins of HDV are still questionable and highly speculative. But this is one of the many fascinating questions that still remain to be unveiled for this awkward and awesome virus.

CONCLUSION

Almost 40 years after its discovery, HDV remains a challenge for clinicians and researchers. It is disconcerting simplicity, with a small RNA genome and a single protein, the Delta antigen, make it an excellent model not only for virologists but also for those interested in RNA and cell biology. The virus bears a number of unique features including a RNA-directed RNA replication mechanism of the genome catalyzed by host RNA polymerase Ⅱ. Enzymatic activities were not identified in Delta antigens thus making difficult the identification of potential targets for specific and effective therapies. Development of such therapies is crucial to reduce the number of chronic patients progressing to cirrhosis and hepatocellular carcinoma. The burden of disease caused by HDV is most probably underestimated since there is a considerable lack of epidemiologic data from several countries where HBV is highly prevalent.

In conclusion, despite considerable progress made in HDV research a significant number of questions remain to be answered concerning fundamental aspects of its biology, pathogenesis, and interaction with the host. The next few years will hopefully bring to light new answers but also new exciting questions, helping understand this fascinating pathogen, and contributing to reducing morbidity and mortality among infected individuals.

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REVIEW

Update on hepatitis B and C virus diagnosis

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Abstract

Viral hepatitis B and C virus (HBV and HCV) are responsible for the most of chronic liver disease worldwide and are transmitted by parenteral route, sexual and vertical transmission. One important measure to reduce the burden of these infections is the diagnosis of acute and chronic cases of HBV and HCV. In order to provide an effective diagnosis and monitoring of antiviral treatment, it is important to choose sensitive, rapid, inexpensive, and robust analytical methods. Primary diagnosis of HBV and HCV infection is made by using serological tests for detecting antigens and antibodies against these viruses. In order to confirm primary diagnosis, to quantify viral load, to determine genotypes and resistance mutants for antiviral treatment, qualitative and quantitative molecular tests are used. In this manuscript, we review the current serological and molecular methods for the diagnosis of hepatitis B and C.

Key words: Diagnostic methods; Genotypes; Hepatitis B virus; Molecular diagnostic techniques; Serological tests; Hepatitis C virus

Core tip: Reliable methods for diagnosing hepatitis B virus (HBV) and hepatitis C virus (HCV) infection are essential to reduce the burden of these infections. Serological and molecular assays are used to identify acute and chronic cases of infection. In this article, the current knowledge about HBV and HCV diagnosis is updated and emphased the characteristics of each techniques to be useful to most laboratory personnel.

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INTRODUCTION

Hepatitis B virus (HBV) infection is caused by a virus that

shows a diameter of 42 nm and comprises an icosahedral capsid surrounded by a lipid envelope containing hepatitis B surface antigen (HBsAg)[1]. Viral capsid is formed by core protein and carries viral genome and polymerase[2]. HBV genome is composed by a circle DNA partially double stranded and has four open reading frames regions overlaped (ORFs): PreC/C that encodes for hepatitis B e Antigen (HBeAg) and core protein (HBcAg); P for polymerase (reverse transcriptase), S for surface proteins [three structures of HBsAg, small (S), middle (M) and large (L)], and X for a transcriptional transactivator protein[3,4]. Despite HBV has a DNA genome, it shows high mutation rates, similar to those observed in RNA viruses and retroviruses[5] what could be due to viral polymerase mistakes associated with the additional step of reverse transcription, necessary for genetic material replication[6]. HBV has been classified in 10 genotypes (A to J) with between approximately 4 and 8% intergroup nucleotide divergence across the complete genome and genotypes A-D, F, H, and I are classified further into subgenotypes[4,7].

In 1989, hepatitis C virus (HCV) was described after extensive research conducted to find the cause of post-transfusion hepatitis[8]. In 1991, Choo *et al*[9] were able to identify complete HCV genomic sequence and thereafter, it was possible to conceive hybridization probes and primers to amplify viral genome by polymerase chain reaction (PCR)[10]. HCV particle is constituted by a spherical envelope which involves an icosahedral capsid. It possess a RNA genome with 9.5 kb and an ORF responsible for encoding viral polyprotein, constituted by structural (core, glycoproteins E1 and E2 and protein P7) and nonstructural (NS2, NS3, NS4a, NS4b, NS5a, NS5b-RNA polymerase) proteins. HCV presents high genome variability due to low proofreading of viral RNA polymerase. This variability allows virus classification into 7 genotypes (1 to 7), based on their genomic features[11].

Worldwide HBV and HCV infection are the principal etiological agents of chronic liver disease[12-14]. Both viruses are transmitted by parenteral route, sexual and vertical transmission is more common for HBV infection compared to HCV[15,16]. Approximately 240 million people are HBV chronic patients while 150 million individuals present HCV infection in the world[13,14].

HBV endemicity is classified as high, intermediate or low according HBsAg prevalence[12,17]. South East Asia, sub-Saharan Africa, China, Indonesia and Nigeria are considered high endemicity regions since HBV chronic infection can be present in more than 8% of the population[17]. Developed countries in Western Europe and North America are classified as low endemicity areas since chronic infection rates range from 0.5% to 2.0% of population. South America and Central countries, Eastern and Southern Europe and South West Asia are considered intermediate areas since HBV prevalence rates between 2% and 7% of population[12,17].

HCV global prevalence is 1.6% corresponding to 115 millions of infected individuals and viremia prevalence is 1.1% corresponding to 80 million of cases[18]. Although HCV infection presents global distribution, different prevalences are observed according geographic area[18,19]. High anti-HCV prevalence is observed in Central Asia (5.4%), Eastern Europe (3.3%), the Midwest of North Africa region (3.1%) and Central and Western Sub-Saharan Africa (4.2% and 5.3%, respectively). Intermediate prevalence rates are found in southern sub-Saharan Africa (1.3%), Central Europe (1.3%), Australia (1.4%), Latin America (1%-1.25%). Low prevalence are observed in Oceania (0.1%), Caribbean (0.8%) and Western Europe (0.9%)[18]. Highest rates of chronic infection can be found in Egypt (22%), Pakistan (4.8%) and China (3.2%) where the main infection mode is the reuse of contaminated needles[13,14,20].

One important measure to diminish the burden of HBV and HCV infection is the diagnosis of acute and chronic cases. In this article, we describe the current serological and molecular methods for HBV and HCV diagnosis.

SEROLOGICAL ASSAYS FOR HBV DIAGNOSIS

Takahashi *et al*[1] described the Australia antigen in 1965 and after this was named HBsAg, while the Dane particle (complete hepatitis B virion) was subsequently identified in 1970. After these discoveries, the identification of HBV antigens and antibodies allowed: (1) clarify the natural history of the disease; (2) evaluate the clinical phases of infection; (3) monitor antiviral treatment; (4) identify infected individuals; and (5) monitor the efficacy of immunization[21-23].

HBsAg is the first serological marker to appear indicating active infection. Chronic infection is characterized by the persistence of this marker for more than 6 mo[24]. Recently, HBsAg quantification has become important to monitor polyethylene glycol interferon treatment[25] since this assay could be used along to HBV DNA test to define the clinical phase of HBV infection and to evaluate the therapy[26]. To date, two chemiluminescent microparticle immunoassay (CMIA) (Architect HBsAg QT and Elecsys HBsAg Ⅱ, Elecsys) could be used for HBsAg quantification showing good agreement[27].

Anti-HBs is a neutralizing antibody, and its presence indicates immunity to HBV infection[28,29]. The simultaneous presence of anti-HBs and HBsAg has been documented in HBsAg positive patients[30,31], probably due to the incapacibility of antibodies to neutralize the circulating virions. In this situation, these people are classified as carriers of HBV infection.

HBeAg marker indicates viral replication and risk of transmission of infection, and seroconversion of HBeAg to anti-HBe is associated with remission of liver disease[32]. However, some anti-HBe reactive subjects continue to have active viral replication and hepatic disease caused by mutations in the pre-core and core

Table 1 Clinical significance of hepatitis B virus serological markers

Marker	Clinical significance
HBsAg	First marker to appear in course of infection Appears one to 3 wk before the onset of symptoms The permanence of this marker for more than 24 wk indicates chronicity
Anti-HBc IgM	Marker of recent infection marker Appears with the onset of symptoms and persists up to 32 wk after infection
Anti-HBc IgG	This marker did not indicate immunity and it is not elicited by vaccination This presence indicates prior contact with the virus
HBeAg	It appears before the onset of symptoms and indicates viral replication independent of disease phase (acute or chronic) This presence indicates high infectivity
Anti-HBe	It appears after the disappearance of HBeAg Their presence suggests reduction or absence of viral replication, except when infection is due to HBV strains with pre-core mutant (not producing the protein "e")
Anti-HBs	It appears one to 3 mo after HBV vaccination or after recovery of HBV acute infection and indicates immunity to HBV infection

HBV: Hepatitis B virus; HBeAg: Hepatitis Be Antigen; HBsAg: Hepatitis B surface antigen; Anti-HBe: Antibodies against HBeAg; Anti-HBs: Antibodies against HBsAg.

region in the HBV genome, which reduces the production of HBeAg[24].

The HBcAg is intracellular and for this reason is not detected in the serum of infected individuals. Antibodies against core protein (anti-HBc) appear shortly after HBsAg in acute infection and persist after acute phase indicating previous exposure. The IgM anti-HBc antibody is the first detected during acute infection, approximately 1 mo after the onset of HBsAg and disappears after 6 mo of infection. Anti-HBc IgG remains detectable in patients cured of hepatitis B and among chronic cases of HBV infection[28,33].

Isolated Anti-HBc can be found in three situations: (1) during the window period of acute phase when the anti-HBc is predominantly IgM; (2) years after acute infection had finished and anti-HBs has diminished to undetectable levels; and (3) when the titer of HBsAg has decreased thereunder the detection level after many years of chronic HBV infection. In order to evaluate the presence of isolated anti-HBc, serum samples should be retested for anti-HBc, HBsAg, anti-HBe, and anti-HBs. If the sample still had an isolated anti-HBc positive result, this sample should be tested for IgM anti-HBc in order to eliminate the possibility of recent HBV infection. HBV DNA testing should be done in chronic liver disease patients to eliminate low-level chronic HBV infection[34,35]. The clinical significance of serum markers detected during the course of infection with HBV is disclosed in Table 1.

Specific serologic assays to detect HBV markers were developed around the 70s[36]. Various serological techniques could be employed, such as radioimmunoassay (RIA), enzyme immunoassay (EIA), Electrochemiluminescence immunoassay (ECLIA) and chemiluminescence immunoassay (CLIA), micro-particle enzyme immunoassay (MEIA), CMIA[36,37].

RIA was the first technique used for HBV diagnosis where one of reactants is conjugated to radioisotopes[38,39]. This method present good sensitivity but high cost and risk to operator. In 1971, Engvall *et al*[40] described a technique named EIA that is similar to RIA where enzymes are attached to one of the reactants in an immunoassay to allow detection through the development of color after the addition of a suitable substrate/chromogen. The colored product is monitored visually or by spectrophotometer where the amount of the substance measured is related to color intensity. The advantages of this technique include highly reproducible results, automation, and low cost[41]. An evaluation of 70 HBsAg kits showed that 17 HBsAg EIA kits present high analytical sensitivity 4 IU/mL, but reduced sensitivity for HBsAg was observed in samples containing genotypes/subtypes *D/ayw3*, *E/ayw4*, *F/adw4* and *S* gene mutants[42].

MEIA is a method that uses very small microparticles in liquid suspension as solid-phase support. Particles are coated with related molecules specific for the measured material. MEIA is executed in less time than other immunological methods due to the presence of active surface area of microparticles what rises the kinetics study and decreases the incubation time. ECLIA uses molecules as conjugated, generate chemiluminescence like, luminol derivatives, nifropenil oxalate derivates or rutenium tri-bipridyl with tripropylamine. The electrochemiluminescence occurs when is applied an electric current in an electrode platinum, creating an electric field that making all materials respond. This reaction hydrolyzes the chemiluminescent substrate, producing an unstable product which after stabilization generates emission of light photons (amplified) what is measured by a photomultiplier[43].

Kim *et al*[44] compared a radioimmunoassay, an ECLIA (Modular E170 analyzer, Roche Diagnostics, Mannheim, Germany) and CMIA (Architect i2000 analyzer, Abbott Diagnostics, Abbott Park, IL, United States) for HBV markers detection showing concordance rates among the three analyzers of 100%, 91.6%, 94.6%, and 82.2% for HBsAg, anti-HBs, HBeAg, and anti-HBe, respectively. High difference results among three immunoassays analyzers (Abbott AxSYM, Roche Modular Analytics E170, and Abbott Architect i2000) for HBV markers detection was observed in samples with low level of serum HBV markers[45]. Xu *et al*[46] also showed that most weak positive results, determined by ECLIA, were negative determined by ELISA.

Huzly *et al*[47] compared the performance of six different automated immunoassays (one MEIA and five chemiluminescence assays) and three EIAs for anti-HBs quantification. The assay specificity ranged between 96.8% and 100% and sensitivity ranged between 93.5% and 100%. There was no difference between anti-HBc-

positive and -negative individuals and, hemolysis or lipemia did not seem to influence the measurement. However, classical EIAs tend to detect lower anti-HBs levels than the automated systems.

EIA is the most widely technique used for detection of HBV serological markers. Although EIAs are sensitive and specific, they are time consuming, need for sophisticated equipment and trained technicians, continuous supply of electricity, and long turnaround time hampering the execution of these assays in field settings and during the household surveys[48]. Due to this situation, rapid point-of-care tests (RPOCTs) were developed for HBV diagnosis. These assays use particle agglutination, immunochromatography, immunodot or immunofiltration. The device contains a solid support (cellulose or nylon membranes, latex microparticles or plastic cards) where viral antigens or antibodies are fixed and results can be read in up to 10 min[49]. Rapid assays offer advantages of simplicity, low need for instrumentation, minimum training to execute the assay and performance at room temperature.

Since 1990s, several RPOCTs for HBsAg detection have been developed. The sensitivity of these assays varies from 43.5% to 100% while specificity varies from 95.8% to 100%[50-54]. A recent systematic review showed that the performance of rapid tests for HBsAg detection was higher in developed countries compared to developing countries what could be due to minimal heterogeneity observed in first than in later. RPOCTs presented analytical sensitivity of 4 IU/mL, but the performance of these assays is poor in seroconversion panels and among individuals infected by several HBV mutants. Thus these tests are not indicated in situations with low concentrations of HBsAg such as in healthy blood donors, general populations, patients recovering from acute HBV infection and those on antiviral therapy[49]. RPOCTs for detection of anti-HBc, anti-HBs, HBeAg, anti-HBe markers have demonstrated sensitivities of 85.5%, 64.2%, 80% and 82.8% and specificities higher than 95% for those assays[55,56].

Actually, the evolution of development of nanoscience and nanotechnology has increased the development of immunosensors for HBV diagnosis. Immunosensors are solid-state affinity ligand-based biosensing apparatus that combine immunochemical reactions to proper transducers. Generally, an immunosensor comprises of a sensing element and a transducer. The sensing element is composed by means of the immobilization of antigens or antibodies, and the binding event is transformed into a measurable signal by the transducer[57]. The goal of immunosensor is to produce a signal proportional to the concentration of analyte[58-60]. Wang and collaborators[61] developed a gold nanorod based localized surface plasmon resonance biosensor that quantify HBsAg until 0.01 IU/mL. This limit of detection is about 40 times lower than the limit of detection of the EIA method. Other immunosensors for HBsAg detection uses magnetic nanoparticle and three dimensional carbon nanotube-conducting polymer network detecting 0.001 to 0.015 ng/mL of HBsAg[62,63].

For HBV antigen or antibodies detection, it is necessary blood sample collection by venipuncture in order to obtain sera or plasma samples. However, venipuncture is difficult in some individuals like drug users, haemodialysis, obese and elder individuals. In addition, the transport of these samples from remote areas to laboratories could be difficult. This situation has led to development of methods for HBV diagnosis in alternative fluids, like saliva or dried blood spot (DBS) samples[64,65].

EIA for HBsAg detection among saliva samples present sensitivities of 74.29% to 95.24% and specificities of 89.88% to 100%[66-70]. Assays for anti-HBs, total anti-HBc in saliva samples showed low sensitivity (< 15%) while assay for detecting IgM anti-HBc assay demonstrated sensitivity and specificity of 100%[71].

Using DBS samples along to immunoassays, sensitivities varies from 78.6% to 98% for HBsAg detection, 90.5% to 97.1% for anti-HBc detection and 74.2% to 97.5% for anti-HBs detection. In all of these studies specificities varies from 88.6% to 100%[72-76]. HBV markers could be detected in DBS samples using EIA until 60 d of storage in room temperature[74]. HBV prevalence has been evaluated using DBS in specific groups at risk for infection, such as drug users, and endemic areas[77-79].

Serological diagnostic tests for HBV have improved in sensitivity, time, need for trained personnel and cost over time. In laboratories with low infrastructure or in field situations, rapid tests could be useful since they can be storage at room temperature, results are available in few minutes and they do not need trained technicians. While in laboratories presenting good infrastructure and with high-demand of results, automated ELISA ECLIAs, MEIAs, CLIAs and CMIAs can be used due to its high sensitivity although trained technicians, sophisticated equipment, and continuous supply of electricity are necessary. The main characteristics, advantages, disadvantages and specific applications of HBV serological assays are disclosed in Table 2.

MOLECULAR METHODS FOR HBV DIAGNOSIS

HBV DNA is detectable at the beginning of infection (1 mo after exposure to HBV) and increases to get a peak approximately 3 mo after the exposure to HBV reaching usually more than 10^8 copies/mL and then progressively decreases in chronic infection or disappears at the resolution of infection. In many chronic patients, when HBeAg seroconversion occurs, low levels of viral load persists (< 10^4 copies/mL)[80].

HBV DNA qualitative and quantitative methods are useful to: (1) diagnose HBV replication in chronic infections; (2) evaluate the prognosis of the disease and follow the risk of progression to cirrhosis and hepatocellular carcinoma; (3) define the beginning of

Table 2 Advantages and disadvantages characteristics of hepatitis B virus serological assays

Technique	Advantages	Disadvantage	Commercial assays	Ref.
RIA	High sensitivity	High cost Risk to operator	IRMA kit (North Institute of Biological Technology, Beijing, China)	[38,39,44]
EIA	Automation High reproducible results Low cost	Time consuming, need sophisticated equipment and trained technicians, continuous supply of electricity, not suitable for field settings	ETI-AB-AUK-3 (DiaSorin) Enzygnost anti-HBs (Dade Behring) Monolisa anti-HBs (Bio-Rad Laboratories)	[41,42,47,48]
MEIA	High sensitivity Faster than other immunological methods	Sophisticated equipment, trained technicians, continuous supply of electricity	Abbott Axsym AUSAB assay	[47]
ECLIA	High sensitivity Results available in few minutes Automation	High cost, sophisticated equipment, trained technicians, continuous supply of electricity	Modular E170 analyzer, Roche Diagnostics	[43,44,46]
CLIA	High sensitivity and specificity Automation	High cost, sophisticated equipment, trained technicians, continuous supply of electricity	Advia Centaur anti-HBs Vitros anti-HBs on the Vitros ECI Immunodiagnostic system (Ortho Clinical Diagnostics) Roche Elecsys anti-HBs on the Modular System (Roche Diagnostics) Liaison anti-HBs on the Liaison system (DiaSorin) Abbott Architect anti-HBs assay on the Architect i2000 system (Abbott)	[47]
CMIA	High sensitivity and specificity Automation	High cost, sophisticated equipment, trained technicians, continuous supply of electricity	Architect i2000 analyzer (Abbott Diagnostics)	[44]
Rapid point-of-care tests	Simplicity, do not need sophisticated equipaments, minimum training to execute the assay, storage and performance at room temperature, results can be read in up to 10 min	Poor performance in seroconversion panels and among individuals infected by several HBV mutants	Determine™ HBsAg (Abbott Laboratories) Virucheck® HBsAg (Orchid Biomedical Systems) Cypress HBsAg (Cypress Diagnostics) Hexagon® HBsAg (Human) Cortez Rapidtest (Cortez Diagnostics) VIKIA HBsAg (Biomériuex) Quick Profile™ (Lumiquick) DRW-HBsAg v2.0 (Diagnostics for the Real WorldTM) AMRAD ICT Binax Advanced quality™ one step test (Intec Products)	[49-56]

HBV: Hepatitis B virus; RIA: Radioimmunoassay; EIA: Enzyme immunoassay; MEIA: Micro-particle enzyme immunoassay; ECLIA: Electro-chemiluminescence immunoassay; CLIA: Chemiluminescence immunoassay; CMIA: Chemiluminescent microparticle immunoassay; HBsAg: Epatitis B surface antigen; Anti-HBs: Antibodies against HBsAg.

antiviral treatment; and (4) monitor antiviral treatment and to identify resistance to nucleoside/nucleotide analogues drugs[81,82].

Molecular assays with high-sensitivity are clearly important for the diagnosis of chronic hepatitis B without HBeAg detection in serum (core promoter, precore stop mutation), and occult HBV, where viral loads can be quite low. The principle of methods to detect and quantify HBV DNA is the signal amplification such as branched DNA technology (bDNA) and hybrid capture or target amplification such as PCR[83-85].

In therapeutic monitoring of HBV, a more sensitive assay with a lower limit of detection of 10 IU/mL is recommended for early detection of viral reactivation. In addition, the assay employed should equally quantify all HBV genotypes[86]. A commercial PCR method to quantify HBV DNA is COBAS Amplicor HBV Monitor test (Roche Molecular Systems, Pleasanton, California, United States) that measure HBV DNA in serum samples, being considered reproducible, with a high degree of accuracy and precision in both intra-assay and inter-assay comparison[87]. This assay presented a limit of detection of 200 HBV DNA copies/mL and has the disadvantage of requiring dilution of samples with high viral load, which is not a practical solution for all laboratories[88].

Methods of signal amplification, like bDNA, allow the direct quantification of HBV DNA in human serum or plasma. In this method, sample DNA is caught by a set of capture nucleotide probes fixed on a microtitulation well. Then, another set of target probes hybridize with both the target DNA and capture probes, and this complex is hybridized with multiple copies of alkaline phosphatase. After incubation with a chemiluminescent substrate, the luminometer measures the results as relative light units and the amount of HBV DNA in each sample is determined by comparison to a standard curve. VERSANT HBV 3.0 Assay (Siemens Healthcare, United States) is a commercial method that uses bDNA technology and presented a detection limit of 2000 HBV DNA IU/mL[83,89] (Table 3).

