HIV drug resistance and hepatitis co-infections in HIV-infected adults and children initiating antiretroviral therapy in Rwanda

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HIV drug resistance and Hepatitis co-infections in HIV-infected adults and children initiating Antiretroviral Therapy in Rwanda

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CHAPTER 1

General introduction

1.1 | Global epidemiology and diversity of HIV

HIV/AIDS is a global disease with an estimated 35.2 million people living with HIV/AIDS worldwide at the end of 20131. Sub-Saharan Africa is still the region most affected by the epidemic worldwide, with 24.7 million HIV cases at the end of 2013 (Table 1). The prevalence among adults in this region is estimated at 4.7% with great variability between sub regions and countries. Thanks to combined antiretroviral therapy (cART) and prevention efforts, HIV incidence in sub-Saharan Africa has declined to 1.8 million new infections in 2012, which is 33% lower than in 20011.

Table 1 | Epidemiology of HIV/AIDS in 2013

Region	Estimated prevalence of HIV infections (adults and children)	Expected adult and child deaths in 2012	Adult prevalence (15–49) (%)
Worldwide	35.2 million	1.6 million	0.8%
Eastern Europe and Central Asia	1.3 million	91 000	0.7%
Western and Central Europe	860 000	7 600	0.2%
North America	1.3 million	20 000	0.5%
East Asia	880 000	41 000	<0.1%
Latin America	1.5 million	52 000	0.4%
Caribbean	250 000	11 000	1.0%
Oceania	51 000	1200	0.2%
Sub Saharan Africa	24.7 million	1.2 million	4.7%

World Aids Day Report 2013: Centers for disease control and prevention. World AIDS Day – December 1, 2013. *MMWR Morb Mortal Wkly Rep.* 2013;62(47):945. http://www.ncbi.nlm.nih.gov/pubmed/24416816

HIV is an enveloped single strand, positive sense RNA virus belonging to the *Lentivirus* genus in the family of *Retroviridae*[2], which is subdivided in two types: HIV-1, representing more than 99% of HIV viruses, and HIV-2, representing less than 1% (Figure 1)3. HIV-2 is restricted to the West African epidemic. Its prevalence decreases over time4, 5, presumably related to lower fitness and pathogenicity[6]. HIV-1 is subdivided into four distinct groups: major (M), outlier (O), non-M /non-O (N) and P with an average of 40-50% genetic diversity between the groups. Group M is responsible for more than 90% of the infections worldwide and is further subdivided into nine genetically distinct subtypes recognized as A, B, C, D, F, G, H, J and K[7]. These subtypes show 25-35% inter-subtype sequence variability in the *env* gene and 10-15% in the pol gene. Apart from these distinct subtypes, mosaic forms of recombinants exist due to the possibility that viruses from two subtypes infect the same cell and mix their genetic material within a host. Recombinant viruses are either circulating recombinant forms (CRF), i.e. viral strains detected in at least three unrelated individuals and capable of establishing an epidemic on its own, or unique recombinant forms (URF), i.e. viral

strains identified in a single individual or in a cluster of associated individuals and which remain a localized phenomenon[7,8].

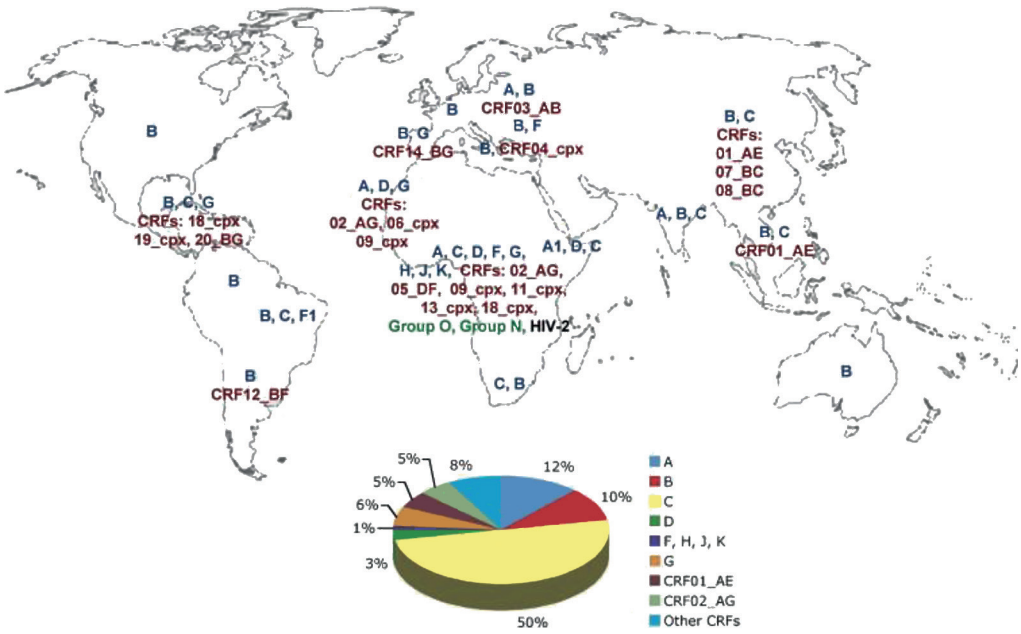

Figure 1 | Distribution of HIV subtypes by geographic regions. The molecular epidemiology of HIV worldwide is related to the zoonotic origin of the epidemic, the introduction of new genetic forms in different areas of the world and the continuous intra-host diversification of HIV through mutation and recombination. The picture of the global HIV subtype distribution is in constant evolution. *Source*: AIDS Rev. 2007 Apr-Jun; 9(2):75-87.

HIV subtype distribution around the globe is highly dynamic with distinct regional epidemics highlighting the introduction and evolution of new subtypes and recombinants (Figure 1)9. HIV diversity is highest in the sub-Saharan Africa region where the prevalence of HIV infection is also the highest[10]. All HIV types, groups and subtypes occur in this region, almost certainly due to West Central Africa being the region where the zoonotic crossover from non-human primates to humans took place[11]. Most HIV infections in West Africa are caused by the HIV-1 A and A/G recombinant subtypes, but a high number of dual infections with HIV-1 and HIV-2 also occur12. The lowest HIV prevalence in Africa has been documented in Northern Africa where the dominant subtype is B, except for Libya where HIV-1 CRF_02AG predominates[11,13]. In East and Central Africa, the predominant subtypes are A, D and C but also recombinant forms have also been identified and currently form up to 30% of the total infection burden in this region[14]. Subtype C predominates in Southern Africa where HIV prevalence is the highest in the world[15,16]. Existing data on HIV subtypes circulating in Rwanda are scarce and indicate a large predominance of subtype A1 followed by subtype C and with a discreet presence of subtype D and A/C recombinants[17,18].

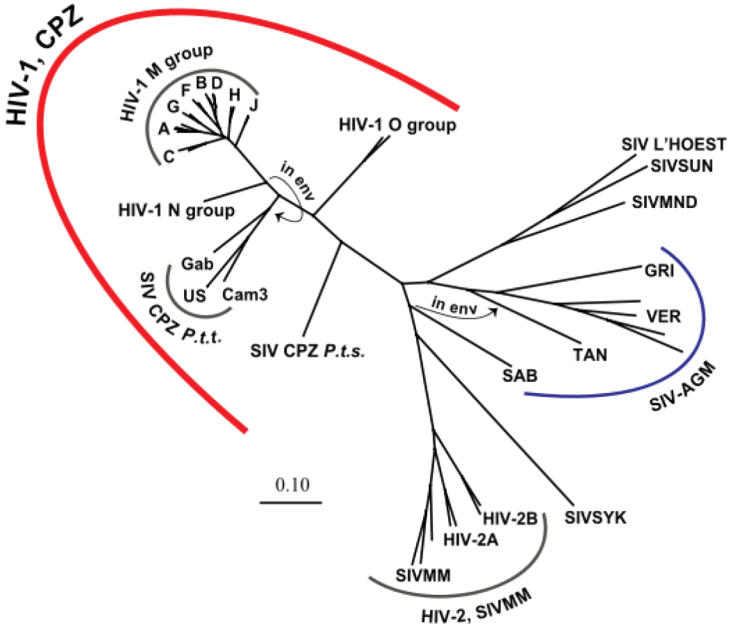

Figure 2 | Phylogenetic relationship of primate lentivirus: Two of the viruses presented in the tree (HIV-1 group N and SIVagm SAB) have mosaic genomes. The small arrows in the tree indicate where the sequences would branch in an *env* gene tree (*source*: Theoretical Biology and Biophysics Group, Los Alamos National Laboratory: Kuiken C, Foley B, Hahn BH, Marx P, Mc Cutchan F, Mellors JH, Mullins J, Wolinsky, Korber B. 1999. Los Alamos National Laboratory, Nm: http/www.hiv.lanl.gov. Accessed on November 2004).

Importantly, the study of HIV genetic distances between subtypes enables reconstruction of the history of HIV introduction and evolution in a given region[19]. Whether or not HIV subtypes bear clinical significance in terms of disease progression6, response to treatment6 or viral transmission 20 is being investigated. Differences in replication capacity or viral fitness may exist between various HIV subtypes and these may potentially become magnified under conditions of drug resistance. The CRF02_AG recombinant virus that is circulating in West Africa has been reported to have a natural V118I mutation in the reverse transcriptase gene, which is responsible for low level resistance to the antiretroviral drug lamivudine, while subtype G carries the mutation V82I in the protease gene, which can impact viral protease inhibitors sensitivity[21]. Group 0 and HIV-2 viruses naturally harbor resistance to non-nucleoside reverse transcriptase inhibitors (NNRTIs) due to respectively intrinsic Y181C and Y18I polymorphysms[22]. HIV-1 subtypes also show variation in the ease of development of resistance to different drugs, such as an increased risk of resistance to non-nucleoside reverse transcriptase inhibitors (NNRTIs), mainly nevirapine (NVP), observed in subtype C as compared to other subtypes[23,24].

1.2 | HIV pathogenesis and antiretroviral treatment

HIV primarily infects CD4+ T-lymphocytes and macrophages after entering the body via mucous membranes or through parental exposure to blood. The infection starts with a primary acute infection, followed by an asymptomatic phase and finally a symptomatic phase. In the absence of treatment, the symptomatic phase eventually leads to acquired immune deficiency syndrome (AIDS) and ultimately death. Primary HIV infection is an acute illness characterized by flu-like symptoms. During this period, the host experiences a seroconversion to HIV antigens and a significant reduction of CD4 count associated with a peak of HIV replication in plasma[25]. During the following chronic phase, the number of CD4+ T-lymphocytes gradually decreases, leading to a progressive loss of cell-mediated immunity and increasing susceptibility to opportunistic infections and neoplasms.

Antiretroviral therapy (ART) is targeted at inhibiting viral replication, thereby reducing viral load to undetectable levels. This in turn allows for restoration of immunity, or at a minimum prevents further deterioration of the immune system, hence reducing HIV-related morbidity and mortality. Currently, five different classes of antiretroviral drugs are available that interfere with different steps of the HIV replication cycle.

Nucleoside analogue reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) inhibit conversion of the viral single-stranded RNA into double-stranded DNA; protease inhibitors (PIs) prevent proteolytic cleavage of the viral precursor protein into mature viral enzymes and structural proteins; integrase inhibitors prevent integration of the transcribed viral DNA into the host genome as proviral DNA; entry inhibitors interfere with the ability of the virus to bind to host cell receptors, (for example, maraviroc binds to the CCR5 co-receptor); and fusion inhibitors inhibit fusion of the virus particle with the host cell membrane (for example, enfuvirtide binds to the gp41 membrane protein). The latter two classes both prevent HIV from entering the host cell in the earliest stage of the viral life cycle[27].

Table 2 | Antiretroviral drug class

	Generic name	Brand name
Nucleoside/Nucleotide Reverse Transcriptase Inhibitors (NRTIs)	Didanosine (DDI)	Videx
	Emtricitabine (FTC)	Emtriva
	Lamivudine (3TC)	Epivir
	Tenofovir (TDF)	Viread
	Zidovudine (AZT, ZDV)	Retrovir
Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)	Delavirdine (DLV)	Rescriptor
	Abacavir (ABC)	Ziagen
	Efavirenz (EFV)	Sustiva
	Etravirine (ETR)	Intelence
	Nevirapine (NVP)	Viramune
Protease Inhibitors (PIs)	Atazanavir (ATV)	Reyataz
	Darunavir (DRV)	Prezista
	Fosamprenavir(FPV)	Lexiva
	Indinavir (IDV)	Crixivan
	Nelfinavir (NFV)	Viracept
	Ritonavir (RTV)	Norvir
	Saquinavir (SQV)	Invirase
	Tipranavir (TPV)	Aptivus
Fusion or Entry Inhibitors	Enfuvirtide (T-20)	Fuzeon
	Maraviroc (MVC)	Selzentry
Integrase Inhibitors	Dolutegravir (DTG)	Tivicay
	Elvitegravir(EVG)	Vitekta
	Raltegravir, RAL)	Isentress

Antiretroviral agents used the in treatment of HIV-1-infection[26]

1.3 | HIV drug resistance

HIV-1 has a high mutation rate due to the error-prone reverse transcription step in the viral life cycle. Approximately one nucleotide mutation occurs per replication cycle which, combined with the high levels of viral replication, results in the daily generation of viral variants containing every possible single and many multiple mutations across the genome28the high viral load, and the errors made during viral replication. Mutations can arise from errors made either by host DNA-dependent RNA polymerase II or by HIV-1 reverse transcriptase (RT. This translates into the aptitude to rapidly generate new HIV-1 variants with growth and/or survival advantages under changing selective

pressures. HIV-1 drug resistance (HIVDR) mutations usually are selected in the genes targeted by the antiretroviral drugs that the patient is taking and these mutations usually decrease viral replication fitness. The emergence of HIVDR mutations occurs when viral replication is incompletely suppressed, for example due to suboptimal adherence to drugs. If the same drugs continue to be used, wild type virus is inhibited while HIVDR mutants overgrow. Other factors such as the drug's intrinsic antiretroviral potency, together with the genetic barrier to resistance also influence the vulnerability to resistance of a cART[29]. Secondary or acquired drug resistance is acquired through drug selection pressure. These resistance mutations can subsequently be transmitted from person to person (which is referred to transmitted or primary resistance)[29]. HIVDR compromises the efficacy of first and second line treatment.

1.4 | The principle of combined antiretroviral treatment

The most effective way to achieve undetectable viral load and to avoid the emergence of HIV drug resistance mutations is to combine highly potent drugs from different classes to which the patient has not been exposed before. The rationale is to target at least two steps in the viral cycle and reduce the risk that the virus becomes resistant. cART is a lifelong treatment, and its efficacy highly depends on patient adherence[27]. In industrialized countries, cART is often provided based on individualized patient information regarding the susceptibility of the HIV strain to various antiretroviral drugs. The clinical response to cART is monitored regularly by assessing CD4 count, HIV viral load and HIVDR as well as indicators of drug toxicity[30]. The cART regimen is adjusted in case of drug toxicity, in the first instance by drug substitution within the same class. Virological failure or emergence of HIVDR mutations generally leads to a switch to second or third line therapy after careful clinical assessment and adherence counseling[31].

The decision to start cART is based on the CD4 count or the clinical stage of HIV disease as standardized by WHO into IV stages[32]. The latest WHO guidelines recommend to start cART when the CD4 count ≤500 cells/mL or the patient has WHO stage III or IV disease, irrespective of the CD4 count 33.The CD4 cell count threshold has been adjusted from 200 cells /mL[34] to 350 cells/ mL to 500 cells/mL over time35350, and 500 cells/mm(3, based on mounting evidence that early initiation of cART is associated with prolonged life and overall better clinical outcomes[36].

1.5 | Public health approach to cART in resource-poor settings

Providing cART based on individualized patient management is too costly to be applied in resource-poor settings. In the late 1990s and early 2000s, national government initiatives in collaboration with UNAIDS, and programs implemented by non-governmental organizations, clearly demonstrated that programs increasing access to cART in low-and middle-income countries were feasible37. WHO proposed a public-health approach that took into consideration weak health care systems and other

constraints as well as the experiences of up-to-date cART programs[38]. The goal of this approach was to make cART rapidly available to millions of patients in need and to shift from an individual-based approach cART treatment to a population-based approach[34,39].

1.5.1 | Standardized and simplified regimens

The simplification and standardization of first- and second-line cART regimen has been the principal achievement of the WHO public health approach. In resource limited settings, antiretroviral drugs that are readily available belong to the NRTI, NNRTI and PI classes. First-line regimens typically contain one NNRTI backed by an NRTI37. PIs are reserved for second-line therapy, supported by an NRTI using two new drugs to reduce cross-resistance.

1.5.2 | Simplified clinical decision-making and standardized monitoring

To facilitate a rapid expansion of cART and reach a high number of patients in need of treatment in resource-limited countries, the WHO has developed a public health approach to treatment based on a decentralized service delivery as well as standardized and simplified guidelines, taking in account that the standard patient care model in industrialized countries based on specialist physician together with an extensive virological monitoring is not possible in resources-poor settings. To overcome the lack of specialist physicians and the absence of wide-range of virological patient monitoring in these settings, the WHO public health model enables healthcare workers with minimum required training to deliver care to large numbers of patients. Clinical decision-making is directed by clinical observation, WHO clinical staging and, if available, hematology, biochemistry and CD4+ T-cell counts[40].

However, to ensure access to cART in the poorest countries, care and drugs have to be provided free of charge at the health facility. To anticipate a possible HIV drug resistance and sustain the efficiency of cART regimens, WHO recommend that cART program factors, for instance prescribing practices, patient retention, drug supply as well as adherence support, be monitored to optimize the quality of patient care and routinely integrated into the national HIV treatment programmes[41].

Although treatment decisions can be made with clinical information alone, it is better informed with immunological (CD4-cell) monitoring. The WHO has outlined immunological classification of established HIV infection and advocated for much wider access to CD4-monitoring technology and viral load monitoring in specialist centers for management of complex cases, and for virological diagnostic efforts for infants, including using dry blood-spots[40,42].

1.5.3 | Decentralized and integrated delivery of care.

In order to achieve universal access to cART treatment and care in HIV epidemic settings, WHO has proposed to decentralize cART programs to the lowest level of health facilities and developed tools to consolidate the decentralization of cART program within integrated HIV service. Most cART should progressively be delivered at the health district level. This approach has been a useful for country adaptation whereby health service management is performed at the central level as well as

at district level. It also includes the provision of training and job-aids for clinical teams; strong follow-up after training based on clinical and counseling mentors and supportive supervision; materials to support patient education and self-management as well as simple patient-monitoring system. Provider initiated HIV-testing and counseling, prevention for both HIV-uninfected and infected, and prevention of transmission within the health setting are included. These tools support the broad delivery of simplified and standardized services provided through district networks with treatment teams, headed by doctors or medical officers, but largely composed of nurses and clinical officers. People living with HIV and other lay providers are trained to join the clinical team, working with community health workers and community-based organisations[40].

1.5.4 | Prevention of mother to child transmission

Prevention of mother to child transmission (PMTCT) of HIV is an important component of ART programs in resource-limited settings. WHO recommends that all HIV positive pregnant women are eligible for lifelong cART when their CD4 count ≤350 cells/μl and/or have a WHO clinical stage of 3 or 427. Under WHO's 2010 PMTCT ART recommendations, countries had the option to choose between two prophylaxis regimens for pregnant women living with HIV with CD4 greater than 350 cells/mm^3: Option A and Option B. Under Option A, women receive AZT starting as early as 14 weeks of gestation. At onset of labor, a single-dose NVP and a first dose of AZT/3TC antiretroviral prophylaxis is prescribed to the woman and is continued through 7 days postpartum to reduce risk of drug resistance. Infants receive a daily dose of NVP from birth until 1 week after cessation of breastfeeding, or until age 4-6 weeks if not breastfeeding or if the mother is on treatment. Option B, on the other hand, consists of a simpler clinical flow in which all pregnant and lactating women with HIV are initially offered first-line cART as early as the 14th week of gestation during pregnancy and when breastfeeding, combined with 6 weeks of daily NVP for the infant, irrespective of infant feeding method[43]. At the end of breastfeeding, women who do not yet require cART according to the guidelines would discontinue the treatment but continue CD4 count monitoring, eventually re-starting cART when the CD4 count falls below 350cells/mm^3. WHO has approved a new option known as Option B+, in which a triple cART regimen is initiated during pregnancy and continued for life.

1.5.5 | Pediatric cART

As in adults, the cART of choice for HIV-1-infected children is also a combination of two NRTIs and a third agent from a different class, either a NNRTI or a ritonavir-boosted PI[33]. Importantly all antiretroviral drugs used in adults and adolescents are approved for use in children and some are available in pediatric formulations[44]. Although the WHO currently recommends early initiation of cART in childhood[42], the treatment of HIV-infected infants in sub-Saharan Africa[45] lags behind as compared to that of older children and adults mainly due to delays in diagnosis. The situation is further complicated by limited choices of cART, lack of appropriate drug formulations and inadequate levels of trained medical personnel[46] to manage this specific population of patients. These difficulties coupled with the high rates of viral resistance among infants that received NVP during PMTCT hampers the roll-out of cART treatment in children[47,48]

1.6 | Outcomes of cART scale-up in resource-limited settings

1.6.1 | Increased cART coverage

As of June 2013, an expected 9.7 million people in low- and middle-income countries were receiving cART[49]. Furthermore, reports about the efficacy of cART rollout in sub-Saharan Africa are promising. Reductions in new HIV cases, AIDS-related deaths, and overall mortality have been related to expanded access to cART in this region50. Despite these achievements, only approximately one half of the patients eligible for immediate cART by WHO criteria were receiving it by the end of 2012[49]. Children represented only 7% of the people receiving antiretroviral therapy and 12% of the people who needed it. Of the 1 900 000 (1 700 000-2 200 000) children estimated to need cART therapy, only 34% (31-39%) had access to treatment versus 64% of adults (60-69%)[43]. Thus, there is a need to scale up provision of this lifelong treatment both in adults and in children.

Despite the undisputable reduction of mortality and morbidity, increase of cART coverage is also associated by the emergence of HIV drug resistance, with more patients experiencing treatment failure and switching to second-line therapy in settings with limited treatment options[39].

1.6.2 | Clinical improvement

The beneficial effects of cART have been reported among HIV-infected adults and children in both resource-rich and resource-limited settings[51]. Improving access to cART has reduced mortality among patients infected by HIV/AIDS. It is well documented that in low- and middle-income countries, the access to cART has increased from 400 000 patients in 2003 to 6.65 million patients in 2010, which is 47% of patients in need of cART in 2010. Sub-Saharan Africa has experienced 30% fewer deaths in 2010 compared to 2004[52]. However in resource-limited settings, the expected gain of cART on reducing mortality has been compromised by a number of factors such as low CD4 count at cART treatment initiation, lack of cART adherence, lack of nutrition support, low body mass index, age, and limited availability of treatment for severe opportunistic infections (e.g. tuberculosis), as well as cART adherence and nutrition support[53,54]. Additionally, early diagnosis and early initiation of treatment increase the potential for better outcome. To achieve the expected gain, countries need to address factors that may promote better survival such identifying patients earlier when they do not yet have AIDS, providing nutrition support, and promoting adherence to cART. Moreover, mortality following cART initiation could also be a signal of development of viral resistance to cART and poor quality of other health services[54,55].

