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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

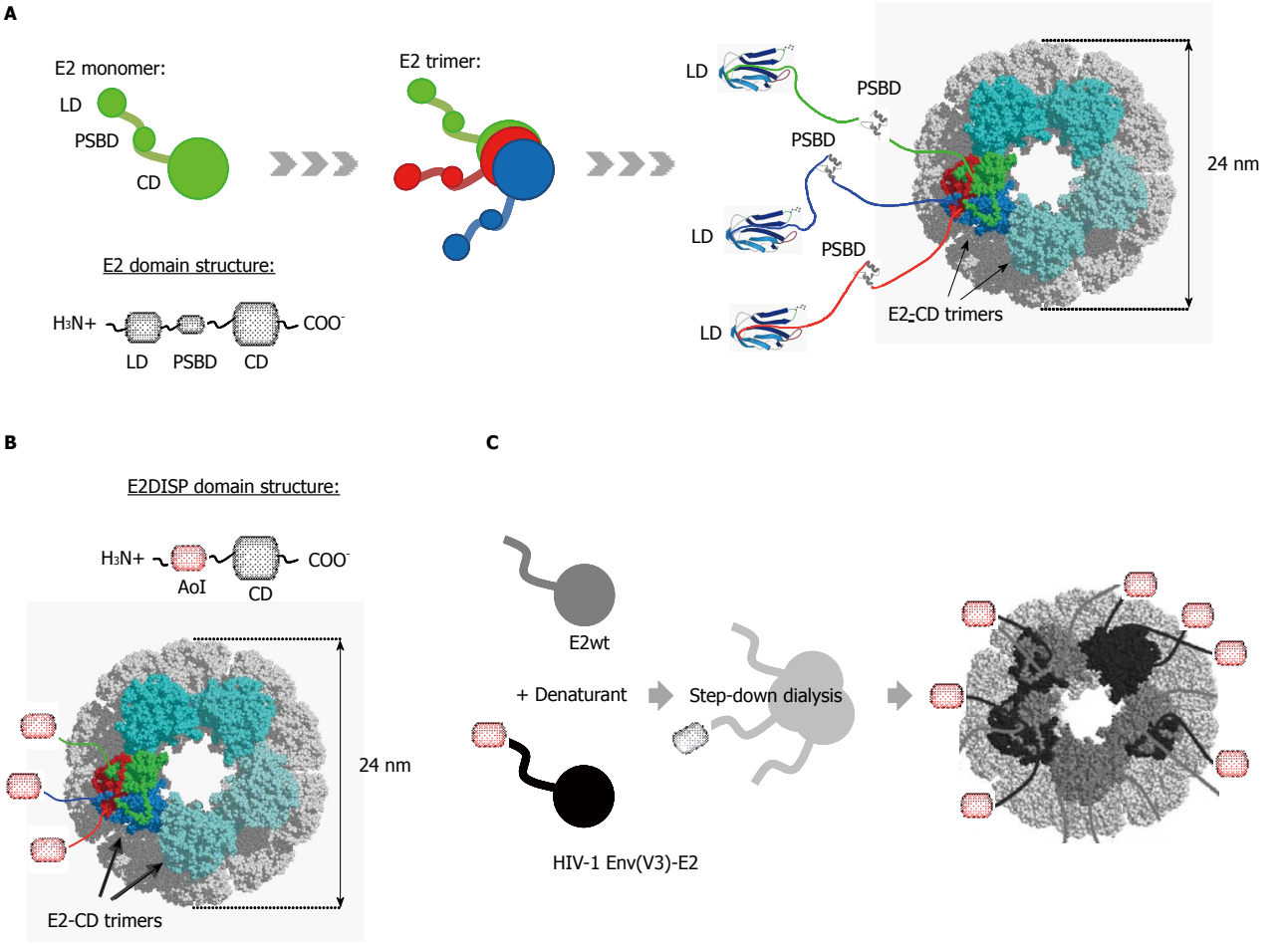

Figure 1 E2 acetyltransferase component from *Geobacillus stearothermophilus* pyruvate dehydrogenase complex. A: Schematic illustration of the native E2 chain with: lypoil domain (LD), peripheral subunit-binding domain (PSBD), and catalytic acetyltransferase core domain (CD). E2 CD forms trimers that 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).

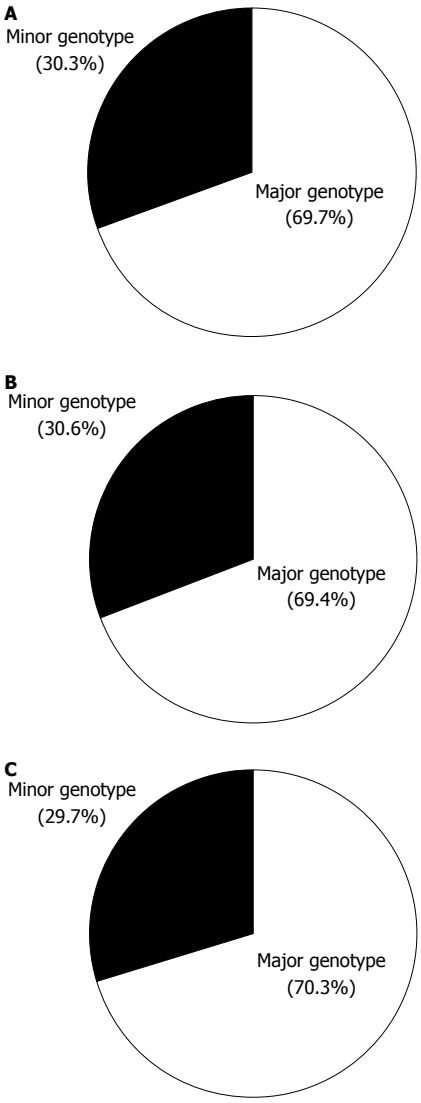

Figure 1 Distribution of interleukin-28B genotypes in Japanese patients infected with hepatitis C virus between February 2010 and April 2014. (A) Total patients (n = 432), (B) HCV genotype 1 patients (n = 314), and (C) HCV non-genotype 1 patients (n = 118). The white and black parts indicate the IL28B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