Cobas Amplicor HBV Monitor assay (PCR technology) is more sensitive than Quantiplex HBV DNA; Bayer

Table 3 Performance characteristics of commercial methods for hepatitis B virus and hepatitis C virus detection and quantification

Assay (manufacture)	Method	Measurable range (IU/mL)	Limit of detection IU/mL (using WHO HBV standard)	Conversion factor (IU/mL to copies/mL)
HBV				
Cobas ampliPrep/Cobas TaqMan HBV test v2.0 (Roche Diagnostics, California, United States)	Semi-automated qPCR	20-1.7 × 10^7	20	5.82
Cobas TaqMan HBV test (for use with high pure system)	Semi-automated real time PCR	29-1.1 × 10^7	6	5.82
Abbott real time HBV (Abbott Diagnostic, Chicago, United States)	Automated real time PCR	10-1 × 10^9	10	3.41
Versant HBV 3.0 assay (Siemens Healthcare, United States)	Branched DNA	2000-1 × 10^8	2000	5.6
HCV qualitative				
Cobas Amplicor HCV v2.0 (Roche)	Semiautomated RT-PCR	50	50	-
Versant HCV RNA qualitative assay (TMA)	Transcription-mediated amplification	< 9.6	5-9.6	-
Quantitative				
Versant HCV RNA 3.0 (Siemens)	Branched DNA	7.7 × 10^6	615	5.2
Cobas Amplicor Monitor HCV v2.0 (Roche)	Semiautomated RT-PCR	5 × 10^5	600	2.7
Real time HCV (Abbott)	Real time PCR	1 × 10^7	10	3.8
Cobas AmpliPrep/Cobas TaqMan (Roche)	Automated real time PCR	6.9 × 10^7	43	3
High Pure/Cobas TaqMan (Roche)	Semiautomated real time PCR	3.9 × 10^8	25	3

WHO: World Health Organization; HBV: Hepatitis B virus; HCV: Hepatitis C virus; PCR: Polymerase chain reaction; RT-PCR: Reverse transcriptase-PCR; qPCR: Quantitative PCR.

Diagnostics (bDNA technology) to detect HBV DNA among HBeAg negative samples and useful for monitoring the viral load during treatment in chronic HBV infection[90]. On the other hand, Cobas Amplicor HBV Monitor presented poor agreement to Digene Hybrid Capture 2 (HC2; Digene Corporation, Gaithersburg, Md.)[88].

Currently, real time PCR has become the standard technique of choice to detect and quantify HBV DNA in clinical practice due to its capacity of detecting low viral loads (10-15 IU/mL) and having a broad dynamic range (upper range of quantification of 7-8 Log10 IU/mL) as shown in Table 2. Moreover, they do not carry over contamination and can be fully automate[91].

At this time, several real time PCR assays are commercially available, such as, Da-an real-time HBV DNA assay (Da-an Gene Co. Ltd, Sun Yat-Sen University, Guangdong, China), COBAS TaqMan HBV test (Roche Molecular Diagnostics, Pleasonton, CA, United States), Abbott RealTime HBV assay (Abbott Molecular, Des Plaines, IL, United States), Artus RealART HBV LC PCR kit (QIAGEN, Hamburg, Germany), AdvanSure HBV real-time PCR assay (LG Life Sciences, Ltd., Seoul, South Korea). Cobas Taqman HBV (Roche) can be used along to automated sample preparation, Cobas AmpliPrep system (CAP-CTM, Roche Molecular System, Pleasanton, CA)[92] and the Abbott HBV (Abbott Diagnostic, Chicago, IL) uses m2000rt amplification platform along to m2000sp device for sample preparation[93]. Characteristics of quantitative methods for HBV DNA are described on Table 3.

Kim *et al*[94] showed good correlations among Abbott RealTime HBV Quantification Kit, the COBAS TaqMan HBV Test, and the VERSANT bDNA 3.0 assay, AdvanSure HBV real-time PCR assay detecting HBV genotypes A-F and without cross reactivity with high HCV RNA levels or high protein concentrations. Qiu *et al*[95] demonstrated good correlation among Abbott and Da-an assay for HBV DNA quantification. Morris *et al*[93] observed good correlation among Cobas Taqman HBV Test and Abbott RealTime HBV for HBV DNA quantification, although Abbott assay presented wide dynamic range without additional dilution or repeating of HBV high titers. In house real time PCR molecular beacon based was builder up by Paraskevis *et al*[96] showing good correlation to COBAS TaqMan HBV test.

HBV DNA detection and quantification are made using serum samples, but alternative fluids, like saliva, DBS samples have been studied as potential fluids for HBV molecular diagnosis[64,75,97-100]. Kidd-Ljunggren *et al*[98] demonstrated that concentration of HBV DNA in saliva is 1000-fold lower than in serum, while Heiberg *et al*[99] could quantify HBV DNA using Cobas TaqMan HBV Test (Roche Diagnostics) in saliva samples. Mohamed *et al*[75] demonstrated sensitivity of 98% and specificity of 100% for the detection of HBV DNA using DBS and performed HBV genotyping and mutation detection among those samples with total concordance between the 10 paired DBS and plasma samples. Jardi *et al*[101] also revealed no decrease in HBV DNA levels or integrity among DBS storage for 7 d at room temperature and 21 d at -20 ℃.

Besides that, the detection of HBV DNA in plasma or serum is also important to determine the occult hepatitis B defined by detectable HBV DNA in peripheral blood or liver in the absence of HBsAg. Tests for occult

HBV detection are recommended in the following situations: (1) in cryptogenic liver disease, particularly when individual presented anti-HBc in serum; (2) prior to immunosuppression, due to the potential for hepatitis flares; and (3) in solid organ transplant donors whose only anti-HBc is detected in serum, due to the potential for transmission[83].

Evidences are increasingly suggesting that the HBV genotyping is important for designing appropriate antiviral treatment and determining HBV disease progression. The disease progress faster in individuals infected by HBV genotype A than genotype D, subjects infected with genotype C progressed to end stage liver disease earlier than those infected by genotype B and higher mortality rates are observed in individuals infected with genotype F than those infected with genotype A or D[102].

HBV genotyping can be determined using several methods: Reverse hybridization, restriction fragment-length polymorphism (RFLP), genotype-specific PCR assays, sequence analysis, microarray (DNAchip), real time PCR and fluorescence polarization assay[102,103]. Among them, PCR-RFLP is widely used to genotype HBV since it is inexpensive and simple. Nevertheless, this technique is poor accurate to identify some genotypes[104]. A commercial method to genotype HBV is the INNO-LIPA® HBV Genotyping (Fujirebio Europe, Tokyo, Japan) based on reverse hybridization that shows high sensitivity and a detection limit of 700 copies of HBV[105,106], but is relatively cost. INNO-LIPA can be completely automated if using the systems Auto-LiPA48 and AutoBlot 3000H and the LiRAS® for LiPA HBV to reading and interpretation of strips. INNO-LIPA® HBV Genotyping and direct sequencing presented the best results when genotyping methods of HBV were compared[107].

To identify amino acid substitutions associated to antiviral resistance to treatment, direct sequence analysis and reverse hybridization methods are used. nowadays, early detection of HBV substitutions conferring resistance to nucleoside/nucleotide analogues could be useful to modify therapy in order to avoid HBV reactivation and hepatitis flare[108]. For this purpose, commercial assays are available, such as the Trugene HBV Genotyping Kit (Siemens Medical Solutions Diagnostics), which is based on direct sequence analysis of a portion of the reverse transcriptase domain of the HBV polymerase gene[109] and can also determine HBV genotype satisfactory[110].

However, despite commercial methods can be used for mutational analysis in HBV DNA sequence, direct DNA sequencing is still the standard method cause yields accurate genotype assignments and also the method of choice for patients infected with recombinant genotypes and drug resistance mutations[110,111].

In the past years, the diversity of HBV quasispecies and minority drug resistance mutations were estimated through cloning of individual amplicons followed by Sanger sequencing. However, the "next generation" ultradeep-sequencing allows direct sequencing of the mixed population sample and relative quantification of individual mutations with extremely high coverage over a relatively short time frame[111-114]. The platforms of ultradeep sequencing include: 454 Sequencing (Roche Diagnostics), Illumina Sequencing (Illumina/Solexa) and Pyromark Sequencing (QIAGEN), SOLiD (Applied Biosystems/Life Technologies), Ion torrent (Life Technologies)[115]. All of them present the principle of sequencing by synthesis, that involves sequencing of a single strand of DNA through synthesis of the complementary strand, one base at a time, and the detection of the individual nucleotide incorporated at each step. Fluorescence or light is only emitted when the nucleotide solution complements the first unpaired base in the template DNA strand[116]. Because these signals are obtained by synthesizing new copies of DNA template, the results can be used for extremely reliable investigation of viral mutations[114].

For the past twenty years fluorescence based quantitative PCR (qPCR) chemistries have revolutionized nucleic acid diagnostics and become the gold standard for viral load quantification. These methods are high sensitivity and could be used to evaluate the prognosis and risk of progression, to define the beginning of antiviral treatment and to monitor antiviral treatment but the major disadvantages is the high cost for commercial assays. Methods to determine HBV genotypes and mutations, like nucleotide sequencing have been developed and recently the arouse of third generation sequencing methods promises to reduce the lengthy manual handling times associated with current ultradeep-sequencing approaches, decreasing the generation of raw data, but increasing both throughput and read length in every facet of medical research.

The main characteristics, advantages, disadvantages and specific applications of HBV molecular assays are disclosed in Table 4.

SEROLOGIC TESTS FOR HCV

The hepatitis C diagnosis is performed through the detection of antibodies anti-HCV in blood samples where a non-reactive test result indicates the absence of HCV infection. A positive result for anti-HCV detection or a suspected case of HCV exposure should be followed by HCV RNA test. Serological diagnosis for HCV infection are based on the detection of direct antibodies against viral antigens (non-structural and structural) in human serum or plasma. As the same as cited for HBV serological diagnosis and immunoassays (like EIA or ECLIA) are generally used for anti-HCV detection. EIA has the advantages of high sensitivity, fast processing, high reliability, ease of automation and relatively low cost[57,117]. This method has undergone some changes over time, seeking to improve their diagnostic capability and therefore increased sensitivity and specificity of the assay. Until now, three generations of EIA for detecting anti-HCV using recombinant proteins or synthetic peptides have been developed[57,117,118].

Table 4 Main characteristics of hepatitis B virus and hepatitis C molecular techniques

HBV and HCV molecular diagnosis	Application	Method	Advantages	Disadvantages	Ref.
HBV DNA qualitative methods	Diagnose infection HBV occult cases identification Screening on blood donors	PCR	Low cost; high sensitivity	It only determines the presence or absence of HBV DNA	[81,82,85]
HBV DNA quantitative methods	Evaluate the prognosis and risk of progression Define the beginning of antiviral treatment Monitor antiviral treatment	bDNA	Wide dynamic range	Low sensitivity to detect low HBV DNA levels	[81,82]
		Hybrid capture	More sensitive than bDNA; less labor-intensive	Low sensitivity to detect low HBV DNA levels; individual probes are required	[83]
		Real time PCR	Capacity of detecting low viral loads; broad dynamic range; do not carry over contamination; can be fully automated	High cost	[85,91]
HBV DNA genotyping methods	Determination of HBV genotype	RFLP	Easily done; low cost; simple, rapid and suitable for large number of samples	Low sensitivity for typing samples with low HBV DNA levels; poor accurate to determine some genotypes	[85,104]
		Genotype specific PCR assays	Automated systems; high sensitivity; easy to perform; suitable for detecting mixed genotype infections	High cost	[107]
		Sequence analysis	Identification of patients infected with recombinant genotypes	Technically demanded; time consuming	[107]
HBV DNA aminoacid substitution identification	Identify antiviral resistance to treatment	Direct DNA sequencing	Accurate	Technically demanded; time consuming; necessity of cloning for identification of mixed population	[107,110,111]
		Commercial methods	Sequencing of mixed population, relative quantification of individual mutations with extremely high coverage	Differences between the statistical and biological/ clinical relevance of HBV mutation maximal sequence read length and PCR amplification bias	[114]
HCV RNA qualitative methods	To confirm chronic hepatitis C in patients with positive HCV antibodies To identify virological response during, at the end or after antiviral therapy To screen blood donations for evidence of infection with HCV	RT-PCR	High sensitivity Equal sensitivity for all genotypes	It only determines the presence or absence of HCV RNA	[121,168]
		Transcription-mediated amplification	High sensitivity; amplifies viral RNA; more sensitivity for detection of genotype 1	It only determines the presence or absence of HCV RNA	[121,168]
HCV RNA quantitative methods	To guide treatment decisions; To evaluate the prognosis; To monitor the antiviral efficacy of treatment	bDNA	Wide range of detection of HCV independent of HCV genotype (615 IU/mL to 8 million IU/mL)	Low sensitivity to detect samples presenting low HCV RNA levels	[172]
		qRT-PCR	Capacity of detecting low viral loads; broad dynamic range; not carry over contamination; can be fully automated	High cost	[170-173]
HCV RNA genotyping methods	HCV genotyping is mandatory for double antiviral treatment (interferon and ribavirin), since patients infected with genotypes 1 or 4 are treated for longer times than patients infected by genotypes 2 and 3	RFLP	Easily done; low cost; simple, rapid and suitable for large number of samples	Low sensitivity for typing samples with low HCV RNA levels; Poor accurate to determine some genotypes	[186,187]
		Probes	Easily done; low cost; useful to detect HCV genotypes and subtypes based on region 5'UTR and core and has a low limit of detection	Identify only subtypes 1a and 1b; discrepant results among subtypes when compared to sequence analysis of NS5B region	[180-184]

		qPCR	Can be fully automated avoiding contamination; determines the viral genotype and subtypes 1a, 1b, 2a, 2b, 3, 4, 5 and 6	High cost	[186,187]
		Direct sequencing	Gold standard; identification of patients infected with recombinant genotypes	Technically demanded; time consuming	[120,182]
HCV RNA aminoacid substitution identification	Identify antiviral resistance to treatment	Direct Sequencing	Identification of antiviral resistance in majority population	Technically demanded; time consuming; necessity of cloning for identification of quasispecies	[188,189,193]
		Deep Sequencing	Identification on resistant variants predominate in the HCV population; powerful tool for obtaining more profound insight into the dynamics of variants in the HCV quasispecies	Need for in-depth knowledge to analyze the results	[112,113,195,196]

PCR: Polymerase chain reaction; HCV: Hepatitis C virus; HBV: Hepatitis B virus; RFLP: Restriction fragment-length polymorphism; RT-PCR: Reverse transcriptase-PCR; qPCR: Quantitative PCR; RFLP: Restriction fragment-length polymorphism; bDNA: Branched DNA technology.

The first generation HCV EIA, which is no longer used, was created using recombinant protein derived from the NS4 region (C100-3 the polypeptide), with a sensitivity of 70%-80% and a poor specificity. The C-100 antibodies are developed approximately 16 wk after HCV infection[57,119-122].

The second generation HCV EIA have included recombinant/synthetic antigens from non-structural NS3 and NS4 (c33c and C100-3) and core (c22-3) regions improving sensitivity to about 95% and diminishing the number of false-positive results. Anti-HCV can be detected nearly 10 wk after HCV infection[57,119,121,122].

Third generation HCV EIA was developed using recombinant antigens from the core region, NS3, NS4 and NS5 regions of the viral genome. These assays allowed anti-HCV detection nearly 4 to 6 wk after infection with specificity and sensitivity greater than 99%[57,121,123-125].

Up to now, there is no reliable and simple diagnostic marker currently available to detect early HCV infection. The avidity of an antibody may be an early and reliable marker of recent viral infection, since antibodies of low avidity are usually indicative of recent infection. Commercial immunoassays for anti-HCV antibody detection have been optimized to evaluate avidity in serum and DBS samples and "in-house" anti-HCV IgG avidity assay has been developed using seroconversion panels and serum samples from chronically infected individuals[126-129].

Serum samples are necessary to investigate the presence of anti-HCV using EIA, but some studies have been conducted using alternative fluids like saliva and DBS. Anti-HCV assays using saliva and DBS samples showed sensitivity and specificity higher than 90% and DBS samples can be stored for a period of 117 d, at room temperature. Then, these samples could be useful tool to increase the access of diagnosis in remote areas or individuals with poor venous access[130-136].

Anti-HCV detection is not useful to identify current or past infection, since this marker will be in serum for life after HCV exposure[125,137]. In this situation, HCV RNA detection is generally recommended, but the cost and availability of this assay difficult the access of this method. Assays for detection of HCV core antigen (HCV Ag) or simultaneous detection of HCV Ag and anti-HCV antibodies (HCV AgAb) were developed. HCV Ag levels seems to correlate well with HCV RNA levels indicating its potential use as an inexpensive technique for diagnosing HCV acute cases[138-141]. Brandão *et al*[133] optimized commercial methods for HCV Ag/Ab in DBS samples showing specificity of 99.71% for Monolisa™ HCV AgAb ULTRA and 95.95% for Murex HCV AgAb and sensitivity of 97.5% for both assays. Larrat *et al*[142] tested Monolisa™ HCV AgAb ULTRA along to oral mucosal transudate demonstrated sensitivity of 71.7% and specificity of 94.3%. These methods were employed among haemodialysis and homosexual individuals showing good performance to early detection of hepatitis C virus infection during window period of HCV infection[143,144].

Other immunoassays for anti-HCV detection include ECLIA, CMIA, CLIA, MEIA. In these assays, HCV antigens are presented on distinct solid phases (microwell, magnetic and paramagnetic particles) and anti-HCV at clinical sample is then identified using a conjugate antibody (anti-human IgG labeled with acridinium or horseradish peroxidase) that catalyzes the oxidation of a luminol, generating light. The system measures the light signal that is normalized in relation to cut-off value [signal/cut-off (S/CO)], or given as relative lights units[145,146]. Most of these assays used antigens from core, NS3, NS4 and NS5 of HCV. Sample volume varies from 10 to 40 microliters and reaction is executed from 18 to 58 min[147].

To confirm positive or indeterminate results by immunoassays, recombinant immunoblot assays (RIBA) can be used as complementary test, especially in low prevalence settings. This test is highly specific due to the presence of recombinant proteins and synthetic peptides of envelope, NS3, NS4, NS5 regions of HCV on a membrane strip. HCV antibodies present in clinical samples should react to these proteins leading to the

appearance of cored dots at specific antigens in the strips. Interpretation may be visual or automated and a positive result is considered when two or more bands are visualized on the nitrocellulose strip, representing a specific antigen-antibody reaction. Indeterminate result is showed when only one band is visualized and negative results are obtained when no bands are observed. The major disadvantage of RIBA is the occurrence of indeterminate results, especially in those specimens with grey-zone results in the screening assays. Currently, this assay has been substituted as a confirmatory test by widely-used molecular techniques, which can additionally distinguish between active and resolved infections[121,147,148].

Nowadays there have been emergent needs for developing highly accurate, rapid and cheap analytical tools. To achieve this goal, many attempts have been focused on the development of rapid point-of-care testing (POCT) such as lateral flow tests. These techniques are based on immunochromatographic assay, lateral flow tests (LFT) or test strips. The advantages of POCT are the time for execution, simplicity and cost-effective[149]. The principle of LFT is similar to ELISA, and the base substrate is nitrocellulose membrane in which the solid phase is immobilized capture binding protein, usually an antibody or antigen. Labels such as latex, colloidal gold, carbon, and recently up-converting phosphorus technology have been employed in LFT development[150,151]. POCT is useful for HCV detection in field situations, particularly among hard-to-reach, high-risk populations, such as drug users or individuals living in remote areas[152-154]. A good performance of POCTs compared to EIA or PCR results were observed with sensitivities and specificities above 90%[152-156]. A recent metanalysis compared seven POCT and OraQuick had the highest test sensitivity and specificity and showed better performance than a third generation enzyme immunoassay in seroconversion panels[156]. This assay could be used along to serum, whole blood and saliva samples what could increase the access of diagnosis in emergency situations[151,154].

Biosensor technology has emerged as alternative technique with low detection limit, higher selectivity and sensitivity, and faster responses for anti-HCV detection. Biosensor employs specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles, or whole cells to detect chemical compounds, usually by means of electrical, thermal, or optical signals. The surface of bioreceptor contains a biological recognition element that interacts to analyte; then the transducer converts the recognition event into a measurable signal; finally, the output from the transducer is amplified, processed and displayed. The concentration of the analyte is proportional to the intensity of signal[57]. The main advantages of biosensors are offering a quantitative test for detection in cases with about 100 copies of hepatitis virus, in addition to automation, multiplexing analysis and throughput. Some biosensors available for anti-HCV detection include surface plasmon resonance, piezoelectric biosensors, microcantilever based biosensors, electrochemical biosensor and apta-sensor[121,149].

EIA is widely used for anti-HCV detection, and has the advantage of high sensitivity, fast processing, high reliability, ease of automation and relatively low cost[57,117], but this technique did not identify if the infection is current or past, and therefore requires confirmation of the results by a more specific method, such as HCV RNA[125,137]. Other immunoassays for anti-HCV detection as MEIA, CMIA, ECLIA and CLIA have the advantage of being performed in less time, with less incubation period and easily automatable. While RIBA can be used as a complementary test to confirm positive or indeterminate by immunoassays. The advantage is that this test is highly specific, due to the presence of recombinant proteins and synthetic peptide regions of the envelope, NS3, NS4, NS5 of HCV. However, its drawback is the occurrence of indeterminate results. Therefore, this assay has been replaced by molecular testing to confirm results, so distinguishing active infection cured[48,121,147].

Every day more, is continuing need to develop highly accurate, rapid and inexpensive analytical tools. Therefore, the immunochromatographic techniques, such as rapid diagnostic tests have been developed and widely used. Present as the short runtime advantages, simplicity and low cost[149] as well as being useful for detecting HCV in difficult access populations, high risk, such as injecting drug users or people living in remote areas[152-154]. The biosensor technology has emerged as an alternative technique with low anti-HCV detection limit, higher sensitivity and selectivity, and faster for the detection of anti-HCV responses. The main advantages of biosensors are automation, multiplexing analysis and fast processing[121,149], but these assays are not widespread and present high costs. Information regarding different aspects of HCV assays is available on Table 5.

MOLECULAR TECHNIQUES FOR DIAGNOSING HCV INFECTION

In hepatitis C infection, the molecular virological techniques play a key role both in diagnosis and in monitoring of HCV antiviral treatment. Due to the difficulty to grow HCV in cellular culture, molecular techniques were used to identify this virus, making HCV one of the first pathogens to be identified solely by molecular diagnostics[8].