1.6.3 | Virological suppression

Potent cART is expected to result in undetectable viral load within 12 months after initiation. cART provided according to the WHO public health approach results in virological suppression rates comparable to those observed in resource-rich settings[56]. To prevent and assess HIV drug resistance in resource-limited countries, WHO recommends a virological suppression rate of over 70% at one year after starting antiretroviral therapy[57]. In practice, rates of virological suppression in resource-poor settings after 12 to-24 months of cART vary greatly[58].

Patient-related determinants of virological failure include suboptimal adherence, lower pre-treatment CD4 T-cell count, comorbidities prior to treatment failure, missed clinic appointments, and interruption or intermittent access to cART[59,53]. Regimen-related factors include drug adverse effects, toxicities, suboptimal pharmacokinetics, suboptimal virological potency, prior exposure to suboptimal regimens, food requirements, high pill burden and/or dosing frequency, adverse drug-drug interactions with concomitant medications as well as prescription errors[60]. The consequences of virological failure are deterioration of the clinical and immunological outcomes and the emergence and accumulation of HIVDR mutations which will require a switch to a more expensive second-line cART regimen.

1.6.4 | HIVDR outcomes

The emergence of HIVDR is limited when cART completely supresses viral replication but is a risk in situations promoting virological failure. As the result of the massive cART roll out, emergence of HIV drug resistance following treatment failure poses a major concern for HIV programs in limited resource settings[61]. Studies conducted in these settings have reported cumulative levels of transmitted drug resistance, commonly related to NNRTIs, during treatment scale up[62]. The increase in NNRTI resistance is of specific concern because this drug class constitutes the basis of current first line treatment regimens and prevention of mother to child transmission[42]. HIV transmitted resistance more than doubles the threat of first line failure and causes the accumulation of more drug resistant mutations in the first year of treatment[60]. Recent studies have reported a high prevalence of transmitted HIVDR in various African countries[63,64,65], with a high 9-12% prevalence reported in Uganda, likely due to the fact that cART was introduced ahead of neighboring countries[41].

Potential determinants that may exacerbate the development of HIVDR include the widespread use of low cost, substandard drugs such as single dose of NVP for PMTCT66 as well as inclusion of stavudine in the first line treatment. However, the latter drug has been phased out in the 2010 WHO recommendations[67]. Restricted access to monitoring of HIV viral load plays a critical role in the emergence of HIV drug resistance[68] as well as treatment interruptions when drug supplies run out[69]. Lastly, suboptimal long term adherence and recurrent drug-drug interactions such as those between NVP and rifampicin in patients co-infected with tuberculosis are also reported to be associated with the development of HIV drug resistance[70].

1.7 | Challenges of cART in resource-poor settings

Although many successes have been achieved in HIV care in low- and middle-income countries, such as increased number of HIV-infected patients receiving cART, extensive decentralization, reduction of morbidity and mortality and accessibility to cheapest drugs, existing failures and difficulties should not be hidden by these successes[71].

1.7.1 | Sustainability

International aid to fight HIV in resource-limited settings has decreased since 2010. A few examples: only $1.05 billion per year was available from the Global Fund to fight AIDS, Tuberculosis, and Malaria (GFATM) in 2010 and 2011 with needs estimated at US$ 24 billion. Overall international aid decreased in 2010 compared to 2009 and 2008, whereas it had previously increased every year since 2004 with the U.S. President's Emergency Plan for AIDS Relief (PEPFAR) allocating $48 billion per 5-years ($9.6 billion/year) since 200472,73. With this reduction of international funds to combat these diseases, domestic funding mechanisms are needed in order to continue the fight against HIV74. Despite international financial mobilization, only 65% of patients in limited resource settings in need of cART had initiated cART at the end of 2012 from the global target of 15 million people on cART set for 2015[75]. Increase of cART coverage and treatment of the maximum of eligible patients who have not yet started cART is still a matter of concern, especially in the perspective of the fact that even more patients are eligible for cART according to the latest WHO guidelines[76]. Countries need to increase the few available resources, in order to meet the increasing needs, as commitment of donors to avail new funds is now challenging.

1.7.2 | Sub optimal standards of care

Despite massive roll-out of cART, mortality at 12 months in patients initiating cART in low-income countries is still increasing and ranging between 8% and 26% 77,78. This is much higher than that observed in high-income countries[22]. The most reported causes of death are tuberculosis, bacterial diseases, cryptococcal meningitis, and poor patient adherence, which are mainly preventable[78,79,80]. Due to poor linkage of recently diagnosed patients to HIV care[81] in sub-Saharan Africa, many patients continue to initiate cART at later stages of HIV disease[82] with a negative impact on the clinical outcome. Lastly, managing lifelong cART treatment and care including residual HIV-related morbidity, particularly tuberculosis, bacterial diseases, cardiovascular disease, cancers, and co-infections, particularly hepatitis B virus (HBV) and hepatitis C virus (HCV), constitute key challenges for the health system[83,84].

1.7.3 | Insufficient virological monitoring

Viral load monitoring is usually not available in resource-limited settings due to its high cost and the substantial laboratory infrastructure and logistics which are difficult to implement and maintain[81]. HIV diagnosis of treatment failure still highly depends on immunologic (i.e. CD4 cell counts) and clinical criteria only[43], which have been shown to be poor predictors of virological failure. HIV-1 infected patients with access to viral load monitoring are reported to be more likely to switch to second-line therapy earlier and at higher CD4 cell counts than those enrolled in programs without viral load monitoring[85]. There is an urgent need to scale-up routine viral load testing by mobilization of equipment and reagents at lower prices. Efforts to avail point-of-care instruments which are easy to maintain and affordable are highly needed[36].

1.7.4 | Low retention in care

Lost-to-follow-up rates in cART programs are very high in african settings, with incidence of patient attrition reaching up to 35%[86] after 3 years of follow-up. Thus, reducing lost-to-follow-up rates, managing lifelong treatment and care for long-term morbidity, including drug toxicity, AIDS morbidity, ensure sustainability of cART provision and being able to face unexpected events such as socio-political and military crisis in some areas are crucial[71].

1.7.5 | Prevention of HIV drug resistance

WHO recommends strategies for the prevention and assessment of HIV drug resistance at the population level in resource-limited settings. These strategies are based on standardized routine minimum-resource assessments and surveys to provide information for supporting optimal use of cART, to minimize cART interruptions and to guide population based selection of cART regimen[36]. They include monitoring for early indicators of poor program performance, surveillance of transmitted drug resistance as well as evaluation of drug resistance acquired during therapy cART36. WHO HIV drug resistance strategy is often not integrated in cART program due to the scarcity of funding. An important challenge for sub-Saharan African countries is to durably implement HIV drug resistance prevention and assessment strategies.

1.8 | Interplay between HIV and hepatitis B and C co-infection

HIV, HBV and HCV infections share common transmission routes and are reported to be the most chronic viral infections worldwide. HBV and HCV co-morbidities pose serious clinical and public health challenges[87,88]. Chronic HBV infection affects over 240 million people worldwide and there are an estimated 150 million cases of HCV infection[89,90]. At the global level, Africa carries the largest burden with 350 million of patients infected by HBV, 170 million infected by HCV and 33 million infected by HIV[91].

Despite the effective decline of the mortality and morbidity rate from HIV/AIDS as the result of cART, liver diseases become leading causes of death due to chronic HBV and HCV infections. Although the direct impact of HIV infection on hepatitis disease progression remains controversial in many studies[92,93,94], interactions between HIV infection and HBV/HCV disease progression are more and more evident[95]. HBV infection is preventable by vaccination and treatable in adults using either TDF, 3TC and FTC. This infection can also be prevented in children from mothers with HBV infection by giving HBV immunoglobulin (HBIG) within 12 hours after birth[96]. No vaccine is currently available to prevent HCV and treatment options are very limited in resource-constraint settings. New highly efficacious drugs are however becoming available in resource-rich settings[97,98].

Although HBV and HCV infections are preventable and to some extent treatable[87], most low and middle income countries do not integrate the management and monitoring of these infections in national cART programs[87]. This is due to the limited access to diagnostic testing, the widespread absence of virological monitoring by HBV and HCV molecular testing, the little understanding of

natural history of co-infections, the limited availability to assess liver disease progression (fibrosis) in most poor settings, as well as the lack of routine screening of pregnant women for HBV and low coverage of universal infant vaccination[99]. In these contexts, HBV and HCV infection remain typically undiagnosed and co-infected individuals only receive cART[87].

1.9 | Overview of the HIV epidemics and ART program in Rwanda

The most recent demographic health survey (DHS) conducted in Rwanda in 2010 reported an HIV prevalence of 3.0% in the population aged between 15 and 49 years. The most affected groups were women aged between 35 and 39 years (7.9%) and men between 40 and 44 years (7.3%). The prevalence varied widely between urban and rural areas, with a prevalence of 7.6% (8.7% in women and 5.4% in men) in urban areas and 2.3% (2.8% in women and 1.6% in men) in rural areas[100]. The key populations at higher risk of HIV in Rwanda include female sex workers, men who have sex with men (MSM), truck drivers and prison inmates. Data from the DHS indicate that in female sex workers, the prevalence of HIV is very high (50.8%), ranging from 57.7% in women 25 years of age and older to 42.1% in women under 25 years of age[100]. Due to lack of funds, data on MSM and prison inmates are not available.

1.9.1 | cART program in Rwanda

Since 2004, a nation-wide care and treatment program has been implemented for HIV-infected individuals. In Rwanda, patients receive their antiretroviral medication free of charge[101,102]. The Rwandan national cART guidelines follow the WHO clinical and immunological eligibility criteria for antiretroviral therapy initiation in adults and adolescents. By the end of 2010, Rwanda was ranked as one of only 3 low- and middle-income countries with generalized HIV epidemics that had achieved and surpassed the WHO's definition of universal cART access with 88% of those in need receiving treatment[36,100]. These accomplishments were made through a multi-pronged national strategy based on decentralization of services, involvement of peer educators and task shifting to nurse-provided care[101,36], as well as collaboration with partner organizations[103]. Despite the high cART coverage achieved in adults, much still needs to be done in the PMTCT program and access of children to cART. Data have reported that only 36.4% of all pregnant HIV-positive women received cART, while only 35% infants and children less than 15 years old initiated this treatment by the end of 2011[104].

1.9.2 | Specific Challenges of the ART programme in Rwanda

Despite the reported high rate coverage of cART treatment program, Rwanda like other Sub Saharan Africa countries is dependent on international funding assistance[71]. According to the Rwandan 2013-2018 HIV national strategic plan[105], external international donors cover up to 90% of the total budget that is needed each year for HIV care and treatment. This includes PEPFAR and GFATM[106,104]. However as international aid is now decreasing, the government of Rwanda is currently struggling to fill the gaps and to meet the increasing needs[105].

Based on recent WHO recommendations and scientific evidence, the country has embarked on the process of decentralization of CD4 count and viral load testing from the national reference laboratory to six referral hospitals using the Facscount instruments for CD4 (Becton Dickinson) count testing and Ampliprep Cobas TaqMan (Roche) 96 platform for viral load testing. However, recurrent stock-out of reagents and consumables as well as lack of proper maintenance of these fully automated monitoring systems make decentralization very challenging. Furthermore, while staff trained in HIV drug resistance testing are available at the National Reference Laboratory, the high cost of this test does not allow the availability of sufficient reagents so satisfy the high demand from all patients in need[105].

Frequent stock-outs of drugs, lack of adequate numbers of skilled staff to ensure proper task shifting, and weak procurement systems are potential threats to the success of the cART program[101]. The national HIV program follows the WHO recommendations to monitor occurrence of HIV drug resistance. Only one component of the WHO strategy is currently being applied in Rwanda: the monitoring of HIV drug resistance early warning indicators (EWIs). EWI comprise the proportion of health facilities who meet the WHO target about drug prescription at the initiation of cART in all health facilities during the last 12 months, the number of health facilities with patients loss to follow up at 12 months of cART initiation, the percentage of patients who are taking an appropriate first-line cART regimen 12 months later per health facility, the evaluation of the percentage of patients picking up prescribed cART drugs on time during their first 12 months of cART, as well as the percentage of months in a designated year in which there were no cART drug stock-outs[107].

The latest data indicate that despite efforts deployed to implement this strategy, staff shortage and sometimes lack of motivation do not allow for a proper reporting system of EWIs. To overcome these obstacles and ensure regular national surveillance system, the Ministry of Health is struggling with the training of more staff and increasing salaries in the health sector[108].

1.10 | Justification of the study

Since the roll-out of cART in Rwanda, few data have been generated on outcomes and outcome predictors of ART in adults and children in the country. This information is crucial to inform the choice of empiric first- and second-line cART regimen and provide data on virological, patient and programmatic factors associated with unfavorable ART outcomes. In addition to that, the extent of chronic hepatitis virus infections and their impact on the cART outcomes are not known in Rwanda, although the country belongs to a region with expected high prevalence.

This work intends to update Rwandan clinicians and policy makers on the outcomes of cART in the country, the trend of HIV drug resistance as well as the burden of the HIV and hepatitis among HIV patients. Options for better management of HIV and hepatitis co-infections as well as ways forward to improve the performance of the ART programme in Rwanda are discussed.

1.10.1 | Outline of the thesis

Clinical, immunological, virological and HIV drug resistance outcomes are fundamental elements of monitoring the success of the therapeutic goals of ART and inform on the performance of the ART program. **Chapter 2** describes these outcomes in adult patients with HIV-1 infection who were followed up for a period of 12 months after initiation of antiretroviral therapy in Kigali, Rwanda. Factors associated with the emergence of virological failure and HIV-1 drug resistance were examined.

Following the widespread use of antiretroviral drugs in developing countries, the risk of acquiring HIV-1 drug resistance during HIV became a public health problem in pediatric populations as well. **Chapter 3** addresses long term outcomes of cART therapy and prevalence of genotypic drug resistance in children and adolescents on combination antiretroviral therapy over 24 month of follow up at Kigali University Teaching Hospital.

Information on baseline HIVDR, HIV-1 subtype distribution, and transmission networks in Rwanda is scarce. **Chapter 4** discusses molecular characterization and phylogeographic analysis of HIV-1 strains isolated in chronically and recently infected individuals in Kigali. These data have implications for prevention strategies as well as for success of first-line treatment.

Information regarding the prevalence and predictors of viral hepatitis co-infections among HIV-infected individuals presenting at the health facilities in Rwanda is limited. Persons at high risk for HIV infection are also likely to be at risk for other infectious pathogens, including HBV and HCV. These viruses may complicate the delivery of cART by increasing the risk of drug-related hepatoxicity and impacting the selection of specific agents. **Chapter 5** assesses the prevalence, incidence and determinants of markers of HBV and HCV infection in adults starting on ART. The study also determines the effect of HBV and HCV infection on HIV disease progression, and discusses the implications associated with ART regimen received by the patients. **Chapter 6** addresses the added value of HBV vaccine in HIV-1 infected children and explores reliable ways of hepatitis B infection prevention in this population.

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CHAPTER 2

Low primary and secondary HIV drug-resistance after 12 months of antiretroviral therapy in human immune-deficiency virus type 1 (HIV-1)-infected individuals from Kigali, Rwanda

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Abstract

Treatment outcomes of HIV patients receiving antiretroviral therapy (ART) in Rwanda are scarcely documented.

HIV viral load (VL) and HIV drug-resistance (HIVDR) outcomes at month 12 were determined in a prospective cohort study of antiretroviral–naïve HIV patients initiating first-line therapy in Kigali. Treatment response was monitored clinically and by regular CD4 counts and targeted HIV viral load (VL) to confirm drug failure. VL measurements and HIVDR genotyping were performed retrospectively on baseline and month 12 samples.

One hundred and fifty-eight participants who completed their month 12 follow-up visit had VL data available at month 12. Most of them (88%) were virologically suppressed (VL ≤1000 copies/mL) but 18 had virological failure (11%), which is in the range of WHO-suggested targets for HIVDR prevention. If only CD4 criteria had been used to classify treatment response, 26% of the participants would have been misclassified as treatment failure. Pre-therapy HIVDR was documented in 4 of 109 participants (3.6%) with an HIVDR genotyping results at baseline. Eight of 12 participants (66.7%) with virological failure and HIVDR genotyping results at month 12 were found to harbor mutation(s), mostly NNRTI resistance mutations, whereas 4 patients had no HIVDR mutations. Almost half (44%) of the participants initiated ART at CD4 count ≤200cell/µl and severe CD4 depletion at baseline (<50 cells/µl) was associated with virological treatment failure ($p = 0.008$).

Although the findings may not be generalizable to all HIV patients in Rwanda, our data suggest that first-line ART regimen changes are currently not warranted. However, the accumulation of acquired HIVDR mutations in some participants underscores the need to reinforce HIVDR prevention strategies, such as increasing the availability and appropriate use of VL testing to monitor ART response, ensuring high quality adherence counseling, and promoting earlier identification of HIV patients and enrollment into HIV care and treatment programs.

Introduction

Improved access to combined antiretroviral therapy (ART) has significantly reduced HIV-related morbidity and mortality worldwide[1]. To date, HIV treatment and care programs in sub-Saharan Africa have implemented a public health approach[2] with good access to a limited number of first and second-line ART regimens and CD4 count monitoring, but little attention paid to HIV viral load monitoring and the detection of HIV drug resistance (HIVDR). In 2010, more than five million HIV-infected Africans were estimated to receive life-saving ART, with Rwanda reporting treatment coverage of 80%[3].

However, ART scale up in resource-poor settings could accelerate HIVDR emergence[4,5,6,7] due to insufficient viral load (VL) monitoring[8], inconsistent drug supply[9], and possible unregulated use of antiretroviral drugs (ARV)[10].

HIVDR can develop because of the error prone nature of HIV replication resulting in a high mutation rate in combination with the ongoing presence of drug-selective pressures. HIVDR strains that emerge after treatment initiation (referred to as acquired or secondary HIVDR) can subsequently be transmitted to previously uninfected patients (referred to as transmitted or primary HIVDR)[11,12]. Transmitted HIVDR increases the risk of virological therapy failure[13] and compromises the efficacy of first-line ART regimens. This is important in a context of limited treatment options.

HIVDR increases direct medical and laboratory costs associated with treatment failure as well as indirect health care costs associated with having to switch patients to more expensive second-line therapy and the ongoing need to develop new drugs. Therefore, HIVDR prevention should be an important public health goal in countries with limited resources.

Recent reports indicate that the lack of VL monitoring during ART leads to late detection of virological failure. Long-term exposure to failing regimens facilitates the emergence and accumulation of acquired HIVDR mutations[8,14,15]. The East African region has a high prevalence transmitted HIVDR[16,17] and prevalence is highest in countries of early ART roll-out and high ART coverage[8,14,18]. Collectively, these observations underscore the need to monitor HIVDR in countries that are scaling up ART in order to remedy programmatic deficiencies in a timely fashion and protect the efficacy of first and second-line ART regimens. In Rwanda and most other resources-constrained countries, routine VL and HIVDR testing is not available to support routine HIV clinical care due to high costs. Moreover, many patients are diagnosed late and commence ART at lower CD4 counts[19], which increases risk of treatment failure[20,21].

Since Rwanda initiated ART roll-out in 2004, few data have been generated on treatment outcomes in general, and prevalence and incidence of transmitted and acquired HIVDR in particular. Here, we describe transmitted HIVDR at baseline, and treatment outcomes and acquired HIVDR one year after ART initiation in a cohort of Rwandan HIV patients. Treatment outcomes were specifically

examined as a function of immunological status at ART initiation, and other potential determinants of virological failure after 12 months of treatment were also examined.

Materials and Methods

Ethics statement

Ethical clearance was obtained from the Rwandan National Ethics Committee. All study participants provided written informed consent prior to study enrolment. Participants had the right to withdraw from the study at any time.

Study design

The present study was part of a larger prospective investigation addressing the secondary effects of HIV treatment and its impact on reproductive health in a cohort of patients initiating ART (Side effect and Reproductive Health in a Cohort on HAART: SEARCH). The study was conducted at the Treatment and Research AIDS Center (TRAC-Plus) outpatient clinic. Patients were enrolled between November 2007 and January 2010, and were followed up for a maximum of 24 months per person. In this report, only data up to month 12 of follow-up and collected before the 15th of September 2010 were analyzed.

Medical visits were scheduled at baseline (ART initiation), week 2, month 1, month 3 and every three months thereafter until month 12. Information on demographics, sexual behavior, clinical history and physical examination was collected at these visits. Pharmacy visits were scheduled at baseline, every week during the first four weeks of ART (week 1 to 4), and every month thereafter (month 2 to 12). During these visits, medications were delivered and treatment adherence was monitored. Laboratory tests (CD4 count, HIV VL and HIVDR genotyping) were scheduled at baseline, month 6 (CD4 count only) and month 12. Study staff minimized loss-to-follow-up by actively contacting participants who had missed a scheduled clinic visit and by providing travel reimbursements to participants.

Study participants

HIV-positive patients attending the TRAC-Plus clinic, who were ARV-naive and immediately eligible for ART according to the Rwandan national guidelines[22], were asked to participate in the study. Other inclusion criteria were being 18 years of age or older, residing and planning to reside within travel distance from the TRAC-Plus clinic for the duration of follow-up, willing and able to adhere to the study protocol, and willing and able to give written informed consent for study enrollment. The main exclusion criteria were being pregnant and laboratory or clinical diagnosis or suspicion of tuberculosis. Previous use of ARVs in the context of prevention of mother-to-child transmission was not an exclusion criterion.

HIV treatment

ART was provided through the national HIV treatment program. Participants received a first-line regimen in accordance with the 2007 Rwanda national ART treatment guidelines, which were in line with WHO recommendations at that time[22,23]. First-line regimens included a combination of either two nucleoside-analogue reverse transcriptase inhibitors (NRTI) and one non-nucleoside reverse transcriptase inhibitor (NNRTI) or a combination of three NRTIs. Response to ART was routinely monitored on the basis of clinical symptoms and CD4 count as recommended by the national guidelines. HIV VL and HIVDR genotyping tests foreseen in the study design were not performed in real time and hence could not be used for the clinical management of study participants. However, the study clinicians could request additional VL and/or CD4 count testing if they suspected clinical or immunological failure to support the decision to switch to a second-line regimen. Results of retrospective VL and HIVDR genotypes were available after study completion and were reported to the study clinicians.

Monitoring of drug adherence

ART adherence was captured in three different ways. First, a standardized questionnaire including questions on frequency of dosing, missed doses, drug sharing and reasons for poor adherence was administered by the study nurse at month 1 and at every three-month visit thereafter. Second, pill counts were recorded at each monthly pharmacy visit. Third, the attendance rates of medical and pharmacy appointments (with a 7 day window) were recorded. Participants were classified as fully adherent if they took more than 95% of their prescribed ART regimen doses and as poorly adherent otherwise.

Blood sample collection and storage

Blood samples for laboratory testing were collected at baseline, month 6 (CD4 count only) and month 12. Five milliliters (mL) of whole blood was collected in EDTA vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ). Fifty microliters (µl) of fresh blood was used for CD4 cell enumeration. Plasma was separated from the cellular fraction by centrifugation and collected into three aliquots of one mL each, within four hours after blood collection. Plasma samples were stored at -80°C until further analysis.

Laboratory investigations

CD4 count

CD4 count was determined at baseline, month 6 and month 12 in whole blood on a single flow-cytometry platform using TruCOUNT® tubes on a FACScalibur instrument (Becton Dickinson, San Jose, CA, USA) and according to the manufacturer's instructions.

Immunological failure was defined as failure to achieve a CD4 gain of at least 50 cells above pre-therapy levels or having an absolute CD4 count of less than 100 cells/µl after one year of therapy[23].