Bujdoso R, Landgraf M, Jackson WS, Thackray AM. Prion-induced neurotoxicity: Possible role for cell cycle activity and DNA damage response. *World J Virol* 2015; 4(3): 188-197 Available from: URL: http://www.wjgnet.com/2220-3249/full/v4/i3/188.htm DOI: http://dx.doi.org/10.5501/wjv.v4.i3.188

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

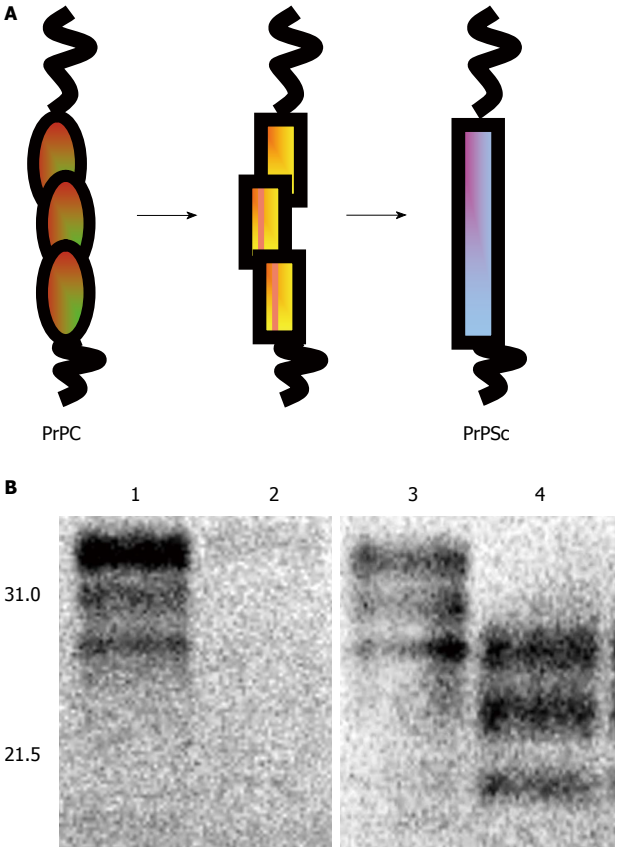

Figure 1 Conversion of PrPC into PrPSc. A: Schematic diagram of the conversion of PrPC into PrPSc. A major structural event occurs in the C-terminal domain of PrPC as it converts from a predominantly α-helical form into one enriched for β-sheet. This conformational change may involve the formation of 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

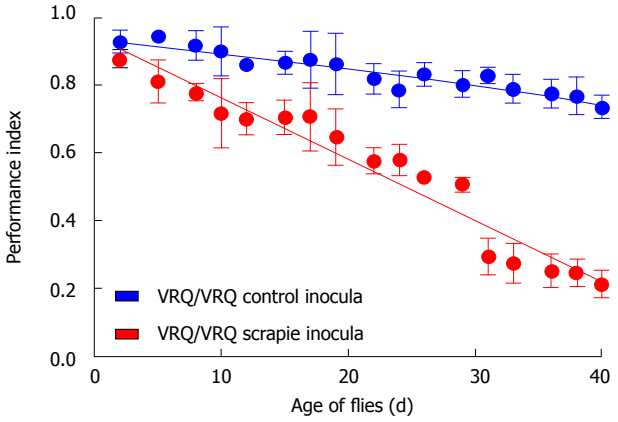

Figure 2 Prion-exposed ovine PrP transgenic *Drosophila* show enhanced locomotor defect. *Drosophila* with pan neuronal expression of ovine VRQ(cyt) were fed VRQ/VRQ scrapie-free (blue circles, blue line) or scrapie-infected (red circles, red line) sheep brain homogenate at the larval stage of development. 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

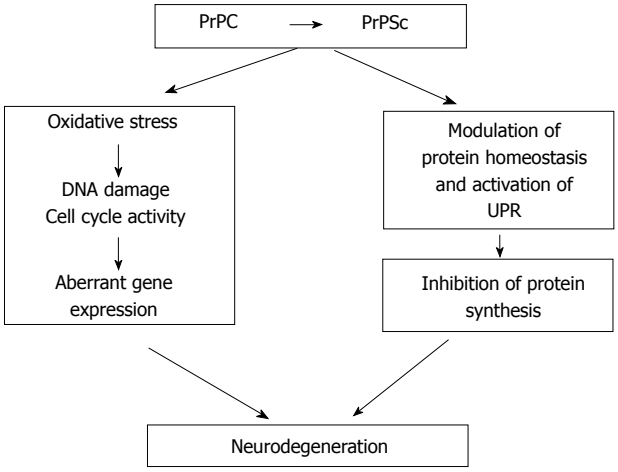

Figure 3 Hypothetical model for prion-induced neurodegeneration. The conversion of PrPC into PrPSc is an essential requirement for the neurotoxicity that occurs during prion disease. Neurodegeneration in post mitotic neurons, stressed by prion replication, may arise through various cellular events including 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 ABCC10rs2125739, CYP2B6 516G > T	Cutaneous rash, SJS/TEN 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 APOA5 (1131T > C, 64G > C), APOC3 (482C > T, 455C > T, 3238C > G) ABCA1 2962A > G APOE (ε2, ε3 haplotypes)	Early onset of lypodistrophy 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.

Brugnaro P, Morelli E, Cattelan F, Petrucci A, Panese S, Eseme F, Cavinato F, Barelli A, Raise E. Non-AIDS definings malignancies among human immunodeficiency virus-positive subjects: Epidemiology and outcome after two decades of HAART era. *World J Virol* 2015; 4(3): 209-218 Available from: URL: http://www.wjgnet.com/2220-3249/full/v4/i3/209.htm DOI: http://dx.doi.org/10.5501/wjv.v4.i3.209

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.