The hepatitis C viral genome can be detected in blood, by nucleic acid testing (NAT). The presence of HCV RNA is a marker for HCV viremia and is detected only in persons who are currently infected. Patients with anti-HCV detected should be evaluated for the presence of HCV RNA in their blood[157]. In this regard, the detection and quantification is useful in clinical practice to: (1) diagnose chronic HCV infection; (2) guide treatment decisions (identify patients who need antiviral therapy and offer them the most adapted genotype treatment);

Table 5 Main Characteristics of serological assays for hepatitis C virus diagnosis

Serological assays		Antigen (region of the genome)	Assay/manufacturer	Sensitivity	Specificity	Ref.
EIA	1st generation EIA	c100-3 (NS3-NS4)	HCV-Ac-EIE Salck	70%-80%	50%-70%	[118,119]
	2nd generation EIA	c100-3 (NS3-NS4), c33-c (NS3), c22-3 (core)	ORTHO HCV ELISA test system	92%-95%		[118,119]
	3rd generation EIA	c100-3 (NS3-NS4), c33-c (NS3), c22-3 (core), NS5	ORTHO HCV 3.0 ELISA (Ortho)/ETI-AB-HCVK Sorin	95%-99%	99%	[57,118]
	4th generation EIA		Monolisa™ HCV AgAb ULTRA (BioRad)/HCV Murex AgAb (Abbott)	100%	99.5%	[117,133,144]
MEIA		HCr43 (Fusion core e NS3), c200 (NS3 - NS4), c100-3 (NS3-NS4), NS5	AxSYM® HCV 3.0 (Abbott)	100%	99.8%	[117,118]
ECLIA		Core, NS3 and NS4 proteins	Elecsys anti-HCV assay (Roche)	100%	99.7%	[117,118]
CLIA		[c22-3 (core), c200 (NS3 - NS4) and NS5]	ARCHITECT i4000 anti-HCV assay (Abbott); VITROS Eci anti-HCV assay (Ortho)	99.5%	98.2%	[145]
CMIA		HCr43 (core - NS3), c100-3 (NS3-NS4)	ARCHITECT® anti-HCV (Abbott)	99.1%	99.6%	[146]
RIBA	RIBA-1	5-1-1 (NS4) e c100-3 (NS3-NS4)		NP	NP	[118]
	RIBA-2	5-1-1 (NS4), c100-3 (NS3-NS4), c33-c (NS3), c22-3 (core)	Chiron RIBA-2.0 RIBA-2	NP	NP	[148]
	RIBA-3	c100-3 (NS3-NS4), c33-c (NS3), c22-3 (core), NS5	Chiron RIBA HCV 3.0 SIA	NP	NP	[118]
Biosensor technology		Core protein (p22 fusion protein), NS3, NS4 and NS5	mBio Diagnostics® company	NP	NP	[57,137]

NP: Not presented; EIA: Enzyme immunoassay; MEIA: Micro-particle enzyme immunoassay; ECLIA: Electrochemiluminescence immunoassay; CLIA: Chemiluminescence immunoassay; CMIA: Chemiluminescent microparticle immunoassay; RIBA: Recombinant immunoblot assays; HCV: Hepatitis C virus; ELISA: Enzyme-linked immunosorbent assay.

(3) monitor the antiviral efficacy of treatment; (4) identify amino acid substitutions responsible for direct acting antiviral drug (DAA) resistance; (5) to confirm the presence of HCV viremia in patients who are seronegative (anti-HCV non reactive) but immunocompromised such as HIV infected individuals; and (6) in babies who are born to HCV positive mothers- once antibody testing in babies can give false positive results up to 18 mo of age[121,158].

Approximately 1 mo before the appearance of total anti-HCV antibodies HCV RNA can be already detected 1-3 wk after infection[81]. Molecular methods are useful to identify the stage of infection. A negative NAT result with positive serological test is usually indicative of a resolved infection or spontaneous resolution[159], but low-level of viremia may occur during chronic infection, for these reasons a second NAT should be performed 6 to 12 mo later. A positive HCV NAT result indicates active infection independent of antibody test results. NAT are used before, during, and after antiviral treatment to indicate whether HCV is present or not, and to determine when and whether treatment should be stopped or continued[160]. In acute infections, the NAT result will become positive within 1 to 3 wk, several weeks earlier than serological tests, as in occupational exposures[120]. This way, the serological window present in HCV infection can be resolved using qualitative and quantitative nucleic acid testing, whereas these techniques have a wide dynamic range of detection, which is well chosen according to the clinical needs (upper range of quantification: 7-8 log10 IU/mL)[161].

HCV detection and quantification is made using sera samples, but saliva, DBS and platelets have been studied as alternative samples[162-165]. The detection of HCV RNA in these samples could be useful to increase the access of molecular diagnosis for HCV infection and to evaluate antiviral response in some groups, like haemodialysis, children, drug users. A systematic review demonstrated good correlation among HCV RNA quantification from DBS and whole plasma[166], but low levels of HCV among saliva and platelets compared to sera samples[162,163].

Qualitative HCV assays comprises viral RNA isolation, complementary DNA (cDNA) production, PCR amplification and detection of PCR amplicons[121]. A large amount of commercial and in-house PCR for HCV uses primers for amplification of 5′ untranslated region (UTR) since this region has above 90% of sequence identity among distinct HCV genotypes, where several fragments are almost undistinguishable among distinct strains[121]. The 5′UTR is the first region to be transcribed and has secondary and tertiary structures that are largely conserved[167]. Furthermore, core and 3′UTR are also used in PCR for detection of HCV[121].

Qualitative NAT are used as the first diagnosis of a suspected acute infection, to confirm chronic HCV infection in patients with antibodies anti-HCV positive, confirmation of virological response during, at the end or after antiviral therapy and to screen blood donations for evidence of infection with HCV[120,168]. These tests usually utilize conventional reverse transcriptase-PCR (RT-PCR) or transcription-mediated amplification (TMA).

In RT-PCR-based assays, HCV RNA is the source for production of a single-stranded complementary cDNA by reverse transcriptase. DNA polymerase amplifies cDNA into multiple double-stranded DNA copies. In TMA assay, viral RNA is isolated from clinical sample and two enzymes (T7 RNA polymerase and reverse transcriptase) amplify this RNA. Hybridization protection assay detects these amplicons by probe hybridization in which only hybridized probes stay chemiluminescent and are detected in a luminometer[168].

Qualitative RT-PCR assays should detect 50 HCV RNA IU/mL or less with the same sensitivity for all genotypes. With the advent of more qPCR that has a lower limit of detection to as low as 30 copies/mL, qualitative assays were replaced especially in diagnostic laboratories[120,169]. These qualitative tests are still very common due to its higher sensitivity, but the main inconvenience is that it only verifies the absence or presence of HCV RNA[121,168].

Some qualitative commercial assays include Cobas Amplicor HCV version 2.0 (Roche Molecular Diagnostics, Pleasonton, CA, United States) and Versant HCV RNA qualitative assay (Siemens Healthcare Diagnostics, Deerfield, IL, United States). Cobas Amplicor HCV is performed according to standard RT-PCR and 50 IU/mL is the limit of detection for all HCV genotypes, while Versant HCV qualitative assay employs TMA and presents analytical sensitivity of 10 IU/mL for most genotypes and 5.3 IU/mL for genotype 1[121].

HCV RNA quantification can be accomplished by target or signal amplification, respectively quantitative RT-PCR (qRT-PCR) and branched deoxyribonucleic acid (bDNA) technology. Due to its good sensitivity (99%) and specificity (98%-99%), the classical techniques for viral genome detection and quantification are progressively being replaced by quantitative PCR[120,169]. When these tests are used to monitor viral load during treatment, it is critical to use the same assay before and during therapy[120,169].

Several tests are commercially available for HCV quantification and those assays employ competitive PCR (Cobas Amplicor HCV Monitor), bDNA technique (Versant HCV RNA), real time PCR (COBAS TaqMan assay and Abbott Real Time HCV test). To quantify HCV by Cobas Amplicor HCV Monitor 2.0, the target and internal standard is amplified in a single reaction tube. The initial quantity of HCV RNA is obtained by comparing the final amounts of both templates. The dynamic range of the Amplicor™ HCV 2.0 monitor assay is 500 to approximately 500000 IU/mL with a specificity of almost 100%, independent of the HCV genotype[170,171]. The Versant HCV quantitative test (Siemens Healthcare Diagnostics) is based on signal amplification by bDNA and present a range of detection of 615 IU/mL to 8 million IU/mL independent of HCV genotype[172]. Martins *et al*[173] compared qualitative (in-house RT-nested PCR and COBAS AMPLICOR HCV Test v2.0 and TMA) and quantitative (COBAS AMPLICOR HCV Monitor Test v2.0 and bDNA) techniques for HCV quantification and detection, and TMA presented the highest rate (87.8%) of HCV detection among qualitative tests being the most sensitive for HCV RNA detection over the early and late phases of HCV infection.

Real time PCR technology allows the direct monitoring of the PCR process due to the detection and amplification of the target nucleic acid at the same time. In order to detect and amplify DNA at the same time, a probe (oligonucleotides containing a quenching molecule and a fluorescent reporter molecule) binds to target cDNA between the two PCR primers and are degraded or released by DNA polymerase during DNA synthesis. When probe is degraded, it occurs the separation of reporter and quencher molecules, which leads to emission of an increased fluorescence signal from the reporter. The quantity of RNA in the starting (first cycle) sample is proportional to the fluorescence signal. Quantification in absolute numbers is obtained by the comparison of kinetics of the target amplification with the amplification kinetics of an internal control of a defined initial concentration[168].

Cobas Taqman HCV present limit of detection and quantification of approximately 15 IU/mL among all HCV genotypes, and a linear amplification range of HCV RNA from approximately 15 to 10000000 IU/mL[174]. Abbott RealTime HCV assay has a lower detection limit of nearly 10 IU/mL, a specificity higher than 99.5% and a linear amplification range from 12 to 10000000 IU/mL independent of the hepatitis C genotype[175-177].

Currently, there are leastwise seven genotypes and more than 80 subtypes of HCV[178,179]. Methods for HCV genotyping are direct sequence analysis, real time PCR, RFLP, and reverse hybridization technology. HCV genotyping is mandatory for double antiviral treatment (interferon and ribavirin), since patients infected with genotypes 1 or 4 are treated for longer times than patients infected by genotypes 2 and 3[180]. Nevertheless, with the accessibility of new and highly effective antiviral therapies, HCV genotyping will not be important in the future.

The first commercial assays for HCV genotyping evaluated exclusively the 5'UTR, which has a high ratio of misclassification particularly on the subtype level. Nowadays commercial tests analyze coding regions, especially non-structural protein NS5B and core protein.

TRUGENE-SIEMENS HCV 5'NC Genotyping Kit (Siemens Medical Solutions Diagnostics, Tarrytown, NY, United States) is useful to detect HCV genotypes and subtypes based on region 5'UTR and has a lower limit of detection of 5000 IU/mL[181,182]. Versant HCV Genotype 2.0 Assay (LiPA) uses oligonucleotide probes specific for the 5'UTR and core regions of the six HCV genotypes and has lower limit of detection of 3700 IU/mL[183]. LIPA is efficient for HCV genotyping, but some divergent results were observed when compared to sequence analysis of the NS5B region at the subtype level (sensitivity of 95.2% for subtype 1b and 96.1% for subtype 1a)[184].

Real time PCR using TaqMan technology as Abbott Real time PCR HCV Assay (Abbott Diagnostics Europe,

Wiesbaden, Germany) determines the viral genotype of the samples for HCV genotypes 1a, 1b, 2a, 2b, 3, 4, 5 and 6 and has limit of detection of 6053 IU/mL[185]. On the other hand, in house methods like RFLP presented a limit of detection using the Probit test ranging from 51 to 3300 IU/mL[161]. RFLP is a useful technique for HCV genotyping in different groups since presents low cost compared to commercial methods[186,187].

The direct sequencing (Sanger sequencing) is the gold standard for determining HCV genotypes and subtypes HCV. Nucleotide sequencing involves sequencing of one or more genes in the HCV genome (mainly the 5′UTR, core, E1, NS3 and NS5) and comparing these sequences to the established genotypes by computer analysis[120]. This provides the most complete information on the variations of the sequences analyzed. Furthermore, it is the most useful method for the study of viral genetic variability[182].

The sequencing technique for HCV genotyping consists of PCR amplification of part of the viral genome, especially these regions: The 5′NC, NS5B and core regions. These regions present diversity for the discrimination of viral genotypes and subtypes and are sufficiently conserved for the development of reliable primers[188]. In addition to genotype determination, direct sequencing is also used for molecular epidemiological studies and to DAA resistance mutations[189-193]. In DAA treatment, the replication is intensively inhibited in drug-sensitive viral population, and the resistant variants gradually predominate in the HCV population[194]. This way, in a near future, the most appropriate treatment for HCV patients will be based on the analysis of the nucleotide or amino acid sequence[195].

Actually, deep sequencing technologies is a promising approach to characterizing viral diversity, they have the ability to generate high throughput screening that provide exceptional resolution for studying the underlying genetic diversity of complex viral populations[112,113,196]. Currently Illumina deep sequencing technology (Illumina Inc. San Diego, CA) and PacBio sequencing technologies (Pacific Biosciences of California, Inc.) are the newest platforms in the market. In HCV studies, deep sequencing technologies are powerful tools for obtaining more profound insight into the dynamics of variants in the HCV quasispecies of human serum. It allows sequencing the complete genome in a short time and is able to generate much more information on the viral genome sequences in internal organs[195].

Due to the rate of 10^{-3} mutations per nucleotide, HCV results in high-circulating quasispecies in infected patients. Recently, it was observed that approximately 15.6% of samples from Pakistan did not match any genotype[197]. Ultra deep sequencing could be useful for identification of these genotypes what is important to determine the pattern of double antiviral therapy (interferon and ribavirin) that is a standard therapy in Pakistan and other countries with few resources.

HCV quantitative methods, like qPCR are useful to monitor antiviral therapy and could detect low viral loads with broad dynamic range. These assays are fully automated and reduced contamination. However, commercial assays present high cost compared to in house qualitative methods. For HCV genotyping, the method most useful is nucleotide sequencing, principally ultra deep sequencing that could identify resistant variants predominate in the HCV population and give information about dynamics of HCV quasispecies. However, it is necessary in-depth knowledge to analyze the results.

The main characteristics, advantages, disadvantages and specific applications of HCV molecular assays are disclosed in Table 4.

CONCLUSION

In this review, we attempted to give information regarding HCV and HBV serological and molecular methods available at clinical and research areas. Most of review articles regarding HBV and HCV diagnosis are relatively old[28,120,148,198] or discuss only one aspect of viral hepatitis diagnosis, for example, rapid tests for HBV[24], rapid tests for HCV[156], serological methods for HBV[49], serological methods for HCV[117], molecular methods for HBV[83] or molecular methods for HCV[120]. In the present review, both serological and molecular methods for HBV and HCV diagnosis were included and new methods such as biosensors and ultra deep sequencing were discussed giving new and updated information about this theme.

Diagnosis of HBV and HCV infection is a key tool to identify acute and chronic cases of infection in order to define preventive measures and to initiate antiviral treatment. Nowadays HBV vaccination and antiviral therapies for HBV and HCV infection have arisen drug resistant, vaccine and diagnosis escape variants that complicate diagnosis and treatment. In this situation, effective diagnosis presenting high sensitivity and specificity is crucial.

Each detection method presents advantages and limitations. EIAs are the most important serological assays used for HBV and HCV detection due to its simplicity, automation and convenience. Nevertheless, they can be time-consuming and expensive and rapid assays have been developed in order to overcome these disadvantages. Molecular techniques are useful to diagnose chronic infection; to identify HBV occult cases; to evaluate the prognosis of disease; to help in treatment decisions and monitor the antiviral treatment efficacy; and to identify resistance mutants to antiviral treatment. Molecular methods present higher specificity and sensitivity and larger dynamic range of detection compared to other diagnostic assays like serological assays. Nevertheless, these methods are relatively expensive and require special instruments and specialized techniques. The choice of each method should be done according advantages and disadvantages and the purpose of diagnosis.

In the near future, biosensors and biochips seems to be useful technologies for serological diagnosis of

HBV and HCV, principally due to real-time diagnosis and early intervention to reduce the burden of diseases. Alternative specimens and rapid assays can also be extremely useful for remote areas, low resource settings and health services with limited laboratory infrastructure. In respect to molecular HBV and HCV assays, digital PCR promises to resolve some of the deficiencies of qPCR by transforming the analog, exponential nature of PCR into a digital and linear signal. For DNA sequencing, ultra deep sequencing will be helpful for analysis of HBV and HCV mutants in order to study the dynamics of viral variants.

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REVIEW

Hepatitis E virus infection: Epidemiology and treatment implications

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Abstract

Hepatitis E virus (HEV) infection is now established as an emerging enteric viral hepatitis. Standard treatments in acute and chronic hepatitis E remain to be established. This study undertakes a review of the epidemiology, treatment implication and vaccine prevention from published literature. HEV infection is a worldwide public health problem and can cause acute and chronic hepatitis E. HEV genotypes 1 and 2 are primarily found in developing countries due to waterborne transmission, while the zoonotic potential of genotypes 3 and 4 affects mostly industrialized countries. An awareness of HEV transmission through blood donation, especially in the immunocompromised and solid organ transplant patients, merits an effective anti-viral therapy. There are currently no clear indications for the treatment of acute hepatitis E. Despite concerns for side effects, ribavirin monotherapy or in combination with pegylated

interferon alpha for at least 3 mo appeared to show significant efficacy in the treatment of chronic hepatitis E. However, there are no available treatment options for specific patient population groups, such as women who are pregnant. Vaccination and screening of HEV in blood donors are currently a global priority in managing infection. New strategies for the treatment and control of hepatitis E are required for both acute and chronic infections, such as prophylactic use of medications, controlling large outbreaks, and finding acceptable antiviral therapy for pregnant women and other patient groups for whom the current options of treatment are not viable.

Key words: Treatments; Blood donors; Adverse effects; Vaccination; Pegylated-interferon; Ribavirin; Hepatitis E

Core tip: Hepatitis E virus (HEV) infection affects individuals in both industrialized and developing countries and can cause acute and chronic hepatitis E. HEV genotypes 1 and 2 are primarily found in developing countries due to waterborne transmission, while the zoonotic potential of genotypes 3 and 4 affects mostly industrialized countries. An awareness of HEV transmission through blood donation, especially in the immunocompromised and solid organ transplant patients, merits an effective anti-viral therapy. The current treatment for HEV infection involving ribavirin and pegylated interferon-α therapy has shown limited efficacy. Although not widely used, an HEV vaccine is available for immunization in China.

Lee GY, Poovorawan K, Intharasongkroh D, Sa-nguanmoo P, Vongpunsawad S, Chirathaworn C, Poovorawan Y. Hepatitis E virus infection: Epidemiology and treatment implications. *World J Virol* 2015; 4(4): 343-355 Available from: URL: http://www.wjgnet.com/2220-3249/full/v4/i4/343.htm DOI: http://dx.doi.org/10.5501/wjv.v4.i4.343

INTRODUCTION

Hepatitis E infection is now regarded as a major cause of fecal-orally transmitted non-A, non-B hepatitis. Hepatitis E virus (HEV) is a non-enveloped, single-stranded RNA virus that contains three open reading frames (ORFs) that encode structural and non-structural proteins[1]. There are 4 major HEV genotypes (1, 2, 3 and 4). Symptomatic HEV infections produce varying clinical presentations depending on the HEV genotypes. Fulminant hepatic failures are caused mainly by genotype 1, while chronic infections have been observed only with genotype 3 thus far[2]. Recent reports of sporadic cases from developed countries in Europe and in the United States resulted from HEV genotype 3, which is zoonotic[3]. In a 2015 survey on the seroprevalence and risk factors for HEV infection among slaughterhouse workers in South Korea, the seropositive rate for HEV IgG was 33.5%[4]. Another report from Thailand in 2014 found that HEV strains identified from acute symptomatic hepatitis E infections were all genotype 3f, which was genetically closely related to a strain isolated in swine[5].

The clinical course of HEV infection among pregnant women is known to be more severe and often led to fulminant hepatic failure and death in up to 20%-25%, specifically in those living in developing countries[6]. The mortality rate has been found to be higher during the 2nd and the 3rd trimester[7]. However, underlying factors influencing high mortality during pregnancy are poorly understood.

Studies in chimpanzees to examine HEV and hepatitis C virus (HCV) infection revealed that HEV showed a lower frequency and a shorter duration of differentially expressed genes compared to HCV in intrahepatic transcriptome analysis. This suggests that HEV may be more susceptible to the innate immunity induced by interferon alpha[8]. However, HEV showed suppression of interferon alpha signaling *in vitro*[9].

MOLECULAR VIROLOGY OF HEV

HEV belongs to the family *Hepeviridae*[10]. It is a small non-enveloped virus 27-34 nm in diameter. The single-stranded positive-sense RNA genome of HEV is approximately 7.2 kb, 7-methylguanine capped at the 5′ terminus, and polyadenylated at the 3′ terminus[11]. Both ends of the genome consist of short 5′ and 3′ untranslated regions, which fold into stem-loop structures and are approximately 58 and 68 nucleotides, respectively[12]. The viral genome contains 3 open reading frames (ORF)[10,13]. ORF1 encodes a 1693 amino acid non-structural polyprotein including: Methyltransferase (MeT), which catalyses the capping of viral RNA; a papain like cysteine protease (PCP) with presumed post-translational protein processing; a helicase (Hel), which supports MeT by catalyzing the first step of RNA capping; an RNA-dependent RNA polymerase (RdRp) required for the synthesis of the genomic RNA; and several uncharacterized domains such as X or Macro, Y and V domain[1,12]. ORF2 located near the 3′ end encodes 599 to 660 amino acids capsid protein[1,10]. It is involved in virion assembly, cell attachment, and immunogenicity. ORF3, which overlaps ORF2, encodes a protein of 114 amino acids shown to be associated with subcellular localization and virion morphogenesis[11,14] (Figure 1).

Replication steps in the HEV life cycle are not easily elucidated due to lack of a suitable cell culture system. However, proposed replication cycle commences with the viral attachment to the surface of target cells (hepatocyte) and binding to the unknown receptor(s). Next, the virus penetrates, uncoats, and releases the genomic RNA. Translation of the nonstructural proteins occurs in the cytoplasm. RdRp synthesizes the negative-sense intermediate RNA from the positive-sense genome, which subsequently acts as a template for

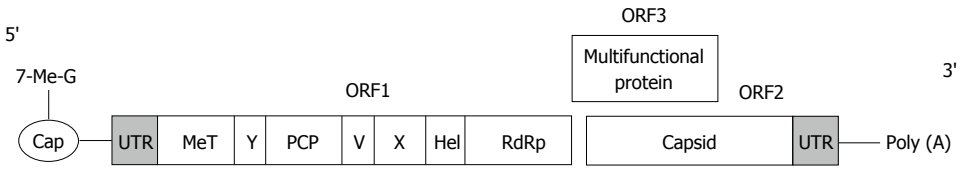

Figure 1 Schematic representation of hepatitis E virus genome[1,12,14]. ORF: Open reading frame; UTR: Untranslated region; MeT: Methyltransferase; PCP: A papain like cysteine protease; Hel: Helicase.

the synthesis of subgenomic RNA and the full-length positive-sense transcripts. The subgenomic RNA is then translated into ORF2 and ORF3 proteins, which facilitates viral assembly and optimizes the host cell environment for viral replication[11,12]. The mechanism of viral egress from the host cells remains unclear[15,16].