HIV-1 viral load

HIV RNA VL was measured at baseline and month 12. Viral RNA quantification was performed on thawed plasma using the Roche CobasAmpliPrep/CobasTaqMan HIV-1 version 2 (Roche Molecular Systems, France) and according to the manufacturer's instructions. The lower limit of detection was 40 copies of HIV RNA/mL. Virological treatment failure was defined as a confirmed VL >1000 copies/mL at month 12.

HIV-1 DR genotyping

HIVDR genotyping was done retrospectively on samples from participants that had completed their month 12 visits and had available VL data at baseline and month 12. Baseline and month 12 samples with a VL >1000 RNA copies/mL were analyzed at the Department of Medical Microbiology (Academic Medical Centre, Amsterdam) using the Viroseq HIV genotyping kit version 2 (Abbot Molecular Inc, IL, USA) on an automated sequencer ABI 3130 XL (Applied Biosystems, Carlsbad, CA, USA). Sequences of amplified viral genes coding for the HIV-1 protease and reverse transcriptase enzymes were assembled, edited using the software provided by Viroseq, and submitted to the Stanford University database. Baseline sequences were categorized according to the WHO list of mutations for surveillance of transmitted drug resistant HIV strains (2009 update)[24]. Month 12 sequences were categorized according to the International AIDS Society-USA drug resistance mutations group (December 2010 list)[25]. HIV-1 subtypes were determined using the REGA HIV-1 Subtyping Tool (Version 2.0) available from the Los Alamos database [26] on the same gene sequences. The sequences generated in this study are available in the GenBank repository with accession numbers KC841660-KC841778.

Statistical methods

Analyses were conducted with STATA version 11 (STATA Corporation, College Station, TX).

Baseline characteristics were reported as percentages for categorical data and means with standard deviations (SD) for continuous data. Differences between the groups were tested using the Pearson Chi-square test, the student's *t*-test and the Fisher's exact test, as appropriate. Stem and leaf plots and Shapiro-Wilks test were used to investigate the normality of data distribution. If variables deviated from the normal distribution, medians and interquartile ranges (IQRs) and non-parametric tests were used.

Potential risk factors for virological treatment failure at month 12 were examined by bivariable and multivariable logistic regression analysis. Factors were included in the multivariable analysis when p-values <0.2 in bivariable analysis[27]. Factors known to be associated with virological failure from the literature, such as age, baseline HIV viral load and treatment adherence were included in the multivariable model regardless of the strength of their association with VL in bivariable analysis[28]. Co-linearity was checked by performing a linear regression analysis instead of the logistic regression analysis to calculate the variance inflation factors, which were all below 2. Missing values were excluded from all analyses. The level of significance was set at $p<0.05$.

Results

Study profile

Two hundred and eighteen HIV-1 positive participants (52.3% women) visited the TRAC-Plus clinic, were eligible for ART and consented to participate in the study (Figure 1). Of these, 5 participants were erroneously enrolled and were excluded from the analysis. Two hundred and thirteen participants were prospectively followed. One patient withdrew his consent. Three of 213 participants (1.4%) died during follow-up: two were reported to have committed suicide (at months 1 and 8) and the third participant discontinued ART after eight months due to social reasons and died from tuberculosis at month 12. Six of 213 participants were lost to follow-up (3%) while 40 active participants in the cohort had not reached 12 months of follow-up at study closure in September 2010. In total, 203 participants of the initial cohort of 213 (95.3%) had been retained in care at the end of the study period, of whom 163 had reached 12 months of follow-up. One hundred and fifty-eight participants had VL results available at month 12 and could be classified into 140 virological treatment successes and 18 treatment failures.

Baseline characteristics of the study participants

At ART initiation, the 158 participants with 12 months of follow-up who were included in this analysis did not significantly differ from the 213 participants who enrolled in the SEARCH study for any of the baseline parameters collected (data not shown). The mean age was 37.9 years (SD = 7.6) and 55.1% were females (Table 1). Of the 158 participants included in the analysis, 52.0% were married 43.0% did not know their sexual partner's HIV status. Only 14.4% of the patients with a known HIV-positive partner were aware of their partner taking ART. Patients with and without virological failure at month 12 were comparable for all socio-demographic parameters (Table 1).

All participants were prescribed an appropriate ART regimen as per the 2007 Rwanda ART National guidelines. A combination of zidovudine (AZT), lamivudine (3TC), and nevirapine or efavirenz (NVP/EFV) was the most commonly (89%) prescribed first-line regimen. The other first-line regimens contained tenofovir (TDF; 4%) or stavudine (d4T; 7%) instead of zidovudine. Women with a previous history of PMTCT all received NNRTI-based regimen. Participants with virological failure at month 12 were more likely to have initiated ART at a more advanced WHO stage 3 or 4 (38.9%) than participants with virological suppression at month 12 (13.4%; $p = 0.01$). Participants with virological treatment failure also had lower baseline CD4 count (median = 129 versus 219 cells/μL; $p = 0.04$) and a higher proportion of baseline HIVDR mutations (1.1% versus 18.8%; $p = 0.001$) than participants with virological suppression.

Characteristics of participants with HIVDR mutations at baseline

HIVDR genotyping was performed in pre-ART plasma specimens from 109 participants and HIVDR mutations were identified in 4 of them (3.6%; 3 women and 1 man). NNRTI HIVDR mutations were detected in all 4 participants. The K103N mutation was the single NNRTI mutation detected in 3 of 4 patients, with one of them also harboring the M184V NRTI mutation (Table 2). One patient

had a combination of A98G, Y181C and G190A mutations. None of the women with baseline HIVDR mutation(s) had a history of ART in the context of PMTCT. The 4 participants with HIVDR mutations at ART initiation received ART regimens that were only partially active. During the 12 months of follow-up, none of these patients was switched to a fully active first-line or second-line regimen.

The HIV-1 subtype distribution in the SEARCH cohort has been described elsewhere [30]. Briefly, the most predominant HIV subtype among the 109 participants was HIV subtype A1 (70.9%), followed by recombinant A1/C (19.8%), subtype C (5.3%) and subtype D (3.0%).

Among the 4 participants with baseline HIVDR, the observed proportions of subtype C (2/4), A1 (1/4) and A1/C (1/4) were significantly different than those observed in the 105 participants with no HIVDR mutation at baseline (Fisher's exact test p = 0.036).

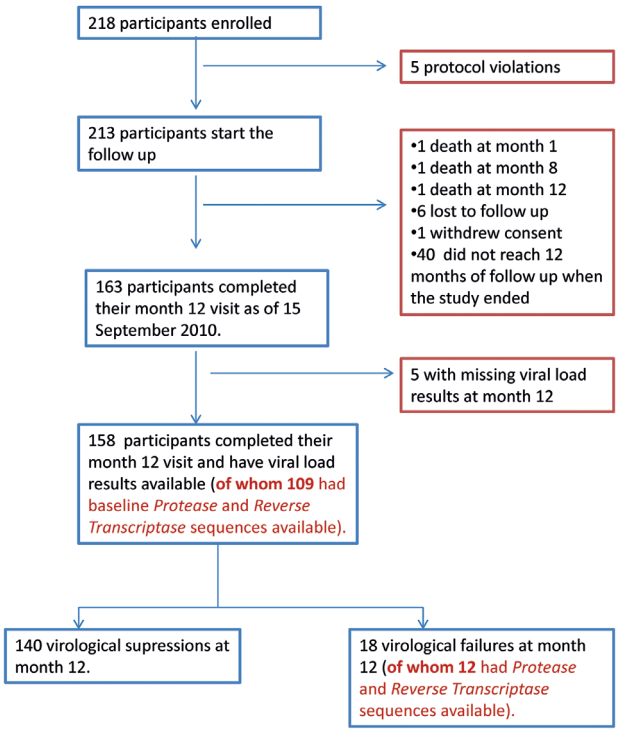

Figure 1 | Study profile. Of the 218 were enrolled in the study, 213 started the 12 month follow-up. One hundred and fifty-eight participants completed their month 12 visit and had viral load results available at baseline and month 12. Of these 140 could be classified as virological successes (VL ≤1000 copies/mL) and 18 as virological failures (VL >1000copies/mL)

Table 1 | Baseline characteristics of participants that received 12 months of ART.

Characteristics	Viral load testing at month 12 N = 158 n (%)	Virological Suppression N = 140 n (%)	Virological failure N = 18 n (%)	p-value
Age in years (mean, sd)	37.9(7.6)	38.3(7.4)	35.1(8.9)	0.09
Gender: Female	87(55.1)	77(55.0)	10(55.7)	0.96
Education level:				
None	12(7.8)	11(8.1)	1(5.6)	0.72
Primary	66(42.9)	58(42.6)	8(44.4)	
Secondary	68(44.2)	59(43.4)	9(50.0)	
Post-secondary	8(5.2)	8(5.9)	0(0)	
Marital status:				
Never married	15(9.9)	15(11.0)	0(0)	0.27
Married	79(52.0)	72(52.9)	7(43.8)	
Divorced	37(24.3)	32(23.5)	5(31.2)	
Widowed	21(13.8)	17(12.5)	4(25.0)	
≥2 sex partners in the last year	10(6.8)1	10(7.7)2	0(0)3	0.24
Alcohol use*	68(43.9)	57(41.6)	11(61.1)	0.12
Condom use during the last sex act	68(44.4)	63(46.3)	5(29.4)	0.20
Age at sexual debut (median years, range)	19(6-31)	20(6-31)	18(12-21)	0.06
Partner HIV status:				
Negative	20(12.8)	19(13.8)	1(5.6)	0.42
Positive	69(44.2)	62(44.9)	7(38.9)	
Unknown	67(43.0)	57(41.3)	10(55.6)	
Partner on ART	29(19.6)4	26(20.0)5	3(16.7)6	0.59
Received ART for PMTCT among females	14(17.5)7	14(19.4)8	0(0)9	0.32

Characteristics	Viral load testing at month 12 N = 158 n (%)	Virological Suppression N = 140 n (%)	Virological failure N = 18 n (%)	p-value
WHO stage: Stage 1	93(60.0)	87(63.5)	6(33.3)	0.01
Stage 2	37(23.9)	32(23.4)	5(27.8)	
Stage 3	21(13.6)	16(11.7)	5(27.8)	
Stage 4	4(2.6)	2(1.4)	2(11.1)	
Median CD4+ T cell count (cells/µl, IQR)	215(129-278)	219(139-272)	129(48-282)	0.04
Median HIV-1 viral load (Log10 RNA copies/mL, IQR)	4.8(4.2-5.2)	4.8(4.2-5.2)	4.9(4.3-5.4)	0.32
ART regimen:				0.22
AZT+3TC+NVP/EFV	140(89.0)	126(90.0)	14(77.7)	
d4T+3TC+NVP/EFV	11(7.0)	8(5.7)	3(16.7)	
TDF+3TC+NVP/EFV	7(4.0)	6(4.3)	1(5.6)	
Baseline HIVDR mutations	4(3.6)10	1(1.1)11	3(18.8)12	<0.001

[1] n = 147, [2] n = 130, [3] n = 17, [4] n = 148 , [5] n = 130, [6] n = 18, [7] n = 81, [8] n = 72, [9] n = 9, [10] n = 109, [11] n = 91 [12] n = 16
Statistical differences between virological treatment failures (n = 18) and virological treatment success after 12 months of ART were determined by student's t test for continuous normally distributed data, Wilcoxson rank sum test for non-parametric continuous data and chi-square and fisher's exact where appropriate for categorical data.
*drinking any quantity of alcohol at least 3 days a week, every week in the last 6 months.

Table 2 | Baseline HIVDR mutations.

Participants Codes	ART regimen at baseline^	Viral load at baseline (RNA copies/mL)	CD4 count (cells/µL)	Major mutations			HIV subtypes	Virological outcome at month 12
				NRTI*	NNRTI **	PI		
1¥	AZT/3TC/**NVP**	68500	112	None	K103N	None	C	Viral suppression
2	AZT/3TC/**NVP**	8300	416	None	K103N	None	C	Viral failure
3	AZT/**3TC**/**NVP**	141000	60	M184V	K103N	None	A1/C	Viral failure
4	AZT/3TC/**EFV**	68500	48	None	A98A, Y181C, G190A	None	A1	Viral failure

^ None of the participants with drug resistance at baseline were switched to alternative first-line or second-line treatment during the 12 month follow-up. The treatment shown was initiated after the baseline HIVDR genotyping.
¥ participant 1 showed baseline mutations, but was virologically suppressed at 12 months (see table 4);
* M184V/I cause high-level in vitro resistance to 3TC
** K103N and K103KN, A98AG and Y181CY cause high-level resistance to NVP and EFV. G190A causes high level resistance to NVP and intermediate resistance to EFV
The drugs in bold and underlined have a reduced sensitivity against the mutated viruses
Viral suppression is defined by VL ≥1000copies/mL

Month 12 treatment outcomes

Treatment substitutions and switches. Forty-one substitutions within the first-line, and two switches from first- to second-line regimens, occurred during the study period (Table 3). The most frequently reported reasons for single drug substitutions within the first-line were: compliance with the change of the Rwandan ART guidelines recommending a TDF backbone instead of an AZT or d4T-based backbone, hepatotoxicity, and compensation for stock-outs of specific drugs. The two participants that were switched from a first- to a second-line regimen presented with virological failure at month 12 (Table 4). Participant 7 had an initial drug substitution within the first-line due to alleged hepatotoxicity at month 3. EFV was replaced by abacavir (ABC) using an NRTI backbone of TDF and 3TC. At month 7, this same patient was switched to a second-line regimen containing TDF, 3TC, lopinavir/ritonavir (LPV/r) based on failure to reach a CD4 count >100cells/µl (from 9 cells/µl at baseline to 11 cells/µl at month 6). Participant 15 was switched from d4T/3TC/NVP to a second-line regimen containing TDF/3TC/LPN/r at month 4 based on worsening of his clinical condition and the recurrence of an oral Kaposi sarcoma. All the other participants remained on the same first-line regimen up to their month 12 visit.

Virological outcomes and related immunological criteria. The proportion of patients with virological failure was higher, although not significantly so, in the group of participants that initiated ART at CD4 count ≤200 cell/µl as compared to the group that initiated therapy at CD4 count >200 cells/µl (12/70 versus 6/81 participants; p = 0.07, Table 3). None of the women that had received NVP or AZT for PMTCT experienced virological treatment failure at month 12. Only nine of the 18 participants with virological failure (50%) also experienced immunological failure at month 12 and would have been correctly identified as failing their treatment (sensitivity = 50%). Conversely, 32 of 41 participants

with virological suppression (23.8%) would have been misclassified as experiencing treatment failure based on immunological criteria at month 12 (specificity = 77%). Although the difference was not significant, acquired HIVDR mutations at month 12 were more frequent in the group of participants with baseline CD4 count ≤200 cells/µl, as compared to participants with baseline CD4 count >200 cells/µl (6/70 versus 2/81; p<0.09, Table 3).

Table 3 | Treatment outcomes during 12 months of follow-up.

Outcomes	Month 12 (n =158) n, (%)	Baseline CD4 count ≤200 (n = 70) n, (%)	Baseline CD4 count >200 (n = 81) n, (%)	p-value
Treatment switches and substitutions				
Switches to second-line treatment				
TDF+3TC+LPV/R	2(1.3)	2(2.9)	0	-
Substitution within first-line treatment				
AZT/d4T to TDF	26(63.4)	14(63.6)	9(56.3)	0.38
AZT to d4T	1(2.4)	0	1(6.3)	
NVP to EFV	10(24.4)	7(31.8)	3(18.8)	
EFV to NVP	1(2.4)	0	1(6.3)	
NVP/EFV to ABC	3(7.3)	1(4.5)	2(12.5)	
Virological outcomes				
Virological suppressed (VL <1000 copies/mL, %)	140 (88.6)	58(82.9)	75(92.6)	0.07
Virological treatment failure (VL ≥1000copies/mL, %)	18 (11.4)	12(17.1)	6(7.4)	
HIVDR mutation at month 12	8^^(5.3)	6(9.0)	2±(11.1)	0.09
Immunological criteria for treatment failure				
Immunological failure among virologically suppressed	32(23.8)	12(20.7)	20(27.0)	0.40
Immunological failure among virologically failed	9/18(50)	5(41.7)	4(66.7)	0.32

^^ n = 152: 140 treatment successes + 12 treatment failures with a HIVDR genotype available
± n = 12 treatment failures with a HIVDR genotype available.

Characteristics of participants with virological failure at month 12

Samples from 12 of the 18 participants with HIV VL >1000 copies/mL at month 12 were successfully genotyped. Eight participants harbored major mutations while no evidence of acquired HIVDR was found in 4 participants (Table 4). HIVDR genotyping could not be performed for 6 participants due to technical difficulties (one participant) or insufficient plasma volume (5 participants; Table 4). Three participants with HIVDR identified at month 12 had pre-existing HIVDR mutation(s) at baseline (Tables 3 and 4), and 2 of them (participants 2 and 4) had acquired additional mutations during follow-up (Tables 2 and 4).

Table 4 | Characteristics of participants with virological failure at month 12.

Participant codes	Viral load (RNA copies/mL)	CD4 (cells/µL)	Immunological Failure	HIV-1 subtype	Major gene mutations			ART regimen	Switched To 2nd line
					NRTI	NNRTI	PI		
			(yes/no)						
2*	9390	386	Yes	C	M184V	K103N	None	AZT/**3TC/NVP**	No
3*	7940	105	Yes	A1/C	M184V	K103N	None	AZT/**3TC/NVP**	No
4*	8100	71	Yes	A1	D67N, K70R M184V, K219Q	A98G, K101Q G190A	None	AZT/**3TC/NVP**	No
5	5480	306	No	A1	M184V	K103N	None	AZT/**3TC/NVP**	No
6	29800	159	Yes	A1	M184V	K103N, V108I G190A	None	AZT/**3TC/NVP**	No
7**	8020	70	Yes	A1	D67N, K70R M184V	Y181I	None	TDF/**3TC**/LPV/r	Yes: month 7
8	7920	137	Yes	A1	None	K103N, Y181C G190A	None	AZT/3TC/**NVP**	No
9	9260	211	No	A1/C	None	K103N, V106M	None	AZT/3TC/**NVP**	No
10	82000	246	Yes	A1	None	None	None	AZT/3TC/EFV	No
11	2380	194	No	A1	None	None	None	TDF/3TC/NVP	No
12	346000	190	Yes	A1	None	None	None	TDF/3TC/EFV	No
13	86300	210	Yes	A1	None	None	None	TDF/3TC/NVP	No
14	7850	242	Yes	A1	NA	NA	NA	TDF/3TC/NVP	No
15	808000	192	No	A1	NA	NA	NA	TDF/3TC/LPV/r	Yes: month 4
16	2500	181	No	D	NA	NA	NA	TDF/3TC/NVP	No
17	63500	449	No	NA	NA	NA	NA	AZT/3TC/NVP	No
18	113000	115	No	D	NA	NA	NA	AZT/3TC/NVP	No
19	1700	552	No	A1	NA	NA	NA	TDF/3TC/NVP	No

A98G reduces NVP susceptibility, Y181C causes high-level resistance to NVP, G190A causes high level resistance to NVP and intermediate resistance to EFV, M184V cause high-level in vitro resistance to 3TC, K103N causes high-level resistance to NVP, and EFV, D67N, K70R and K219Q cause resistance to AZT and d4T

* These participants showed mutations at baseline (see Table 2); ** Participant sample failed sequencing at baseline; NA- Not applicable since these samples failed to sequence at both baseline and month 12. The drugs in bold and underlined have a reduced sensitivity against the mutated viruses.

All cases of acquired HIVDR involved at last one NNRTI mutation, with the K103N mutation being the most frequently observed (6/8), followed by the G190A mutation (3/8) and the Y181I mutation (2/8). The K101Q, V108I and V106M mutations were each observed once as a single mutation (Table 4). Combined NNRTI and NRTI mutations were seen in 6 of 8 participants and involved M184V alone in 4 cases or M184V in combination with thymidine analogue mutations (TAMs) in 2 cases (Table 4). The 2 TAMs cases were of pathway 2 and included the D67N, K70R and K219Q mutations for participant 4 and the D67N, K70R mutations for participant 7, respectively. Among the participants with virological failure at month 12 and a known HIV subtype, the proportions of subtypes A1 (12/17), C(1/18), D (2/17) and recombinant A1/C (2/17) was significantly different than the subtype distribution among patients with treatment success (Fisher's exact test p = 0.026).

Patients with virological failure at month 12 were not less adherent than the rest of the group regardless of the method used to measure adherence. Levels of antiretroviral treatment adherence in this cohort have been analyzed elsewhere[29].

Factors associated with virological failure at month 12

Bivariable analyses indicated that treatment adherence by pill count (OR 2.25, 95% CI 0.81−0.62) and baseline viral load (OR 1.32, 95% CI 0.74−2.36) were not significant risk factors for virological failure at 12 months. HIV subtype was not a significant risk factor for virological failure at month 12 either when comparing subtype C to subtype A1(OR 3.65 (95% CI 0.34−39.09) and when comparing recombinant subtype A1/C to subtype A1 (OR 3.65 (95% CI 0.34−39.09).

In the multivariable model, participants with advanced HIV disease defined by WHO HIV clinical stage 3 and 4 were more than 5 times more likely to have virological failure compared to those with WHO HIV clinical stage 1 (OR 6.31: 95% CI 1.43−27.83, p = 0.02). In addition, severe immunosuppression at ART initiation (CD4 count <50 cells/µl) was significantly associated with virological failure at month 12 (OR 10.99: 95% CI 1.86−64.91, p = 0.008, see Table 5). The CD4 count at month 6 was not an indicator of virological failure at month 12., However, the odds of having a virological failure at month 12 was 5 times higher in participants with CD4 count ≤200 cells/µl (95% CI 1.8−14.1) when compared to the others.

Table 5 | Factors associated with virological failure at month 12.

		Crude Odds Ratio	95% CI	p-value	*Adjusted Odds Ratio	95% CI	p-value
Age (years)		0.94	(0.88–1.01)	0.09	0.96	(0.89–1.04)	0.32
Adherence (pill count)		2.25	(0.81–6.22)	0.12	3.01	(0.91–9.99)	0.07
WHO stage	1	reference			reference		
	2	2.27	(0.65–7.94)	0.2	1.86	(0.45–7.57)	0.38
	3&4	5.64	(1.69–18.77)	0.005	6.31	(1.43–27.83)	0.02
Baseline CD4 count (cells/µL)	>200	reference					
	50-200	1.68	(0.53–5.29)	0.37	1.77	(0.49–6.36)	0.38
	<50	10.42	(2.45–44.36)	0.002	10.99	(1.86–64.91)	0.008
Baseline viral load (Log10 RNA copies/mL)		1.32	(0.74–2.36)	0.35	0.91	(0.49–1.66)	0.76

*Adjusted model includes age, baseline viral load, adherence, WHO stage and baseline CD4 count, n =138 (6 participants missing adherence data, 1 participant missing age, 3 participants missing WHO stage data, 7 missing baseline CD4 count and 3 participants missing baseline viral load results). Adherence is used as a binary variable: adherence/non adherence

Discussion

To our knowledge, this is the first prospective study describing virological and HIVDR outcomes in a cohort of HIV-1 patients initiating first line therapy in Rwanda. The study achieved WHO-suggested targets of virological suppression in at least 70% of patients, and no more than 20% of patients lost to follow up, 12 months after ART initiation[5,31]. These results may partly reflect the elite character of the cohort and the good performance of the TRAC-plus clinic and may not be fully representative of other public HIV clinics in Rwanda.