Méndez C, Ahlenstiel CL, Kelleher AD. Post-transcriptional

gene silencing, transcriptional gene silencing and human immunodeficiency virus. *World J Virol* 2015; 4(3): 219-244 Available from: URL: http://www.wjgnet.com/2220-3249/full/v4/i3/219.htm DOI: http://dx.doi.org/10.5501/wjv.v4.i3.219

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.*

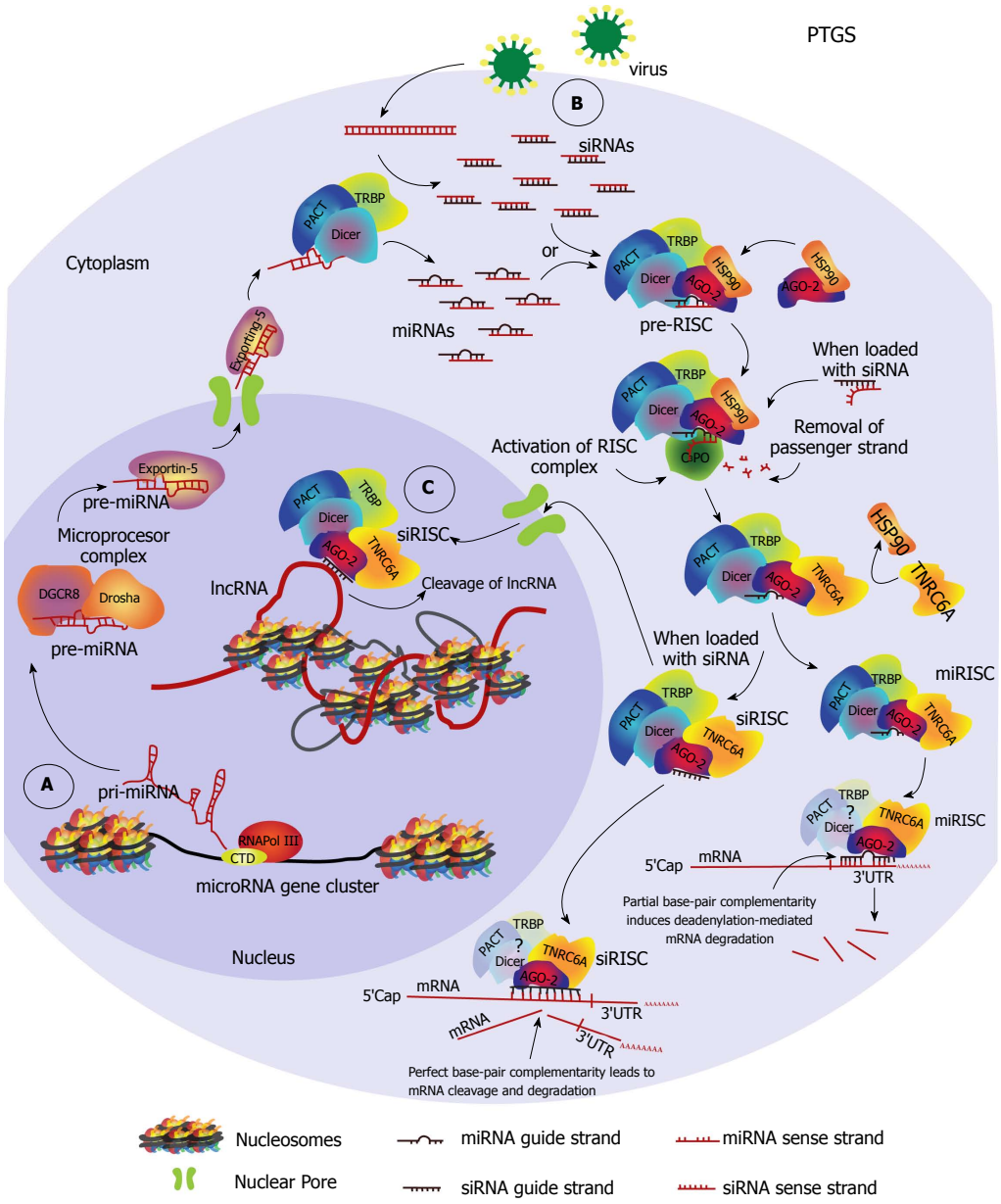

Figure 1 Cytoplasmic and nuclear post-transcriptional gene silencing pathways. A: A primary-microRNA (pri-miRNA) is transcribed by the RNA Polymerase III (RNA Pol III) from a miRNA gene cluster. The pri-miRNA is then processed by the microprocessor complex into the precursor-miRNA (pre-miRNA) which is 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