IMMUNE RESPONSES IN HEV INFECTION

Investigation of host immune responses against virus infection are essential to understand host immunity and for vaccine development. Several studies have examined the host immune responses following HEV infection and the roles of immune components responsible for causing liver damage as a consequence of HEV infection. Although the mechanisms of hepatic injury enhanced by the host immune responses to HEV infection are still unclear[17-19], we review here the available literatures on HEV infection and how it affects host immunity and induction of liver injury.

Once HEV enters the body, viral clearance involves recruitment of immune cells against infection. These cells recognize HEV in the early stages through pattern recognition receptors. HEV components are then detected by toll-like receptors and retinoic acid-inducible gene-Ⅰ like receptors. Subsequent recruitment of adapter proteins MyD88 and TRIF mediate interferon regulatory transcription factor 3 (IRF3) and nuclear factor κB (NF-κB) to produce type Ⅰ interferons and pro-inflammatory cytokines vital to antiviral response[20].

Type Ⅰ interferons are cytokines, which play important roles in innate immunity against virus infection. Interferon-inducible genes are up-regulated in HEV infected PLC/PRF/5 cells. Replication of HEV in this cell type was inhibited when cells were treated with interferon (IFN)-α2b[21]. A549 cells infected with HEV genotype 3 showed inhibition of IFN-α induced the signal transducer and activator of transcription 1 (STAT1) phosphorylation. Additionally, HEV ORF3 protein enhances IFN-β production induced by poly (I·C), a double-stranded RNA analogue[22]. HEV ORF3 was also shown to bind STAT1 resulting in inhibition of STAT1 phosphorylation[9]. A study in hepatocyte cells demonstrated that HEV infection inhibited IFN-β production induced by poly (I·C). Meanwhile, HEV ORF1 was identified as an IFN antagonist. It inhibited phosphorylation and ubiquitination of various proteins involving in interferon production such as IRF3, retinoic acid-inducible gene 1 and TANK-binding kinase 1[23]. ORF1 also activates the promoter activity of a chemokine, chemokine (C-X-C motif) ligand 8[24]. These reports collectively demonstrated that HEV proteins are involved in the activation of host antiviral cytokines, although some viral components could also inhibit interferon signalling, which facilitate HEV evasion from the host innate immune defenses.

Natural killer (NK) cells and NKT cells are responsible for killing virus-infected cells. Comparison of the numbers and function of NK/NKT cells from HEV infected patients and healthy controls showed that the percentage of activated NK cells was higher in patients than in controls[18]. However, there was no difference in the target cell cytotoxicity of NK cells from both groups. A study of lymphocytes taken from liver biopsy demonstrated that CD56 cell counts were higher in HEV patients than in patients with hepatitis A, B and C virus infections[25]. This group also showed increased $CD8^+$ cells in liver failure cases caused by HEV and other viruses.

IFN-γ is a cytokine responsible for activation of NK and T cell function. IFN-γ expression was up-regulated in peripheral blood mononuclear cells (PBMCs) from HEV infected patients following HEV ORF2 stimulation[26]. In addition, PBMCs from acute viral hepatitis cases elicited higher responses to recombinant HEV ORF2 peptides than PBMCs from the liver failure group[27]. Studies in hepatocytes demonstrated that HEV ORF2 inhibited NF-κB activation[28].

Besides the innate immune responses, the alteration of cell-mediated immune responses by HEV infection had also been reported. HEV infected patients had increased $CD8^+$ and $CD4^+CD8^+$ cells compared with healthy individuals. In addition, proportion of IFN-γ secreting cells in response to recombinant ORF2 and ORF3 were higher in patients than in controls. These data provided evidence of effector T cell responses induced by HEV components[29].

The studies of clinical manifestations in patients with self-limited acute viral hepatitis and acute liver failure support the roles of immune responses in liver injury. HEV infected patients with acute liver failure had higher antibody titers and higher levels of cytokines such as tumor necrosis factor-α, IFN-γ, interleukin-2 (IL-2) and IL-10. HEV RNA was detected in patients with self-limited acute hepatitis group but not in liver failure cases. This observation suggests that the immune responses rather than the virus are responsible for liver damage[30].

For humoral immune response, the incubation period is around 15-60 d after infection[31]. IgM antibodies increase rapidly and then begin to subside after 3

mo[32,33]. Anti-HEV IgG antibodies start to increase when anti-HEV IgM is first detected and may persist for years[17,34,35]. Presently, the viral detection by serology tests has been developed for HEV. Examination of blood and stool of 10 patients with acute HEV infection can identify HEV RNA, but only for a short period[36]. Experimental infection in chimpanzee demonstrated that HEV RNA was detectable in blood around 22 d after inoculation. In human volunteer, HEV RNA could be detected in blood sooner than in feces[37]. Naturally acquired humoral immune response in the body not only increased rapidly after infection, but also afforded protect from infection during an outbreak[38].

Regulatory T cells (Treg) and IL-10, components of the immune system which modulate immune response, were examined in acute hepatitis E patients, recovered individuals and healthy controls. Both percentage of Treg cells and IL-10 levels were higher in acute hepatitis patients than in recovered and healthy individual groups[39,40], implicating a role for Treg in the immune response induction as a result of HEV infection.

The regulation of protection in the body involves a complex network of innate and adaptive immune response. Immune responses are shown to promote tissue injury in various infectious diseases. During viral infection, both innate and adaptive immune responses are elicited in order to eliminate pathogens. Even so, several viruses are able to evade eradication by the host immunity. Prolonged or hyper-immune responses to eradicate persistent infection, however, could result in tissue injury. Knowledge gained from HEV studies thus far have mostly relied on recombinant HEV proteins instead of viral infections due to the lack of an efficient cell culture system. Moreover, most HEV infections are self-limited. Future studies including patients presented with various degrees of clinical manifestations should allow better understanding of the immunity or immunopathology induced by HEV infection.

EPIDEMIOLOGY OF HEV

HEV is a leading cause of hepatitis transmitted *via* fecal-oral route[41]. The first outbreak of HEV occurred in India around 1955-56, which affected as many as 29000 persons[42]. Other large outbreaks were reported in China, India, Somalia and Uganda[1]. Outbreaks were observed to occur after heavy rainfall and floods, which allowed contamination of human excreta with drinking water sources. Alternatively, outbreaks sometimes follow the dry summer season when water in the rivers or streams is reduced, resulting in increased concentration of fecal contamination in water[43].

It is known that humans could be infected with all 4 HEV genotypes[44]. In developing countries where HEV is endemic (Indian subcontinent, Asia, Middle East and Africa)[11], HEV genotypes 1 and 2 are common and restricted to human. In developed countries, these two genotypes were diagnosed only in persons who had recently traveled to highly endemic areas[45]. It is estimated that 21.1 million people, or 71% of the world population, are infected with HEV genotypes 1 and 2. Furthermore, HEV infection results in approximately 3 million symptomatic acute cases each year and 70000 deaths worldwide[1,44,46]. Individuals could be infected with HEV genotypes 1 and 2 from drinking contaminated water[47], and unfortunately people affected in large HEV outbreaks were found using water from a common source for cooking, drinking, and bathing. HEV RNA has also been detected in the sewage-contaminated water source. The limitation of inadequate public infrastructure may facilitate the spread of HEV infection, such as in refugee or military camps[43]. Areas affected by natural disasters such as earthquakes and monsoon storms are also at risk of HEV epidemic. Displaced populations with limited access to clean drinking water, lack of sanitary facilities, overburdened health-care infrastructure, and immunologically naive population lacking protective antibodies combined will increase the likelihood of HEV transmission[48]. Large outbreaks of HEV have occurred in Nepal, which in 2014 involved more than 10000 cases[49].

HEV infection does not only affect developing countries. HEV genotypes 3 and 4 are autochthonous in several industrialized countries. Occasional foodborne outbreaks have occurred in Europe, North America, Japan and New Zealand from consuming undercooked meat contaminated with HEV[50-53]. HEV genotypes 3 and 4 are found in human and other animal species such as pig, wild boar, and shellfish[54-56]. Specifically, HEV genotype 3 is zoonotic in developed countries from reports in pig farmers, individuals who came into close contact with animal reservoir and who consumed raw meat or meat products from deer, wild boar, and pig. HEV RNA could also be detected in food products such as liver and sausage. Viral sequence extracted from uncooked meat or sausage were very similar (99.7%-100% identity) to sequences of virus recovered from HEV-infected patients. In addition, HEV genotype 4 infection could be detected in both human and swine in Eastern Asia and Europe[57,58]. These evidence supported foodborne zoonotic HEV transmission, which occurred from consuming infected meat[59-63].

Several studies suggest that HEV genotypes 3 and 4 could be transmitted across multiple species. In animal models, pathogen-free pigs could be experimentally infected with human HEV genotypes 3 and 4. Furthermore, swine HEV genotypes 3 and 4 inoculated in rhesus monkey and chimpanzee resulted in seroconversion and virus shedding in feces. Anti-HEV IgG seroconversion in the pigs was observed within 2 wk. These experiments suggest that human HEV genotypes 3 and 4 could replicate in pigs and likely originated from swine[64]. In contrast, pigs inoculated with HEV genotypes 1 and 2 were not infected. This suggests that HEV genotypes 1 and 2 were host-restricted compared to genotypes 3 and 4[65]. Therefore, zoonotic transmission to humans is possible for HEV genotypes 3 and 4[65-67]. Other routes of transmission

Table 1 Global prevalence of anti-hepatitis E virus IgG in different populations

Regions	Prevalence (%)	Ref.
Low-to-medium income		
Kashmir region	49.6	Khuroo *et al*[35]
India	23.8-28.7	Mathur *et al*[76]
Myanmar	32.0	Nakai *et al*[77]
Egypt	67.7	Stoszek *et al*[71]
Bangladesh	22.5	Labrique *et al*[78]
China	19.7	Dong *et al*[79]
Mexico	36.6	Alvarado-Esquivel *et al*[80]
Thailand	14.0	Gonwong *et al*[75]
Nigeria	42.7	Junaid *et al*[81]
Industrialized		
Germany	17.0	Wenzel *et al*[82]
United States	6.0	Teshale *et al*[83]

including HEV-infected blood transfusion, mother-to-child, person-to-person, and sexual intercourse were also documented but relatively uncommon[68-70].

Seroprevalence of anti-HEV IgG varies in different parts of the world. In developing countries, positive anti-HEV IgG serology might reach as high as 70%[71]. Being male, having pet animals or frequent contact with animal reservoirs, residing in the endemic area, or consuming liver or other organ meats were highly associated with positive HEV serology. Veterinary and slaughterhouse workers also have high seropositive rates for anti-HEV IgG compared to individuals with no occupational exposure to swine[72,73]. In swine-dense areas, individuals were more likely to be HEV-seropositive compared to those living in areas with few pig farms[74,75]. The seroprevalence of anti-HEV IgG antibodies is shown in Table 1.

CLINICAL MANIFESTATIONS AND DIAGNOSTIC CRITERIA

HEV infection produces wide-ranging clinical manifestations. Most cases of HEV-related acute viral hepatitis resolve within 1 to 2 mo. However, HEV sometimes leads to acute liver failure, chronic infection, or extrahepatic symptoms[17]. An acute hepatitis from HEV is indistinguishable from other forms of acute viral hepatitis and is usually asymptomatic or self-limiting in most individuals[34]. However, acute hepatitis E infection can be severe and prolonged among the immunocompromised and even healthy individuals[84]. Severe acute hepatitis E infections have been described in pregnant women, elderly men, and persons with pre-existing chronic liver disease[85-87]. Among people with the chronic liver disease, acute hepatitis E can adversely lead to acute-on-chronic liver failure[88]. Acute HEV infection with pronounced symptoms resulted in higher mortality rates of 1%-4% and up to 11%, compared to the mortality rates of acute HAV (1%) or HBV (1.5%) infection based on CDC viral hepatitis surveillance data (2010)[84]. Extrahepatic manifestations including pancreatitis, arthritis, aplastic anemia, and neurologic complications have all been reported[11,89,90].

Hepatitis E, especially genotype 3, is known to cause chronic infection among the immunocompromised, especially solid organ recipients. Recently, however, there are reports of chronic hepatitis E infection in elderly immunocompetent patients[91,92]. Diagnosis of acute hepatitis E infection is established in patients with clinically relevant symptoms of acute hepatitis with positive level of anti-HEV IgM or a level of anti-HEV IgG that is twice the baseline concomitant with detectable HEV RNA in the serum and/or stool[93]. Chronic hepatitis E is defined by the persistent increase in liver enzyme levels and polymerase chain reaction-detectable HEV in the serum and/or stool over 6 mo[94].

TREATMENT OF ACUTE HEPATITIS E

Currently, there is no indicated treatment of acute hepatitis E. Nevertheless, treatment with ribavirin showed significant clinical improvements by reducing the symptomatic period (Table 2). Considering the high mortality caused by acute hepatitis E in acute-on-chronic liver failure, ribavirin therapy provides significant benefits for those with poor prognosis or at high risk of fulminant liver failure such as underlying chronic liver disease. Moreover, a high percentage of immunosuppressed individuals [such as human immunodeficiency virus (HIV)-positive individuals or recipients of organ transplant] who eventually develop chronic hepatitis E necessitate early and effective treatment of acute hepatitis E[84,88,95-98].

TREATMENT OF CHRONIC HEPATITIS E

In a case-control study of solid-organ transplant recipients performed in France, 22 out of 38 individuals who tested positive for HEV genotype 3 (58%) developed chronic infection. Compared to the control group of 148 individuals who had no markers for HEV infection, one independent factor associated with HEV infection was the consumption of game meat (68% *vs* 47%, OR = 2.32)[3]. Moreover, a retrospective analysis of data from Europe and the United States found that among 85 recipients of solid organ transplants who had HEV infection, 56 patients (65.9%) developed chronic hepatitis E[94]. The main factor associated with developing chronicity assessed by multivariate analysis in this study was tacrolimus use for immune-suppression (OR = 1.87; 95%CI: 1.49-1.97). The reduction of immunosuppressive drugs, however, enabled HEV clearance in one-third of the individuals.

When reduction of immunosuppression is impossible or when clearing HEV by immunosuppression could not be achieved, 2 alternative therapies for chronic hepatitis E may be pursued: (1) Ribavirin monotherapy (dose 29-1200 mg/d, for 1-18 mo)[96,99,100]; and (2) Pegylated (Peg)-IFN-α for 3-12 mo[101,102].

It was reported that among 59 patients with chronic HEV after solid organ transplantation, HEV clearance was observed in 95% of the patients at the end of

Table 2 Recent evidences on the outcome of therapies against acute hepatitis E infection

Ref.	Type of study	Patient profile	HEV genotype	Ribavirin regimen	Results
Gerolami *et al*[84]	Case report	61-year-old man, 7 d after admission ALT 4565 IU/L	3	1200 mg/d for 21 d	At day 21 of treatment, ALT normalized, RNA almost undetectable
Péron *et al*[88]	Case report	79-year-old man with chronic liver disease, acute kidney failure	3f	200 mg/d for 3 mo	Serum HEV RNA negative at 1 mo therapy, stopped dialysis at 2 mo
		A patient with chronic liver disease	3f	1000 mg/d for 10 d	Viral load 4.07 log copies/mL declined to 2.54 log copies/mL at day 6, Hgb 12.6 g/dL declined to 11.6 g/dL at day 6 of treatment
Del Bello *et al*[95]	Case report	65-year-old man, liver transplant recipient Guillain-Barré syndrome with severe necrotizing myositis	3f	400 mg/d adapted to GFR (40 mL/min) for 3 mo	HEV RNA undetectable by day 15, progressive recovery of mobility
Pischke *et al*[96]	Case from prospective case series	42-year-old woman had traveled to Eritrea and acquired severe acute hepatitis E	1e	For 6 wk (dose: Undefined)	Rapidly improved liver function and cleared HEV
Robbins *et al*[97]	Case report	39-year-old man HIV (+) CD4 51/mm^3 prothrombin index 45%	3c	1200 mg/d (15 mg/kg per day) for 12 wk	Gradual normalization of LFT-HEV RNA decreased to < 100 copies/mL at 1 mo of treatment
Riveiro-Barciela *et al*[98]	Case report	68-year-old man with Waldenström's macroglobulinemia	3f	800 mg/d for 12 wk	Achieved SVR after 12 wk; no ribavirin-related side effect reported

LFT: Liver function test; ALT: Alanine transaminase; Hgb: Haemoglobin; GFR: Glomerular filtration rate; SVR: Sustained virological response; HIV: Human immunodeficiency virus; HEV: Hepatitis E virus.

ribavirin therapy and that 78% of the patients achieved sustained virologic response (defined by undetectable serum HEV RNA at least 6 mo after cessation of therapy). These patients received ribavirin for a median of 3 mo. Longer treatment duration allowed 4 patients who had recurrence to achieve sustained virologic response[100]. The mechanism that ribavirin acts against HEV is not clearly understood[103]. However, a recent study suggests that ribavirin exerts an antiviral effect against HEV by depleting intracellular guanosine 5'-triphosphate pools[104]. Further research on this mechanism as well as other possible mechanisms of action of ribavirin is to be revealed. Meanwhile, overall data showed that ribavirin provided therapeutic effect in the treatment of chronic hepatitis E with the only significant adverse effect being anemia (Table 3).

Treatment with Peg-IFN-α has been reported (Table 4). The duration of therapy ranged from 3 mo to 1 year. Most of the cases are from solid organ transplant recipients, all of them showed the favourable outcome in liver enzyme levels as well as viral RNA suppression. However, 2 out of 6 transplanted patients developed acute allograft rejection after 3-mo Peg-IFN therapy[101,109].

The slight synergistic effect for ribavirin combined with Peg-IFN-α was observed in a recent study *in vitro*[104]. Successful combination therapy had also been reported for a chronic HEV infection in an HIV-positive patient[110]. Decreasing the ribavirin dosage may help reduce anemia and other treatment-associated side effects[111].

HEPATITIS E VACCINE

Current data demonstrated that immunity acquired from infection or vaccination can protect individuals from symptomatic hepatitis E. In a large cohort study, the risk of infection was highest among the baseline seronegative placebo group participants (2.04%). The risks of HEV infection in population with pre-existing immunity or vaccine-induced immunity were significantly reduced to 0.52% and 0.30%, respectively[112]. Two recombinant hepatitis E vaccines developed from HEV genotype 1, by Glaxo SmithKline and Xiamen Innovax Biotech, have had short-term efficacy in clinical trials[113,114]. The latter vaccine, commercially available as Hecolin, has been in use in China since 2012. Its long-term efficacy was 86.8% during the 4.5-year follow-up period[93]. Estimated long-term persistence of anti-HEV IgG from hepatitis E vaccine is predicted to be from 8 years to nearly life-long based on mathematical assumptions[115]. The only currently licensed hepatitis E vaccine (Hecolin) is approved for use in China in those aged 16-65 years available in prefilled syringe for intramuscular injection at 0, 1, and 6 mo. Expansion of vaccine coverage to other HEV endemic country is necessary and might significant decrease burden of the disease[85]. In addition to improved personal hygiene, sanitation, and health education, vaccination might play a crucial role in the future prevention and control of HEV infection.

HEV IN BLOOD DONORS: CLINICAL IMPLICATIONS

Experiments involving the transfusion of blood plasma from anti-HEV IgM positive and anti-HEV IgG negative blood donors to rhesus monkey demonstrated that the

Table 3 Treatment of chronic hepatitis E virus with ribavirin regimen

Ref.	Type of study	Patient profile	Ribavirin regimen	Result	Adverse effects
Kamar *et al*[99]	Prospective case series	6 kidney transplant recipients, HEV RNA (+) for median of 36.5 mo	600-800 mg/d for 3 mo adapted to GFR, Hgb	SVR in 4/6 patients; relapse in 2/6; AST, ALT normalized all	Anemia led to blood transfusion and RBV dose reduction in 2/6 patients
Mallet *et al*[105]	Case report	A kidney and pancreas transplanted man, a women with idiopathic $CD4^+$ T lymphocytopenia	12 mg/kg daily for 12 wk	Both cleared HEV after 4 wk of treatment and remained undetectable, LFT normalized	Anemia in 1st patient led to Ribavirin dose reduction to 200 mg/d
Pischke *et al*[96]	Prospective case series	Organ transplant recipients 11 subjects	600-1000 mg/d for 5 mo, dose reduction according to Hgb or anemia	9/11 showed SVR	Anemia, the mean Hgb decline was 3.4 g/dL (range 0-7.9 g/dL)
Neukam *et al*[106]	Case report	2 HIV (+) male with liver cirrhosis with severe immunosuppression	Oral ribavirin 1200 mg/d (case 1) 1000 mg/d (case 2) for 24 wk	LFT normalized-Liver stiffness improved HEV RNA was detected after the end of treatment in both patients	-
Giordani *et al*[107]	Case report	60-year-old man with lymphocytic leukemia	1000 mg/d in 2 doses (400 and 600 mg), for 3 mo	HEV cleared and sustained over 6 mo after therapy	Mild anemia (Hgb 10.5 mg/dL)
Kamar *et al*[100]	Retrospective, multicentre case series	37 kidney, 10 liver, 5 heart, 5 kidneys and pancreas, and 2 lung transplant recipients with chronic HEV	Median dose of 600 mg/d (range 29-1200), for a median of 3 mo (range 1-18 mo)	At the end of the therapy, 95% cleared HEV, 18% recurred after cessation of therapy is stopped, 78% showed SVR	Anemia required dose reduction (29%); use of erythropoietin (54%); required blood transfusion (12%)

LFT: Liver function test; AST: Aspartate aminotransferase; ALT: Alanine transaminase; SVR: Sustained virological response; Hgb: Haemoglobin; GFR: Glomerular filtration rate; HEV: Hepatitis E virus; HIV: Human immunodeficiency virus.