A significant proportion of patients in need of ART presented late to HIV care services (25% were already at WHO stage 3 and 4, and 44% had a CD4 count ≤200 cells/µl). In addition, higher frequency of virological failure and acquired HIVDR mutations was associated with lower CD4 count at baseline. Initiation of ART at a more advanced stage of HIV disease is common in sub-Saharan Africa and negatively impacts HIV care and treatment program outcomes[19,20,21,32]. Recent findings from Rwanda indicate that earlier ART initiation could be achieved by improving pre-ART retention and linking HIV screening to HIV care and treatment[33].

As previously documented, immunological criteria were poorly correlated with virological treatment failure[34]. Clinical decisions based on immunological criteria alone would have led to an unnecessary switch to second line therapy in one in four virologically suppressed participants and would have delayed a switch to second line therapy in 50% of those with virological failure. These

results corroborate previous reports and confirm the added value of regular viral load monitoring to detect virological failure in a timely fashion [15, 34]. The appropriate use of targeted VL at month 12 would have increased the positive predictive value of detecting virological failure from 22 to 100% as compared to using immunological criteria alone. In addition, recent cost-effectiveness studies suggest that HIVDR genotyping at treatment failure could also have economical and clinical benefits in settings characterized by low CD4 count at treatment initiation and a relatively high frequency of wild-type viruses among patient failing therapy[35]. Genotyping of patients failing treatment at month 12 would have allowed for deferring costly second-line therapy in at least 4 participants with persistent wild-type virus infections and in whom HIV might be re-suppressed by improving ART adherence. In the SEARCH study, however, targeted VL monitoring was requested for only 9 of 41 participants who were suspected treatment failures at month 12, highlighting possible difficulties in interpreting CD4 counts or potential barriers to the utilization of VL testing by clinicians. Encouraging clinicians to use available laboratory-based monitoring methods to support clinical decision-making could contribute to improved quality of HIV care and reduced HIVDR. HIV sequencing capacity is currently being established at the National Reference Laboratory in Kigali. This would enable genotyping as part of ART monitoring and could improve the long term success of ART programs in Rwanda.

The 11% of participants that developed virological failure after 12 month of treatment is comparable to findings from 12 low- and middle-income countries of Asia and Africa, reporting an average of 9.4% patients experiencing treatment failure after one year[31]. Although pre-therapy drug resistance was more frequent in participants failing therapy, it was not associated with virological failure at 12 months in bivariable analysis, possibly due to the small number of cases. The low level of transmitted HIVDR in our cohort is comparable to findings from WHO-designed surveys in the region[8,31], agrees with the relatively recent history of ART scale up in Rwanda[18,36], and suggests that a change in first-line ART is not warranted in the near future.

Among the 18 participants failing therapy, at least four (22%) did not show any evidence of HIVDR mutations, indicating that they are failing therapy for reasons other than drug resistance. One reason might have been sub-optimal adherence although none of the treatment adherence measures collected in this study was identified as a predictor for treatment failure. Eight of the 12 study participants (66.6%) genotyped at treatment failure had evidence of HIVDR, which is comparable to the 63.7 % reported in more than 2000 HIV patients initiating first-line ART using the WHO approach in Eastern Africa between 2006 and 2010[31]. Our results indicate that reliable measurements of drug adherence are needed.

Overall, the NRTI and NNRTI transmitted and acquired mutation patterns that we identified were consistent with previous reports in similar settings[14,37,38]. The most frequent NRTI mutation (M184V) and NNRTI mutations (K103N, 190G and Y181C) described in our study are known to be common in cases of treatment failure[31]. They are associated with the use of 3TC, EFV and NVP, which have low genetic barriers towards resistance. The frequent association of M184V with at least one NNRTI

resistance-associated mutation (6/10) is also in accordance with the results of other studies[39]. M184V causes resistance to 3TC and FTC, enhances the susceptibility to AZT, and delays the emergence of mutations associated with AZT and d4T such as TAMs[40,41,42].

In 2 of 4 participants with HIVDR mutations at baseline, exposure to a failing regimen during 12 months was associated with the accumulation of additional HIVDR mutations, including the emergence of TAMs, also in association with M184V. M184V and TAMs confer cross-resistance to NRTIs and their relatively high overall prevalence in this cohort may have consequences for second line treatment responses in Rwanda. NRTI cross-resistance has the potential to significantly reduce the activity of the NRTI backbone of standard second line regimens. More specifically, TAMs have the capacity to reduce the efficacy of TDF containing-NRTI backbones. Functional PI monotherapy will lower the barrier for PI resistance[43]. Although poor adherence cannot be completely ruled out, the reduced activity of the NRTI backbone might have contributed to continuing viral replication in the face of second line treatment, which was observed in the 2 participants that were switched early.

Although our findings may not be generalized to all HIV clinics in Rwanda, they indicate that efforts to minimize HIVDR are needed. These should include improved availability and utilization of VL-based monitoring of ART response, and evaluation of the potential added value of HIV genotyping at treatment failure. In addition, high quality patient support for treatment adherence as well as earlier initiation of therapy will contribute to protecting the efficacy of second line and subsequent therapy and improving overall treatment outcome.

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CHAPTER 3

Long-term effectiveness of combination antiretroviral therapy and prevalence of HIV-drug resistance in HIV-1 infected children and adolescents in Rwanda

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Abstract

Objective: To determine the long-term outcomes of treatment, and prevalence of genotypic drug resistance in children and adolescents on combination antiretroviral therapy (cART).

Methods: A cross-sectional study (September 2009 to October 2010) in which clinical, immunologic and virologic outcomes were assessed at a single study visit and through patient records in a cohort of HIV-infected children and adolescents. Risk factors for clinical and immunologic responses, and virologic outcome were evaluated using logistic regression; and the accuracy of clinical and immunologic criteria in identifying virologic failure were assessed.

Results: 424 patients were enrolled with a median age of 10.8 years (range:1.7−18.8); and a median duration on cART of 3.4 years (range:1.0−8.1). 33% were stunted and 17% underweight. 84% (95% CI 79−87) of children >5 years had CD4 ≥350 cells/mm^3 and in 74% (95% CI 62−84) of younger children CD4% was ≥25. CD4 values and age at cART initiation were independently associated with CD4 outcomes; 124(29%) had HIV-1 RNA ≥1000copies/mL, with no significant predictors. Sensitivity for weight-for-age and height-for-age and CD4 cells (<350/mm^3) remained under 50% (15−42%), CD4 cells showed the best specificity, ranging from 91%−97%.
Of 52 samples tested, ≥1 mutations were observed in 91% (NRTIs) and 95% (NNRTIs); 1 to 2 TAMs was detected in 16 (31%) and ≥3TAMs in 7(13%).

Conclusion: Nearly one in three children showed virologic failure, and >10% of the subgroup of children with treatment failure in whom genotyping was performed demonstrated multiple HIV-drug resistance mutations. Neither clinical condition nor CD4 cells were good indicators for treatment failure.

Introduction

Combination antiretroviral treatment (cART) for HIV-1 infected children has had a great impact on the reduction of child morbidity and mortality as well as improved quality of life[1-5]. In Rwanda, 468 children were receiving cART in 2005 whereas the number had risen to 7,356 in 2011[6-7]. Despite this, only 52% of children in need were on treatment in 2011, while 93% of adults were[7].

Reports on cART in children in lower and middle-income countries (LMIC) so far have indicated good clinical and immunologic outcomes, illustrated by an overall improvement of growth and nutritional status, and recovery of CD4 cells. From the limited data collected on virologic outcomes, however, the risk of virologic treatment failure at the same time seems to be substantial[8-12]. This may be due to several factors, including high viral loads and severe immunosuppression before initiating treatment, exposure to sub-therapeutic drug concentrations, and weak links between primary health care and HIV-specific treatment for children[13-17]. In addition, prior exposure to single-dose nevirapine(NVP) as part of prevention of HIV mother-to-child transmission (PMTCT) have been shown to affect the treatment outcomes of children on cART as a result of archived resistance mutations[18-19]. Most studies on safety and effectiveness of cART in pediatric and adolescent populations in LMIC have focused on clinical and immunological evaluations within the first 12 months after initiation of treatment[13,15,17]. Therefore the primary aim of this study was to determine the long-term effectiveness of cART in Rwandan children and adolescents and to determine the accuracy of clinical and immunologic criteria in identifying virologic failure and genotypic drug resistance.

Methods

Study design, setting and patient population

A cross-sectional study was conducted in which clinical, immunologic, and virologic outcomes of cART were assessed at single study visit in a cohort of HIV-infected children and adolescents. Additional retrospective review of charts and electronic patient records was conducted. The prevalence of HIV drug resistance was determined in sub-group of children who failed treatment. The study visit was conducted between September 2009 and October 2010, at the Treatment and Research AIDS Center outpatient clinic and Kigali University Teaching Hospital, Department of Pediatrics/Centre of excellence. At the time of the data collection for the study, the TRAC-plus clinic and KUTH provided care to 884 HIV-infected children and adolescents of whom 444 were receiving cART, see Figure 1.

Patients were excluded if >18 years or not willing to participate in the study. Participants were on either first or second line ART in accordance with Rwandan National guidelines. First line cART consisted of two nucleoside reverse transcriptase inhibitors (NRTI) and one non-nucleoside reverse transcriptase inhibitor (nNRTI), and second line regimen consisted of two different NRTIs and a boosted protease inhibitor (PI)[20].

The study was approved by the Rwandan National Ethical Committee (RNEC). Written consent was obtained from the parents or guardians of the enrolled children, and in addition assent was required from children aged 12 years and above as recommended by the RNEC.

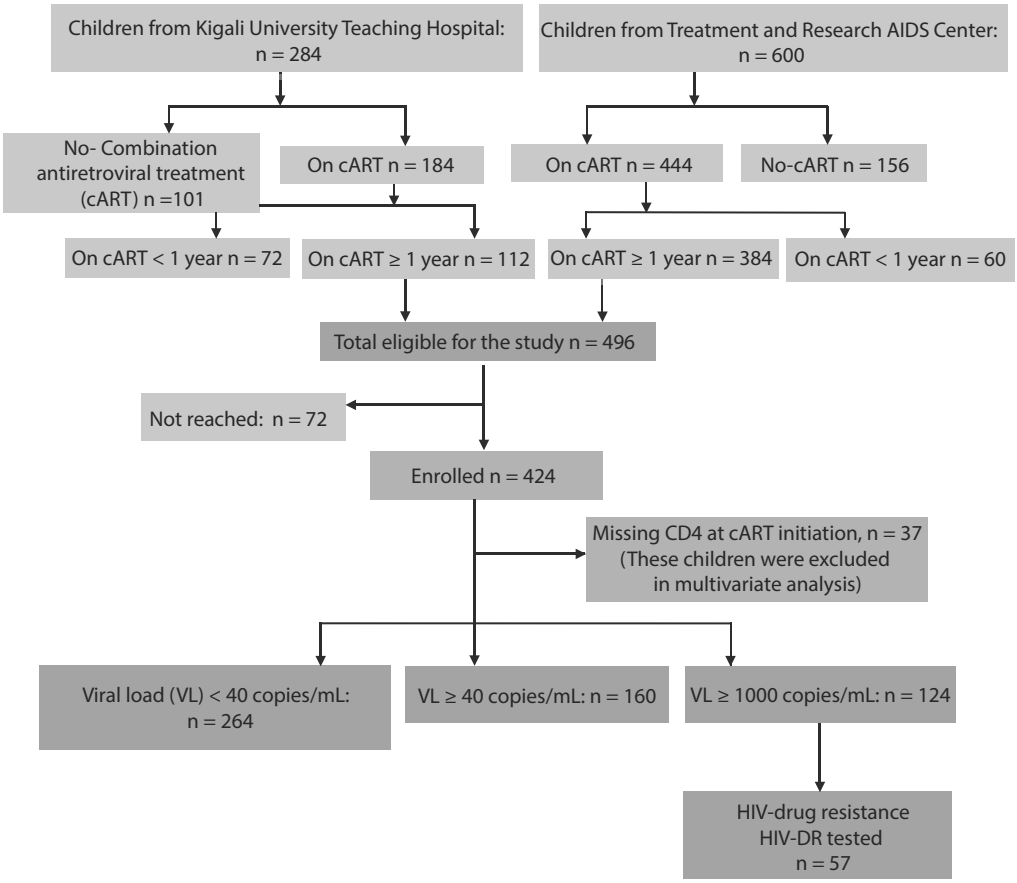

Figure1 | Flowchart summarizing patient inclusion

Data collection

The following data were retrospectively collected from patient files: age, sex, mode of HIV transmission, CD4 count before cART initiation, World Health Organization (WHO) clinical stage and weight at cART initiation and past HIV-specific drug history (e.g. PMTCT), cART start date, initial regimen, changes to second line and reasons for switching. At the study visit, a clinical exam was conducted by study physicians and data was collected regarding current cART regimen, height and weight. Blood was drawn for CD4 count and viral load determinations.

HIV drug resistance (HIV-DR) genotyping was conducted in a selected group of children demonstrated to have virologic failure (defined as HIV-RNA ≥1000 copies/mL)[20]. We selected every 2nd patient with virologic failure, if no sample available from that patient, the previous patient on the list was selected.

At the study visit, adherence, and specifically during the last 30 days was assessed using a standardized adherence questionnaire. For children ≥12 years, both the child and caretaker were interviewed, in younger children only the caretaker was interviewed. The pharmacy refill forms from the past month were also checked to verify self-reported data. Children were considered as fully adherent if they had taken more than 95% of their prescribed doses and non-adherent if less than 95%.

Laboratory investigations

CD4 lymphocyte count and HIV-1 RNA quantification were determined at the National Reference Laboratory in Kigali, Rwanda. CD4 count and percentage were performed by flow-cytometric measurement using FACS calibur (Becton Dickinson, San Jose, CA, USA), while plasma HIV-1 RNA was quantified using the Roche Cobas AmpliPrep/Cobas TaqMan HIV-1 (Roche Molecular Systems, France) with a lower limit of detection of 40 copies of HIV RNA/mL. A cut-off of HIV-1 RNA ≥1000 copies/mL was chosen for HIV drug resistance and genotyping. Patients were still taking the failing regimen when venipuncture was performed

HIV-1 genotypic resistance analysis

As a result of financial constraints, half of the children with HIV viral load (VL) ≥1000 copies/ml were selected for HIV-DR genotyping (see Figure 1). HIV-1 genotyping was performed on plasma samples stored at -80°C, using the US Food and Drug Administration licensed Viroseq HIV-1 Genotyping System (Abbott Molecular, Abbott Park, Il, USA). The laboratory procedures for sample extraction, amplification and sequencing were performed according to the instructions of the Viroseq HIV-1 Genotyping System. Prior to electrophoresis of the genotyping products on an automated sequencer (model ABI 3130 XL; Invitrogen, Foster City, CA, USA), each sequencing reaction was purified to remove unincorporated dyes and salts using CentriSep plates according to manufacturer instructions. Sequences were assembled and edited using Viroseq software (version 5.4). The obtained consensus sequences were subsequently submitted to the Stanford University HIV Drug Resistance Database for the identification of Drug Resistance Mutations (DRMs): http://hivdb.stanford.edu/.

Outcome evaluations

Clinical outcomes included weight-for-age (WAZ) for underweight (index of acute malnutrition) and height-for-age (HAZ) for stunting (index of chronic malnutrition). Epinfo version 3.5.1 was used to convert weight and height into WHO z-scores. In accordance with the NCHS/CDC/WHO international reference standards, children with a z-score less than -3.0 standard deviation (SD) below the mean were considered as severely malnourished and children with a *z*-score between -2.99 and -2.0 SD below the mean were considered as moderately malnourished.

Clinical outcome was also assessed through an evaluation of clinical complaints and a physical assessment; a clinical detrimental outcome was defined as the presence of any stage 3 or 4 clinical condition as defined in the WHO pediatric HIV classification[21] or any other severe medical conditions.

Immunological outcome was evaluated through assessment of the CD4 T-cells; a poor immunologic status was defined as a CD4 T-cell percentage <25 in children <5 years[21], and as a CD4 T-cell count <350 cell/mm^3 in children ≥5 years of age; a decrease of CD4 T-cells or failure to increase was defined as poor immunological outcome. Virologic success was defined as HIV-RNA below <40 copies/mL and HIV-RNA ≥1000 copies/mL as virologic failure according to the Rwandan ART guidelines[20]. Treatment failure was defined as having one or more of the following outcomes: clinical detrimental, immunological and virologic failure.

Children were defined as having NRTI-mutations if one of the following was present: the 69 insertion complex, M184V/I and the thymidine analogue associated mutations (TAMs) including M41L, D67N, K70R, L210W, T215F/Y, and K219Q/ E; NRTI multidrug resistance was defined by the presence of the 69 insertion or at least three TAMs.

Children were defined as having NNRTI-associated mutations if one of the following mutations was present: K103N/S, Y181C/I/V, K101P/E, A98G, E138A/E, V179IT, G190A/S, V108IV, V179D, V106IV, V109I and K101AEKT.

Statistical analysis

Continuous variables were reported as mean with SD for normally distributed data and median with interquartile range (IQR) or range when appropriate for non-normally distributed data. Univariate analysis associations were compared using Chi^2, ANOVA and Kruskall-Wallis tests, as appropriate.

Two logistic regression models were developed to assess risk factors for clinical response: gender, WHO clinical stages, WAZ and HAZ at cART initiation and at study visit, CD4 cells at cART initiation, cART duration and regimen), one with CD4 count/percent as the outcome (CD4 <350 or CD4 ≥350 cell/mm^3, for children ≥5 years; CD4% <25 or CD4% ≥25 for children <5), and a second model with virologic failure as the outcome (VL <1000 (0) and VL ≥1000 (1) copies/mL). Factors were analyzed for their relations to both outcomes. The univariate threshold p-value for inclusion in the multivariate model was 0.10 or clinically relevant as determined by the literature[22]. Variables in multivariate analyses included WHO clinical stages(I/II vs. III/IV), age at cART initiation, adherence, CD4+ cell at cART initiation, treatment switchers, PMTCT exposure, cART duration, cART regimen and CD4-T cells changes. Odds ratio with 95% confidence intervals (95% CI) are presented and considered significant if the 95% CI did not include one. All continuous exposure variables were plotted by logit with the outcomes (CD4 >350 cell/mm^3, CD4% ≥25 and VL ≥1000 copies/mL) to determine linear relationships. In multivariate analyses, children without available baseline data including CD4, weight and WHO stages were excluded in the model.

To assess the performance accuracy of clinical and immunologic criteria in identifying virologic failure sensitivity, specificity and positive and negative predictive values for virologic failure were determined. Statistical analyses were performed using STATA version 11 (STATA Corporation, College Station, TX).

Results

Characteristics of study population at study enrollment

Four hundred and twenty four children were included in the study (Figure 1). General characteristics are presented in Table 1. All children were reported (by parents/guardians) to have acquired HIV perinatally. Median age at study visit was10.8 years and median (range) duration between cART initiation and data collection was 3.4 (1.0−8.1) years.

Initial cART regimens included: AZT/3TC/NVP (41%), d4T/3TC/NVP (33%), AZT/3TC/EFV (20%) and d4T/3TC/EFV (6%). Regimen changes occurred in 105 (25%) children for the following reasons: 47 (45%) out of 105 children switched from mostly NVP to efavirenz(EFV) because of side effects, 29 (28%) children switched from NVP to EFV because of concurrent tuberculosis treatment. In 23 children, tuberculosis treatment was prescribed based on positive sputum or a suspicious chest x-ray; for the other 6 children tuberculosis diagnostic data were not available. Six children (6%) switched to a 2nd line regimen (at the time: ABC/ddI/lopinavir/ritonavir) because of suspected treatment failure. The reason for drug substitution/switches was not recorded for 22 (21%) children.

Data on exposure to anti-retrovirals during PMTCT were available for 258 (60%) of children; among these children 69 (27%) were reported to have been exposed to single-dose NVP.

At the time of the study visit, 62(15%) children presented with moderate malnutrition and 11 (3%) with severe malnutrition; and 140 (33%) children were stunted.

Data on CD4 T-cell counts are summarized in Table 1. For children ≥5 years of age at study visit, 84% (95% CI 79−87) had a CD4 ≥350 cells/mm^3 and 69% (95%CI 64−73%) had a CD4 ≥500 cells/mm^3; for those under 5, 74% (95% CI 62−84) had a CD4 percentage ≥25%. More than half of children had undetectable HIV-1 RNA (<40 copies/mL). 124 (29%) children had treatment failure and all but one were still on their first-line therapy. From 258 children with PMTCT data available, 78 (30%) were on a NNRTI containing regimen and had treatment failure. Thirty-eight (49%) of these 78 children were exposed to NVP during PMTCT.

96% of children or their caregivers reported to be fully adherent. Only 58% of results from pharmacy refill forms were available; when the pill count data were compared to the self-report, it appeared that adherence was less than 95% in 17% of these children. We did not find any association between non-adherence and treatment failure.

Table 1 | General characteristics of the children.

Characteristics	Virologic success (HIV 1-RNA <1000 c/mL)	Virologic failure (HIV 1-RNA ≥1000 c/mL)
Demographic	**Population**	
Total number	424	124 (29%)
Female, n (%)	219 (52%)	67 (54%)
NVP exposure	69 (27 %)	38 (30%)
Duration of combination of Antiretroviral therapy (cART)	**Median [interquartile rage (IQR)]**	
Median (IQR) age at cART initiation , years	6.9 (3.3 to 9.8)	7.0 (3.0 to 10.6)
Median(IQR) age at study visit, years	10.8 (6.8 to 13.4)	11.4 (6.7 to 13.9)
Median(IQR) duration on cART, in years	3.4 (1.9 to 4.8)	3.4 (1.9 to 3.8)
Age	**N (%)**	
Children under 5 years of age	157(37%)	46(29%)
Children 5 years and above	267(63%)	78(29%)
cART regimen at study visit	**N (%)**	
2 NRTIs + NVP	278 (65%)	90 (71%)
2 NRTIs + EFV	138 (33 %)	33 (26%)
2 NRTIs + PI	6 (2%)	1 (1%)
Clinical	**Median(IQR)**	
Weight for age z-score, prior to initiation of cART (median, IQR)	-1.87 (-2.65 to -0.80)	-1.87 (-2.63 to -0.93)
Weight for age z-score, at study visit (median, IQR)	-1.183 (-1.64 to -0.8)	-1.20 (-1.84 to -0.46)
Height for age z-score, at study visit (median, IQR)	-1.37 (-2.34 to -0.53)	-1.46 (-2.12 to -0.67)
Tuberculosis treatment	29 (7%)	15 (12%)
Immunologic	**Median(IQR)**	
Children ≥5 years of age: CD4 T-cells at study visit, (median cells/mm^3 (IQR)	690 (451 to 984)	479 (296 to 806)
Children <5 years of age: % CD4 T-cells at study visit, median (IQR)	32 (24 to 40)	29 (22 to 40)
CD4 increases	317(81.91)	83(72.81)
Virologic	**Median (IQR) or n (%)**	
HIV-1 RNA (copies/mL), median (IQR)	8945 (1140 to 69600)	26050 (5210 to 97050)
HIV-RNA ≥ 40 copies/mL, n (%)	160 (38%)	NA
HIV-RNA ≥ 1000 copies/mL, n (%)	124 (29%)	

Clinical, immunological and virological treatment outcomes

From the children who had weight recorded at both cART initiation and study visit (n = 381); the median WAZ increased from -1.8 (IQR: -2.6, -0.8) at cART initiation to -1.2 (IQR: -1.8, -0.4).