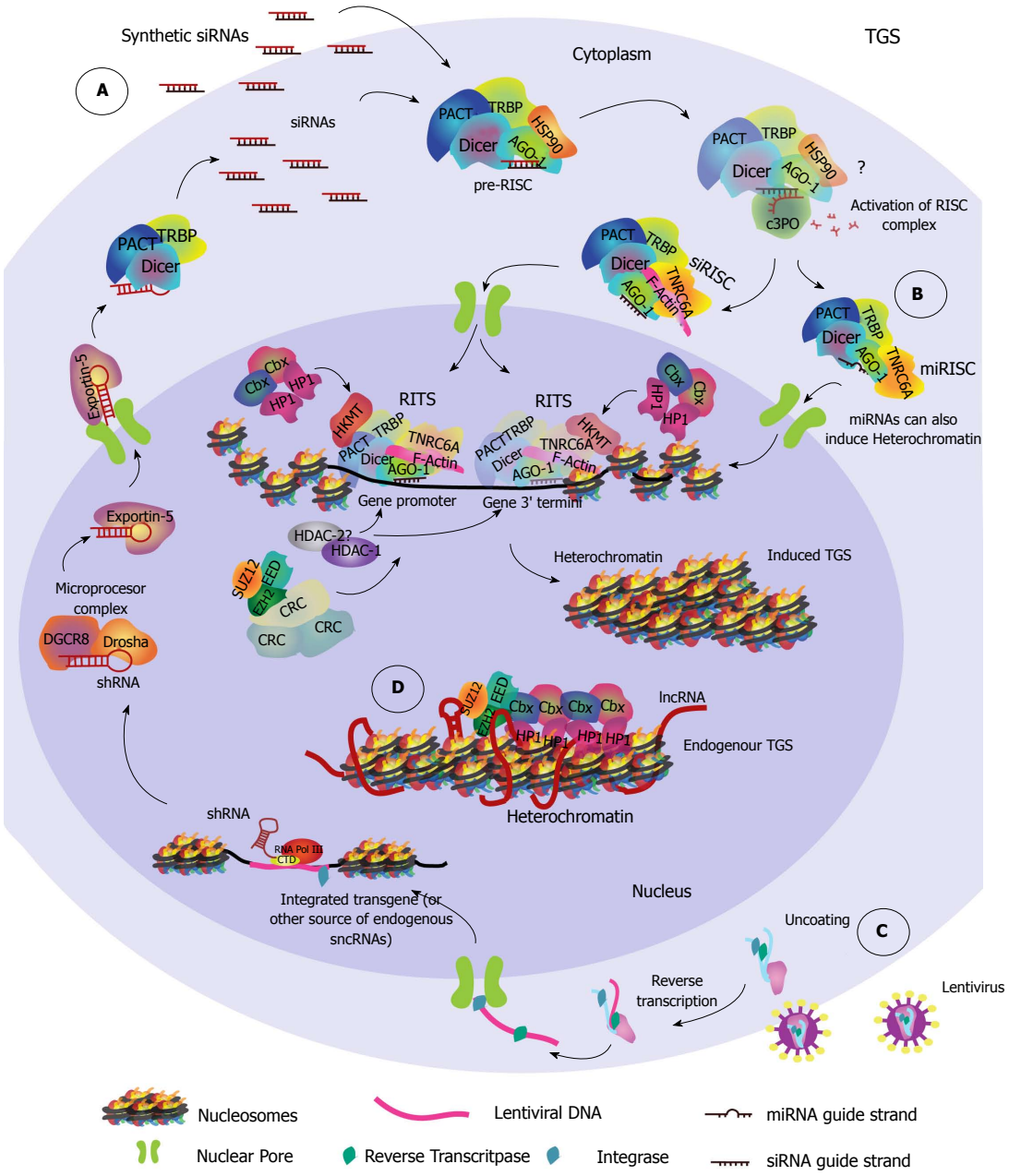

Figure 2 Endogenous and induced transcriptional gene silencing pathways. A: Synthetic siRNAs that have been designed to target the promoter region or the 3' end termini of a gene are loaded into AGO-1, forming the pre-RISC complex. It is currently unknown if removal of passenger strand is required, nonetheless 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

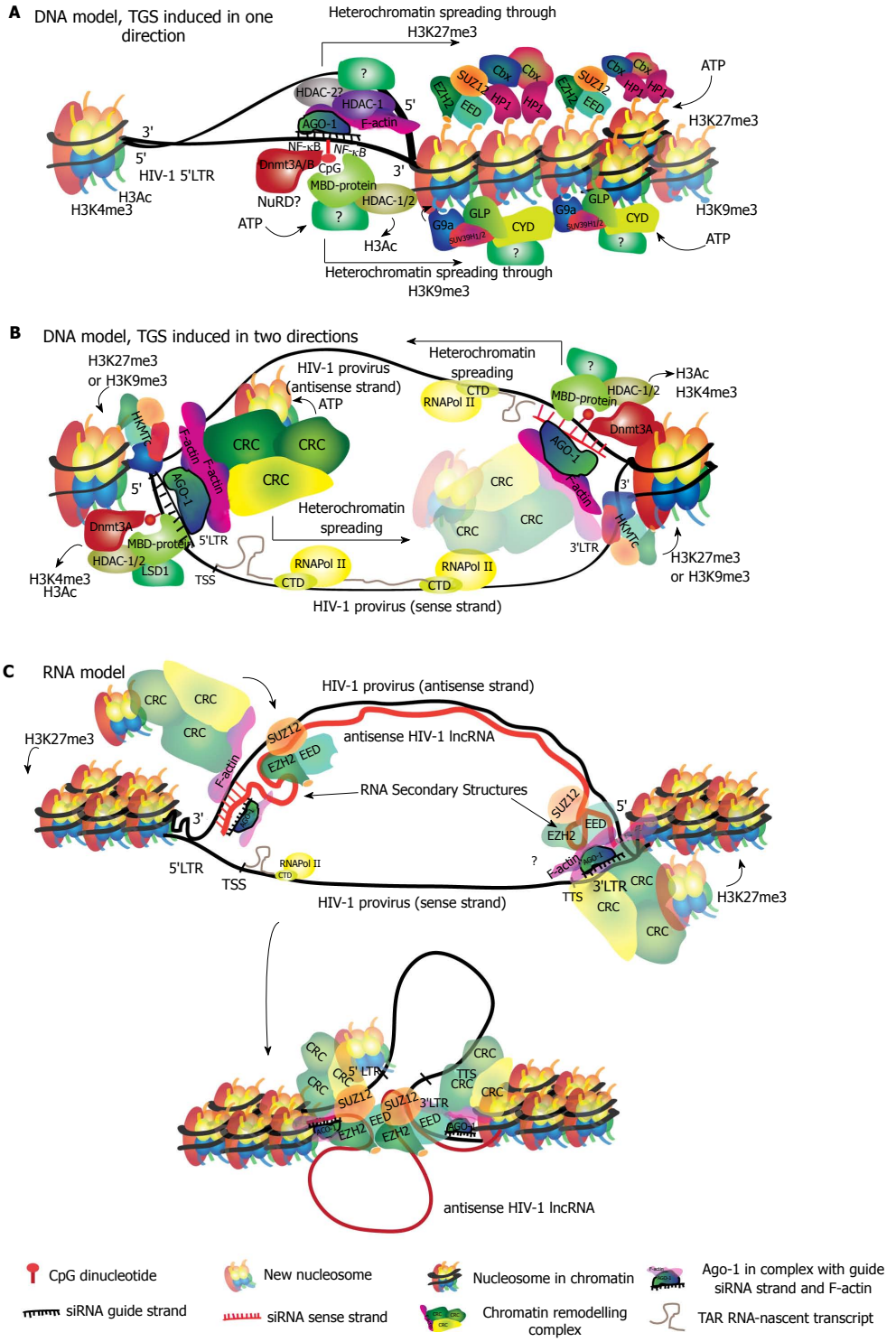

Figure 3 Models describing possible molecular mechanisms of siRNA-induced transcriptional gene silencing in human immunodeficiency virus 1. A: DNA model in which the siRNA guide-strand finds its target in the 5'LTR promoter of the HIV-1 genome binding directly to the DNA. This binding triggers the recruitment 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.