Table 4 Treatment of chronic hepatitis E virus with pegylated interferon-α therapy

Ref.	Patient profile	Peg-IFN-α regimen	Result	Adverse effects
Kamar *et al*[101]	29-year-old man with liver transplantation	Peg-IFN-α-2a for 12 wk (135 μg/wk)	Liver enzyme levels decreased. HEV RNA levels remained undetectable until week 12	At week 12, signs of acute humoral rejection in liver biopsy
	26-year-old man with liver transplantation	Peg-IFN-α-2a for 12 wk (135 μg/wk)	HEV RNA levels undetectable by week 12; liver enzyme levels normalized by week 12	
	58-year-old man with liver transplantation liver cirrhosis from chronic HEV infection	Peg-IFN-α-2a for 12 wk (135 μg/wk)	HEV RNA was redetected 2 wk after completion of treatment; Liver enzyme levels normalized by 3 mo of therapy	
Haagsma *et al*[102]	37-year-old woman with liver transplantation	Peg-IFN-α-2b for 52 wk (80 μg/wk declined to 60 μg/wk)	Serum HEV RNA sustained undetectable during 3 mo follow-up; serum liver enzyme became normalized	
	59-year-old man with liver transplantation	Peg-IFN-α-2b 150 μg/wk, dose reduction due to leukopenia	HEV viral load and aminotransferases declined, but Peg-IFN discontinued from lack of further efficacy, HEV RNA level undetectable at 4 wk after the discontinuation of Peg-IFN and aminotransferase normalized	Leukopenia
Alric *et al*[108]	57-year-old man with hairy cell leukemia	Discontinued at week 16, Peg-IFN-α-2b 1 μg/kg per week for 3 mo	Achieved a complete virologic response by week 4	
Kamar *et al*[109]	24-year-old man with kidney transplantation, kidney failure from chronic HEV infection	3-mo Peg-IFN-α-2a 135 μg/wk	Serum RNA undetectable after 5 mo, SVR for 6 mo after treatment	Acute rejection of the kidney allograft by month 3 of Peg-IFN therapy

Peg-IFN: Pegylated interferon; SVR: Sustained virological response; HEV: Hepatitis E virus.

virus was transmissible[116]. Therefore, many developed countries are now focused on studying the prevalence of viral transmission from blood donation[117-124] (Table 5). The demand for pathogen-free blood and blood components is highly needed for hospital patients and individuals requiring continuous blood transfusion (*e.g.*, thalassemia patients). Thus, these patients are at-risk for being infected with HEV from donated blood. The development of screening methods for detecting HEV in donated blood involves both serology test and nucleic acid test. Novel techniques are being developed to increase the efficiency and sensitivity to identify HEV rapidly even at low viral concentration[125-127]. Many countries are aware of the necessity to screen the blood supply for HEV among blood donors and have begun to implement diagnostic tests for HEV. For example, Japan has started monitoring for HEV by comparing the increase in the alanine aminotransferase as a biomarker

Table 5 Incidence of detectable hepatitis E virus in blood donors (hepatitis E virus-RNA)

Year of study	Countries	Technique used for detection	No. of tests	Ratio of positive detections	Ref.
2005	China	Real-time fluorescence RT-PCR	10741	1:1094	Ren *et al*[117]
2011	England	PCR	42000	1:7000	Ijaz *et al*[118]
2011	German	Real-time RT-PCR	18100	1:4525	Baylis *et al*[119]
2011	Sweden	Real-time RT-PCR	95835	1:7986	Baylis *et al*[119]
2011	United States	Real-time RT-PCR	51075	None detected	Baylis *et al*[119]
2011	German	Real-time RT-PCR	16125	1:1241	Vollmer *et al*[120]
2011-2012	The Netherlands	Real-time PCR	45415	1:2672	Slot *et al*[121]
2012-2013	England	RT-PCR	225000	1:2848	Hewitt *et al*[122]
2012	France	RT-PCR	53234	1:2218	Gallian *et al*[123]
2013	Spain	Transcription-mediated amplification assay	9998	1:3333	Sauleda *et al*[124]

RT-PCR: Reverse transcription-polymerase chain reaction.

in the surveillance of HEV[128]. Germany is looking for a new approach to detecting HEV to find alternative ways to blood screening test[120]. Other countries have also implemented screening test but only focus on the suspected cases[129]. In Thailand, there were two reports on the incidence of HEV among blood donors. First, a study in 1996 reported the prevalence of HEV transmission among adults in different parts of Thailand, in which the studied population also included blood donors. The study found 9%-22% positive rates for anti-HEV IgG[130]. Another study found HEV seroprevalence around 8.7% among blood donors in 4 Northern provinces of Thailand[131], which was similar to those in other countries (Table 5). Thus, HEV poses a significant public health problem especially in blood donation even if the prevalence and virulence of the disease are lower than other infections[132].

CHALLENGES IN THE TREATMENT OF HEPATITIS E

Although the recent reports of treatment of HEV infection are showing beneficial outcomes, there are still areas to be overcome in the treatment of HEV infection. First of all, as described by previous articles, there are known severe side effects of current regimen with Peg-IFN-α and ribavirin. For Peg-IFN-α, the severe side effects include influenza-like symptoms[133] and acute rejection of allografts for solid organ transplant recipients[101]. For ribavirin monotherapy, it has the side effect of severe hemolytic anemia, sometimes resulting in treatment failures probably caused by dose reduction[100,111]. Furthermore, both ribavirin and Peg-IFN-α cannot be administered in pregnancy. Also, ribavirin use requires close monitoring of hemoglobin levels and other hematological parameters that make it difficult to apply in developing countries[111].

There has been a case report showing resolution of acute liver injury cause by hepatitis E with steroid use that was initially intended for immunosuppression[134]. Also, there have been reports of fulminant hepatic failure from HEV in women taking oral contraceptives[135]. Known that immunosuppression can cause persistent infection of hepatitis E, the relation between steroid hormone use and the clinical course of hepatitis E is not clear as the immune pathogenesis of hepatitis E infection itself being not explained thoroughly[2]. The various manifestations of hepatitis E according to the potential immune and hormonal status including pregnancy need to be explored precisely in their relation and the pathogenesis for the future direction of developing treatment regimen of hepatitis E. Larger research for establishing appropriate standard treatment as well as supportive treatment and steroid use are still in need in order to minimize limitations and side effects of the current administration of ribavirin and Peg-IFN-α monotherapy, with appropriate vaccination for the high-risk populations for controlling epidemics in resource-limited settings, and for pregnant women living in developing countries where the acute infections are threatening extremely great number of women and new-borns.

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Abstract

Entry of acquired immune deficiency syndrome virus into the host immune cell involves the participation of various components of host and viral cell unit. These components may be categorized as attachment of the viral surface envelope protein subunit, *gp120*, to the $CD4^+$ receptor and chemokine coreceptors, *CCR5* and *CXCR4*, present on T cell surface. The viral fusion protein, *gp41*, the second cleaved subunit of Env undergoes reconfiguration and the membrane fusion reaction itself. Since the $CD4^+$ T cell population is actively involved; the ultimate outcome of human immunodeficiency virus infection is total collapse of the host immune system. Mathematical modeling of the stages in viral membrane protein-host cell receptor-coreceptor interaction and the effect of antibody vaccine on the viral entry into the susceptible host cell has been carried out using as impulsive differential equations. We have studied the effect of antibody vaccination and determined analytically the threshold value of drug dosage and dosing interval for optimum levels of infection. We have also investigated the effect of perfect adherence of drug dose on the immune cell count in extreme cases and observed that systematic drug dosage of the immune cells leads to longer and improved lives.

Key words: Human immunodeficiency virus; Acquired immune deficiency syndrome; Antibody vaccine; Perfect drug adherence; Impulsive differential equation

Core tip: Use of single-cell antibody-cloning techniques uncover naturally arising, broad and potent human immunodeficiency virus (HIV) neutralizing antibodies. These antibodies can protect against infection and suppress new HIV infection. This antibody vaccination gives new ideas about the fight against the HIV infection. From the analytical study of the effect of antibody vaccination we found the threshold value of drug dosage and dosing interval for optimum levels of infection. We have also investigated the effect of perfect adherence of drug dose on the immune cell count in extreme cases and observed that systematic drug dosage of the immune cells leads to longer and improved lives.

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INTRODUCTION

Over the last two decades there has been extensive research on the area of human immunodeficiency virus (HIV) infection invading the human immune system. According to the World Health Organisation (WHO), almost 75 million people have already been infected with the HIV virus and about 36 million people have died of HIV. Globally, 35.3 million people were living with HIV at the end of 2012. An estimated 0.8% of adults aged 15-49 years worldwide are living with HIV, although the burden of the epidemic continues to vary considerably between countries and regions. Sub-Saharan Africa remains most severely affected, with nearly 1 in every 20 adults living with HIV and accounting for 71% of the people living with HIV worldwide. $CD4^+$ T lymphocyte count is the only way to discover the disease progression monitoring during anti retroviral treatment (ART). From the premature days of infection, $CD4^+$ T-lymphocyte cells have been acknowledged as most important for HIV disease progression[1]. A healthy human adult has about 1000 $CD4^+$ T cells per microliter of blood[1], and when the number of $CD4^+$ T cells is reduced below 200/μL, as HIV infected patient considered as acquired immune deficiency syndrome (AIDS) patients[2,3]. The infection process of the human cells by the human immunodeficiency virus (HIV) is very complicated process. When HIV invades into the body, it targets the immune cells, mainly the $CD4^+$ T cells. HIV virus can easily hit into $CD4^+$ T cells by a binding process between the envelope proteins (*gp*41/*gp*120) on the surface of HIV with both the CD4 receptor and the chemokine coreceptor. The entry process is initiated by binding of *gp*120, a cleaved subunit of viral surface envelope protein, *Env*, to the $CD4^+$ receptor present on the T cell surface. However, this binding will be futile unless a conformational change is induced in *gp*120 resulting in exposure of a coreceptor binding site and enabling it to bind to as N-terminus of chemokine coreceptor, namely *CCR*5 and *CXCR*4, when a heterotrimeric complex of *gp*120 - C_{D4}-coreceptor is formed. Basically, the chemokines are small soluble paracrine signaling molecules involved in trafficking and recruitment of leukocytes to sites of injury and inflammation. Chemokines and their receptors contribute significantly to disease progression in HIV-afflicted patients. Co-receptor availability and expression determine host susceptibility to infection. These coreceptors are determinants of viral tropism. After that, viral fusion occurs in the target cell membrane and the genetic material viral RNA gets entry into the $CD4^+$ T cell. This genetic material has a reverse transcriptase enzyme. By the reverse transcription process the RNA genome is reverse-transcribed to a DNA copy, and thus the cell becomes infected. This provirus can enter into the host cell genome where it can stay in an actively infected state or a latently infected state.

Virus specific antibodies are created by a complex differentiation pathway that includes B cell proliferation, isotype switching, germinal center formation and affinity maturation[4]. As a result, the presence of specific antibody secreting plasma cells and antibody production tends to continue long after the infection. In this outlook we consider B cell responses by assuming that antibodies are produced in response to free virus and that antibody production diminishes at a certain rate. But the antibody response diminishes during the course of infection and thus the immune system cannot fight against the HIV.

A recent vaccine experiment shows the use of single-cell antibody-cloning techniques that uncovered naturally arising, broad and potent HIV neutralizing antibodies (bNAbs)[5]. It is observed experimentally that these antibodies can defend against infection and suppress HIV infection in animals. The invention of this antibody vaccination gives new ideas about the fight against the HIV infection. The most contemporary approach to study the drug dynamics is determined by use of impulsive differential equations. Perfect or imperfect drug adherence and drug holidays can make easy the development of resistance. In recent years the effects of perfect adherence to antiretroviral therapy have been studied by impulsive differential equations[4,6-10]. Using this method, the dosing period and threshold values of dosage can be obtained more precisely. Also the effect of maximal acceptable drug holidays can be found by using impulsive differential equations[4]. Impulsive differential equations result if drug effect as well as that of the metabolites are assumed to decay with time in an exponential manner during each cycle and are assumed to change instantaneously at dosing times, t_j for different drug doses and can result in either implicit or explicit models[6-8].

This article is arranged in the following manner: In the first section, we formulate the basic mathematical model on the basis of antibody responses against the virus and applying impulsive differential equations, we show how antibody responses on the human immune system. Analytical and numerical studies have been performed in the next sections. Lastly, we discuss the implication of the results which were found in the earlier sections.

THE MODEL

In this research article, we have modified the explicit mathematical model as proposed by Roy *et al*[11], considering the perfect adherence behavior of CTL vaccination in HIV infected patients. Here G_1 represents the concentration of viral *Env* subunit, *gp*120 *in vivo*, and C_{D4} denotes the concentration of C_{D4} receptor on T cell surface. Let C_1 be the concentration of the dimeric complex of *gp*120 and C_{D4} receptor, G_2 be the concentration of viral fusion protein, *gp*41, and C_{CR} be the concentration of the chemokine

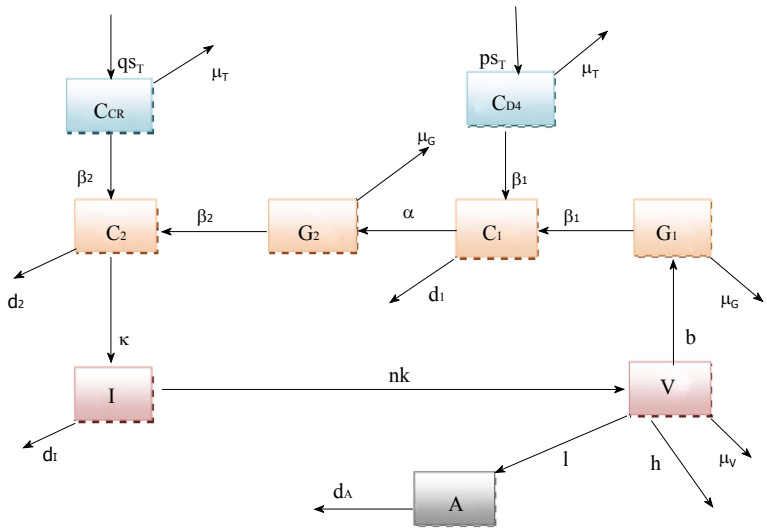

Figure 1 Schematic explanation for the model (1) showing the effect of high drug levels.

coreceptor on CD4$^+$ cells. Also, C_2 be the concentration of the combination of *gp*120 and *gp*120 - C_{D4} chemokine coreceptor ternary complex, *I* denote the concentration of infected CD4$^+$ T cells, and *V* denote the concentration of HIV virus. The model is represented in Figure 1. Here *A* stands for concentration of antibody response. We suppose that antibody responds proportionally to the production of free virus particles at a rate *IVA*. Antibody responses neutralize free virus particles at a rate *hVA*. In presence of antibody vaccination through perfect adherence, the model is given by:

$$dG_1/dt = bV - \mu_G G_1 - \beta_1 G_1 C_{D4}$$
$$dC_{D4}/dt = ps_T - \mu_T C_{D4} - \beta_1 G_1 C_{D4}$$
$$dC_1/dt = \beta_1 G_1 C_{D4} - d_1 C_1$$
$$dG_2/dt = \alpha C_1 - \mu_G G_2 - \beta_2 G_2 C_{CR}$$
$$dC_{CR}/dt = qs_T - \mu_T C_{CR} - \beta_2 G_2 C_{CR}$$
$$dC_2/dt = \beta_2 G_2 C_{CR} - d_2 C_2$$
$$dI/dt = kC_2 - d_I I$$
$$dV/dt = nd_I I - \mu_V V - hVA$$
$$dA/dt = lVA - d_A A \qquad \text{for} \quad t \neq t_k \qquad (1)$$

$$\triangle A \equiv A(t_k^+) - A(t_k^-) = \bar{A} \qquad \text{for} \quad t = t_k \qquad (2)$$

In the system (1), *b* represents the multiplication capacity of *gp*120 in response to virus. The parameter a represents the successful exposure of *gp*41, since it is exposed only after attachment between *gp*120 and the C_{D4} receptor is complete. The parameter μ denotes the decay of *gp*120 and *gp*41, and β_1 denotes the bonding force between *gp*120 and the C_{D4} receptor. Further β_2 denotes the bonding force between the dimeric complex of *gp*120 and C_{D4} receptor and also the chemokine coreceptor. The source of susceptible CD4$^+$ T cells is represented by s_T, and *p* and *q* denote the number of C_{D4} receptors and chemokine coreceptors on one CD4$^+$ T cell respectively. The death rate of healthy CD4$^+$ T cell μ_T and the death rate of infected CD4$^+$ T cells is d_I. The dissociation rate of C_1 and C_2 are d_1 and d_2 respectively and for the sake of simplicity, it is assumed that after dissociation of C_1 and C_2, they will not return to their respective components. Here *n* represents the number of virus particles that are produced by one infected CD4$^+$ T cell. The clearance rate of free HIV virus is represented as μ_V. Antibody responses by *A* are produced at a rate *l* and d_A indicates the death rate of antibody responses. The concentration of antibodies (bNAbs) is represented by $A_{(t)}$ in plasma. The drug dose, $\bar{A}$, that is taken at each impulse time t_j (j = 1, 2, 3...) is kept constant. Since vaccination may be taken at either regular or irregular intervals, we have considered. The impulse time t_j to be fixed. The system (1) together with the system (2) represent the dynamics with vaccine. The vaccination interval $\tau = t_{j+1} - t_j$ is fixed. Here we have only analyzed the models (1) - (2).

ANALYSIS OF THE MODEL

In absence of drug

To study the model (1) together with (2), we first analyze the model in absence of drug. When drug is not administered to the system, we have observed that there exist two equilibrium point: (1) The disease free equilibrium point $\bar{E}$; and (2) The endemic equilibrium point E^*.

The disease free state: In absence of drug, there may exist disease free equilibrium point $\bar{E}$ which is given by

$$\bar{E}\,(0, ps_T/\mu_T, 0, 0, qs_T/\mu_T, 0, 0, 0, 0, 0) \qquad (3)$$

Now for the system (1) and (2) the Jacobian matrix is $J = [J_1|J_2]$, where

$$J_1 = \begin{bmatrix} -(\mu_G + \beta_1 C_{D4}) & -\beta_1 G_1 & 0 & 0 & 0 \\ -\beta_1 C_{D4} & -(\mu_T + \beta_1 G_1) & 0 & 0 & 0 \\ \beta_1 C_{D4} & \beta_1 G_1 & -d_1 & 0 & 0 \\ 0 & 0 & \alpha & -(\mu_G + \beta_2 C_{CR}) & -\beta_2 G_2 \\ 0 & 0 & 0 & -\beta_2 C_{CR} & -(\mu_T + \beta_2 G_2) \\ 0 & 0 & 0 & \beta_2 C_{CR} & \beta_2 G_2 \\ 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 \end{bmatrix}$$

and

$$J_2 = \begin{bmatrix} 0 & 0 & b & 0 \\ 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 \\ -d_2 & 0 & 0 & 0 \\ k & -d_I & 0 & 0 \\ 0 & nd_I & -(\mu_V + hA) & -hV \\ 0 & 0 & 0 & -d_A \end{bmatrix}$$

The characteristic equation for the disease free equilibrium $\bar{E}$ is

$$(\Lambda + d_A)(\Lambda + \mu_T)^2 G(\Lambda) = 0. \quad (4)$$

where

$$G(\Lambda) = \begin{bmatrix} \Lambda + \mu_G + \beta_1\bar{C}_{D4} & 0 & 0 & 0 & 0 & -b \\ -\beta_1\bar{C}_{D4} & \Lambda + d_I & 0 & 0 & 0 & 0 \\ 0 & -\alpha & \Lambda + \mu_G + \beta_2\bar{C}_{CR} & 0 & 0 & 0 \\ 0 & 0 & -\beta_2\bar{C}_{CR} & \Lambda + d_2 & 0 & 0 \\ 0 & 0 & 0 & -k & \Lambda + d_I & 0 \\ 0 & 0 & 0 & 0 & -nd_I & \Lambda + \mu_V \end{bmatrix}$$

we get,

$$G(\Lambda) = \Lambda^6 + \xi_1\Lambda^5 + \xi_2\Lambda^4 + \xi_3\Lambda^3 + \xi_4\Lambda^2 + \xi_5\Lambda + \xi_6 = 0, \quad (5)$$

where,

$$\xi_1 = d_1 + d_2 + d_I + 2\mu_G + \mu_V + \beta_1\bar{C}_{D4} + \beta_2\bar{C}_{CR} > 0,$$
$$\xi_2 = (\mu_G + \beta_1\bar{C}_{D4})(\mu_G + \beta_2\bar{C}_{CR}) + (d_1 + d_2 + d_I + \mu_V) \times (2\mu_G + \beta_1\bar{C}_{D4} + \beta_2\bar{C}_{CR}) + (d_1 + d_2)(\mu_V + d_I) + \mu_V d_I + d_1 d_2 > 0,$$
$$\xi_3 = (\mu_G + \beta_1\bar{C}_{D4})(\mu_G + \beta_2\bar{C}_{CR})(d_1 + d_2 + d_I + \mu_V) + (2\mu_G + \beta_1\bar{C}_{D4} + \beta_2\bar{C}_{CR})\,[(d_1 + d_2)(\mu_V + d_I) + (d_I\mu_V + d_1 d_2)] + [d_1 d_2(\mu_V + d_I) + d_I\mu_V(d_1 + d_2)] > 0,$$
$$\xi_4 = (2\mu_G + \beta_1\bar{C}_{D4} + \beta_2\bar{C}_{CR})[d_1 d_2(\mu_V + d_I) + d_I\mu_V \times (d_1 + d_2)] + (\mu_G + \beta_1\bar{C}_{D4})(\mu_G + \beta_2\bar{C}_{CR}) \times [(d_1 + d_2)(\mu_V + d_I) + (d_I\mu_V + d_1 d_2)] + d_1 d_2 d_I\mu_V > 0,$$
$$\xi_5 = (\mu_G + \beta_1\bar{C}_{D4})(\mu_G + \beta_2\bar{C}_{CR})\,[d_1 d_2(\mu_V + d_I) + d_I\mu_V\,(d_1 + d_2)] + d_1 d_2 d_I\mu_V(2\mu_G + \beta_1\bar{C}_{D4} + \beta_2\bar{C}_{CR}) > 0,$$
$$\xi_6 = d_1 d_2 d_I\mu_V(\mu_G + \beta_1\bar{C}_{D4})(\mu_G + \beta_2\bar{C}_{CR}) - nkab\beta_1\beta_2 d_I\bar{C}_{D4}\bar{C}_{CR}. \quad (6)$$

For $\xi_6 > 0$ there exist no positive roots and all roots are negative. Hence, the basic reproduction R_0 is,

$$R_0 = \frac{nkab\beta_1\beta_2 pqs_T^2}{d_1 d_2\mu_V(\mu_G\mu_T + ps_T\beta_1)(\mu_G\mu_T + qs_T\beta_2)}$$

Remark: At disease free equilibrium point $\bar{E}$, the system is locally stable if the basic reproduction number $R_0 < 1$ and the system is unstable when $R_0 > 1$.

The endemic state: There exists another equilibrium in the form of endemic equilibrium (E^*),

$$(G^*_1, C^*_{D4}, C^*_1, C^*_2, C^*_{CR}, C^*_2, I^*, V^*, A^*) \quad (7)$$

where,

$$C^*_{D4} = \frac{ps_T}{\mu_T + \beta_1 G_1^*}, \quad C^*_1 = \frac{ps_T\beta_1 G_1^*}{d_1(\mu_T + \beta_1 G_1^*)},$$

$$V^* = \frac{d_A}{\iota}, \quad I^* = \frac{kG^*_2}{d_I},$$

$$C^*_{CR} = \frac{qs_T}{\mu_T + \beta_2 G_2^*}, \quad A^* = \frac{(nd_I I^* - \mu_V V^*)}{hV^*},$$

$$C^*_2 = \frac{\beta_2 G^*_2 C^*_{CR}}{d_2},$$

$$G^*_1 = \frac{-\zeta_2 + \sqrt{\zeta_2^2 + 4\zeta_1\zeta_3}}{2\zeta_1},$$

$$G^*_2 = \frac{-\eta_2 + \sqrt{\eta_2^2 + 4\eta_1\eta_3}}{2\eta_1}, \quad (8)$$

and,

$$\zeta_1 = \iota\beta_1\mu_G,\ \zeta_2 = \iota(ps_T\beta_1 + \mu_G\mu_T) - bd_A\beta_1,\ \zeta_3 = bd_A\mu_T,$$
$$\eta_1 = \mu_G\beta_2,\ \eta_2 = (qs_T\beta_2 + \mu_G\mu_T - \alpha\beta_2 C^*_1),\ \eta_3 = \alpha\mu_T C^*_1 \quad (9)$$

The endemic state with antibody response exist if the concentration of the combination of *gp41* and *gpl20-CD4*-chemokine coreceptor ternary complex (C_2) satisfy the condition

$$C^*_2 > \frac{\mu_V d_A}{nkl} \quad \text{and} \quad R_0 > 1$$

In this article, our main aim is to justify the effect of antibody vaccination mathematically. So we have not carried out the stability analysis for the endemic state. However we have derived the existence condition for the endemic state. Moreover we have carried out the numerical illustration for the endemic state and verified that the endemic state exist when $R_0 > 1$.