Data on absolute CD4 T-cells both at cART initiation and at study visit were available in 341/368 children ≥5 years. Among them 86% (293/341) had increased their CD4 cells, with a median (IQR) increase from 313 (163−522) to 690 CD4 cells/mm^3 (451−984). For those <5 years, data on CD4 cell percentage at cART initiation and study visit were available for 46 out of 56 children, 98% had an increased CD4 cell percentage with a median (IQR) change from 16% (14−19) to 31% (28−34).

Children who started cART with higher CD4 cells were found to have high probability of immunological recovery; 89% of children (≥5years) who started cART with ≥200 CD4 cells/mm^3 had ≥350 CD4 cells/mm^3 at study visit while only 70% of children who started with <200/mm^3 CD4 cells reached a count of ≥350 cells/mm^3, (p = 0.01). In multivariate analyses, age and CD4 cells at cART initiation were associated with immunological success. Other factors were not associated with immunological outcome, Table 2.

Viral load data were only available at study visit and not at cART initiation. In a multivariate analysis, children who had CD4 cells <100/mm^3 at cART initiation (AOR: 2.05, 95% CI 1.07−3.91) and children who did not have any increase or those who decreased their CD4 cells as compared to their initial CD4 cell count were more likely to have virologic failure. None of the other patient characteristics were significantly associated with virologic failure (see Table 3).

Accuracy of clinical and immunological values for indicating virologic failure

To better understand the use of clinical factors in determining patients with virologic failure clinical factors were determined to discriminate those with virologic failure. WAZ, HAZ (less than -2) and CD4 cells <350/mm^3 at study visit showed low sensitivity ranging from 19% to 32% and a specificity ranging from 66% to 94% in detecting treatment failure (Table 4).

Genotypic resistance

As a result of cost limitations, drug resistance genotyping was only completed for a selection of 57 out of 124 children (46%) with virologic failure. This represents 13% of all children in the study. One or more NRTI's major mutations were observed in 91% of the samples. Mutations conferring resistance to lamivudine (M184V) were present in 55 of 57 children. Furthermore, the presence of 1 to 2 TAMs, conferring intermediate resistance to abacavir (ABC), stavudine (d4T), tenofovir (TDF) and zidovudine (AZT/ZDV), was detected in 16 (31%) children. At least one major NNRTI associated drug resistance mutation was detected in 95% of the samples, with equal distribution of these mutations between EFV and NVP treated individuals. In 7 (13%) out of 52 children with major mutation 3 or more TAMs were present, conferring resistance to all first line NRTI's. Data on PMTCT exposure were available for 41 children out of 57 tested for HIV drug resistance, 7 out of these 41 children were exposed to PMTCT.

Table 2 | Predictors of CD4 T-cell recovery

Variables	Children ≥5 years of age at Combination Antiretroviral Therapy (cART) initiation (n = 244)				Children <5 years of age at cART initiation (n = 143)			
	Univariate		*Multivariate		Univariate		*Multivariate	
	Odds Ratio (OR)	95% confidence interval(95% CI)	OR	95% CI	OR	95% CI	OR	95% CI
Demographic								
Sex, female	1	0.5 to 1.8	NE		0.9	0.8 to 1.1	NE	
Age at study visit (per 1 year increase)	0.8	0.7 to 0.9	0.7	0.6 to 0.9	0.9	0.8 to 1.1	3.0	1.4 to 6.8
Clinical								
World Health Organization stage at cART initiation	1.0	0.7 to 1.6	0.8	0.6 to 1	0.9	0.5 to 1.9	1.2	0.4 to2.0
Weight for age >-2 at cART initiation	0.9	0.7 to 1.1	NE		1	0.8 to 1.2	NE	
Weight for age >-2 at study visit	1.8	0.6 to 1.1	NE		1.1	0.8 to 1.5	NE	
Height for age z-scores >- 2 at study visit	0.9	0.4 to 2.0	NE		0.6	0.9 to 2.6	NE	
Immunologic								
CD4 T-cell count ≥200/mm^3 at cART initiation	2.4	1.3 to 4.6	2.7	1.3 to 5.7				
CD4 T-cell percentage ≥10 at cART initiation					4.7	1.3 to 17	16.4	2.5 to 105
Treatment								
cART duration, per year increase	1.0	0.9 to 1.3	NE		0.8	0.6 to 1.0	NE	
cART regimen (NVP vs. EFV)	0.9	0.6 to 1.6	0.9	0.5to 1.6	1.9	0.8 to 4.7	0.3	0.0 to 2

Variables: WHO stages, age at study visit, and CD4 T- cells at cART initiation were included regardless of their univariate significance[21]; self-report adherence was greater than 95% for all children and hence not added to the model.

Table 3 | Predictors of treatment failure [viral load (VL) ≥1000 copies/mL] at follow-up (Total enrolled n = 424)

Covariates	Treatment failure no (%)	Treatment success no (%)	Odds Ratio (95%) confidence interval	Adjusted Odds Ratio* (95%) confidence interval
Age at Combination Antiretroviral Therapy (cART) initiation				
<3 years	38(39.6)	58(60.4)	1.8(1.1–3.0)	1.6 (0.9–3.0)
≥3 years	86(26.2)	242(73.8)	1	
cART duration				
≥5 years	81(33.9)	158(66.1)	1.7(1.1–2.6)	1.0(0.6–2.2)
<5 years	43(23.2)	142(76.8)	1	1
Weight for age (WAZ) at cART initiation (n = 390)				
≤-2	62(35.4)	113(64.6)	1.7(1.1–2.6)	1.3(0.7–2.2)
>-2	53(24.7)	162(75.4)	1	
WAZ at study visit (n = 415)				
≤-2	24(32.9)	49(67.1)	1.2(0.7–2.1)	–
>-2	97(28.4)	245(71.6)	1	
Height for age (HAZ) at study visit (n = 393)				
≤-2	32 (25.2)	81(30.4)	0.8(0.5–1.2)	0.9(0.7–1.2)
>-2	95(74.8)	185(69.6)	1	
World Health Organization at cART initiation (n = 422)				
3&4	112(30.4)	256 (69.6)	1.5(0.8–3.0)	1.2(0.6–2.7)
1&2	12(22.2)	42(77.8)	1	1
CD4 T-cells/mm3 at cART initiation(n = 387)				
<100	21(42.1)	29(54.9)	2.0(1.10–3.5)	2.1(1.1–3.9)
≥100	89(27.0)	248(73.0)	1	1
CD4 T-cells increased as compared to initial CD4 (n = 387)				
No	31(44.3)	39(55.7)	2.2(1.3−3.8)	2.5(1.3–4.6)
Yes	83 (26.2)	234(73.8)	1	
Initial cART regimen (n = 424)				
d4T/3TC/EFV	7(29.2)	17(70.8)	1.1(0.4−2.9)	
AZT/3TC/EFV	18(20.7)	69(79.3)	0.7(0.4–1.3)	
d4T/3TC/NVP	53(37.9)	87(62.1)	1.7(1.0–2.7)	
AZT/3TC/NVP	46(26.6)	127(73.4)	1	
cART regimen at study visit(n = 424)				
Nevirapine based	99 (31.8)	210 (68.0)	1	
Efavirenz based	24(21.8)	84(77.8)	0.6(0.4–1.0)	
PI based	1(14.3)	6(85.7)	1.1(0.1–12.0.)	
cART drug regimen switched				
Yes	36(34.3)	69(65.7)	1.4(0.9–2.2)	1.8(1.0–2.9)
No	88(27.6)	231(72.4)	1	1

Covariates	Treatment failure no (%)	Treatment success no (%)	Odds Ratio (95%) confidence interval	Adjusted Odds Ratio* (95%) confidence interval
Tuberculosis diagnosed				
Yes	7(22.8)	22(77.2)	0.95(0.5–1.8)	0.97(0.5–1.9)
No	35(23.7)	113(76.4)	1	1
Unknown	68(33.2)	116(66.8)	1.9(1.2–3.1)	1.86(1.0–3.5)
PMTCT exposure (n = 424)				
Yes	25(36.2)	44(63.8)	1.22(0.7–2.2)	
No	60(31.8)	129(68.3)	1	
Unknown	39(23.5)	127(76.5)	0.66(0.4–1.1)	
Adherence by pill count (n = 246)				
Adherent	49(24.1)	154(75.9)	1	
Missed ≥5%	15(34.9)	28(65.11)	1.68(0.8–3.4)	

*For the multivariable model, only a total of 344 patients were included, due to select drop out of predictor variables

Table 4 | Accuracy of clinical parameters and CD4 T-cell count indicating virologic failure ≥1000 c/mL) in children ≥5 years of age (n = 350 children).

Parameters	Sensitivity (95% CI)	Specificity (95% CI)	Positive Predictive Value (95% CI)	Negative Predictive Value (95% CI)
Weight for age z-score <-2 at study visit	19(15–23)	83(79–87)	32(28–36)	71(67–78)
Height for age for z-score <-2 at study visit	28(24–32)	66(61–71)	25(21–29)	70(65–74)
CD4 T- cells at study visit (<350/mm^3)	32(24–42)	94(91–97)	13(10– 17)	95(83–98)

Table 5 | Distribution of Resistance Mutations in 52 children with HIV-RNA ≥1000 c/mL.

Regimen	Non-Nucleoside Reverse Transcriptase Inhibitors mutations											
	K103N/S no (%)	Y181C/I/V no (%)	K101P/E no (%)	A98G no (%)	E138A/E no (%)	V179IT no (%)	G190A/S no (%)	V108IV no (%)	V179D no (%)	V106IV no (%)	V109I no (%)	K101AEKT no (%)
AZT/3TC/EFV n = 17	7(37)	2(11)	2(11)	2(11)	1(5)	0	3(16)	1(5)	0	1(5)	0	0
AZT/3TC/NVP n =25	6(24)	7(28)	4(16)	0	0	2(8)	5(20)	0	1(4)	0	0	0
d4T/3TC/EFV n = 5	1(20)	0	1(20)	0	1(20)	0	0	0	0	0	1(20)	1(20)
d4T/3TC/NVP n = 27	7(26)	8(30)	1(4)	3(11)	1(4)	0	7(26)	0	0	0	0	0
	Nucleoside Reverse Transcriptase Inhibitors mutations											
	M184V no (%)	**T215Y/F no (%)**	**K70R no (%)**	**M41L no (%)**	**K219Q no (%)**	**D67N no (%)**	**L210W no (%)**					
AZT/3TC/EFV n = 25	11(42)	3(12)	4(15)	1(4)	3(12)	3(12)	1(4)					
AZT/3TC/NVP n = 29	15(52)	3(10)	5(17)	1(3)	2(7)	2(7)	1(3)					
d4T/3TC/EFV n = 3	2(67)	1(10)	0	0	0	0	0					
d4T/3TC/NVP n = 35	21(64)	5(15)	1(3)	4(12)	1(3)	1(3)	0					

Discussion

In this study, medium- and long-term data on cART from a large cohort of Rwandan HIV- infected children and adolescents are presented. The results show that most children are in a good clinical condition with an adequate immunologic response, but the proportion of children with virologic failure and with multiple drug resistance is high and of major concern.

The impact of HIV on growth and the ability of cART to reverse weight and height deficiencies and body composition abnormalities have been clearly documented, even in settings where malnutrition is highly prevalent[1,10,23-24]. Our findings support these data, as we observed a significant increase of WAZ, with 64% underweight at cART initiation reduced to 18%. Good clinical condition and improvement of WAZ scores was also observed in children whose viral load was not fully suppressed. This supports data from others[25-27] that children may have a good clinical outcome while viral load is not well controlled and unrecognized virologic failure may occur if clinicians rely solely on clinical observation and CD4 cell measurement. A substantial proportion (33%) of children was still found to have stunting at study visit. Sutcliffe et al., in a cohort of 152 Zambian children likewise reported an improved WAZ score, but a HAZ score below -2 throughout 2 years of follow-up in almost half of the children[27]. Advanced malnutrition prior to treatment initiation may explain this high proportion of children with stunting.

Various pediatric studies have indicated that CD4 cells double usually within one year of treatment initiation[10-12]. Overall, in children above 5 years of age, CD4 cells increased substantially from cART initiation to follow-up and all children less than 5 years of age had an increase of CD4 %. However, more than 1 in 10 children older than 5 years of age did not have a CD4 cell increase. Furthermore, the proportion of children with an increase of their CD4 cells ≥350 cells/mm^3 was higher in those starting cART while CD4 were still above 200 cells/mm^3. These findings are in line with previous experiences in HIV-infected children from LMIC, where early cART initiation was reported to significantly improve patient outcomes including immune response. In a cohort of Ugandan children Musoke et al. demonstrated a significant association between CD4 cells at baseline and immune outcome; a late start of cART when the child is already severely immunesuppressed resulted in poor CD4 cell recovery[28]. Similarly, Puthanakit et al. showed that Thai children who started cART with CD4 cells >15% had a better immune outcome[29]. These findings support the strategy to initiate cART before dysregulation of the thymic function is partly irreversible and implies that an earlier start of treatment may preserve the thymic output and thereby may contribute substantially to improved immunologic outcome[30-31]. In this light the Rwandan ART policy recently moved to recommend treatment of all children under 5 years of age regardless of clinical conditions and CD4 count and to start treatment in children older than 5 years of age when CD4 values decrease below 500 cells/mm^3 instead of 350 cells/mm^3[3,32].

Treatment failure and drug resistance are major concerns in both pediatric and adult programs. Our data support these concerns, as the proportion of children with virologic failure was high. Though

children were clinically well and most had adequate CD4 T-cells, nearly one in three children had a virologic failure. Similarly, Barth et al. found that 38% of children had virologic failure after a median treatment time of 2.6 years[23]. In contrast, the prevalence of virologic failure was 17% in another South African study after 3 years of treatment and 15% in a Thai study after 2.7 years of treatment[12;33]. In our study routine viral load determination was not part of the patient monitoring during previous years while in the aforementioned studies the viral load was monitored every six months. Adherence interventions and an earlier treatment switch have likely reduced the proportion of children with virologic failure in these studies.

Treatment failure was more common in children who started cART treatment with low CD4-T cells and in children who did not increase their CD4 T-cells, which is in agreement with other reports[34]. Severe immunosuppression associated with irreversible thymic dysregulation and occurrence of opportunistic infection may explain slower virologic suppression and higher risk of virologic rebound leading to treatment failure.

Among children with virologic failure, only one third showed CD4 T-cell counts <350 cells/mm^3; indicating that the CD4 T-cell count was not a good indicator of virologic outcome with a sensitivity of only 32% but a specificity of 94%. Other experiences in both adults and children in LMIC have supported this finding by reporting virologic failure without evidence of immunological or clinical failure in a high proportion[23;33;35]. As maintaining HIV-RNA suppression is a main goal of cART, these results underscore the limitations of clinical and immunological parameters for timely detection of virologic failure. As a consequence, children will be exposed to prolonged periods of viremia during treatment, resulting in accumulation of HIV drug resistance mutations, which may limit future treatment options.

Data on PMTCT were available for only sixty percent of 124 children with virologic failure. Exposure to NVP during PMTCT was not associated with virologic failure; however, lack of accurate and complete ascertainment of NVP exposure in this study significantly limits any firm conclusion to be drawn from this observation.

The consequences of prolonged continuation of non-suppressive cART were clearly visible from the genotypic results, with 90% of children with virologic failure and available genotyping determinations showed major NRTIs and NNRTI-associated resistance mutations. More than 1 in 10 of these children had at least 3 TAMs resulting in extensive resistance to all first line NRTIs available in Rwanda. Results from other pediatric and adult studies in LMIC showed similar patterns of HIV drug resistance[36-39]. The high numbers of children with M184V mutations conferring resistance to 3TC and the K103N/Y181C mutations conferring resistance to NVP and EFV agree with the low genetic barrier reported for these agents[40-43]. The proportion of children with multi-resistant NRTI (≥3TAMs) was higher than reported previously from pediatric studies in a Malawi (3%), Tanzania (5%), Kenya (5%) and Uganda (7%)[36-39;44]. This difference may be explained by the study design, duration of cART, and frequency of viral load monitoring. Our study was a partially retrospective assessment; earlier

detection of virologic failure by routine viral load monitoring and timely regimen switching could have prevented or reduced the accumulation of TAMs. In our study few (1.4%) participants were on second line treatment.

Poor adherence can be attributed to as the main cause of treatment failure[45-46]. Unfortunately, due to the retrospective nature of the study, we were unable to determine the impact of poor adherence on treatment failure and viral resistance in this patient group. To improve long term treatment results it would be important to determine the main barriers to adherence and the best strategies to support adherence in Rwandan children and adolescents. However, this is a complex field, and barriers are largely influenced by the setting. Recent Rwandan studies indicated adolescents in boarding schools, and double orphans as particular vulnerable groups, that need extra care and attention[47;48]. Various interventions to promote adherence have been investigated with mixed results. Among these are interventions to reduce stigma, promote disclosure, decentralized and home-based care, social support networks, educational sessions of children and caregivers, use of adherence support tools such as pill boxes and health information technology, and child-friendly regimens.

This study had some limitations; the retrospective design may account for possible transient viral load blips, and longitudinal data are needed to assess the clinical, immunological and virological changes overtime, as well as treatment failure and regimen changes to 2nd line. From the total eligible children, 14% were not traceable; and only 344 children could be included in multivariate analysis due to select drop out of predictor variables. The limited number of children with available information on PMTC exposure thwarted knowledge on the impact of NVP exposure to the HIV drug resistance.

Routine adherence assessment was suboptimal through self-report, while only 58% had monthly pharmacy refill data available; longitudinal data with pill counts and other adherence measurements would provide more valid information. Unfortunately, we were unable to complete all drug resistance and genotype testing for those with VL failure; although the selection should allow the data to be representable.

In summary, this study shows that cART has efficacy across clinical and immunological indicators in Rwandan HIV-infected children and adolescents. However, while the country has good access to cART and a good drug supply, the observed proportion of children with virologic failure is worrisome, with more than 10% of children with virologic failure. Genotyping demonstrated multiple HIV-drug resistance mutations, conferring reduced susceptibility to all approved NRTIs. Clinical condition and CD4 cell count were poor indicators of virologic treatment failure emphasizing the need for affordable viral load testing for timely detection of virologic failure to prevent ongoing accumulation of HIV drug resistance mutations that jeopardize further treatment options. Promotion of adherence in children and adolescents are urgent in the light of the current recommendations to initiate cART in patients older than 5 years of age with CD4 cells <500/mm^3, and in all children less than 5 years of age regardless of clinical condition and immune status.

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CHAPTER 4

Molecular and phylogeographic analysis of human immuno-deficiency virus type 1 strains infecting treatment-naive patients from Kigali, Rwanda

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Abstract

This study aimed at describing the genetic subtype distribution of HIV-1 strains circulating in Kigali and their epidemiological link with the HIV-1 strains from the five countries surrounding Rwanda.

One hundred and thirty eight pol (RT and PR) sequences from 116 chronically- and 22 recently-infected antiretroviral therapy (ART)-naïve patients from Kigali were generated and subjected to HIV drug resistance (HIV-DR), phylogenetic and recombinant analyses in connection with 366 reference pol sequences from Rwanda, Burundi, Kenya, Democratic Republic of Congo, Tanzania and Uganda (Los Alamos database). Among the Rwandan samples, subtype A1 predominated (71.7%), followed by A1/C recombinants (18.1%), subtype C (5.8%), subtype D (2.9%), one A1/D recombinant (0.7%) and one unknown subtype (0.7%). Thirteen unique and three multiple A1/C recombinant forms were identified. No evidence for direct transmission events was found within the Rwandan strains. Molecular characteristics of HIV-1 were similar between chronically and recently-infected individuals and were not significantly associated with demographic or social factors.

Our report suggests that the HIV-1 epidemic in Kigali is characterized by the emergence of A1/C recombinants and is not phylogenetically connected with the HIV-1 epidemic in the five neighboring countries. The relatively low level of transmitted HIV-DR mutations (2.9%) reported here indicates the good performance of the ART programme in Rwanda. However, the importance of promoting couples' counseling, testing and disclosure during HIV prevention strategies is highlighted.

Introduction

Rwanda is one of the African countries most affected by HIV/AIDS[1]. Important fluctuations of the national HIV prevalence have been observed in this country since the first HIV infection case was reported in 1983[2]. The initial estimation of the national HIV prevalence rate in between 1988 and 1989 was 17.8 % among urban and 1.3% among rural populations[3,4]. In 1996 and immediately after the civil war, the national HIV prevalence reached 27% among the urban and 6.9% among the rural population[5,6]. This peak has been followed by an overall decline of the national HIV prevalence since the late 1990's, most likely due to an improved serosurveillance strategy and to the important resources devoted to the fight against HIV/AIDS in Rwanda.

Frequent human migration in the region, might have resulted in complex routes of HIV transmission. Population groups have traditionally been moving inside and outside Rwanda for economical, spatial, and political reasons since the early 20th century. In the past decades, war and political turmoil have led to further population shifts in the country with millions of Rwandans fleeing their home to surrounding countries[7,8]. Nowadays, 80% of the Rwandan population returned from the diaspora[9,10] mainly to urban areas.

Similarly to Rwanda, the five neighboring countries where Rwandan populations have been moving in and out are all characterized by a high HIV prevalence: from 8% in Kenya to 20% in Uganda[11]. The region is also characterized by a very heterogeneous epidemic. Uganda and Kenya show a predominance of subtype A and D[12,13,14]. In Burundi, the epidemic is largely due to subtype C[15] while subtype A and C predominate in Tanzania[16,17]. The Democratic Republic of Congo, the presumed epicenter of the epidemic, shows a highly heterogeneous distribution of eight HIV subtypes, with a predominance of subtypes A, G and D and multiple unique circulating recombinant forms[18,19]. In Rwanda, information on HIV-1 subtypes distribution is scarce and indicates a large predominance of subtype A (or A1) followed by subtype C and with a discreet presence of subtype D and A/C recombinants[20,21]. HIV-1 diversity translates into the classification of the virus into groups, subtypes, sub-subtypes and circulating recombinant forms that are generally associated with particular geographic region, risk groups and mode of transmission. Different HIV-1 subtypes are reported to impact on the viral fitness, immunogenicity and pathogenicity, which might have consequences for prophylaxis and therapeutic interventions[22]. Studying the distribution of HIV subtypes within a given population provides the opportunity to study patterns and trends of the epidemic and track routes of HIV transmission[23].

The high mutation rate of HIV can also lead to the emergence of drug resistant strains under the selective pressure of antiretroviral (ARV) drugs. Drug resistant viruses can subsequently be transmitted with serious implications in terms of efficacy of first line antiretroviral therapy (ART). Recent and particularly alarming data from neighboring Uganda[24] suggest that baseline HIV drug resistance (HIV-DR) may increase in Rwanda via population movements across the border. Although the roll out of prevention of mother-to-child transmission (PMTCT) and ART have been initiated in

Rwanda in 1999 and 2004[25], respectively, information on the level of transmitted HIV-DR circulating in the country is still scarce.

Genotypic HIV resistance tests provide a source of HIV pol sequence data that are used to screen for HIVDR mutations and determine HIV-1 subtypes. The pol sequences can also be used to conduct phylogeographic analyses, a useful tool to examine the epidemiology of HIV in terms of relationships between groups of infected persons.