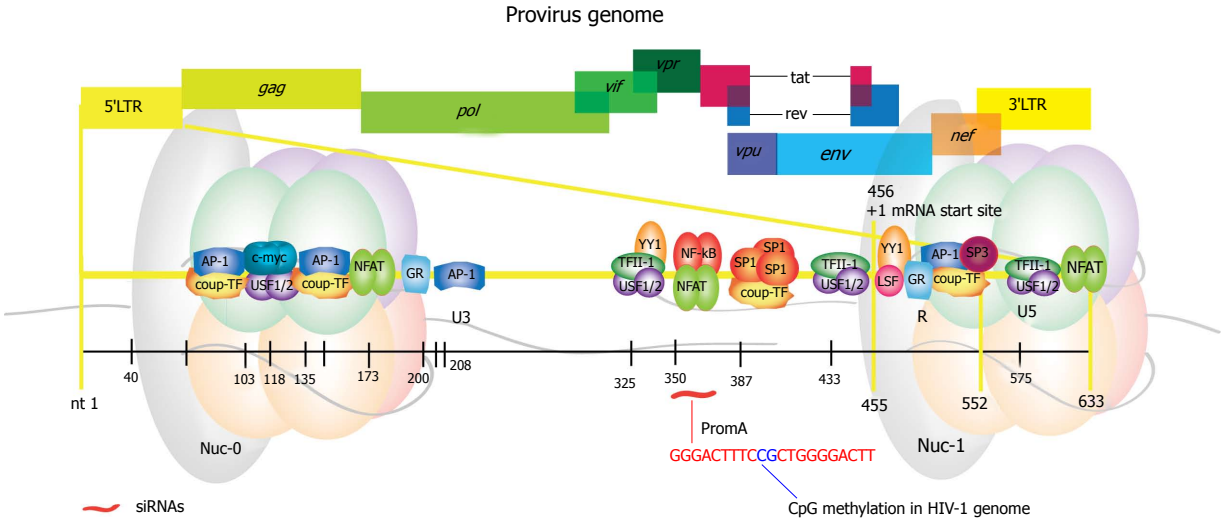

Figure 4 Map of the human immunodeficiency virus 1 genome showing in magnification the 5'LTR region with the location of transcription factor binding sites. The specific coordinates within the HIV-1 genome for each of the shown DNA regulatory elements is listed on Table 1. 5'LTR: 5' long terminal repeat; 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$^{-/-}$IL2rγ$^{-/-}$ 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 $\geqslant$ 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.

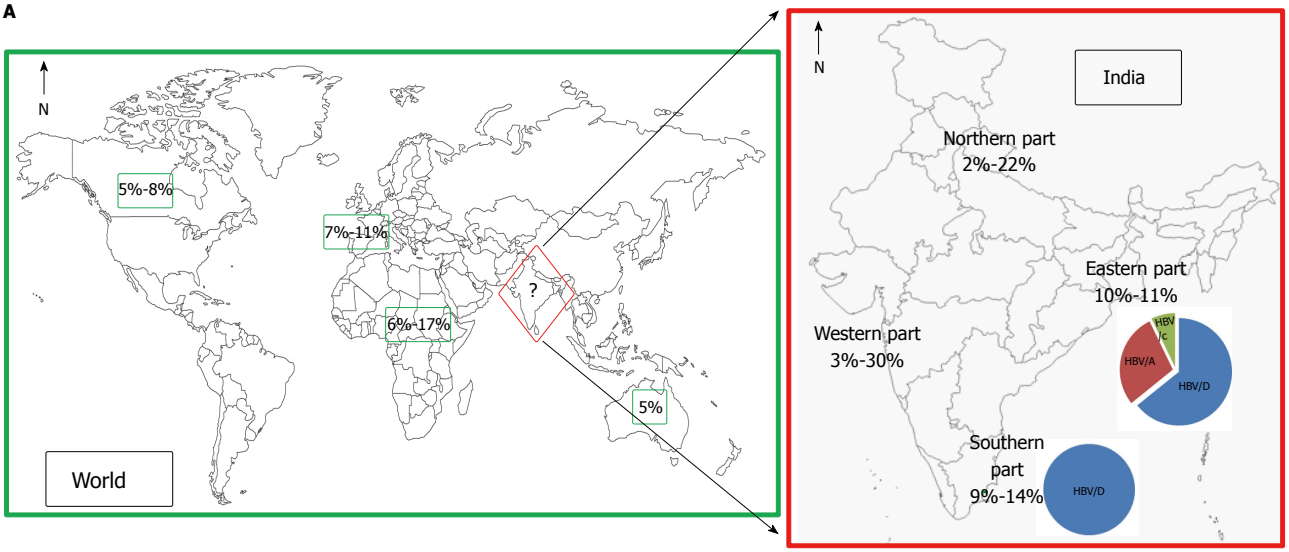
The aforementioned negative impacts on natural history of HBV might be the consequences of influence of HIV on the diversity of HBV genome, modification of host immune response and ART related complications. Some reports could be found to address the genetic diversity of HBV among HIV co-infected individuals[23,38-43].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

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.

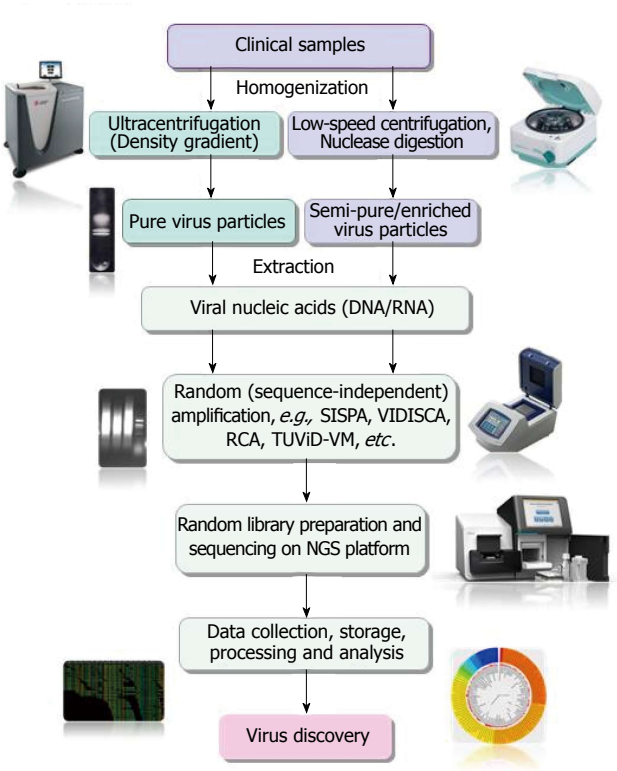

Figure 1 Diagrammatic representation of main steps of clinical virus discovery by next-generation sequencer based technologies.