THE SYSTEM WITH PERFECT ADHERENCE

To study the impulsive system it is assumed that the vaccine is taken at regular intervals with length $\tau = t_{k+1} - t_k$. For a impulsive cycle $t_k \leqslant t \leqslant t_{k+1}$, the solution is

$$A(t) = A(t_k^+)e^{\int_{t_k}^{t}[lV(u) - d_A]du} \qquad t_k < t \leqslant t_{k+1}$$
$$\geqslant A(t_k^+)e^{-d_A(t - t_k)} \quad (10)$$

Suppose that $A(t_k^-)$ denots the value immediately before the impulse and $A(t_k^+)$ is the value immediately after. Calculating the least value of the concentration of $A(t)$ for the perfect adherence with fixed interval length ($\tau > 0$) we get

$$A(t) = A(t_k^+)e^{-d_A(t - t_k)} \quad (11)$$

This is the required concentration of antibody response to control the virus.

If $A(0) = \bar{A}$, then we have

$$A(t_1^+) = \bar{A}$$
$$A(t_2^-) = \bar{A}e^{-d_A\tau}$$
$$A(t_2^+) = \bar{A}(1 + e^{-d_A\tau})$$
$$A(t_3^-) = \bar{A}(1 + e^{-d_A\tau})e^{-d_A\tau}$$
$$A(t_3^+) = \bar{A}(1 + e^{-d_A\tau} + e^{-2d_A\tau})$$
$$\vdots$$
$$A(t_p^+) = \bar{A}(1 + e^{-d_A\tau} + e^{-2d_A\tau} + e^{-3d_A\tau} + \ldots + e^{-(p-1)d_A\tau})$$
$$= \bar{A}\,\frac{1 - e^{-pd_A\tau}}{1 - e^{d_A\tau}} \quad (12)$$

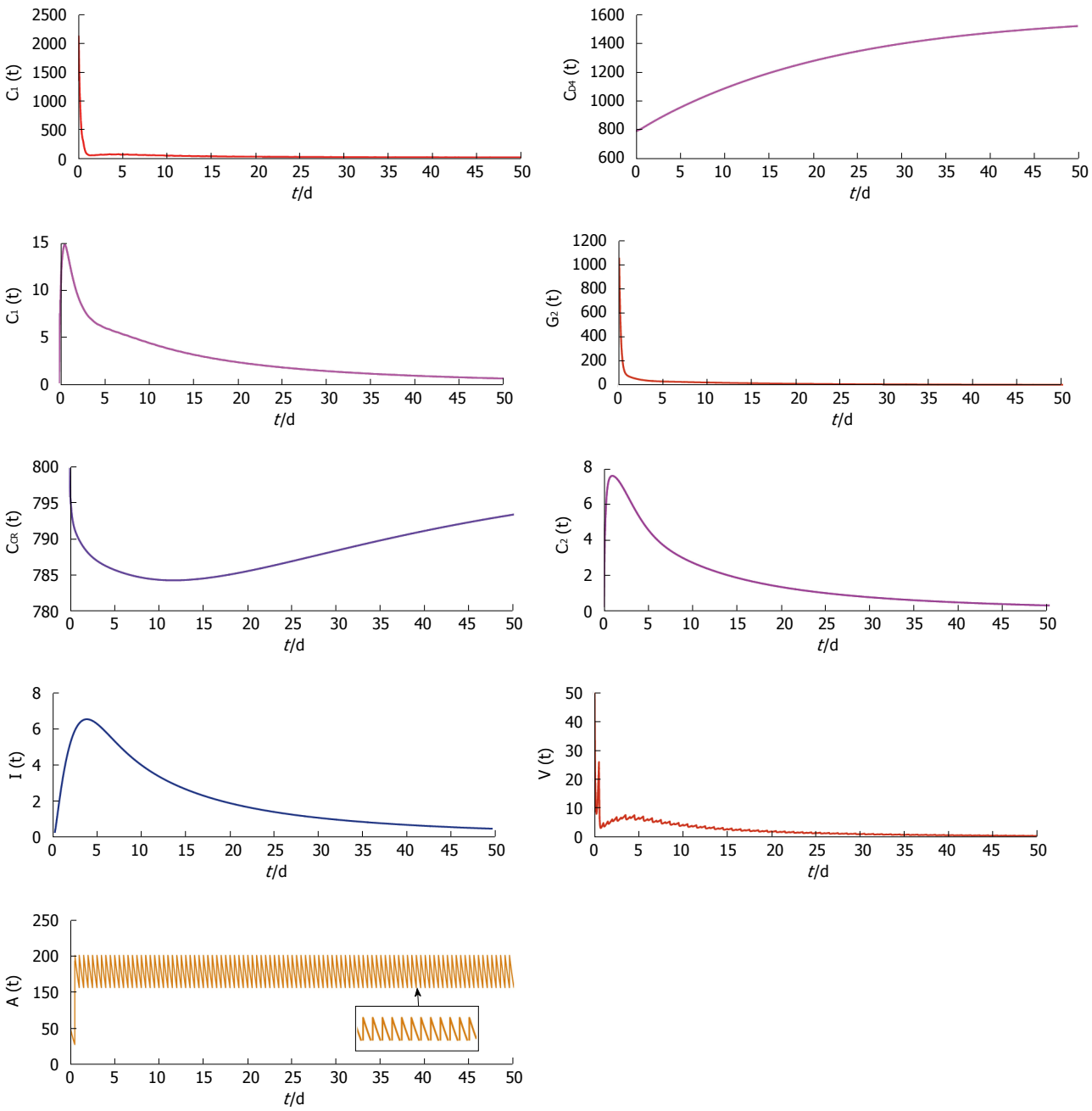

Figure 2 Trajectories showing the concentration changes with time of the model variable under restrained antibody vaccination with τ = 0.5 and $\overline{A}$ = 100.

Hence,

$$\lim_{p\to\infty} A(t_p^+) = \frac{\overline{A}}{1 - e^{-d_A\tau}} \quad (13)$$

The periodic end points of the trajectories are

$$\frac{\overline{A}}{1 - e^{-d_A\tau}} \text{ and } \frac{\overline{A}e^{-d_A\tau}}{1 - e^{-d_A\tau}}$$

For perfect vaccination, the antibody response after the nth vaccination is

$$A(t_n^+) = \frac{\overline{A}}{1 - e^{-d_A\tau}}$$

For perfect adherence, to control the virus and avoid resistance, the minimum value (A^*) of the periodic orbit must satisfy:

$$A^* < \frac{\overline{A}e^{-d_A\tau}}{1 - e^{-d_A\tau}} \quad (14)$$

Which implies that

$$\tau = \frac{1}{d_A} ln\left(\frac{A^* + \overline{A}}{A^*}\right) = \tau_{max} \quad (15)$$

Remark: If we can restrict the dosing interval of τ satisfying the condition $0 \leqslant \tau < \tau_{max}$ (for fixed vaccination) then the disease can be controlled. However, if $\tau > \tau_{max}$, the disease progression continues, even if drug is administered at fixed intervals. Thus maintenance of optimum dosage regimen is essential in order to control

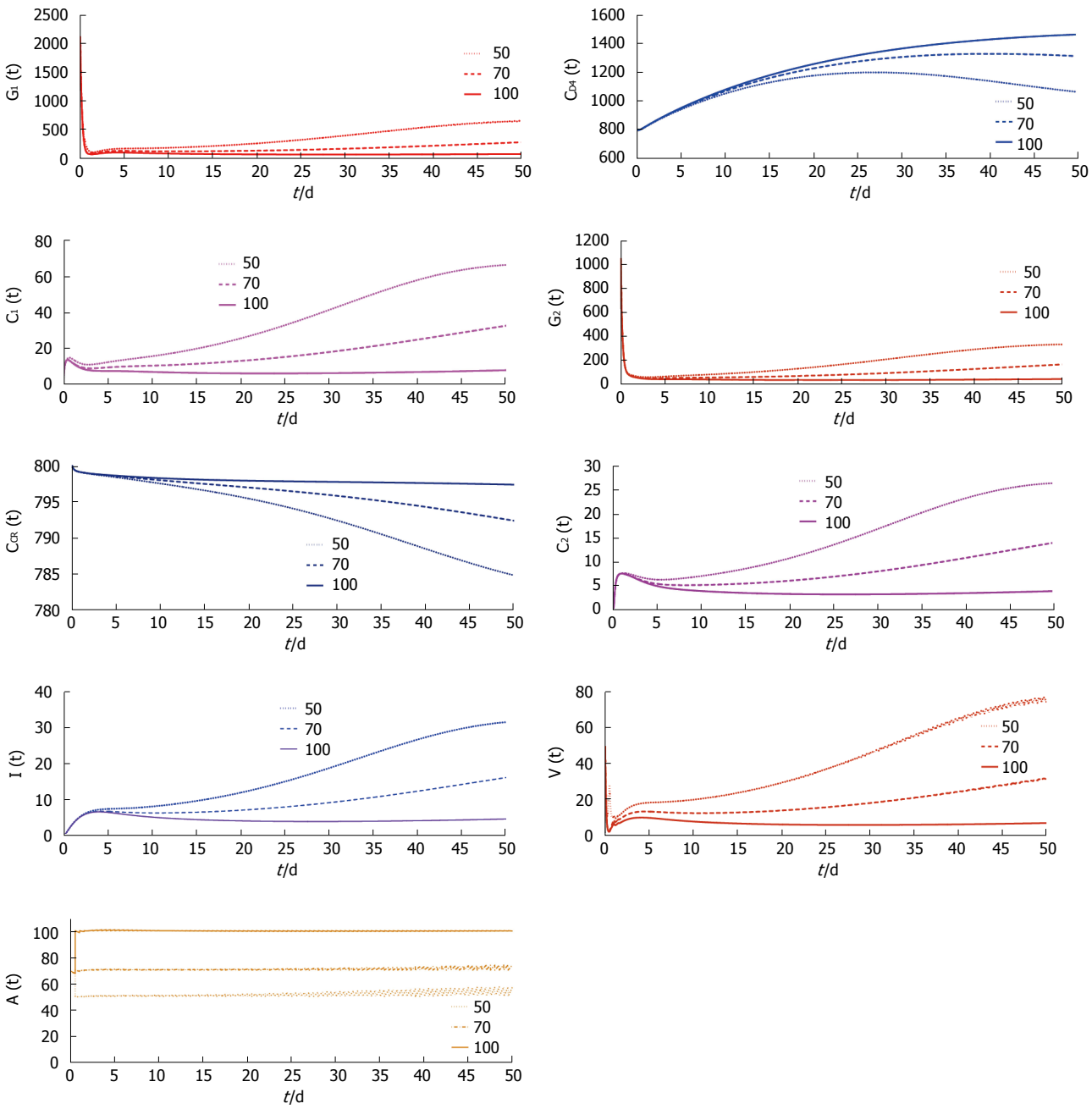

Figure 3 Trajectories showing the concentration changes with time of the model variable under different antibody vaccination concentration with τ = 0.5.

the disease effectively.

NUMERICAL SIMULATION

In our numerical illustration, we have described the perfect drug adherence of antibody vaccination. All the parameters are taken from Table 1. We have assumed the initial condition as $G_1(0) = 2100$, $C_{D4}(0) = 800$, $C_1(0) = 0$, $G_2(0) = 1050$, $C_{CR}(0) = 800$, $C_2(0) = 0$, $I(0) = 0$, $V(0) = 50$, $A(0) = 100$ and the unit of the concentration is mm^{-3}.

In Figure 2 we observe that in the presence of vaccination ($\bar{A}$ = 100) with frequent dosing interval (*i.e.*, τ = 0.5), the virus population and infected $CD4^+$ T cells population reduces. From this illustration, it can be predicted that proper antibody vaccination can restrict the infection process in HIV transmission. Now if the dosage of vaccination is not sufficient, then the effect of vaccination cannot be observed on the virus population and infected cell population.

In Figure 3, it is clearly observed that in presence of low vaccination (*i.e.*, for $\bar{A}$ = 50 or $\bar{A}$ = 70), the disease transmission process persists. Only adequate increment of the dosage of vaccination can restrict the disease progression. From these two figures we can predict that sufficient dosage of vaccination with frequent dosing interval can restrict the disease progression.

From Figures 4 and 5 we try to find out the effect of

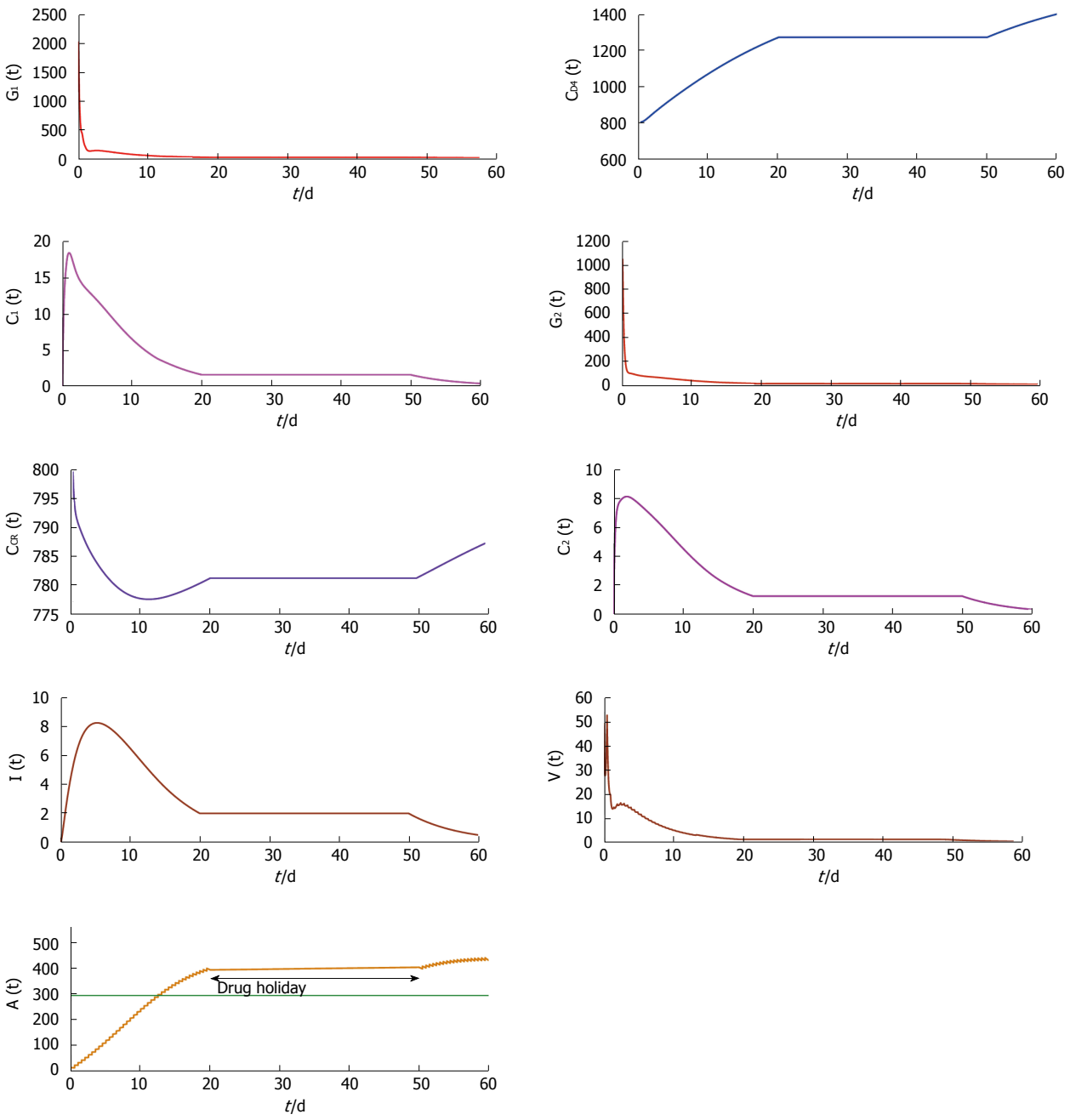

Figure 4 Trajectories showing the concentration changes with time of the model variable under restrained antibody vaccination with τ = 0.5 along with drug holidays for a period of 30 d.

drug holidays. In Figure 4, we restrict the drug holidays 30 d, where as in Figure 5 the drug holidays is 10 d only. It is observed that when the drug holiday is 30 d the virus population attains its minimum value after 55 d. But if we restrict the drug holidays 10 d, the virus population attains its minimum value after 35 d. We also observe that for a 10 d drug holiday the C_{D4} receptor and C_{CR} receptor attains its maximum concentration within 40 d. Whereas C_D receptor and C_{CR} receptor attains its maximum concentration within 60 d for 30 d drug holidays. Thus most unpleasant circumstances occur in case of long-term effects of the drug holidays.

Figure 6 show that phase plane plotted against dosing interval and antibody responses. From this figure it is clearly observed that the antibody responses attain its maximum value if the drug dosing interval is frequent. Also the antibody responses reduce for the outsized dosing interval.

CONCLUSION

Antibody vaccination in AIDS therapy has been studied as a new policy. Here vaccination is delivered to the host system in an impulsive mode to reactivate the antibody response. Dosage of vaccination and dosing interval has been effectively studied by this present mathematical

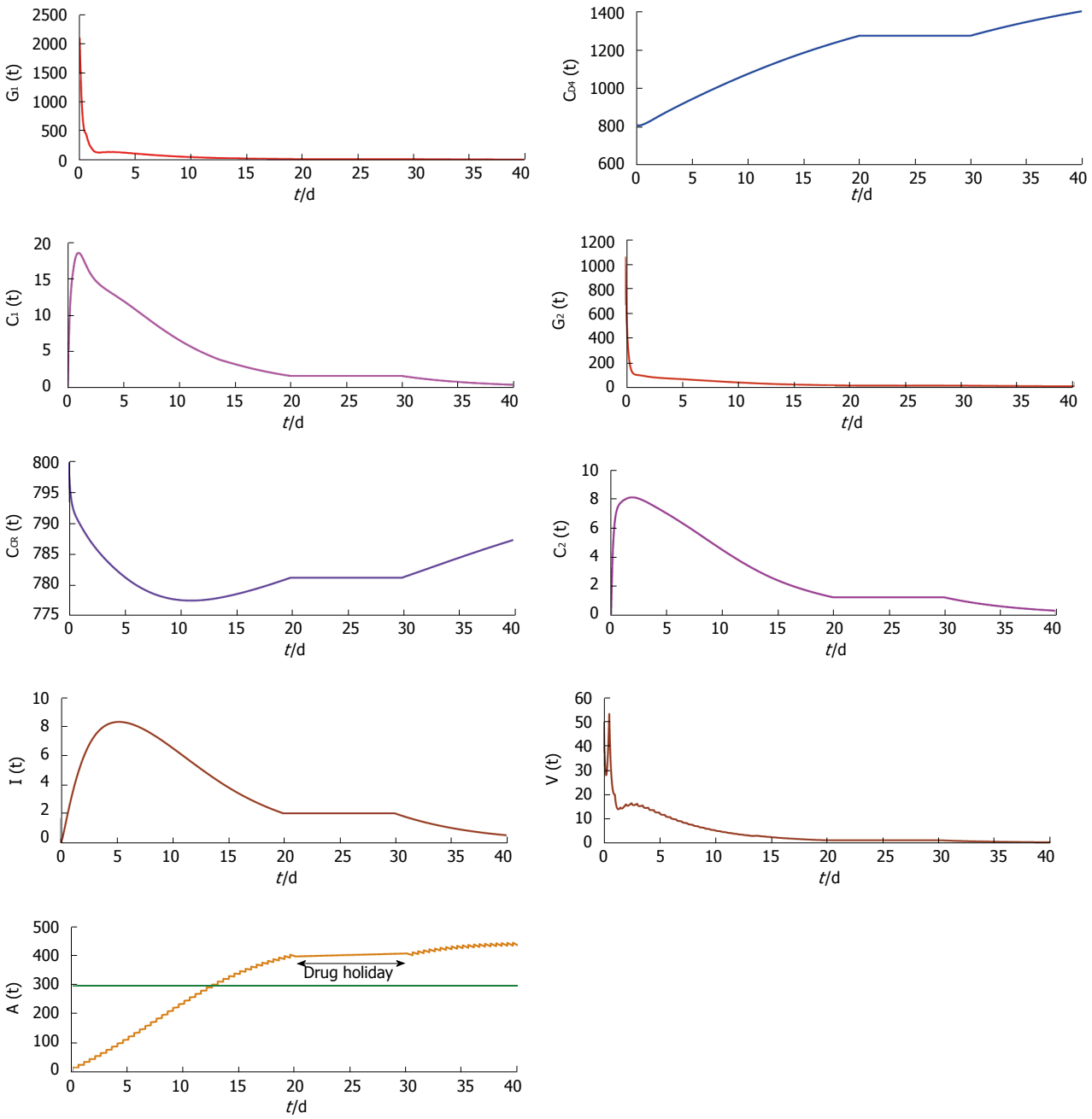

Figure 5 Trajectories showing the concentration changes with time of the model variable under restrained antibody vaccination with τ = 0.5 along with drug holidays for a period of 10 d.

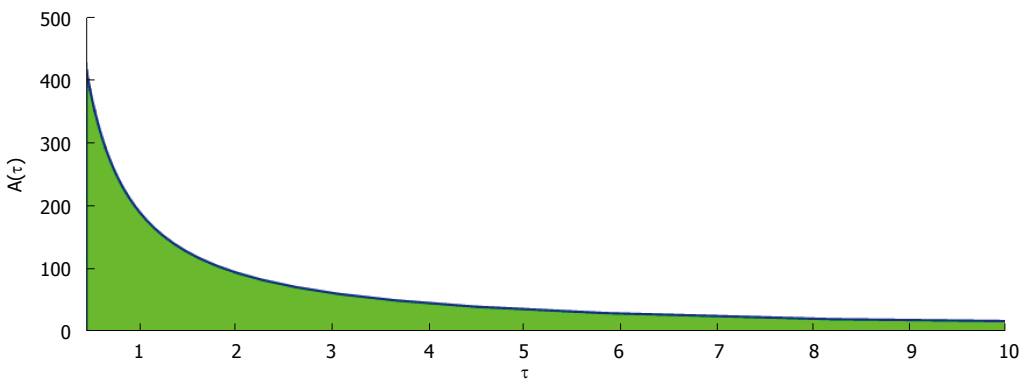

Figure 6 Phase plane showing the concentration of antibody responses against fixed dosing interval.