The heterogeneous HIV-1 subtype distribution in East Africa facilitates the study of HIV epidemiology in the region. To our knowledge, little data on the molecular characteristics of HIV have been generated for Rwanda. In addition, few studies have specifically examined the phylogeographic characteristics of HIV circulating in Rwanda, in relation to the current HIV epidemics of the five neighboring countries. From a public health point of view, the identification of the main entry or focal points of HIV transmission is important to support the implementation of efficient prevention strategies that target relevant populations.

This study was conducted to describe the genetic subtypes of HIV-1 strains derived from infected individuals from Kigali, Rwanda using protease (PR) and reverse transcriptase (RT) genes sequences. To gain more insight into the dynamic of the epidemic in Kigali, our aim was to identify clusters of infections amongst chronically and recently HIV-infected Rwandans. We also analyzed the epidemiological links between HIV strains circulating in Rwanda and in its five neighboring countries. In addition, the pol sequence information was used to examine whether HIV-DR mutations are being transmitted within and across the borders of Rwanda.

Demographic and social factors associated with particular HIV genetic characteristics, as well as differences between chronic and recent infections were investigated.

Material and Methods

Ethics statement

Written informed consent was obtained from all patients. Patients aged 18 to 20 also obtained parental/guardian consent for participation. Illiterate persons were requested to provide a thumbprint witnessed by a person independent of the study staff. The studies were approved by the Rwandan National Ethical Committee (RNEC).

Study population

Chronically HIV-1-infected individuals were recruited from two sources:

1. The voluntary counseling and testing (VCT) program at the outpatient clinic of the Treatment and Research AIDS Center (TRAC/Plus) in Kigali between November 2007 and September 2009. The recruitment took place in the frame of the SEARCH (Side Effect and Reproductive Health in a

cohort on HAART) study examining the impact of highly active antiretroviral treatment (HAART) on various aspects of reproductive health in HIV-infected men and women and the incidence of clinically important adverse effects of HAART. HIV testing was done by First Response Rapid Test (Premier Medical Corporation, India) and Uni-Gold Rapid Test (Trinity BiotechPlc, Ireland), with Capillus HIV-1/HIV-2 Rapid Test (TrinityBiotech Plc, Ireland) as the tie-breaker. Rapid test-positive results were confirmed by Murex HIV Ag/Ab Combination ELISA (Abbott Laboratories, Germany). Individuals testing positive for HIV-1, clinically stable and immediately eligible for ART according to the Rwandan national guidelines[26] were asked to participate in the study. Other inclusion criteria included being ART-naïve; residing, and planning to reside within travel distance from the TRAC clinic; willing and able to adhere to study protocol and able to give informed consent for enrolment in the study. For each patient eligible for ART, the chronic character of infection (i.e infection older than 180 days) was retrospectively confirmed by the presence of any of the following: CD4 count <350 cells/mL, WHO stage ≥II or first positive HIV serology more than six month ago. The main exclusion criteria were: not immediately eligible for ART, not ART-naïve, any clinical suspicion or any laboratory diagnosis of active tuberculosis, no evidence for chronic HIV-1 infection or being pregnant. Previous use of ARV for PMTCT was not an exclusion criterion. Patients were retrospectively selected for this study based on their completion of the 12 month visit post initiation of therapy and the availability of plasma samples for genotypic analysis.

2. A cross-sectional HIV testing survey amongst women with a high risk for HIV through sexual exposure, i.e individuals having exchanged sex for money at least once in the last month and/or currently having sex with multiple partners plus having sex at least twice per week. Study participants were recruited at Projet Ubuzima, a not-for-profitorganization for medical research, with an onsite research clinic and laboratory in Kigali, Rwanda and are described elsewhere[27,28]. Samples from participants who tested positive for HIV using the testing algorithm described above, were further investigated using the capture enzyme immunoassay (BED) (CalypteH Biomedical Corporation, Oregon, US)[29] and AxSYM Avidity Index method (Ax–AI) (Abbott, USA)[30,31]. The BED assay measures the ratio of HIV-specific immunoglobulin (IgG) antibody to total antibody; a low proportion indicates infection within the past 155 days, i.e., recent infection (95% confidence interval (CI): 146–165) The Ax-AI method measures the "avidity" or the strength of the HIV antibody-antigen bond; avidity is weak among individuals infected during the past 180 days (i.e., recent infection). The BED and the Ax-AI assay testing in these patients were performed according to the manufacturers' instructions and have been described elsewhere[24]. Patients that were simultaneously positive with the BED and the Ax-AI assays (i.e, not compatible with recent infection) were considered as chronically infected. Patients that were simultaneously negative with the BED and the Ax-AI assays were considered as recently infected.

Recently HIV-1-infected individuals were recruited from the cross-sectional survey described above and selected in two ways:

1. Women who tested ELISA positive in the cross-sectional survey, that were concordant negative for both the BED and the Ax-AI assay and hence classified as recent infections. Only non pregnant women were selected.

2. Women who tested ELISA negative in the cross sectional HIV testing survey described above. HIV negative women that were not pregnant were eligible to participate in prospective observational HIV incidence cohort described elsewhere[24,25]. Negative plasma were pooled and tested by HIV RNA PCR (Cobas Amplicor) to exclude HIV acute infection. Study participants returned for quarterly follow-up visits for one year and then for a single visit during the second year of follow up. All women were receiving counseling and they were screened for HIV during their visits, using the algorithm described above. Women that seroconverted during the study were confirmed by PCR test; as well their previous follow-up sample was re-checked to confirm negative status. This certified seroconversion within a three month period, hence recent infection.

Demographic data

Age, gender, marital status, information on the sexual behavior and previous use of PMTCT were collected from all study participants at study entry. A clinical examination to screen for the presence of HIV symptoms was also done.

Laboratory testing

HIV-DR genotyping was done at the Department of Medical Microbiology of the Academic Medical Center of the University of Amsterdam. All the other laboratory tests were conducted at the National Reference Laboratory in Kigali

Blood Samples

For the chronically infected individuals recruited from the VCT site and eligible for ART, the analysis was done on baseline samples, i.e collected just before the initiation of ART. For the chronically infected women recruited from the cross sectional survey, the analysis was done on the sample collected at the time long-term infection was diagnosed. For the recently infected persons identified from the cross sectional survey, the analysis was done on the sample collected at the time recent infection was diagnosed. For the recently infected women selected from the longitudinal HIV incidence study, the laboratory investigations were done on a sample collected within six month after seroconversion

Five mL of whole blood was collected in EDTA vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ). Fifty µL of fresh blood was used for CD4+ T cell enumeration. Subsequently and within four hours after sample collection, plasma was separated from the cellular fraction by centrifugation and collected into three aliquots of one mL. Plasma was stored at -80°C until further analysis.

Transportation of frozen plasma to the Netherlands was done according to international standards of packaging ,shipping and IATA recommendations.

CD4 count

CD4+T lymphocytes counts were measured in whole fresh blood on a single flow-cytometry platform using TruCOUNT® tubes on a FACScalibur instrument (Becton Dickinson, San Jose, CA, USA), according to manufacturer's instructions.

HIV-1 viral load

Plasma HIV-1 RNA (pVL) quantification was performed on thawed plasma using the Roche CobasAmpliPrep/Cobas TaqMan HIV-1 test (Roche Molecular Systems, France), according to the manufacturer's instructions. The lower limit of detection was 40 copies of HIV RNA/ml.

HIV-1 DR genotyping

HIV-1-DR genotyping was performed using the FDA-licenced ViroSeq genotyping kits (Abbot Molecular Inc, IL, USA) and an ABI automated sequencer (Applied Biosystems, Carlsbad, CA, USA), according to the manufacturer's recommendations. The quality of the RT and protease gene sequence data was assessed using the Sequence Quality Assessment of the Stanford University HIV Drug Resistance Database (http://sierra2.stanford.edu/).

The PR and RT sequences were screened for mutations associated with drug resistance using the ViroSeq v5.4 software. After editing, data were submitted to the Stanford University database and categorized according to the WHO list of mutations for surveillance of transmitted drug resistant HIV strains (2009 update)[32].

Phylogenetic analysis

Sequences were aligned with the CLUSTAL W sequence alignment tool implemented in BioEdit Sequence Alignment Editor Version 7.0.9. The alignments were manually adjusted to preserve in-frame insertions and deletions. Phylogenetic analyses were performed with the MEGA5 software package distributed by Sudhir Kumar, Arizona State University, Tempe (MEGA 5 software package available at: www.megasoftware.net. (accessed February 8, 2011). Distances were estimated with the Kimura 2 method. Phylogenetic trees were generated with the neighbour-joining method and bootstrap resampling with 1000 replicates, using HIV-1 group M (A to K, CRF01-AE, and CRF02-AG) reference sequences from the Los Alamos database. Bootstrap values >80 were considered significant.

Sequences that could not be assigned to an HIV-1 subtype in the phylogenetic analysis were defined as unclassified (U) and further investigated for possible recombination events. Bootscanning analysis was performed in order to identify the presence of recombination breakpoints using SimPlot (version 3.5.1), a window size of 200 nucleotides, a step size of 20, and the Kimura (two-parameter) distance model. Putative parental strains of subtypes A and C were selected from our own data. The

outgroup strains subtype B, D, F1 and G were selected from the Los Alamos HIV database. The same sequences were analysed with the REGA HIV-1 subtyping tool on the Stanford database to confirm the recombination events.

From the phylogenetic trees we examined transmission events (defined as bootstrap values = 100) within our cohort as well as phylogenetic links (defined as bootstrap values >80) of the Rwandan strains with similar HIV-1 subtypes and subtype recombinants circulating in the five neighbouring countries. For this purpose, reference sequences derived from the five countries surrounding Rwanda were selected from Los Alamos HIV sequence database (Los Alamos National Laboratory HIV Databases [http://hiv-web.lanl.gov]. All available pol fragments with a length of at least 1280 nucleotides, a known collection date and a classification into subtypes and inter subtype recombinant relevant for the Rwandan context, were retrieved from the database. Only one sequence per single individual was selected. For practical reasons, the analysis was done in three separate phylogenetic trees with sets of reference sequences of the same genotype(s).

Statistical analysis

Baseline characteristics were reported as percentages for categorical data. The normality of data distribution was assessed by the Kolmogorov Smirnov test. Continuous variables were reported as mean with standard deviation or median with interquartile range (IQR) or range. The Pearson chi-square test was used to investigate whether the distribution of categorical data differed between groups. For numerical data, differences between groups were assessed using the student-*t* test or ANOVA test (for multiple groups) in case of normal distribution. For data not normally distributed, differences between groups were analyzed using the Mann-Whitney U test or the Kruskall Wallis for more than two groups.

Results

Study population

Two hundred and eighteen patients were identified with HIV infection and were immediately eligible for ART at the VCT site (Figure 1). Of these, 158 completed their month 12 follow up visit and fulfilled the criteria for long term infection based on the presence of any of the following: CD4 count <350 cells/mL, WHO stage ≥II or first positive HIV serology more than six months ago and as described in the method section. Paired VL data at baseline and month 12 as well as adequate volume of plasma for the laboratory investigations were available in 118 patients. One hundred and eleven out of 118 plasma subjected to HIV-DR genotyping yielded a PCR product and were included in the analysis.

Eight hundred high risk women participated in the cross-sectional survey at Projet Ubuzima. One hundred and ninety two women had a positive HIV serology and 190 were tested in parallel with the BED and Ax-AI assays (Figure 1). One hundred and twenty one were classified as long-term infections

based on simultaneous positivity with the two serological assays. Twenty three were classified as recent infections based on simultaneous negativity with the two assays. Forty-six samples with discordant results with the BED/Ax-AI algorithm were categorized as indeterminate and excluded from the analysis. Out of the 121 long term infections, five patients had adequate volume of plasma available for HIV-DR genotyping, had a PCR product generated and were all included in the analysis. Thirteen patients out of the 23 recent infections had adequate volume of plasma available for HIV-DR genotyping, which all yielded a PCR product.

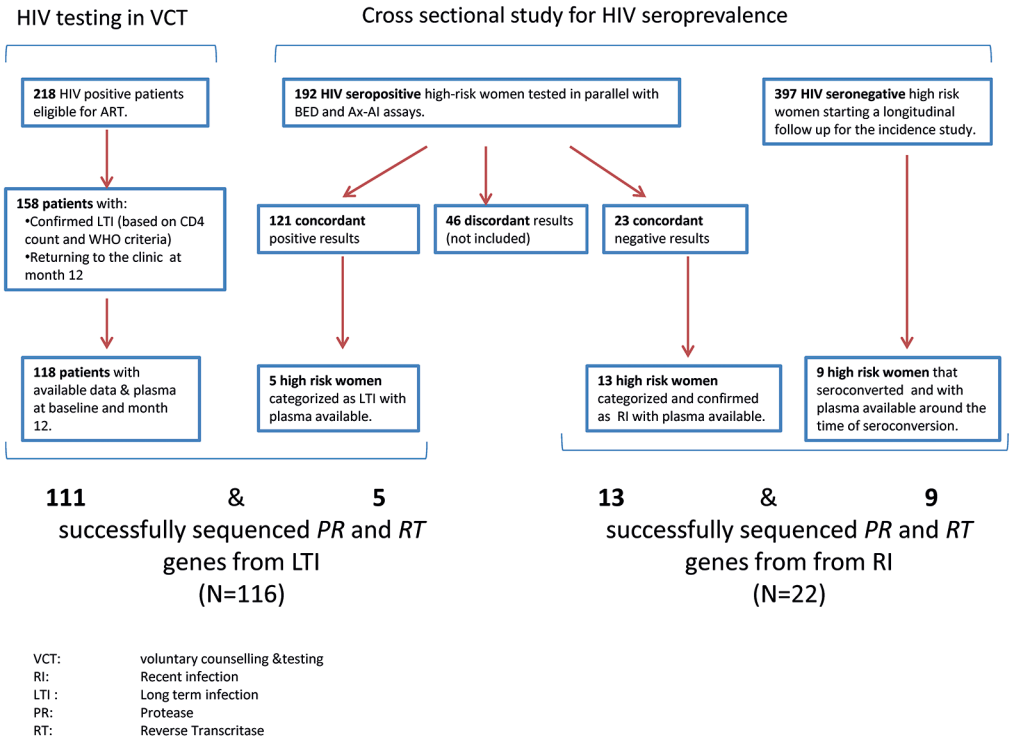

Figure 1 | Selection of recently and chronically infected participants

Left: chronically-infected patient were selected from two sources:
•118 of 218 HIV patients eligible for ART and identified through VCT, were selected for HIVDR genotyping based on the completeness of the month 12 follow up visit, the viral load data and the availability of plasma. For seven samples, a pol sequence could not be generated during HIV-DR genotyping.
•from the cross sectional survey, five of 121 female participants classified as long-term infections had plasma available for HIVDR genotyping. In total, 116 HIV sequences of chronically infected participants were available for the analysis.
Right: out of the patients participating in the cross sectional survey, 13 recent infections based on concordant negative results of the BED/Ax-AI assays were identified and had enough plasma available for HIVDR genotyping. Nine seroconverters were identified from the subsequent longitudinal incidence study and tested for HIVDR genotyping. In total, 22 pol sequences could be generated from recently infected individuals.

Of the 800 high risk women participating in the cross-sectional survey, 397 were confirmed HIV negative and were eligible for the subsequent longitudinal HIV incidence study[27]. Nineteen of these participants seroconverted during the two years follow-up (Figure 1). Nine seroconverters had an adequate volume of plasma available within a six-month period from seroconversion to conduct HIV-DR genotyping (Figure 1) and a PCR product was obtained for all samples.

A total of 116 pol sequences from chronically infected subjects and 22 HIV-1 pol sequences from recently infected individuals were included in the analysis.

Comparison between chronically and recently infected participants

The clinical and demographic characteristics of the 116 chronically and 22 recently infected subjects are displayed in Table 1. The data indicate that among the chronically infected group, men and women had comparable characteristics except for weekly income, which was significantly higher for men (median US$ 21 versus 8, $p = 0.006$) and for marital status showing a higher proportion of married (median = 65.3 versus 33.8%) and a lower proportion of widow(er)s (median = 15.2 versus 38.5%) among men as compared to women ($p = 0.009$). Although chronically infected individuals (men and women) reported HIV positive partners (56.8%) more frequently than recently infected women (4.8%, $p<0.001$), only 63% of them had knowledge about the HIV status of their partner compared to 100% of the women in the high risk group. ttehir paofOverall, recently infected high-risk women differed from the chronically infected population with respect to most of the demographic parameters reported, reflecting the difference in social status and behaviors of this group as compared to the general population. Significantly higher CD4 count (median = 648 versus 221 cell/mL, $p<0.001$) substantiated the earlier stage of infection of recently infected women as compared to the rest of the study population. Lower plasma VL (median = Log10 4.1 versus Log10 4.8 HIV-1 RNA copies/mL, $p = 0.008$) could indicate that a majority of recent infections were included in the study posterior to the peak of viral replication, when the immune response against HIV begins to mount and prior to the re-increase of viral load during the later stage of the disease.

HIV-1 subtype classification and distribution among the Rwandan samples

In total, 111 out of 138 sequences could be assigned a pure HIV-1 subtype. Twenty seven variants could not be classified initially and were further analyzed by bootscan analyses. Out of these, 25 sequences were characterized as A1/C recombinants. Thirteen of these showed a unique recombination pattern between subtypes A1 and C, whereas similar recombination pattern were found in one group of eight and two groups of two individuals, respectively (see Figure 2 A, B, C). One of the last two sequences without an assigned HIV-1 subtype was characterized as A1/D (HENN730) recombinant and the other remained unclassified (H0172, data not shown). These results were confirmed using the REGA HIV-1 subtyping tool on the Stanford database.

The HIV-1 subtype distribution among the Rwandan samples indicated a majority of A1 (71.7%), followed by A1/C recombinants (18.1%), C (5.8%), D (2.9%), one A/D recombinant (0.7%) and one 'U' subtype (0.7%, Figure 3a).

Table 1 | Characteristics of the study population.

	Chronically infected				Recently infected	
	Men (N = 51)	Women (N = 65)	P1	Total (N = 116)	Women (N = 22)	P2
Demographics						
Age in years Mean (sd)	38.4(7.5)	36.7(8.6)	0.25	37.5(8.2)	24.9(4.4)	<0.001
Marital status (%)			0.009			<0.001*[1]
Never married	7(15.2)	15(23.1)		22(19.8)	16(72.7)	
Married	30(63.3)	22(33.8)		52(46.9)	1(4.6)	
Divorced	2(4.3)	3(4.6)		5(4.5)	4(18.2)	
Widow(er)	7(15.2)	25(38.5)		32(28.8)	1(4.5)	
Reported HIV positive partner n (%)	22(55.0)	20(58.8)	0.82	42(56.8)*2	1(4.8)	<0.001*[1]
Weekly income median USD (IQR)	21(8–33)	8(0.2–21)	0.006	12.5(4–29)	18(13.25)	0.007*[3]
Household size median (IQR)	4(3–6)	5(4.7)	0.13	5(3–6)	2(1–3)	<0.001*[3]
Education level n(%)			0.05	*[4]		0.0021
Never went to school	0	6(9.4)		6(5.3)	3(13.6)	
Some primary school	9(18.0)	10(15.6)		19(16.7)	12(54.6)	
Completed primary school	19(38.0)	12(18.8)		31(27.2)	4(18.2)	
Some secondary school	16 (32.0)	23(35.9)		39(34.2)	3(13.6)	
Completed secondary school	4(8.0)	11(17.2)		15(13.1)	0	
Post secondary school	2(4.0)	2(3.1)		4(3.5)	0	
Age at sexual debut median years (range)	19.5(18–21)	18(16–22)	0.52	18.5(17–21)	17(15–18)	0.009*[3]
Presence of HIV symptoms n (%)	21(42.0)	29(46.0)	0.18	50(44.3)	9(40.9)	0.77

	Chronically infected				Recently infected	
	Men (N = 51)	Women (N = 65)	P1	Total (N = 116)	Women (N = 22)	P2
Laboratory data						
Baseline CD4 count	168(116-267)	223(110-301)	0.14	211(110–280)	648(493–822)	<0.001*[3]
Median cell/mL (IQR)						
Baseline viral load	4.9(4.5-5.3)	4.8(4.2-5.2)	0.23	4.8(4.2–5.2)	4.1(3.6–4.6)	0.008*[3]
Median log10RNA copies/mL (IQR)						

One hundred and sixteen chronically (51 men and 65 women) and 22 recently (all women) infected persons participated in the study. Data are reported as total number and percentages (%); median and interquartile range (IQR) or mean and standard deviation (sd). P1 compares men versus women within the group of chronically infected participant. P2 compares chronically versus recently infected participants. *[1] Difference between groups was calculated using the Fisher exact test. *[2] Only 74 chronically infected participants (63.7%) were aware of the HIV status of their partner. *[3] Difference between groups was calculated using the Mann-Whitney test. *[4] Education level was reported in 114 chronically infected participants (98.2%) infected participants.

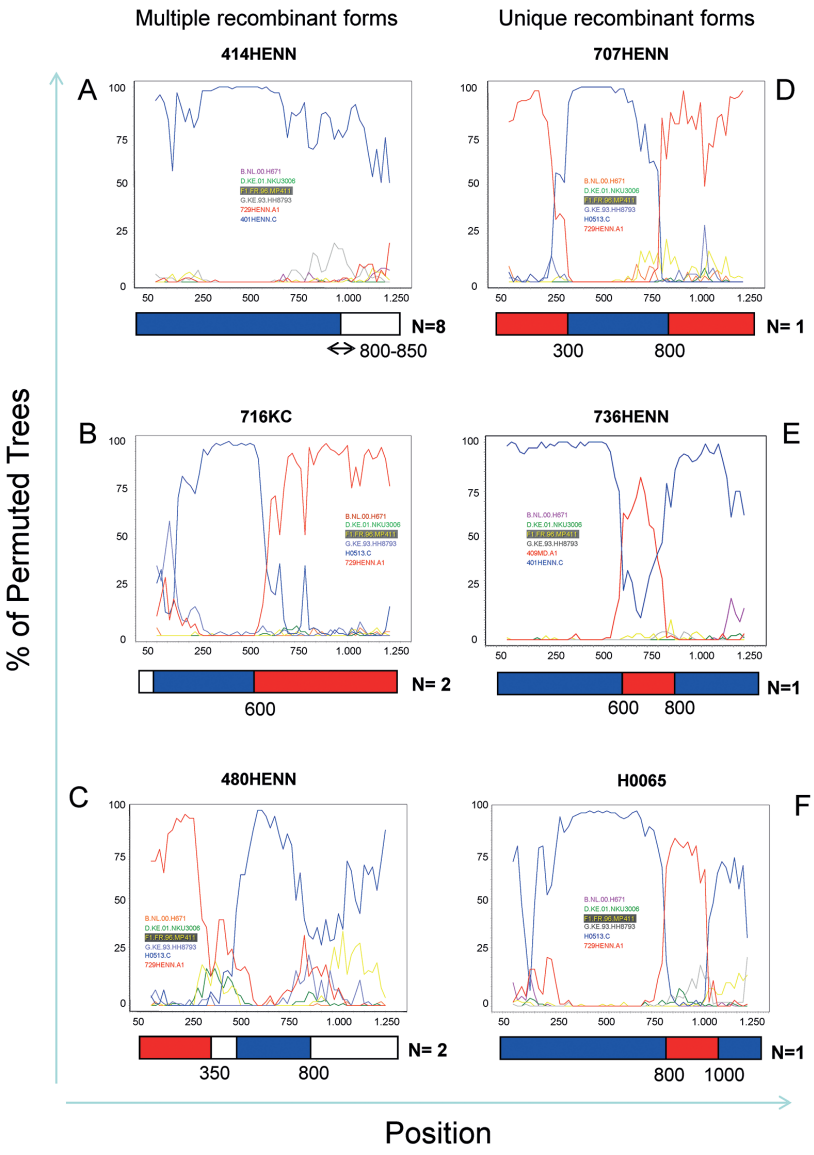

Figure 2 | Bootscan analysis of RT and PR sequences of A/C recombinants from Rwanda. Bootscan analyses of representative multiple (left side: A, B, C) and unique (right side: D, E, F) A1/C recombinant forms. The analysis was performed using SimPlot 3.5.1 configured with 1000 bootstrap replicates, 200 basepair (bp) window and a step size of 20 bp. The x-axis shows aligned nucleotides of the sequences analyzed and the y-axis shows the percentage of permuted tree, i.e. the bootstrap value. The reference strains that were used in the analysis are indicated in color. Parental reference sequences, subtype B, D, G, and F1, were selected from the Los Alamos database and parental subtype A1 (729HENN) and C (H0513) sequences were taken from this study. The breakpoint position(s) and the subtype designation is shown schematically at the bottom of each bootscan, with the number of sequences harboring a similar recombination pattern indicated in bold (e.g, figure A, N = 8). 414HENN is an example of a group of 8 similar recombinants (**A**). 716KC & 427HENN (**B**) and 480HENN & 754HENN (**C**) shared similar bootscan patterns. Graphs C to F show results for observed unique recombinant variants.