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

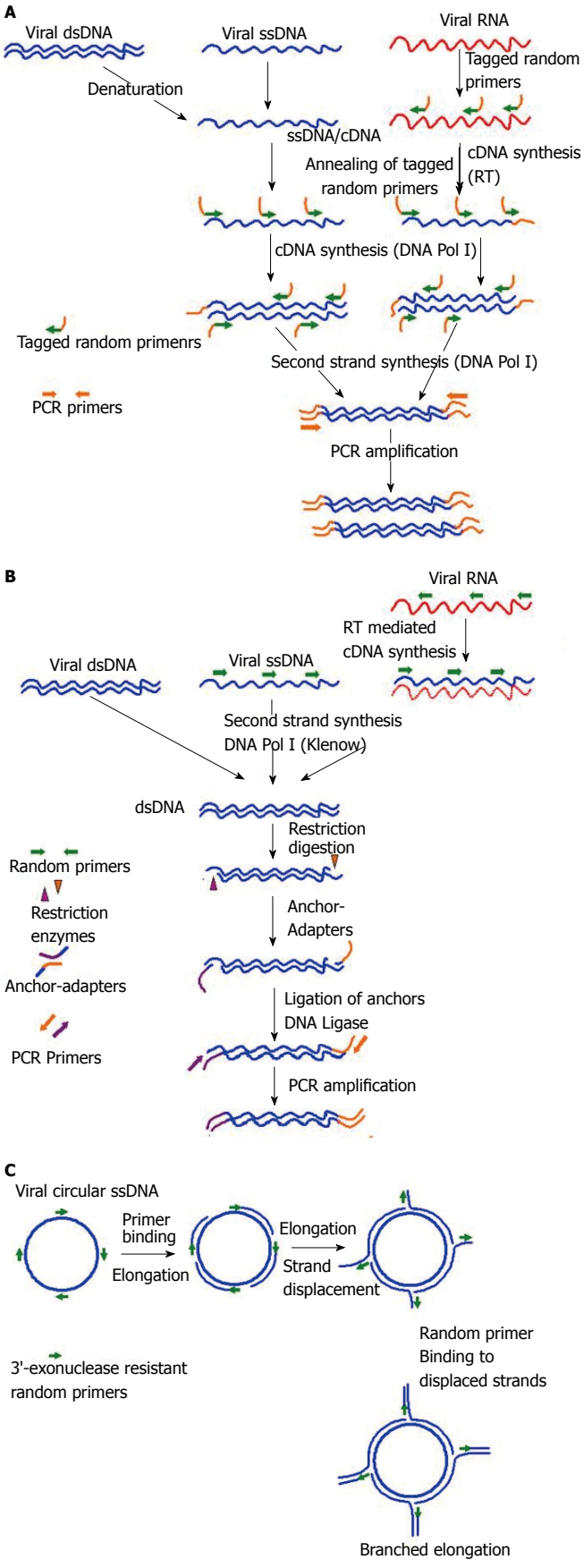

Figure 2 Different virus nucleic acid enrichment techniques. A: Sequence-independent single-primer amplification. Initially viral RNA and ssDNA is transcribed into complementary DNA (cDNA) using reverse transcriptase (RT) and DNA Pol I respectively, with the help of tagged-primers having defined sequence 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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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,

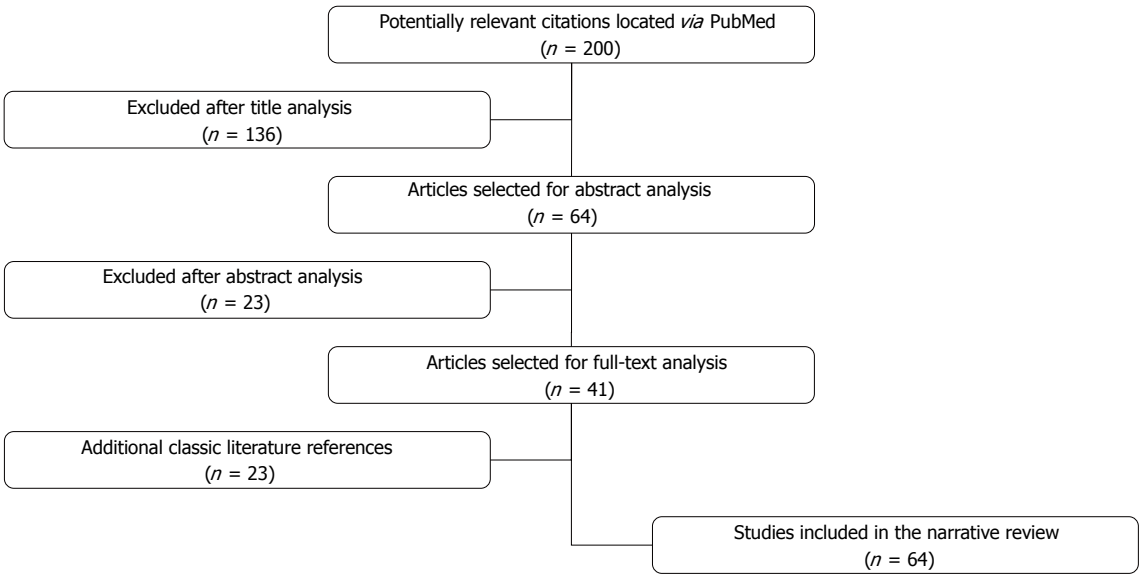

Figure 1 Flow chart representing the process of article selection.