Table 1 List of parameters for system (1) - (2)

Parameter	Value (units)	Ref.
b	42 (per day)	[6]
β_1	10^{-5} (/mm^3/d)	[6]
μ_G	5 (per day)	[9]
p	1 (per day)	[6]
S_T	80 (/mm^3/d)	[9]
μ_T	0.05 (per day)	[6]
d_1	0.4 (per day)	[6]
α	25 (per day)	[6]
β_2	10^{-5} (/mm^3/d)	[6]
q	0.6 (per day)	[6]
d_2	0.4 (per day)	[6]
κ	0.6 (per day)	[6]
d_I	0.5 (per day)	[9]
δ	0.01 (per day)	[7]
n	540	[6]
μ_V	3 (per day)	[7]
ι	0.01 (per day)	[12]
d_A	0.5 (per day)	[7]

model. It has been observed that when basic reproduction ratio lies below one, we expect the system attain its disease free state. However, at $R_0 > 1$, the system switches to endemic equilibrium. These conjectures have been supported by the results of numerical simulations.

It has been observed that the length of the dosing interval and the drug dose play a very decisive role in maintaining stable disease free equilibrium. From analytical as well as numerical finding it has been observed that the antibody responses attain its maximum value if the drug dosing interval is frequent. But the antibody responses reduce for the large dosing interval. Also we have observed that the drug holiday plays a pivotal role during the treatment schedule. From numerical findings we can predict that extensive drug holidays is unsafe for the treatment. Analytically and numerically, it has been observed that viral entry into the host cell is also inhibited with uninfected host CD4$^+$ T cell population remaining unaffected. This happens because the antibody vaccination when administered following the best possible antibody responses can act against the free virus to neutralize free virus particles. This particular situation keeps the infected cell population at a very low level.

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ORIGINAL ARTICLE

Basic Study

Rapid genotyping of human rotavirus using SYBR green real-time reverse transcription-polymerase chain reaction with melting curve analysis

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Abstract

AIM: To develop a real-time reverse transcription-polymerase chain reaction (RT-PCR) assay to genotype rotavirus (G and P) in Alberta from January 2012 to June 2013.

METHODS: We developed and validated a different approach to perform rotavirus G and P genotyping using a two-step SYBR green RT-PCR (rt-gPCR) by selecting genotype-specific primers of published conventional RT nested PCR (cnRT-PCR) assay and optimizing the amplification conditions. cDNA was first synthesized from total RNA with SuperScript™ Ⅱ reverse transcriptase kit followed by amplication step using monoplex SYBR green real-time PCR. After the PCR reaction, melting curve analysis was used to determine specific genotype. Sixteen samples previously genotyped using cnRT-PCR were tested using the new assay and the genotyping results were compared as sensitivity analysis. Assay specificity was evaluated by testing other gastroenteritis viruses with the new assay. The amplicon size of each available genotype was determined by gel-electrophoresis and DNA sequences were obtained using Sanger-sequencing method. After validation and optimization, the new assay was used to genotype 122 pediatric clinical stool samples previously tested positive for rotavirus using electron microscopy between January

2012 and June 2013.

RESULTS: The new rt-gPCR assay was validated and optimized. The assay detected G1 to G4, G9, G12 and P[4] and P[8] that were available as positive controls in our laboratory. A single and clear peak of melting curve was generated for each of specific G and P genotypes with a T_m ranging from 80 ℃ to 82 ℃. The sensitivity of rt-gPCR was comparable to cnRT-PCR with 100% correlation of the 16 samples with known G and P genotypes. No cross reaction was found with other gastroenteritis viruses. Using the new rt-gPCR assay, genotypes were obtained for 121 of the 122 pediatric clinical samples tested positive for rotavirus: G1P[8] (42.6%), G2P[4] (4.9%), G3P[8] (10.7%), G9P[8] (10.7%), G9P[4] (6.6%), G12P[8] (23.0%), and unknown GP[8] (0.8%). For the first time, G12 rotavirus strains were found in Alberta and G12 was the second most common genotype during the study period. Gel electrophoresis of all the genotypes showed expected amplicon size for each genotype. The sequence data of the two G12 samples along with other genotypes were blasted in NCBI BLAST or analyzed with Rota C Genotyping tool (http://rotac.regatools.be/). All genotyping results were confirmed to be correct.

CONCLUSION: rt-gPCR is a useful tool for the genotyping and characterization of rotavirus. Monitoring of rotavirus genotypes is important for the identification of emerging strains and ongoing evaluation of rotavirus vaccination programs.

Key words: Rotavirus A; Melting temperature; Real-time polymerase chain reaction; SYBR green; Genotyping

Core tip: Genotyping rotavirus is essential for monitoring strain shifts in rotavirus surveillance and vaccine evaluation. Current conventional semi-nested real-time reverse transcription-polymerase chain reaction (RT-PCR), the most commonly used rotavirus genotyping assay is a labor-intensive, complex multi-step procedure and has long turn around-time. The newly developed SYBR Green real time RT-PCR assay is simple, fast and has comparable sensitivity and specificity as conventional semi-nested RT-PCR. This new assay was used to genotype clinical samples which tested positive for rotavirus from January 2012 to June 2013 and new emerging G12 strains were identified in Alberta, Canada.

Tong Y, Lee BE, Pang XL. Rapid genotyping of human rotavirus using SYBR green real-time reverse transcription-polymerase chain reaction with melting curve analysis. *World J Virol* 2015; 4(4): 365-371 Available from: URL: http://www.wjgnet.com/2220-3249/full/v4/i4/365.htm DOI: http://dx.doi.org/10.5501/wjv.v4.i4.365

INTRODUCTION

Rotavirus group A is a major cause of severe acute gastroenteritis in children worldwide, with most children contracting the infection by five years of age[1]. Rotavirus vaccines are most efficacious in protecting children from severe rotavirus gastroenteritis, especially in areas with very low or low child and adult mortality[2]. Two effective rotavirus vaccines, RotaTeq® (Merck and Co., Inc.), a bovine-human reassortant vaccine covering the G1-G4 genotypes along with P[8] and Rotarix® (GlaxoSmithKline, Inc.), a monovalent attenuated human G1P[8] vaccine, were recommended by the WHO in 2009 for the routine immunization of infant globally[3].

The viral genome consists of 11 double-stranded RNA segments, which encode six structure proteins (VP1-4, VP6 and VP7) and six non-structure proteins (NSP1-6). The traditional classification of rotavirus using serotyping has mostly been replaced with G and P genotyping that are based on the diversity of VP7 and VP4 gene sequences, respectively. Twenty-seven G genotypes (G1-G27) and 35 P genotypes (P[1]-P[35]) have been described and six G (G1-4, G9 and G12) and three P (P[4], P[6], and P[8]) genotypes predominate globally with some regional differences[4,5]. Introduction of vaccines or natural evolution of rotavirus may alter antigenic properties of circulating strains in the regions. The antigenic drift between G and P genotypes could result in a decrease of the effectiveness of vaccines against infection in the near future[6,7]. Therefore, understanding the presence and distribution of G and P genotypes and monitoring the emerging and recombination of rotavirus genotypes are very important prior to and after the introduction of rotavirus vaccines.

Various molecular methods have been developed for rotavirus genotyping. Conventional reverse transcriptase (RT) and nested polymerase chain reaction (cnRT-PCR) broadly used since the 90s[8-10] have been improved with more sensitive and specific primers[11,12]. These improved cnRT-PCR methods recognize a broad cluster of various rotavirus G and P genotypes with good specificity. However, there are several drawbacks of these type of assays including: (1) multi-step maneuvers and the requirement of two runs of PCR reactions and gel electrophoresis; (2) labor intensive protocols and a long turn-around time for final results; (3) difficulty with result interpretations due to non-specific amplicons or multiple amplicons in samples with co-infections; and (4) risk of cross contamination of PCR end-products because of the need for open tube maneuvers. With the 2010 recommendation of use of rotavirus vaccine for healthy infants in Canada[13], a rapid and accurate genotyping assay is needed for the evaluation of the vaccine programs in many provinces.

Recently, real time RT-PCR assays in a closed tube system with their rapid turn around-time, sensitivity and specificity and low risk of cross contamination have been implemented in both research and diagnostic

Table 1 Polymerase chain reaction primers used for G and P typing of human rotavirus by reverse transcriptase polymerase chain reaction

Primer	Sequence (5′-3′)	Amplicon size (bp)	Direction	Ref.
G-type				
VP7-R	AAC TTG CCA CCA TTT TTT CC		Antisense	Iturriza-Gómara *et al*[10]
G1	CAA GTA CTC AAA TCA ATG ATG G	618	Sense	Gouvea *et al*[8]
G2	CAA TGA TAT TAA CAC ATT TTC TGT G	521	Sense	Gouvea *et al*[8]
G3	ACG AAC TCA ACA CGA GAG G	682	Sense	Iturriza-Gómara *et al*[10]
G4	CGT TTC TGG TGA GGA GTT G	452	Sense	Gouvea *et al*[8]
G8	TTR1 TCG CAC CAT TTG TAA TT	756	Sense	Aladin *et al*[12]
G9	CTT GAT GTG ACT AY[1]A AAT AC	179	Sense	Iturriza-Gómara *et al*[10]
G10	ATG TCA GAC TAC AR[2]A TAC TGG	266	Sense	Gouvea *et al*[8]
G12	GGT TAT GTA ATC CGA TGG CG	396	Sense	Aladin *et al*[12]
P-type				
VP4-F	TAT GCT CCA GTN[3] AAT TGG		Sense	Simmonds *et al*[11]
P[4]	CTA TTG TTA GAG GTT AGA GTC	362	Antisense	Gentsch *et al*[9]
P[6]	TGT TGA TTA GTT GGA TTC AA	146	Antisense	Gentsch *et al*[9]
P[8]	TCT ACT GGR[2] TTR[2] ACN[3] TGC	224	Antisense	Iturriza-Gómara *et al*[10]
P[9]	TGA GAC ATG CAA TTG GAC	270	Antisense	Gentsch *et al*[9]
P[10]	ATC ATA GTT AGT AGT CGG	462	Antisense	Gentsch *et al*[9]
P[11]	GTA AAC ATC CAG AAT GTG	191	Antisense	Iturriza-Gómara *et al*[10]

[1]Y: C or T; [2]R: A or G; [3]N: A, G, C or T.

laboratories for the detection of rotavirus in stool samples associated with gastroenteritis[14-16]. To date, rotavirus genotyping using TaqMan real time PCR assay has been reported[17]. The protocol could successfully genotype G1P[8], G2P[4], G3P[8], G4P[8] and G9P[8] with only two multiplex reactions, however the other genotypes and combinations were still unable to be typed. Melting curve analysis uses the melting temperature of double-stranded PCR products to determine the identity of the PCR products and also can detect the presence of nonspecific PCR products or primer-dimers. Melting curve analysis is commonly used in SYBR green RT-PCR (rt-gPCR) to determine PCR products and omits the need for gel electrophoresis. SYBR green real-time PCR with melting curve analysis has been used to type Dengue virus as a genotyping tool[18]. This study is to develop and validate a simple and rapid rotavirus genotyping assay using SYBR Green RT-PCR to detect a broad range of rotavirus strains and to use the new assay to characterize the rotavirus strains circulated in Alberta from January 2012 to June 2013.

MATERIALS AND METHODS

Development of rt-gPCR assay

Sample preparation, RNA extraction and RT reaction: Archived rotavirus positive pediatric stool samples (n = 16) obtained from the Provincial Laboratory for Public Health (ProvLab) and previously genotyed by the cnRT-PCR[10] were used for the development and validation of the rt-gPCR assay. Viral RNA was extracted from 200 μL of 10% stool suspension (10% W/V with PBS buffer) using MagaZorb® total RNA Mini-Prep kit (Promega, Madison, United States) on an automaton extractor, KingFisher™ mL Magnetic Particle Processors, (Thermo Scientific, Mississauga, Canada) according to manufacturer's instructions. Using SuperScript™ Ⅱ Reverse Transcriptase kit and random primer (Life technologies, Ontario, Canada), cDNA was synthesized from 5 μL RNA at 42 ℃ for 1 h, 75 ℃ for 15 min after heating 5 min at 97 ℃ and stored at -20 ℃ before use.

rt-gPCR: A group of published primers used in cnRT-PCR for genotyping rotavirus G types (G1-4, G8, G9-10) and P types (P[4], P[6], P[8], P[9], P[10], and P[11]) were selected for the new assay development[8-10] (Table 1). Another two sets of primers for genotyping G8, and G12 which were not previously used in our laboratory was also added in the new rt-gPCR assay[12]. 5 μL of 1:10 diluted cDNA was applied to 20 μL of reaction mixture containing 2 μL of LightCycler® FastStart DNA Master SYBR Green Ⅰ (Roche Diagnostics, Quebec, Canada), 4 mmol/L $MgCl_2$, 0.2 μmol/L each of VP7-R and specific G-typing primers or 0.4 μmol/L each of VP4-F and specific P-typing primers. The rt-gPCR was performed using LightCylcer® 1.0 (Roche Diagnostics, Quebec, Canada) with four-step experimental run protocol: (1) denaturation program (10 min at 95 ℃); (2) 45 cycles of amplification program (10 s at 95 ℃; 10 s at 53 ℃ (P-typing), 57 ℃ (G-typing), and 25 s of extension at 72 ℃); (3) melting curve program (0 s at 95 ℃, 120 s at 65 ℃ and 0 s at 95 ℃ with ramp rate at 0.1 ℃ per second); and (4) cooling program down to 40 ℃. Since different genotype specific primers would yield amplicons of different size with various GC content percentages, the temperature (T_m) and melting curve of the amplicon of specific rotavirus G or P genotype would be different. Thus melting T_m profiles were used to identify specific rotavirus G or P genotypes in our design. For data analysis, the T_m, fluorescence (d[F1]/dT) under the melting curve window was selected as the parameters for evaluation. A sample would be assigned to a specific genotype when the reaction T_m matched with known genotype controls, and the fluorescence d[F1]/dT was

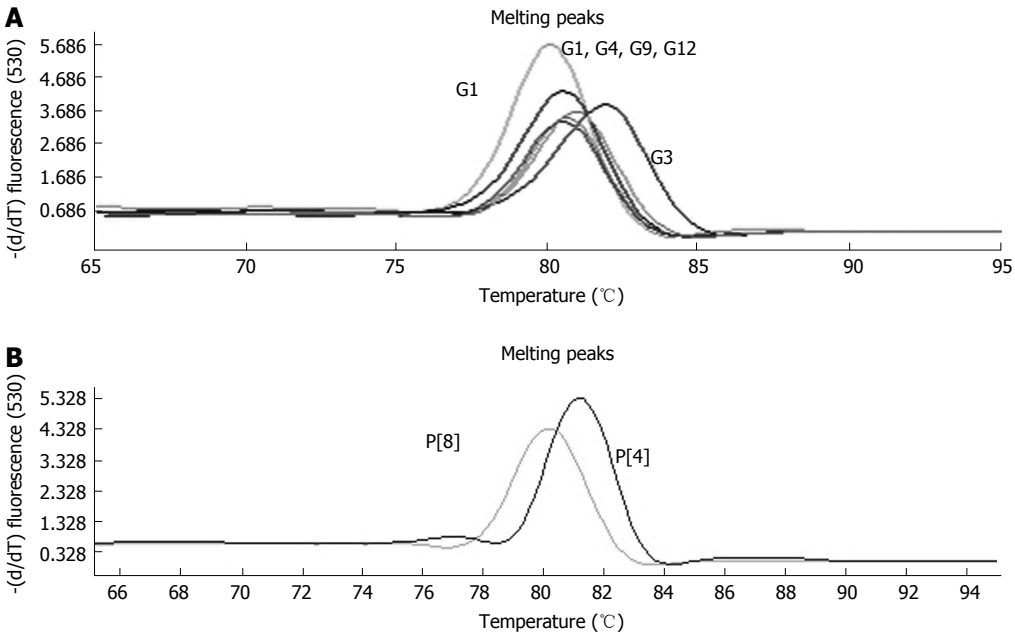

Figure 1 Melting cure analysis of different genotypes using reverse transcriptase polymerase chain reaction (A: G-typing: G1, G2, G4, G9, G12, G3; B: P-typing: P[8], P[4]). The peak of curve in X-axis is the melting temperature of each genotype DNA fragment; Y-axis indicates the SYB green fluorescence density.

above 1.0. The cycle threshold (Ct) of amplification curve was used to provide a relative quantification. Positive controls of specific G and P genotypes were included in each rt-gPCR run as reference genotype and for quality control.

Assay sensitivity and specificity: The sensitivity of rt-gPCR assay was compared with cnRT-PCR using ten-fold serial dilutions from neat (undiluted) to 10^{-6} of samples with known G and P genotypes. Other common gastroenteritis viruses including norovirus, sapovirus, adenovirus, and astrovirus were also tested using the G and P primers to determine the specificity of the rt-gPCR assay.

DNA sequencing: Six G-types including G1 to G4, G9, G12 and two P-types P[4], P[8] detected by the rt-gPCR assay were sequenced with modification as described previously[19]. Briefly, the positive PCR products were run in 2.0% agarose gel and purified with QIAquick Gel Extraction kit (Qiagen, Hilden, Germany) then sequenced using 3730 Genetic Analyzer (Applied biosystems, Foster City, United States) at University of Alberta.

Genotyping clinical samples

ProvLab provides routine diagnostic testing of stool samples submitted for testing of gastroenteritis viruses using electronic microscope (EM). A total of 122 stool samples with rotavirus identified by EM between January 5, 2012 and June 8, 2013 were genotyped using the validated rt-gPCR assay. The purpose was to determine the performance of the assay for rapid genotyping of rotavirus in the clinical setting.

Data analysis

SD for the T_m of each genotype was calculated to show the variations in T_m. The sequence data of amplicons of G1 to G4, G9, G12, P[4] and P[8] were blasted in NCBI or analyzed with Rota C Genotyping tool (http://rotac.regatools.be/).

RESULTS

Development of rt-gPCR assay

All 16 positive control samples previously genotyped by cnRT-PCR were confirmed using the rt-gPCR assay with 100% concordant results. Using the rt-gPCR assay, a single and clear peak of melting curve was yielded for each of specific G and P genotypes with a T_m ranging from 80 ℃ to 82 ℃ (Figure 1). More than 30 of the G1, G3, G9, P[8] and more than 20 of the G2, G4, G12,P[4] replicates and/or different samples at different runs were used to calculate to mean T_m and SD. Each genotype T_m showed very small variation during different PCR runs among different samples. The mean T_m (℃ ± SD) for each of the genotypes were: G1 at 80.0 ℃± 0.20, G2 at 80.9 ℃ ± 0.49, G3 at 81.7 ℃ ± 0.22, G4 at 80.7 ℃ ± 0.20, G9 at 80.9 ℃ ± 0.45, G12 at 80.6 ℃± 0.37, P[4] at 80.7 ℃ ± 0.50, and P[8] at 80.0 ℃ ± 0.34 (Figure 2). Multiplex rt-gPCR could not be performed to simultaneously detect all G or P genotypes because the T_m generated from each genotype was very close. A lower T_m peak (< 77 ℃) indicating primer-dimer was observed in the amplification reactions. All rt-gPCR genotype products showed expected amplicon sizes in the gel electrophoresis (Figure 3).

In the sensitivity comparison by serial ten-fold dilution of positive samples, both rt-gPCR and cnRT-

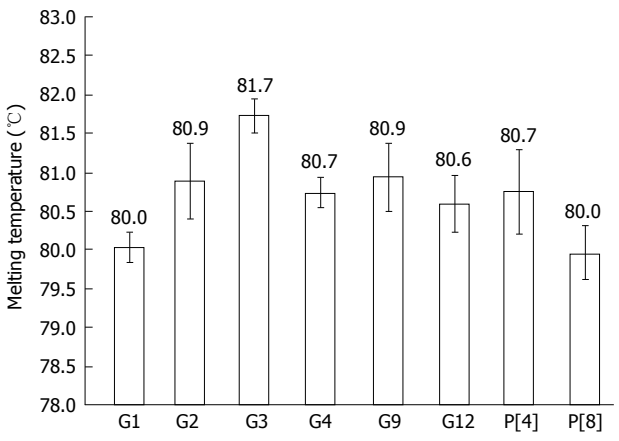

Figure 2 Melting temperatures of different rotavirus genotypes using reverse transcriptase polymerase chain reaction. The mean T_m (℃ ± SD) for each genotype (G1 to G4, G9, G12, P[4] and P[8]) were calculated from more than 20 replicates of different samples at different polymerase chain reaction runs.

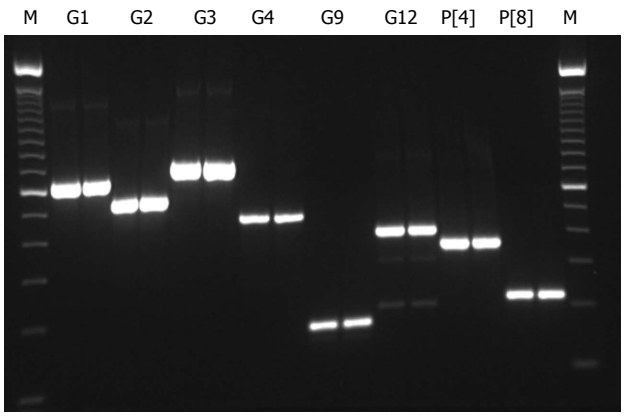

Figure 3 Agarose gel electrophoresis of different rotavirus genotype using reverse transcriptase polymerase chain reaction. Amplicon sizes for different genotypes (G1: 618 bp; G2: 521 bp; G3: 682 bp; G4: 452 bp; G9: 179 bp; G12: 396 bp; P[4]: 362 bp; P[8]: 224 bp. M: 100 bp DNA ladder (life technologies).

PCR detected G2, G3, G4, G9 G12, P[8] at 10^{-5} dilution. cnRT-PCR detected G1 and P[4] at 10^{-6} dilution while rt-gPCR detected at 10^{-5} dilution. For the specificity test, no cross-reaction with other gastroenteritis viruses including norovirus, sapovirus, adenovirus, and astrovirus was observed using the rt-gPCR assay.

The assay was optimized after testing a range of annealing temperatures and primer concentrations. A non-specific amplification was observed for G types at low temperatures (53 ℃) but not the P types. The issue with non-specific amplifications was resolved by increasing the annealing temperature to 57 ℃ and decreasing the concentration of primers to 0.2 μmol/L.