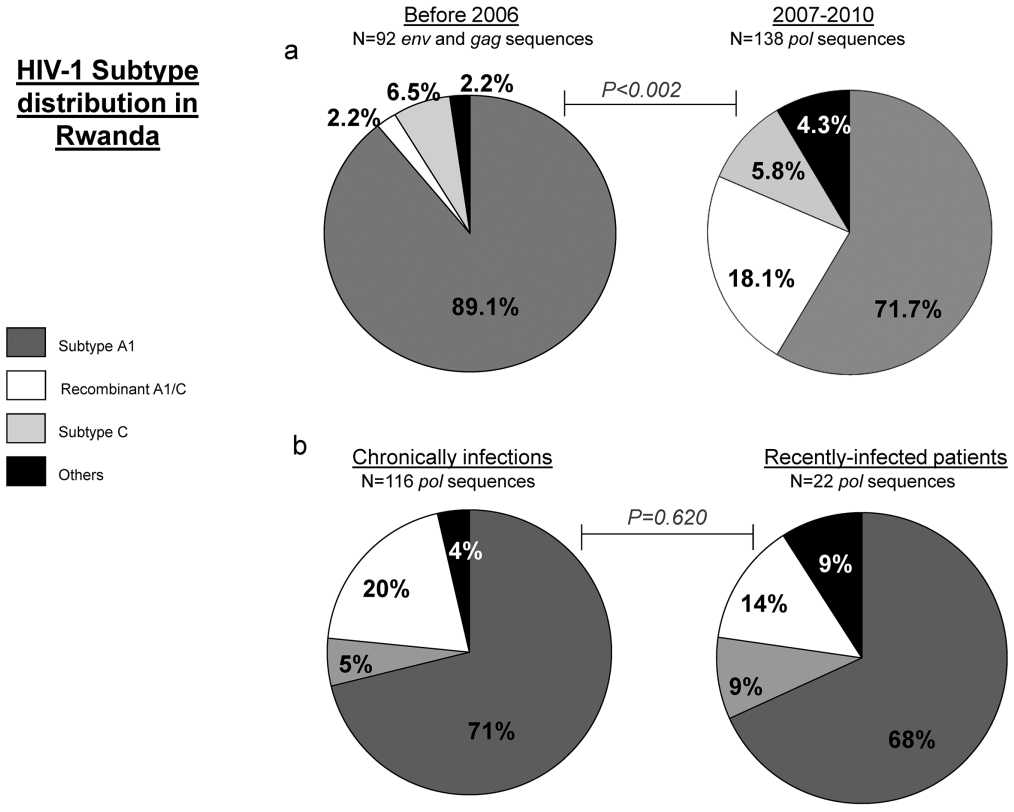

Figure 3 | HIV-1 subtype distribution in Rwanda. The pie chart on the left shows the HIV-1 subtype distribution of 92 gag and env sequences isolated between 1988 and 2006 in Rwanda and available from the Los Alamos database. The classification of subtype A into the A1 sub-subtype was not routinely done before 2006. The pie chart on the right shows the HIV-1 subtype distribution of 138 pol sequences from the chronically and recently infected study participants enrolled between 2007 and 2010. The HIV-1 subtype distribution before 2006 and in our cohort was significantly different as calculated by the Pearson Chi square ($p<0.002$). **b**) Subtype distribution in chronically infected study participants (on the left) and recently (on the right) infected participants enrolled in our study between 2007 and 2010. No statistically significant differences in the subtype distribution were found.

HIV-1 subtypes were similarly distributed among recently as compared to chronically infected persons (Figure 3b). No chronically-infected woman from the normal risk group were identified within the population infected by subtype D, A/D recombinant and 'U'. The age distribution among each subtype indicated a lower median age among subtype D-infected (24 years) as compared to subtype A1-infected (37 years) persons, but this difference was not significant ($p = 0.053$, data not shown).

In order to determine whether changes in subtype distribution occurred over time, we analyzed the subtype distribution of HIV sequences available from the Los Alamos database and isolated before the initiation of the present study. A total of 92 unique sequences (80% env and 20% gag sequences) collected in Rwanda between 1988 and 2006 were selected from the Los Alamos database. The subtype distribution showed a large predominance of subtype A and A1 (89.1%) and modest frequencies of subtype C (6.5%), A/C recombinants (2.2%) and others subtypes (2.2%, Figure 3a). Proportions of HIV-1 subtypes within HIV sequences isolated before 2006 were significantly different from the distribution observed in our cohort (Pearson Chi-square $p<0.002$), mainly showing a spread of A1/C (18.1% in 2010 versus 2.2 % before 2006) recombinant strains over the parental subtype A (71.7% in 2010 versus 89.1% before 2006), while the proportion of C strains remained relatively stable (5.8 % in 2010 and 6.5% before 2006).

Frequency of transmitted HIV drug resistance mutations in the Rwandan cohort

Of the 138 sequences analyzed, four (2.9%) showed evidence of transmitted HIV drug resistance mutations (data not shown). Two sequences presented NNRTI mutations only and two sequences had simultaneous NNRTI and NRTI mutations. Two variants with HIV drug resistance mutation were classified as subtype A1 and two as subtype C and three out of the four sequences were derived from women with no self-reported history of PMTCT. All four sequences with HIV drug resistance mutations were derived from the chronically infected patients.

Relationships between viruses infecting the Rwandan cohort

The relationship between the Rwandan HIV-1 sequences was investigated. We identified three pairs of individuals carrying related subtype A1 viruses. i.e with bootstrap value of 100 (Figure 4, zoomed inserts): 1) two chronically infected women, 2) two chronically infected men, 3) one chronically and one recently infected woman. The possibility of direct virus transmission between individuals carrying related viruses was not supported neither by phylogenetic data nor by the same gender character of the patient pairs.

Routes of HIV-1 transmission between Rwanda and neighboring countries

The possibility of exchange of HIV-1 variants between the neighboring countries and Kigali was examined by comparing the Rwandan pol sequences with sequences from the five neighboring countries selected from the Los Alamos database. Eight hundred and sixty-six pol sequences collected between 1992 and 2009 were included in the analysis. The number, origin and sampling year of the reference sequences are depicted in Table 2. The analysis was done separately for 1) subtype A or A1& C; 2) subtype D and 3) A1/C recombinants.

In the first analysis no country-specific clusters were found for subtypes A1 and C variants. The Rwandan sequences were distributed throughout the reference sequences from the neighboring countries with no evidence for direct relationships (Figure 4).

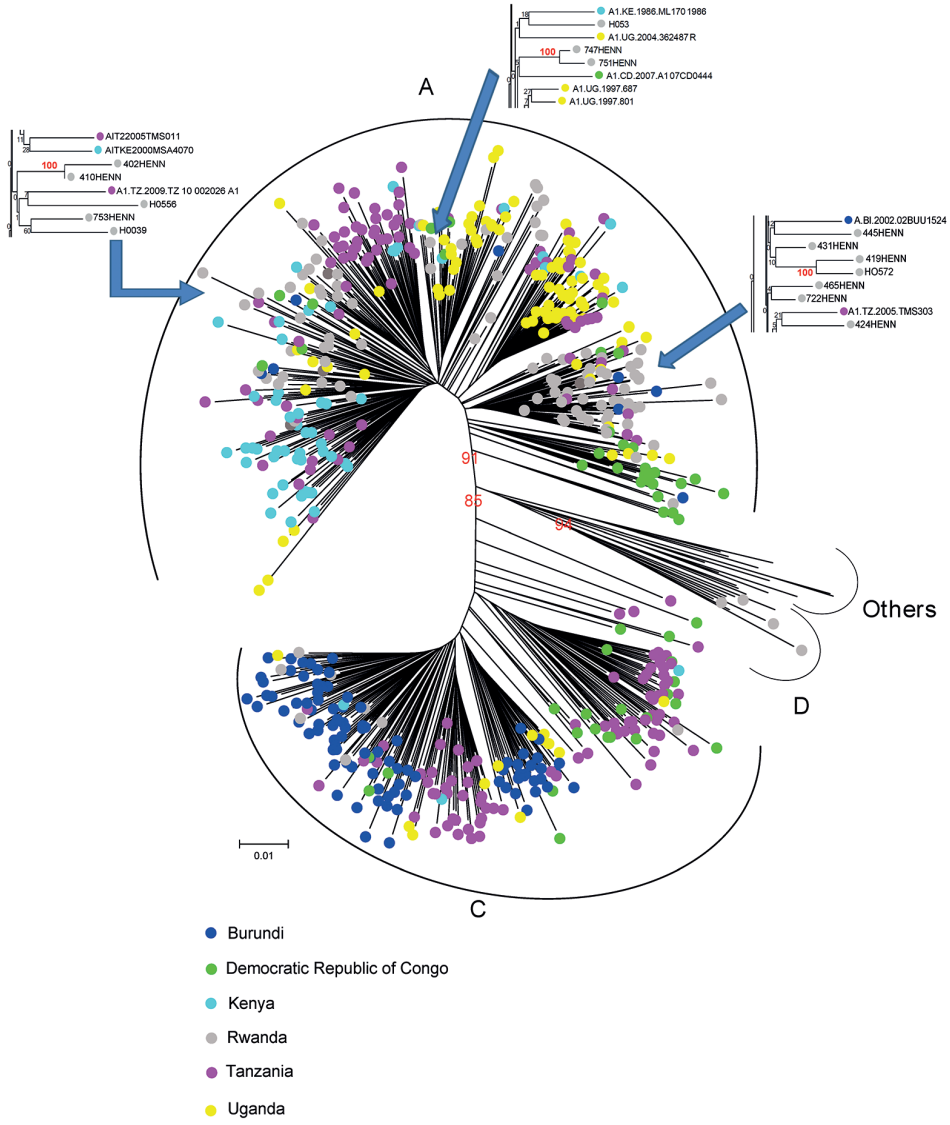

Figure 4 | **Phylogenetic tree for subtype A1 and C**. HIV-1 A1 (N = 94) and C (N = 8) pol sequences obtained from the Rwandan study participants were analyzed in connection to reference sequences from the Los Alamos database originating from Rwanda (N = 4) and the five surrounding countries collected between 1992 and 2009 (272 A1 and 212 C). The four subtype D (analysis 2, data not shown) and the 25 A1/C recombinant sequences were further examined in two separate analyses including 376 subtype D reference sequences and 6 A1/C reference sequences, respectively (analysis 3 data not shown). Country of origin of each sequence is color coded, with Rwanda in grey. The bootstrap values >85% are indicated in red. Related sequences found within subtype A1 (bootstrap value 100) are shown in detail in the three rectangular phylogenetic tree zoomed inserts.

Table 2 | Reference pol sequences from the Los Alamos database included in the phylogenetic analyses

	HIV subtypes							
	A or A1		C		D		A1/C	
Countries	**N**	**Sampling year(s)**	**N**	**Sampling year(s)**	**N**	**Sampling year (s)**	**N**	**Sampling year (s)**
Burundi	8	2002	92	2002	2	2002	0	
Democratic Republic of Congo	29	1997,2002 2007	23	2002, 2007	20	1983–1985, 1997, 1998 2002, 2007	1	2002
Kenya	55	1986, 1994 1997, 1999–2002 2004–2007	3	1991, 2000, 2004	11	1993, 1996 1999, 2001, 2006	0	
Rwanda	4	1992, 1993	0		0		2	1992
Tanzania	83	1997, 2001, 2005 2007–2009	82	1997, 1998, 2001, 2002, 2005, 2007–2009	64	2001, 2005, 2007-2009	2	2005
Uganda	93	1985, 1992, 1997–1999, 2004–2006	12	1997, 2002, 2004	276	1991, 1994, 1997–2005	1	2003
Total	**272**		**212**		**376**		**6**	
	<——————————>				<——————>		<——————>	
	Analysis 1 (Figure 4)				Analysis 2 (data not shown)		Analysis 2 (data not shown)	

1480 n long references sequences of the subtype identified in the Rwandan cohorts were selected from the Los Alamos database. The table indicates the HIV-1 subtypes, respective number of sequences, country of origin and year of sampling. In earlier years subtype A was not yet subdivided into sub-subtype A1. The phylogenetic analysis was done in three separate trees involving subtype A or A1 and C (analysis 1 also shown in Figure 4), subtype D (analysis 2) and A1/C recombinant (analysis 3).

The phylogenetic analysis of the four Rwandan subtype D and the 25 A1/C recombinant variants identified in our cohort did not show evidence of clustering with the reference sequences from the Los Alamos database (data not shown).

Thus, Rwandan strains sequences could not be linked to any of the strains isolated from the surrounding countries included in the analysis. No time-associated trend could be identified.

Discussion

Our aims were to gain insight into the situation and the trends of the current HIV-1 epidemic in Rwanda. We set out to describe the genetic subtype distribution of HIV-1 strains infecting ART-naïve patients from Kigali and estimate its evolution over time in connection with the HIV-1 epidemics of the five surrounding countries.

Our data suggest that while subtype A1 remains the predominant HIV clade circulating in Kigali, the distribution of HIV-1 subtypes has evolved from the situation previous to 2006. Notably, we found a higher percentage of pol A1/C recombinant viruses in our cohorts, as compared to the frequencies observed in env and gag sequences isolated in Rwanda before 2006 and available from the Los Alamos data base. Very few pol sequences derived from sub-Saharan African patients have been generated during the pre-ART era. Although subtype classification might be discordant in different parts of the genome, comparing gag and env to pol sequences provides an approximation of changes in HIV-1 subtypes distribution over time. The indication of a possible shift in HIV-1 subtype distribution in Kigali was not corroborated by any significant differences in HIV-1 subtype distribution between the groups of recently versus chronically infected persons. The false recent rate associated with the combined BED/Ax-AI algorithm in the present study population is reportedly low (2.1%)[27], with no expected ambiguity in the characterization of recent and long term infections in our study. However, the actual times of infection for each patient might still be too adjacent, with the lack of clear-cut differences in the subtypes of viruses infecting the two groups. Alternatively, the changes in HIV-1 subtype distribution over time could be related to some biases in the selection of HIV sequences in our study and those available in the Los Alamos database, which were not primarily chosen to be representative of the HIV-1 distribution in Rwanda at a particular time point. Interestingly, the trends towards an expansion of A1/C recombinants confirmed the results of Servais et al on a group of 43 HIV-1 infected pregnant women recruited in Kigali in 2000[21].

The four subtype D strains identified in our cohort were shown to be associated with relatively younger individuals, as compared to other subtypes. Two of these patients were male and two were females from the high risk behavior groups, with no clear self-reported socio-demographic links. Although younger age represents a correlates of new HIV infections[33], the possible implication of subtype D in relatively more recent infections remains speculative, given the limited number of subtype D infections identified in this study.

The further characterization of recombination events in our cohort suggested at least two mechanisms underlying the increased frequency of A1/C recombinants. On one hand, the identification of three similar patterns of recombination in 12 patients suggests that A1/C variants spread through de novo infections. On the other hand the characterization of 13 unique recombination patterns indicates that random recombination events within the pol gene are ongoing upon co-infections of individual patients with parental A1 and C strains. The possibility of an accidental emergence of A1/C recombinants from circulating A1 and C parental strains seems unlikely given the relatively low

frequency of C strains in Rwanda from 1992 (6.5%) throughout 2010 (5.8%). Moreover, the apparent spread of A1/C recombinants in our cohort contrasts with the epidemiological evidence for the dominant spread of subtype C strains over the other HIV variants in Africa, India, China and South America[34]. A growing body of evidence suggests that the asymmetric spread of certain HIV subtypes in some regions of the world is associated with a superior replicative fitness and higher efficiency of transmissibility[35-39]. A1/C recombinant strains are reported to be emerging in South Africa where subtype A1 is rare[40]. Interestingly a unique A1/C recombinant was also described in Canada, closely related to another A1/C variant from Rwandan origin[41]. Altogether, these observations suggest that A1/C recombinant strains may display biological advantages over their parental A1 and C strains. Further research is needed to assess the epidemiological significance of A1/C recombinants.

Although the study participants were all coming from Kigali and its surroundings the analysis did not support neither a common origin nor the possibility of any direct transmission of the HIV subtypes and subtype recombinants identified in the cohorts. Three pairs of participants with potential epidemiological links were identified but no large networks of transmission could be documented within the present data set. Clusters of HIV transmission reflect a "founder effect" which is commonly observed within specific risk groups such as intravenous drug users (IDUs) or men who have sex with men (MSM)[42-45]. In populations where HIV transmission occurs mainly through heterosexual contacts like in Rwanda, evidence for transmission clusters is generally more limited[46]. This could be related to the existence of complex sexual networks, but also to sampling issue. Study populations recruited in areas of relatively high HIV prevalence are often sparsely sampled (albeit being representative) with a reduced probability of including patients belonging to the same transmission network(s) and a bias toward the underreporting of infection clusters[42].

The lack of close relationship between the HIV sequences from patients in our cohort highlights the complexity of HIV dissemination networks, which represents a challenge for HIV prevention. In addition, our data underscores further social barriers to HIV control. First, only half of the study participants knew the HIV status of their partner(s). Second, there was no partnership history amongst the study participants. This suggests that HIV status remained frequently undisclosed within relationships and that partners do not tend to seek treatment in the same health care facility. Our observations are in accordance with previous reports from Rwanda and other sub Saharan countries, emphasizing the fear of partner reaction and an overall stigma associated with HIV infection in the community[47]. In a context where the majority of HIV transmission occur between co-habiting partners[48], this observation has important implications for HIV prevention and care.

The ART programme was initiated in Rwanda in 2004. The level of baseline HIVDR mutations under 5% is in the range of what has been described in countries where ARVs are available for a comparable duration of time[49-51]. This low frequency of transmitted HIV is an indirect indicator of the correct use of ART in Rwanda[52], confirming the virologic efficacy of the first line recommended regimen[24]. The data do not provide evidence of exchange of HIV strains harboring drug resistance mutations with Uganda, a current hot spot of emerging HIV-DR[23].

Given the frequent population movements traditionally taking place in the Eastern African region[6-8], we postulated that migration might (have) play(ed) a role in the past and present HIV epidemic in Rwanda. We did not find evidence that any of the HIV variants identified in our cohort clustered with the corresponding subtypes or subtype recombinants from the five near-by countries. The possibility that subtype D strains have been recently introduced from Uganda where it largely circulates[53,54] to Kigali was not substantiated by bootstrap analyses, involving no less than 276 Ugandan reference D strains. With Kigali being the largest city of the country and with the highest HIV-1 prevalence5 our study population might illustrate the situation of HIV diversity at the national level, although inference of the results to the situation at the national level needs to be drawn with caution given the limited size of our sample. Notwithstanding, our observations suggests the notion of a fenced, independent evolution of the Rwandan epidemic with little influence of cross border migration events. However, given the uneven availability of up-to-date pol sequences from East and Central Africa in the Los Alamos database, our results may have been biased. Therefore, the existence of genetic links between HIV strains from Rwanda and surrounding countries cannot totally be ruled out.

In conclusion, the data presented here indicate that in Kigali, the HIV-1 epidemic is mainly driven by A1 variants, evolves toward a clear spread of A1/C recombinants and a possible minor emergence of subtype D strains among younger individuals. The present set of data did not show evidence of a major influence of population migrations in and out the five main surrounding countries on the current HIV epidemic in Rwanda. Future phylodynamic analyses of high-density population samples may be envisaged as contemporary HIV-1 sequences from Rwanda and surrounding countries become increasingly available. These studies will contribute to a more highly-defined description of HIV-1 transmission networks in Rwanda.

Our report highlights the relatively low level of primary HIV-DR mutations in the country suggesting the good performance of the ART program in Rwanda. However, the data also indicate that national HIV prevention strategies may benefit from more focus on health education and behavioral interventions that include promotion of couples' counseling, testing and disclosure.

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CHAPTER 5

High seroprevalence of HBV and HCV infection in HIV-infected adults in Kigali, Rwanda

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Abstract

Background: Data on prevalence and incidence of hepatitis B virus (HBV) and hepatitis C virus (HCV) infection in Rwanda are scarce.

Methods: HBV status was assessed at baseline and Month 12, and anti-HCV antibodies at baseline, in a prospective cohort study of HIV-infected patients in Kigali, Rwanda: 104 men and 114 women initiating antiretroviral therapy (ART) at baseline, and 200 women not yet eligible for ART.

Results: Baseline prevalence of active HBV infection (HBsAg positive), past or occult HBV infection (anti-HBc positive and HBsAg negative) and anti-HCV was 5.2%, 42.9%, and 5.7%, respectively. The active HBV incidence rate was 4.2/1,000 person years (PY). In a multivariable logistic regression model using baseline data, participants with WHO stage 3 or 4 HIV disease were 4.19 times (95% CI 1.21−14.47) more likely to have active HBV infection, and older patients were more likely to have evidence of past exposure to HBV (aRR 1.03 per year; 95% CI 1.01−1.06). Older age was also positively associated with having anti-HCV antibodies (aOR 1.09; 95% CI 1.04−1.14) while having a higher baseline HIV viral load was negatively associated with HCV (aOR 0.60; 95% CI 0.40−0.98). The median CD4 increase during the first 12 months of ART was lower for those with active HBV infection or anti-HCV at baseline. Almost all participants (88%) with active HBV infection who were on ART were receiving lamivudine monotherapy for HBV.

Conclusion: HBV and HCV are common in HIV-infected patients in Rwanda. Regular HBsAg screening is needed to ensure that HIV-HBV co-infected patients receive an HBV-active ART regimen, and the prevalence of occult HBV infection should be determined. Improved access to HBV vaccination is recommended. Active HCV prevalence and incidence should be investigated further to determine whether HCV RNA PCR testing should be introduced in Rwanda.

Introduction

In sub-Saharan Africa, 65−98% of the population will have lifetime exposure to hepatitis B virus (HBV) and 8−20% will become a chronic carrier[1]. The predominant modes of transmission are perinatal and horizontal (in early childhood), but transmission via unprotected sexual intercourse or intravenous drug use in adults also occurs[2,3]. The proportion progressing from acute to chronic HBV is primarily determined by the age at infection: approximately 90 percent for perinatal infection, 20−50 percent for early childhood infection, and less than 5 percent for adult infection[4]. Hepatitis C virus (HCV) is a parenterally transmitted virus[5]. Sexual and vertical transmission of HCV is considered inefficient but co-infection of HIV and HCV increases the risk of perinatal transmission of either virus[5].