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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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

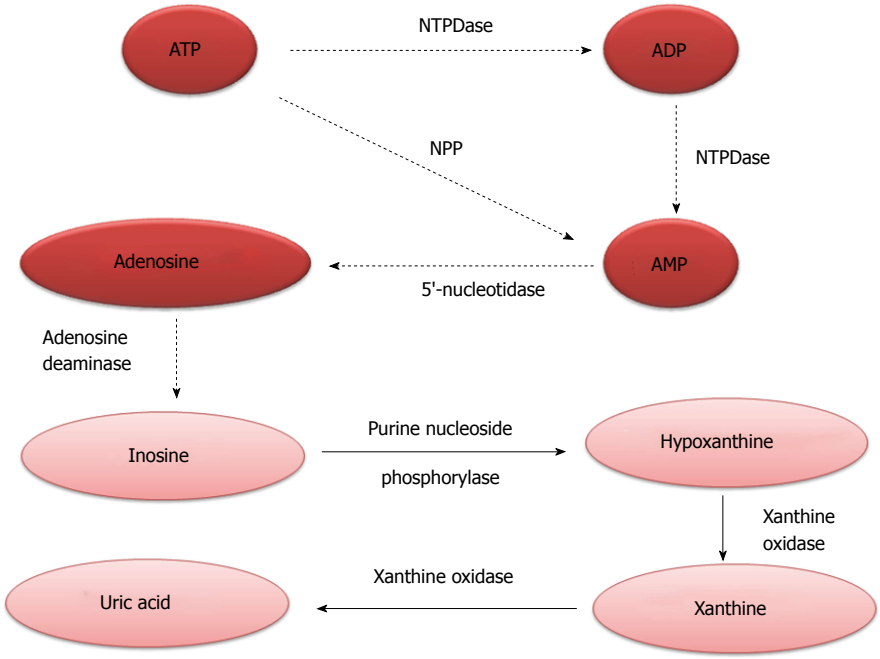

Figure 1 Schematic representation of the purinergic pathway. ATP is broken down to ADP and AMP by NTPDase or directly to AMP by pyrophosphatase/phosphodiesterase (NPP). AMP is converted to adenosine by 5′-ectonucleotidase (CD73). Adenosine deaminase (ADA) transforms adenosine into inosine which is converted 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

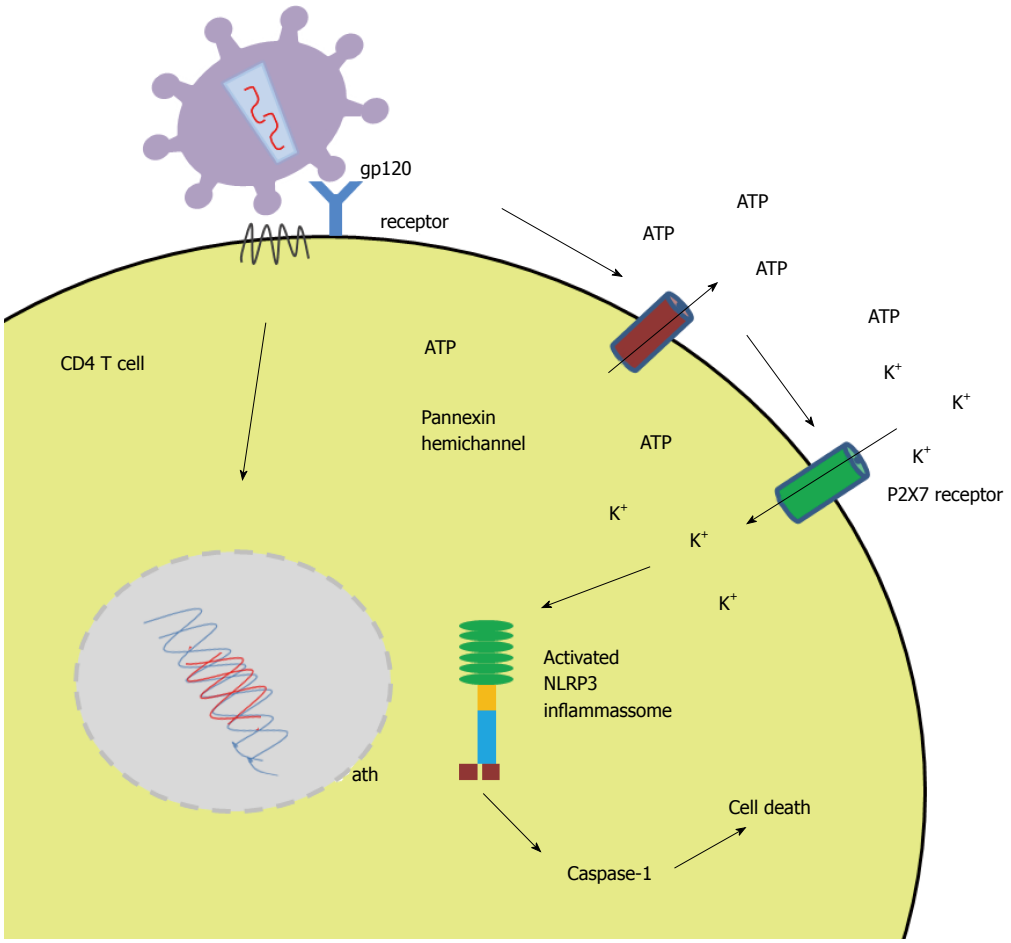

Figure 2 Schematic illustration of ATP release and activation of NOD-like receptor family, pyrin domain containing 3 inflamassome in a human immunodeficiency virus infected CD4 T cell. Once human immunodeficiency virus gp120 binds to its receptor ATP release is stimulated through pannexin hemichannels 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