DNA sequence analysis

With Rota C Genotyping tool or sequence analysis using NCBI BLAST, all genotyping results generated using rt-gPCR were confirmed to be correct. Two of the G12 positive samples yielded clear reading of 329bp and 335bp respectively had 100% nucleotide identity using NCBI BLAST but could not be directly analyzed using the Rota C Genotyping tool because of the minimal requirement of 500 bp by the tool. The genotyping results of these two samples were further confirmed by analyzing the hits generated using NCBI BLAST with the Rota C tool. By blasting the sequence of these samples in NCBI, the first ten hits showed genotype G12 with 100% nucleotide identity. One of the hits [Rotavirus A strain RVA/Human-wt/ZWE/MRC-DPRU1858/2011/G12P[8] segment 9 capsid glycoprotein VP7 (*VP7*) gene, complete cds, Genebank: KP753228.1] was further analyzed by the Rota C tool and was confirmed to be G12.

Clinical sample genotyping

Of the 122 stool samples tested positive for rotavirus by EM, 121 (99%) were characterized by G and/or P genotypes using the rt-gPCR assay except for one sample (1%) that was not typeable. During the study, five G-types were identified, G1, G2, G3, G9 and G12 while only two P types, P[4] and P[8] were found circulating in the province. The predominant G/P combination was G1P[8] at 42.6% (n = 52), followed by G12P[8] (23.0%, n = 28), G3P[8] (10.7%, n = 13), G9P[8] (10.7%, n = 13), G9P[4] (6.6%, n = 8), and G2P[4] (4.9%, n = 6). One sample was typed as P[8] but rt-gPCR for G genotyping did not yield any result. The only untypeable EM positive sample was retested using our in-house rotavirus RT-PCR assay[16] and was found to have a very high Ct of 39 indicating low viral load and possible sample degradation. No mixed infection of different genotypes was found.

DISCUSSION

We developed a new rt-gPCR assay for genotyping rotavirus and compared the results to the cnRT-PCR assay. The rt-gPCR assay has the same specificity as the cnRT-PCR assay. The sensitivity of rt-gPCR was comparable to cnRT-PCR with 100% correlation of known G and P genotypes samples. The only difference between the two assays was the detection of G1 and P[4] at 10^{-6} dilution only by cnRT-PCR, which was an expected result as nested PCR could be more sensitive. More importantly, this degree of difference in sensitivity is not significant for clinical applications as high viral load of rotavirus is usually excreted in acute gastroenteritis[15]. Due to the availability of genotypes in our laboratory, the most common G (G1 to G4, G9, G12) and P (P[4] and P[8]) types were validated.

While the rt-qPCR assay has many advantages, including a simpler protocol, shorter turn around-time and lower risk of cross contamination, the multiple monoplex real-time PCR reactions required to genotype respective G and P types have high reagents cost. For cost-saving, a laboratory can design a stepwise testing algorithm to first test for the more common G and P types to reduce reagent and labor costs. Based on our local data and other reported rotavirus genotypes in Canada, we would suggest: For P typing, first test

for P[8] and if P[8] is negative, then test for P[4]; for G typing, first test for G1 to be followed by G12 and G9, then G3, G2 +/- G4. This stepwise strategy would cover the range of G and P genotypes for most clinical samples.

The first G12 strain was identified in the Philippines in 1987 followed by reports of sporadic detection in other countries[20-22]. G12 is currently recognized as a globally emerging rotavirus genotype that appears to be spreading more rapidly in recent years[23]. Predominant of G12 was reported in Nepal in 2011, Cameroon in 2010/11 and rotavirus outbreak associated with G12 was found in United States in 2006-07[24-26]. In our study, G12 was detected as the second most prevalent genotype using rt-gPCR in Alberta. G12 could be detected by the new rt-gPCR assay but would be missed by the cnRT-PCR assay. We still believe that G12 was an emerging rotavirus genotype in 2012 as only 1.4% of EM rotavirus positive samples were untypeable from previous nine years in Alberta (data not shown). No G12 has been previously reported in Canada[27]. To our knowledge, this is the first report of G12 genotype detected in Canada. In addition, G9P[4] which was rarely detected in previous years increased dramatically in 2012. These findings emphasize the importance of ongoing surveillance for circulating rotavirus genotypes. In conclusion, the newly developed rt-gPCR assay with optimized primer selection enhanced the detection of broader genotypes which makes this assay a useful tool for the characterization and monitoring of strain shifts in rotavirus surveillance and the evaluation of vaccination program.

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COMMENTS

Background

Genotyping rotavirus is very important for the characterization and monitoring of strain shifts in rotavirus surveillance for the evaluation of vaccination program. The most commonly used rotavirus genotyping assay is a conventional nested reverse transcriptase (RT) polymerase chain reaction (cnRT-PCR) which has been used and revised for more than 30 years. The labor-intensive, complex multi-step procedure and high potential contamination risk due to nested PCR format make this method falling behind current demand.

Research frontiers

An accurate, easy-to-perform and rapid rotavirus genotyping tool is needed. Several published studies developed new genotyping PCR assays using multiplex Taqman real time PCR assay to replace conventional nested PCR but has limited sensitivity and can detect only a few G or P types.

Innovations and breakthroughs

Using SYBR Green based RT-PCR with melting curve analysis and melting temperature (T_m) to genotype rotavirus is simple, fast and provides accurate and broad identifications of genotypes. Gel electrophoresis required by traditional conventional nested PCR genotyping assay is not needed and eliminates the risk of contamination from handling post-PCR products. The new assay showed similar sensitivity and specificity as the conventional nested RT-PCR.

Applications

The new assay identified common genotypes circulating in Alberta, Canada as well as the emergence of rotavirus G12 in 2012-2013. G12 has never been reported in Canada. During the study, the most predominant rotavirus genotypes were: G1P[8] (42.6%), G12P[8] (23.0%), followed by G3P[8] (10.7%), G9P[8] (10.7%), G9P[4] (6.6%), and G2P[4] (4.9%). These new findings support the importance of ongoing surveillance and characterization of rotavirus genotypes.

Terminology

Rotavirus G-typing is genotyping viral gene sequences diversity which encoding structure protein VP7; Rotavirus P-typing is genotyping viral gene sequences diversity which encoding structure protein VP4; SYBR green is a commonly used fluorescent DNA binding dye, binds all double-stranded DNA and detection is monitored by measuring the increase in fluorescence throughout the cycle. SYBR Green master mixes are designed for quantitative real-time PCR using a set of two PCR primers that flank the target region; Melting curve analysis is the temperature-dependent dissociation between two DNA-strands can be measured using a DNA-intercalating fluorophore such as SYBR green. Melting T_m can be used for determination of the identity of the target.

Peer-review

The article describes a new assay for genotyping the human rotavirus. The results presented seem pretty convincing and conclusions are valid.

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ORIGINAL ARTICLE

Retrospective Study

Prevalence of adenovirus and rotavirus infection in immunocompromised patients with acute gastroenteritis in Portugal

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Author contributions: Almeida S and Baldaque I performed the experimental tests for Adv and RV detection; Ribeiro J, Ferreira D, and Arrabalde C were responsible for the data collection; Ribeiro J and Sousa H analyzed data; Ribeiro J and Ferreira D wrote the paper; Sousa H supervised the study; all authors have provided the final approval of the manuscript.

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Abstract

AIM: To characterize the prevalence of rotavirus (RV) and adenovirus (AdV) infections in immunocompromised patients with acute gastroenteritis.

METHODS: The presence of RV and AdV (serotypes 40 and 41) was evaluated in 509 stool samples obtained between January 2009 and December 2010 from 200 immunocompromised patients (83 females and 117 males; median age 21 years old, range 0-72. The diagnosis of infection was performed as a routine procedure and the presence of RV and AdV (serotypes 40 and 41) was determined by immunochromatography using the RIDA® Quick Rota-Adeno-Kombi kit (r-Biopharm, Darmstadt, Germany). The data analysis and description of seasonal frequencies were performed using computer software IBM® SPSS® (Statistical Package for Social Sciences) Statistics version 20.0 for Mac. The frequencies of infection were compared into different age and gender groups by χ^2 test.

RESULTS: The study revealed 12.4% AdV positive samples and 0.8% RV positive samples, which correspond to a prevalence of 6.5% and 1.5%, respectively. AdV was more frequent between October 2009 and April 2010, while RV was identified in April 2010 and July 2010. The stool analysis revealed that from the 509 samples, 63 (12.4%) were positive for AdV and 4 (0.8%) positive for RV, which by resuming the information

of each patient, lead to an overall prevalence of AdV and RV of 6.5% (13/200 patients) and 1.5% (3/200 patients), respectively. The stratification of the analysis regarding age groups showed a tendency to an increased prevalence of infection in paediatric patients between 0-10 years old. Considering the seasonal distribution of these infections, our study revealed that AdV infection was more frequent between October 2009 and April 2010, while RV infection was characterized by two distinct peaks (April 2010 and July 2010).

CONCLUSION: The overall prevalence of AdV and RV infection in immunocompromised patients with acute gastroenteritis was 8% and AdV was the most prevalent agent.

Key words: Viral gastroenteritis; Adenovirus; Rotavirus; Immunocompromised host; Stool samples

Core tip: Acute gastroenteritis has been associated with significant rates of morbidity and mortality among immunocompromised patients. Rotavirus (RV) and adenovirus (AdV) are described as common agents of viral gastroenteritis causing acute diarrhoea. This is the first study in Portugal to characterize the prevalence and seasonal features of RV and AdV infections in immunocompromised patients with acute gastroenteritis. Results revealed 12.4% AdV positive samples and 0.8% RV positive samples, which correspond to a prevalence of 6.5% and 1.5%, respectively. Our results also demonstrate the importance of to add more screening methods for other emergent enteric viruses, in order to avoid the morbidity and mortality of the immunocompromised patients.

Ribeiro J, Ferreira D, Arrabalde C, Almeida S, Baldaque I, Sousa H. Prevalence of adenovirus and rotavirus infection in immunocompromised patients with acute gastroenteritis in Portugal. *World J Virol* 2015; 4(4): 372-376 Available from: URL: http://www.wjgnet.com/2220-3249/full/v4/i4/372.htm DOI: http://dx.doi.org/10.5501/wjv.v4.i4.372

INTRODUCTION

Acute gastroenteritis, one of the main causes of morbidity and mortality in the world, is a consequence of microbial infections, which in industrialized countries are mainly caused by viruses, such as rotavirus (RV), enteric adenovirus (AdV), astrovirus and human calicivirus[1-3].

RV is the most common cause of severe diarrhoea in children under 5 years of age, adults in close contact with infected children, hospitalised patients and the elderly[3,4]. Data from United States reveal that RV infection is responsible for approximately 3 million cases of acute diarrhoea in children each year[5]. RV infection is associated with high rates of morbidity throughout the world and high rates of mortality in developing countries, where gastroenteritis caused by RV account for more than 800000 deaths per year due to poor nutrition and lack of health care[6]. In the majority of countries, RV infections occur dispersed throughout the year, nevertheless in temperate climates is characterized by a peak during the winter months[1-3].

AdV, especially serotypes 40 and 41 (enteric AdV), are frequently related with high morbidity and mortality in immunocompromised patients with the most susceptible groups to be children and patients submitted to transplantation[7-9]. Enteric AdV have been related with acute diarrhoea in variable frequency (ranging from 1.4% to 10%), depending on the geographic location and type of patients[10,11]. AdV is known for its large distribution worldwide and the majority of the studies refer it to be equally distributed during all the seasons of the year[8,9].

Immunocompromised patients constitute an important group for prevention of gastrointestinal infections[7,12,13]. In fact, the incidence of infection-related post-transplant diarrhoea has been reported to be up to 40%, with viruses being the most common pathogens[2,3,12]. Considering the importance of gastroenteritis prevention in immunocompromised individuals, the diagnosis of AdV and RV at early stages of the disease is extremely important, in order to reduce morbidity and mortality.

The aim of this study was to characterize the prevalence of RV and AdV infection in immunocompromised patients with acute gastroenteritis from the North region of Portugal treated at the Portuguese Institute of Oncology of Porto.

MATERIALS AND METHODS

Population

This study was performed as a cross-sectorial retrospective hospital-based case study with 509 stool samples obtained between January 2009 and December 2010 from immunocompromised patients diagnosed with acute diarrhoea at Portuguese Institute of Oncology of Porto (IPO Porto). Samples were obtained from 200 immunocompromised patients with different haematological malignancies (median age 21 years old, range 0-72): 83 female (median age 15 years old, range 0-65) and 117 male (median age 39 years old, range 0-72).

RV/AdV detection

The diagnosis of infection was performed as a routine procedure at the Virology Service of IPO Porto. The stool specimens were tested as soon as possible after collection and the presence of RV and AdV (serotypes 40 and 41) was determined by immunochromatography using the RIDA® Quick Rota-Adeno-Kombi kit (r-Biopharm, Darmstadt, Germany) according to manufacturer instructions. The faecal samples were diluted in the dilution buffer supplied with the kit. This is a ready-to-use test based on a nitrocellulose membrane sensitized with

Table 1 Adenovirus and rotavirus results discriminated by year *n* (%)

	AdV positive	RV positive	Negative
Total (n = 509)	63 (12.4)	4 (0.8)	442 (86.8)
Year 2009 (n = 189)	24 (12.7)	-	165 (87.3)
Year 2010 (n = 320)	39 (12.2)	4 (1.3)	277 (86.5)

AdV: Adenovirus; RV: Rotavirus.

antibodies directed against RV and AdV (test lines).

Statistical analysis

Data analysis and description of seasonal frequencies were performed using computer software IBM® SPSS® (Statistical Package for Social Sciences) Statistics version 20.0 for Mac. The frequencies of infection were compared into different age and gender groups by χ^2 test.

RESULTS

In total, 509 stool samples were tested and it was possible to detect 63 (12.4%) positive samples for AdV and 4 (0.8%) positive samples for RV (Table 1). Comparing the number of cases during the period between January 2009 and December 2010, it was possible to observe a substantial increase in 2010, however the infection rates were similar.

Considering only patients-related data, where we have combined the results of all samples obtained from each individual, the overall frequency of AdV and RV infection was 6.5% (13/200 patients) and 1.5% (3/200 patients), respectively. The prevalence of infection was characterized with stratification of individuals according to age groups and genre and the results showed a tendency to an increased prevalence of infection in paediatric patients between 0-10 years old (Figure 1 and Table 2).

Considering the seasonal distribution of these infections (Figure 2), our study revealed that AdV infection was more frequent between October 2009 and April 2010, while RV infection was characterized by two distinct peaks (April 2010 and July 2010).

DISCUSSION

Immunosuppression treatments lead patients to be more susceptible to opportunistic infections, either bacterial or viral. Many studies have shown that RV and AdV are the most frequent pathogenic virus during acute diarrhoea in immunocompromised patients[3,7,9,13]. The overall distribution worldwide has been described in literature[5,14], although little is known about the epidemiology of these viruses in Portugal, and therefore we aimed to add epidemiological information of the prevalence of these infections in immunocompromised patients with haematological diseases treated at IPO Porto.

Firstly, it is important to refer that our results should

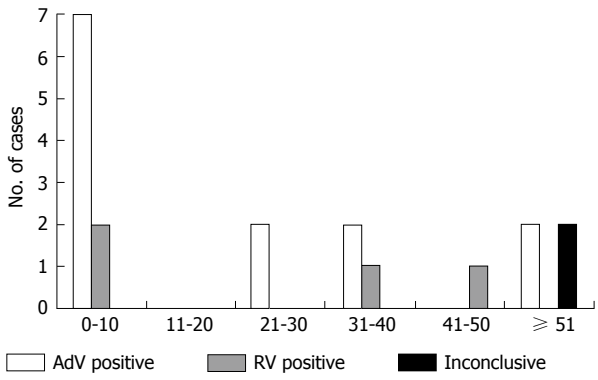

Figure 1 Age-associated distribution of adenovirus and rotavirus prevalence. AdV: Adenovirus; RV: Rotavirus.

be discussed considering the limitations of the diagnostic test. This test is used to screen only infections by RV and enteric AdV, which could limit the identification of positive cases since the range of viruses involved in acute gastroenteritis is larger. Nevertheless, the frequencies that we have obtained were considerably low when compared with the number samples. These results might be explained by the fact that other virus such as Norovirus, or even bacteria, could be the cause of the acute gastroenteritis[15-18]. Moreover, acute diarrhoea is sometimes overestimated since chemotherapeutic agents and radiotherapy may also promote it[19]. In addition, there are no reports of these viruses in Portuguese immunocompromised patients, thus the fact that results are distinct from other studies remain to be further clarified[20].

Recent data indicate that enteric AdV infection in immunocompromised patients remains constant along the year[3,8], and the RV infection is more prevalent during the winter and beginning of the spring[2-4]. These features were not observed in our population, and in fact, our study showed that enteric AdV was more prevalent between November 2009 and April 2010, with a seasonal peak in August 2010. Between November 2010 and December 2010 there were no positive cases, but the number of suspected samples was also lower than in the same period the year before.

In contrast with literature, RV incidence was very low, with only four positive cases to be identified, and therefore no seasonal prevalence was estimated[2,3]. However, it is possible to observe that positive cases were identified in April and June/July months. These data are in agreement with previous studies performed in Portugal, which describe a higher prevalence of RV infection in paediatric population[21,22]. Rodrigues *et al*[22] refers that the prevalence of RV presents clear differences between years and that in 2010 there were two distinct peaks with significant RV activity occurring much later and lasting into July.

To the best of our knowledge, this is the first study to characterize the prevalence of RV and enteric AdV in immunocompromised patients from Portugal. Despite the lower incidence of both viral infections, this study

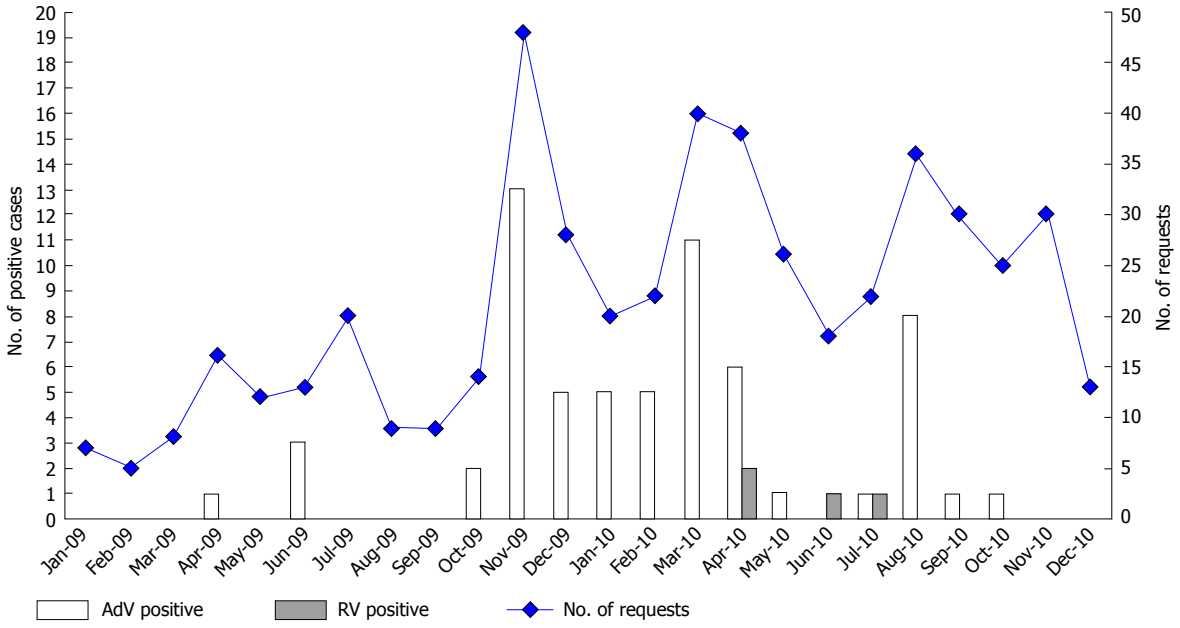

Figure 2 Seasonal distribution of adenovirus and rotavirus infection throughout the study period, taking into account the number of requests. AdV: Adenovirus; RV: Rotavirus.

Table 2 Characterization adenovirus and rotavirus infection by age group and genre *n* (%)

	AdV			RV		
	Positive	Negative	IC	Positive	Negative	IC
Total (*n* = 200)	13 (6.5)	185 (92.5)	2 (1.0)	3 (1.5)	196 (98.0)	1 (0.5)
Age group						
0-10 (*n* = 60)	7 (11.7)	53 (88.3)	-	2 (3.3)	58 (96.7)	-
11-20 (*n* = 21)	-	21 (100.0)	-	-	21 (100.0)	-
21-30 (*n* = 19)	2 (10.5)	17 (89.5)	-	-	19 (100.0)	-
31-40 (*n* = 19)	2 (10.5)	17 (89.5)	-	1 (5.3)	18 (94.7)	-
41-50 (*n* = 19)	-	18 (94.7)	1 (5.3)	-	19 (100.0)	-
⩾ 51 (*n* = 62)	2 (3.2)	59 (95.2)	1 (1.6)	-	61 (98.4)	1 (1.6)
Genre						
Male (*n* = 117)	9 (7.7)	107 (91.4)	1 (0.9)	1 (0.9)	115 (98.2)	1 (0.9)
Female (*n* = 83)	4 (4.8)	78 (94.0)	1 (1.2)	2 (2.4)	81 (97.6)	-

AdV: Adenovirus; RV: Rotavirus; IC: Inconclusive.

emphasizes the importance of vigilance and prevention of viral infections in the gastrointestinal tract. Moreover, as the results reveal a lower incidence of RV and enteric AdV, it is extremely important to add more screening methods for other emergent enteric viruses, in order to avoid the morbidity and mortality of the immunocompromised patients.

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COMMENTS

Background

Acute gastroenteritis is one of the main causes of morbidity and mortality in the world, especially for immunocompromised patients. Immunosuppression treatments lead patients to be more susceptible to opportunistic infections, either bacterial or viral. Many studies have shown that rotavirus (RV) and adenovirus (AdV) are the most frequent pathogenic virus during acute diarrhoea in immunocompromised patients.

Research frontiers

Characterization of RV and AdV prevalence and seasonal distribution in immunocompromised patients with acute gastroenteritis.

Innovations and breakthroughs

The overall prevalence of AdV and RV infection in immunocompromised patients with acute gastroenteritis was 8%. AdV was the most prevalent with 6.5% (13/200 patients) followed by RV with a prevalence of 1.5% (3/200 patients). Results revealed a lower prevalence of RV and enteric AdV than expected.

Applications

The lower incidence of RV and enteric AdV observed in the authors' study pointed that it is extremely important to add more screening methods for other

emergent enteric viruses, in order to avoid the morbidity and mortality of the immunocompromised patients.

Terminology

This study provides the first update on the prevalence of RV and AdV infection as agents of acute gastroenteritis in immunocompromised patients.

Peer-review

This is an interesting study on the cause of diarrhea by viral agents. The study is well-designed and the manuscript is well-written.

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