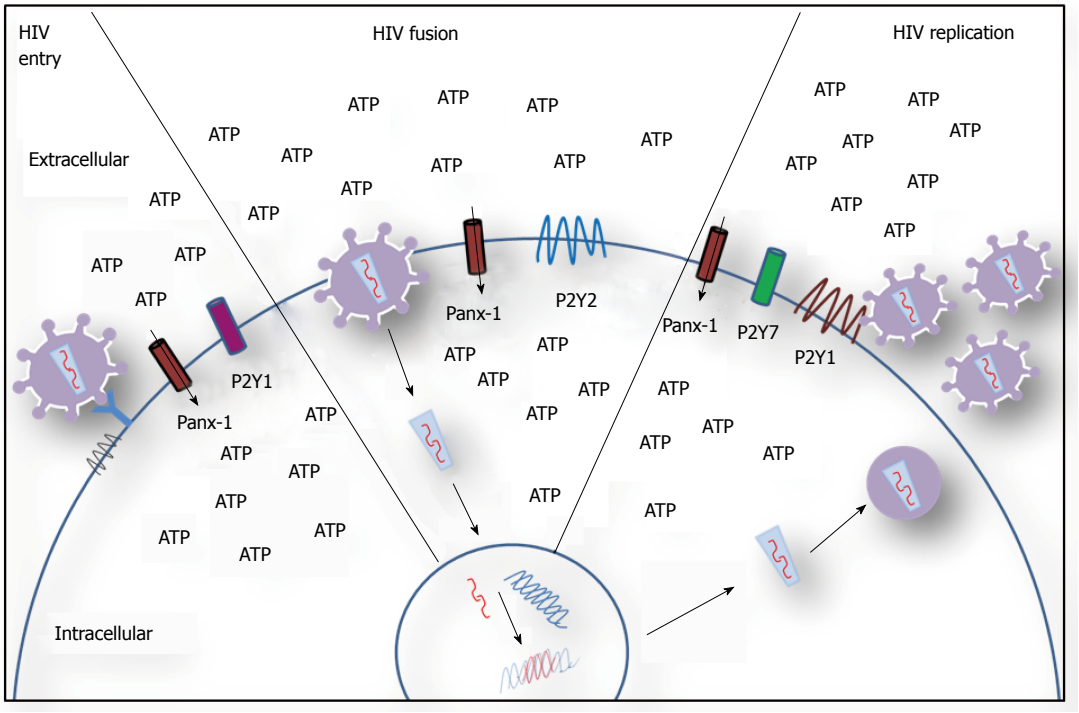

Figure 3 Schematic representation of purinergic receptors involvement in human immunodeficiency virus infection. Pannexin hemichannels (Panx-1) are open in response to ATP release and activation of purinergic receptors, facilitating viral entry, fusion and replication. The blockage of viral entry by 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

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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.

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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.

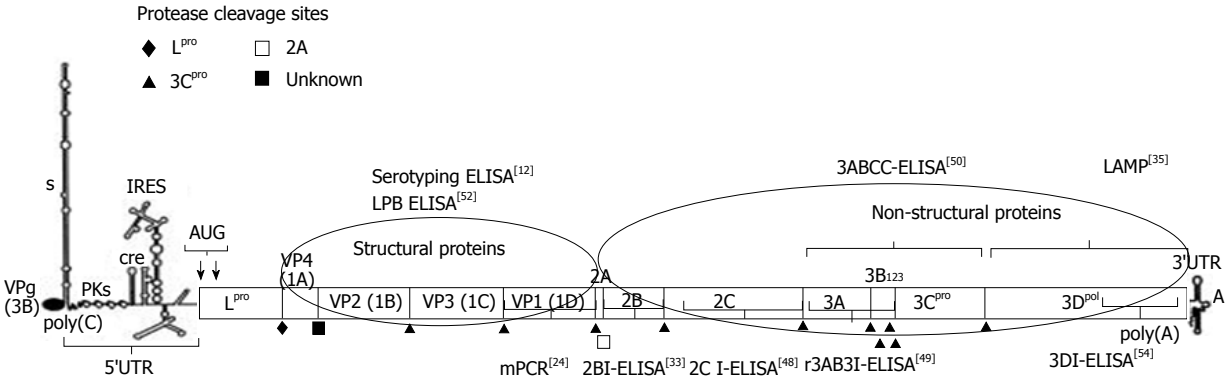

Figure 1 Genome structure of the foot-and-mouth disease virus. Describes in details the different regions of foot-and-mouth disease virus (FMDV) which codes four structural and 8 non-structural proteins. The regions of genome encoding structural proteins are targeted for FMDV serotype determination by 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.

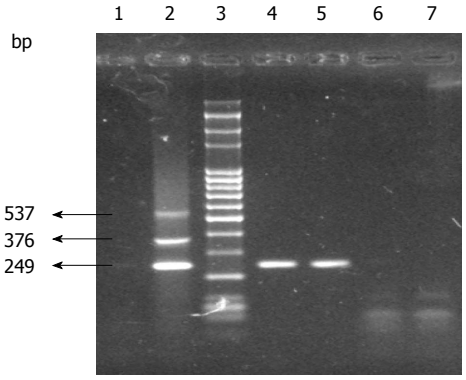

Figure 2 Depicting the foot-and-mouth disease virus serotyping by gel based multiplex polymerase chain reaction assay. Foot-and-mouth disease virus serotype determined by gel based ready-to-use lyophilized one-step realtime-polymerase chain reaction. Lane 1; negative control; Lane 2: Positive control 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.

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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.

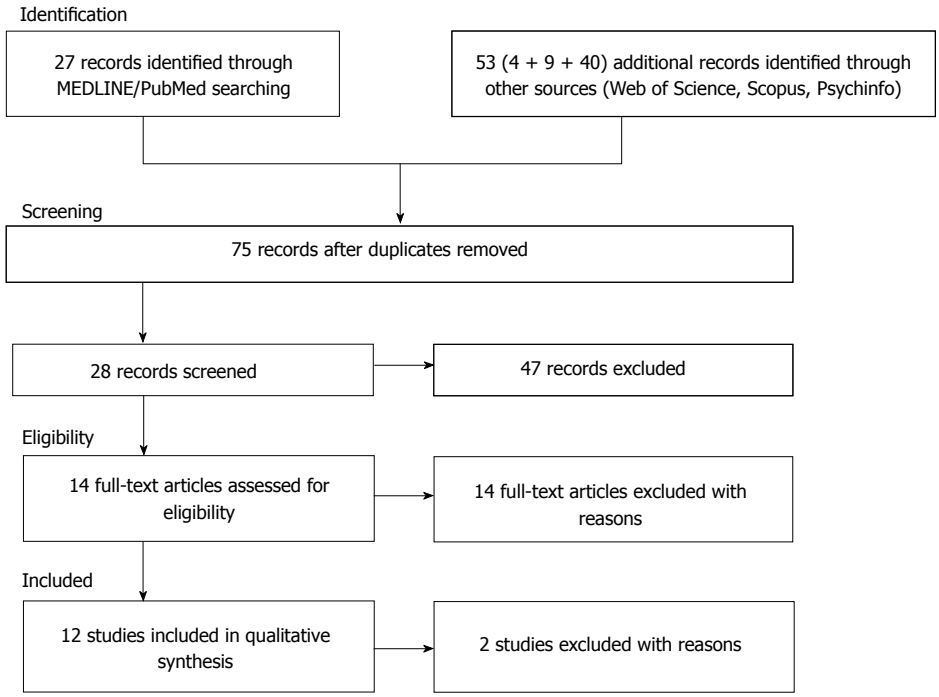

Figure 1 Flowchart of the search and selection process.

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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