Between 2 and 20% of HIV-positive individuals in sub-Saharan Africa are also infected with HBV[6-8]. The consequences of co-infection are increased liver-related morbidity and mortality, increased viral replication of either virus and, in the context of antiretroviral therapy for HIV (ART), immune reconstitution inflammatory syndrome (IRIS) and hepatotoxicity[9,10]. In recent years, lamivudine, emtricitabine, and tenofovir have been approved for the management of HIV/HBV co-infection. This raises a number of possibilities and concerns related to the management of these infections. Studies have demonstrated that anti-HBV-active ART makes it possible to achieve suppression of HBV replication in a significant number of co-infected patients[11]. On the other hand, the HBV status of most HIV patients in Africa is not known, which means that many are unknowingly receiving mono-therapy for HBV infection in the context of their ART for HIV. Mono-therapy with lamivudine has been shown to induce HBV resistance in 24% of HBV mono-infected patients after one year, increasing to 71% after 5 years of treatment[12]. Moreover, resistance to lamivudine confers partial or complete cross-resistance to the HBV inhibitors emtricitabine, telbivudine and entecavir, thus limiting future treatment options for HBV infection. In contrast, an ART backbone of tenofovir/emtricitabine or tenofovir/lamivudine could be expected to suppress HBV replication effectively while inducing less HBV resistance[13].

HIV and HCV co-infections are likely to be less common in sub-Saharan Africa due to the differences in transmission routes but only limited data are available[14]. HIV infection, alcohol consumption, and older age at the time of HCV infection have been shown to be associated with a higher rate of liver fibrosis progression[4]. Treatment for HCV with pegylated interferon is rarely available in sub-Saharan African public health care settings.

Data on the prevalence and incidence of HBV and HCV infection in Rwandan HIV-infected adults are scarce. One study among HIV-infected pregnant women in 2007 found a seroprevalence of 2.4% for active HBV and 4.9% for anti-HCV antibodies[15]. Since 2011, the Rwandan government recommends that all HIV patients co-infected with HBV receive an ART regimen containing tenofovir and lamivudine or emitricitabine[16] but HIV patients are not routinely tested for HBV. We observed HIV patients receiving care at a public HIV clinic in Kigali, Rwanda, between 2007 and 2010, and documented various behavioral, clinical, and laboratory endpoints (the SEARCH study). Within this

context, we determined the prevalence and determinants of active and past HBV infection and anti-HCV antibodies and the incidence of active HBV infection, assessed the effect of HBV and HCV infection on HIV disease progression, and described the ART regimens that patients with active HBV co-infection are currently receiving.

Methods

The SEARCH study (Side Effects and Reproductive Health in a Cohort on HAART) was a prospective cohort study in Kigali, Rwanda, to evaluate ART adherence and outcomes, and the impact of ART on various aspects of reproductive and sexual health, including HBV and HCV. The SEARCH study was conducted at the HIV outpatient clinic of the Center for Treatment and Research on AIDS, Tuberculosis and Malaria (TRAC-Plus), which is now part of the Institute of HIV/AIDS, Disease Prevention and Control (IHDPC) within the Rwanda Biomedical Center. Ethical approval was obtained from the Rwandan National Ethics Committee. All study participants provided written informed consent prior to enrollment, were free to withdraw from the study at any time, and continued to receive care within publicly-funded HIV treatment programs at the end of their study participation.

Study design and population

The SEARCH study was designed to enroll 100 women and 100 men initiating ART and 200 women who did not yet qualify for ART according to the Rwanda Ministry of Health guidelines because they had a CD4 count higher than 350 cells/μl and were asymptomatic[16]. All eligible patients attending the TRAC-Plus clinic between November 2007 and January 2010 were offered enrollment into the study. Patients were followed up for 6 to 24 months, until the study was closed in August 2010. Only data collected during the first 12 months of follow-up are presented in this paper. The study visit schedule followed the national ART program schedule as much as possible so that study participants could easily be transferred from SEARCH to the national ART program when the study ended. Women not eligible for ART were seen at baseline and 3-monthly intervals thereafter, and they were switched to the ART cohort when they became eligible for ART. For patients initiating ART, clinic visits were scheduled at baseline (ART initiation) and at week 2 and months 1, 2, 3, 6, 9, and 12 after ART initiation. In addition, pharmacy visits were scheduled monthly.

The study population consisted of ART-naïve HIV patients, 18 years or older, seeking care at the TRAC-Plus clinic. ART eligibility was determined by a TRAC-Plus committee according to the Rwanda Ministry of Health guidelines and clinic protocols. Study eligibility criteria also included residing within travel distance from the TRAC-Plus clinic, and being willing and able to adhere to the study protocol and give informed consent. The main exclusion criteria were pregnancy and diagnosis or clinical suspicion of tuberculosis.

Study procedures

All participants were interviewed regularly throughout the study to collect information on demographics, sexual and contraceptive behavior, nutritional status, medical and reproductive history, use of medications, and symptoms (with a focus on those potentially related to HIV infection, ART use or reproductive health problems). Clinical assessments were also carried out at most visits and included a general physical exam, a speculum exam in women, and targeted assessments for neuropathy and lipodystrophy. Blood samples were collected at all clinic visits. They were hand-carried from the clinic to the laboratory next door within 4 hours after collection. CD4+ T-cell counts on EDTA blood samples were always done on the day of sample collection. All other samples were centrifuged to separate plasma from blood cells, aliquoted and stored at -80°C on the day of sample collection until further processing and testing.

All laboratory tests relevant to this paper were carried out at the National Reference Laboratory in Kigali, Rwanda. At study enrollment, the presence of HIV infection in each study participant was confirmed using a 4th generation HIV ELISA. CD4+ T cell counts (FACSCalibur, Becton Dickinson, San Jose, CA, USA) were done every 3 months for women who did not yet qualify for ART and every 6 months for those initiating ART. Plasma HIV RNA viral loads (COBAS AmpliPrep/COBAS TaqMan HIV-1 Test versions 2.0, Roche Molecular Diagnostics, Pleasanton, CA, USA) were done at ART initiation and every 12 months thereafter. The lower limit of detection was 40 HIV RNA copies/ml.

An hCG urine pregnancy test was done every 6 months and testing for various sexually transmitted infections (STIs) every 6 or 12 months. Herpes simplex type 2 (HSV-2) serology was done every 12 months using HerpeSelect test kits (Focus Diagnostics, Cypress, CA, USA). The liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (COBAS Integra Chemistry Analyzer, Roche Diagnostics, Mannheim, Germany) were determined at all visits post ART initiation.

HBV and HCV testing was conducted on all available baseline and Month 12 specimens after data collection had been completed and the study had been closed. About 4 mL plasma in EDTA Vacutainer tubes had been stored at -80°C. After thawing, specimens were tested for HBV surface antigen (HBsAg) using the Murex HBsAg version 3 kit, anti-HBV core antibody (anti-HBc) using the Murex anti-HBc total kit, and anti-HCV IgG antibody (anti-HCV) using the Murex anti-HCV version 4 kit (for all: Abbott Murex, Dartford, UK). However due to a kit shortage, 38 specimens were tested on the Abbott ARCHITECT i2000/ SR (Abbott Diagnostics, Lake Forest, IL, USA) using test kits with similar specificity and sensitivity as the Murex kits. All assays were performed according to manufacturer instructions. HBV DNA PCR and HCV RNA PCR were not available in Rwanda at the time of the study. Participants with clinically relevant abnormal test results for HBV and HCV were referred to Kigali University Teaching Hospital, Department of Gastro-enterology for clinical follow-up.

Statistical analysis

Data were analyzed using STATA version 11.0 (StataCorp, College Station, TX, USA). Stem and leaf plots and Shapiro-Wilk tests were used to investigate the normality of data distribution. The Pearson Chi-square test, student's *t*-test or ANOVA test were used to test for differences between groups in the case of normally distributed data, and non-parametric tests were used otherwise.

A positive test for HBsAg, regardless of anti-HBc test result, was interpreted as active HBV infection (acute or chronic). A positive test for anti-HBc in combination with a negative test for HBsAg is referred to as a past infection in this manuscript, but we could not differentiate past infections from occult infections. Any exposure to HBV was defined as a positive test for anti-HBc with or without a positive HBsAg test. A positive test for anti-HCV antibodies was interpreted as evidence of past or ongoing HCV infection. Incident HBV infection was defined as having a positive HBsAg test at Month 12 among participants with a negative HBsAg test at baseline. Virological failure to ART was defined as having more than 40 HIV RNA copies/mL 12 months after ART initiation.

Potential determinants of HBV infection were assessed in bi- and multivariable models using multinomial logistic regression with active HBV infection (HBsAg positive regardless of anti-HBc result), past HBV infection (anti-HBc positive and HBsAg negative), and never having been HBV exposed (negative for both) as the outcome variable. Potential determinants of anti-HCV antibodies were assessed in bi- and multivariable binary logistic regression models. In all multivariable models, potential determinants were selected based on evidence from published studies (age, gender), Akaike's information criteria, and a p-value of less than 0.2 in bivariable models. No evidence for collinearity was found in least squares regression models containing the same variables as our logistic regression models (all variance inflation factors were below 2). Sensitivity analyses were conducted to determine the robustness of the models by excluding participants who were anti-HCV positive at baseline from the analyses with HBV as the outcome. This did not alter any of our conclusions and results are therefore not presented.

Results

Four hundred and eighteen participants were enrolled into this study: 104 men and 114 women initiating ART, and 200 women who were not yet eligible for ART. A total of 16 participants were excluded from the baseline analyses due to erroneous enrollment or missing blood specimens or test results (Figure 1). During follow-up, 4 participants died, 13 transferred from the pre-ART to the ART group, 11 were lost to follow-up, 103 had not yet reached the Month 12 visit when the study was terminated, and 18 had missing HBV test results at Month 12. The analysis sample therefore consisted of 402 participants at baseline, and 253 of them (63%) had HBV test results at 12 months of follow up.

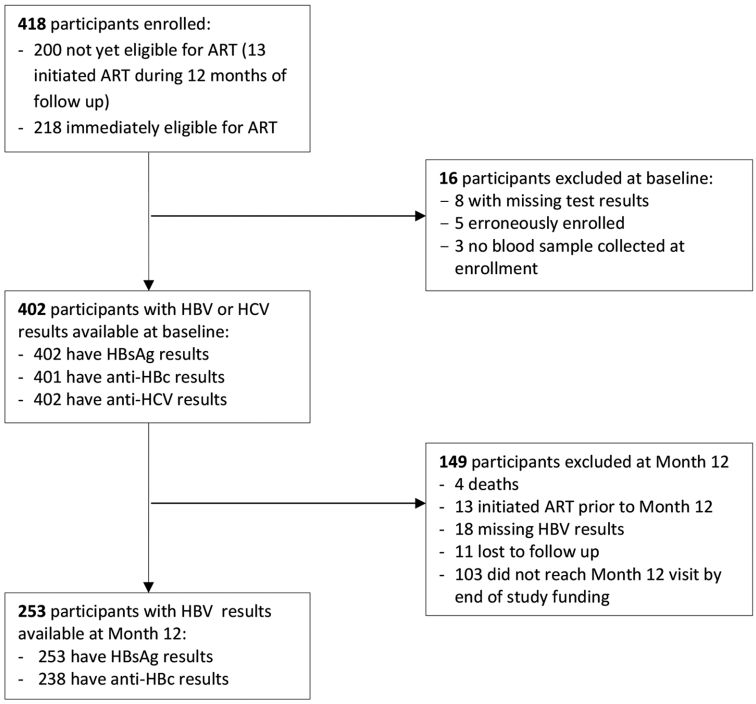

Figure 1 |

Baseline characteristics

The mean age of study participants was 34.6 years, and those who had never been exposed to HBV were significantly younger than those who had been exposed to HBV or HCV (Table 1). More than half (56.9%) of the participants were married, and most had attended primary (50.2%) or secondary (35.8%) school. Eight percent of the participants reported to have two or more sexual partners, 47.0% used a condom during their last vaginal sex act, and 38.1% regularly consumed alcohol (defined as alcohol use on more than 3 days per week) during the previous 6 months. Injection drug use was not reported by anyone.

Most participants had a normal body mass index (BMI; 57.3%) and those with an abnormal BMI were more often overweight or obese (32.9%) than underweight (9.8%). The majority of participants (85.8%) had positive HSV-2 serology. Most participants were in WHO clinical stage 1, but the distribution of clinical stage varied significantly between the HBV and HCV outcome groups (see Table 1 and further). All participants who initiated ART at baseline and who had a Month 12 follow-up visit were given a first-line regimen that contained lamivudine but not tenofovir, which was in agreement with the national treatment guidelines at that time (Table 1). Participants initiating ART had a baseline median CD4 count of 222 cells/µl (interquartile range (IQR) 142–289) and a median

HIV RNA viral load of 4.81 log10 copies/ml (IQR 4.22–5.33). Participants not initiating ART had a baseline median CD4 count of 513 cells/µl (IQR 413–651) and a median HIV RNA viral load of 3.69 log10 copies/ml (IQR 2.92–4.41).

Table 1 | Baseline characteristics of HIV-infected participants by HBV and HCV status

Baseline Characteristic	Active HBV n (% of 21)	Past HBV n (% of 172)	Never Exp to HBV n (% of 208)	HCV n (% of 23)	Total n (% of 402)
Mean age yrs (SD)[1]	37.6(6.7)	35.5(8.7)	33.6(7.5)	40.9(9.7)*	34.6(8.0)
Gender					
Male	7(33.3)	47(27.3)	44(21.2)	4(17.4)	99(24.6)
Female	14(66.7)	125(72.7)	164(78.8)	19(82.6)	303(75.4)
Marital status[1]					
Never married	2(9.5)	17(10.1)	19(9.3)	1(4.5)	38(9.6)
Married	8(38.1)	96(56.8)	120(58.8)	10(45.4)	224(56.9)
Divorced	5(23.8)	26(15.4)	26(12.8)	6(27.3)	57(14.5)
Widowed	6(28.6)	30(17.7)	39(19.1)	5(22.7)	75(19.0)
Educational level[1]					
None	2(10.5)	15(8.9)	22(10.7)	4(18.2)	39(9.9)
Primary school	6(31.6)	87(51.5)	104(50.7)	11(50.0)	198(50.2)
Secondary school	10(52.6)	58(34.3)	73(35.6)	6(27.3)	141(35.8)
Post secondary school	1(5.3)	9(5.3)	6(3.0)	1(4.5)	16(4.1)
Median weekly income in USD (IQR)	12.5(2.1–20.8)	8.3(0–20.8)	6.3(0–16.7)	6.3(0–12.5)	8.3(0–20.8)
Median household size (IQR)	6(4–7)	5(3–7)	4(3–6)	6(4–8)	4(3–6)
Median age at sexual debut in years (range)[1]	18(12–31)	18(13–30)	18(6–30)	18(14–30)	18(6–31)
≥2 sexual partners last 6 mo[1]	0(0.0)	18(11.2)	14(7.0)	1(4.4)	32(8.4)
Condom use last vaginal sex	11(55.0)	83(49.7)	91(44.2)	6(26.1)	185(47.0)
Regular alcohol use[2]	11(52.4)	68(40.5)	71(34.5)	5(21.7)	151(38.1)
Body mass index (BMI)[1]					
Normal (18.5–24.9 kg/m^2)	9(47.4)*	83(51.6)*	123(62.8)*	11(50.0)	216(57.3)
Underweight (<18.5)	3(15.8)	21(13.0)	13(6.6)	1(4.5)	37(9.8)
Overweight (25–29.9)	2(10.5)	40(24.8)	41(20.9)	8(36.4)	83(22.0)
Obese (>= 30)	5(26.3)	17(10.6)	19(9.7)	2(9.1)	41(10.9)
Positive HSV-2 serology	16(76.2)	150(87.7)	176(85.0)	22(95.7)	343(85.8)

Baseline Characteristic	Active HBV n (% of 21)	Past HBV n (% of 172)	Never Exp to HBV n (% of 208)	HCV n (% of 23)	Total n (% of 402)
WHO clinical stage[1]					
Stage 1	10(50.0)*	118(69.8)*	157(77.3)*	12(52.2)	285(72.5)
Stage 2	5(25.0)	38(22.5)	28(13.8)	7(30.4)	71(18.1)
Stage 3	4(20.0)	12(7.1)	17(8.4)	4(17.4)	33(8.4)
Stage 4	1(5.0)	1(0.6)	1(0.5)	0(0.0)	4(1.0)
ART groups3					
No ART	5(23.8)	78(45.3)	113(54.3)	10(43.5)	196(48.8)
AZT/D4T, 3TC, NVP/EFV	14(66.7)	72(41.9)	79(38.0)	10(43.5)	166(41.3)
TDF, 3TC, NVP/EFV	2(9.5)	22(12.8)	16(7.7)	3(13.0)	40(9.9)
Median CD4 count cells/µl (IQR)	276(195–487)	332(230–509)	389(224–535)	359(220–493)	359(221–518)
Median log10 HIV RNA copies/mL (IQR)	4.41(3.97–5.23)	4.24(3.25–4.91)	4.38(3.58–5.00)	4.01*(2.49–4.61)	4.31(3.43–4.99)

* p<0.05 comparing the 3 HBV groups or comparing anti-HCV positive versus negative
[1] Missing values. Age: n = 397; marital status: n = 394; educational level: n = 394; age at sexual debut: n = 393; sexual partners in last 6 months: n = 379; BMI: n = 377; WHO clinical stage: n = 393.
[2] Regular alcohol use was defined as alcohol use more than 3 times per week during the last 6 months
[3] AZT = zidovudine; D4T = stavudine; 3TC = lamivudine; NVP = nevaripine; EFV = efavirenz; TDF = tenofovir diphosphate.

Prevalence and incidence of HBV and HCV

The baseline prevalence of active HBV was 5.2% (21/402; 95% CI 3.0−7.4), past HBV 42.9% (172/401; 95% CI 38.0−47.8) and never having been exposed to HBV 51.9% (208/401; 95% CI 47.0−56.7). Of the 21 cases of active HBV at baseline, 6 were HBsAg-negative at Month 12, 10 were still HBsAg-positive, and 5 were not retested. Between baseline and Month 12, one new case of active HBV was detected, giving an incidence rate of 4.2/1,000 person years of follow-up (PY) (95% CI 0/1,000–12.5/1,000 PY). The baseline prevalence of anti-HCV was 5.7% (23/402; 95% CI 3.44−8.0%). At baseline, 3 participants tested positive for both HBsAg and anti-HCV and 12 for both anti-HBc and anti-HCV.

Baseline determinants of HBV and HCV infection

In the multivariable model, participants with active HBV infection presented with a more advanced WHO clinical stage (stage 3 or 4 compared to stage 1; adjusted RR 4.19; 95% CI 1.21−14.47) than participants who had never been exposed to HBV (Table 2). Participants with past HBV infection were more likely to be older than those who had never been exposed to HBV with a 3% increased risk for each year increase in age (adjusted RR 1.03; 95% CI 1.01−1.06). Baseline CD4 counts and HIV viral loads were not significantly different between the three HBV outcome groups (Tables 1 and 2).

In the multivariable HCV model, a year increase in age led to a 10% increased odds of being anti-HCV positive (adjusted OR 1.10, 95% CI 1.05−1.17) (Table 2). A more advanced WHO clinical stage was positively associated with anti-HCV (stage 3 or 4 compared to stage 1; adjusted OR 4.05; 95% CI

1.12−14.58) but a higher baseline HIV viral load was negatively associated with anti-HCV (adjusted OR 0.60; 95% CI 0.40−0.98).

Table 2 | Determinants of HBV and HCV infection at baseline

	Crude[1]			Adjusted[2]		
	RR	**95% CI**	**p**	**RR**	**95% CI**	**p**
Active HBV (n = 21) compared to never exposed (n = 208)						
Age in years	1.06	(1.01–1.22)	0.03	1.05	(0.98–1.12)	0.10
Female vs male	0.54	(0.20–1.41)	0.20	0.65	(0.19–2.22)	0.49
WHO stage 2 vs 1	2.80	(0.89–8.82)	0.08	2.27	(0.63–8.19)	0.20
WHO stage 3,4 vs 1	4.36	(1.34–14.18)	0.01	4.19	(1.21–14.47)	0.02
Baseline CD4 cells/µl 200−350 vs >350	1.63	(0.54–4.95)	0.39	0.83	(0.23–3.13)	0.79
<200 vs >350	2.13	(0.70–6.54)	0.18	0.88	(0.22–3.47)	0.85
Past HBV (n = 172) compared to never exposed (n = 208)						
Age in years	1.03	(1.00–1.06)	0.03	1.03	(1.01–1.06)	0.02
Female vs male	0.71	(0.44–1.44)	0.16	0.79	(0.44–1.40)	0.42
WHO stage 2 vs 1	1.81	(1.05–3.11)	0.03	1.70	(0.95–3.06)	0.08
WHO stage 3,4 vs 1	0.96	(0.45–2.04)	0.92	0.81	(0.37–1.79)	0.60
Baseline CD4 cells/µl 200−350 vs >350	1.36	(0.84–2.21)	0.21	1.14	(0.66–1.96)	0.63
<200 vs >350	1.25	(0.73–2.14)	0.42	0.95	(0.50–1.82)	0.90
Anti-HCV (n = 23) compared to no anti-HCV (n = 379)						
Age in years	1.10	1.04–1.16	<0.001	1.10	1.05–1.17	<0.001
Female vs male	1.56	0.53–4.78	0.41	1.45	0.43–4.92	0.55
WHO stage 2 vs 1	2.49	0.94–6.57	0.06	2.49	0.87–7.19	0.09
WHO stage 3,4 vs 1	2.76	0.84–9.04	0.09	4.05	1.12–14.58	0.03
Baseline HIV RNA (log10 copies/ml)	0.68	0.48–0.95	0.03	0.60	0.40–0.98	0.01

[1] The following variables were also considered for their association with HBV and HCV but were not statistically significant: multiple sexual partners, age at sexual debut, condom use, weekly income, regular alcohol use, positive HSV2 serology, and baseline HIV viral load. BMI was not included in any of these models because of the high likelihood of reverse causality. [2] The multivariable models contained all variables listed in the table for each outcome.

ART regimens taken by HIV-HBV co-infected patients

Among those with active HBV at baseline (n = 21), 16 were in the group that initiated ART immediately: 14 initiated an ART regimen containing lamivudine but not tenofovir and 2 initiated a regimen including tenofovir. Only the 14 patients not using tenofovir were retested at Month 12: 4 were HBsAg-negative and anti-HBc positive at Month 12 and 10 developed persistent infection. One person switched to tenofovir at the Month 12 visit but this switch was related to drug toxicity and not to the patient's HBV status. Of the 5 HIV-HBV co-infected patients who did not initiate ART at

baseline, only 2 were retested at Month 12. Both of them were HBsAg-negative and anti-HBc positive at Month 12.

Effects of HBV and HCV on liver enzymes and HIV disease progression

Liver enzymes were monitored only after participants had initiated ART. No statistical differences in median ALT and AST levels at baseline and Month 12 were found between the different HBV and HCV outcome groups (Table 3).

Among those who had initiated ART, participants with active HBV at baseline achieved a median CD4 increase of only 28 cells/µl/year while participants who had never been exposed to HBV achieved a median increase of 113 cells/µl/year ($p = 0.04$). There was no statistically significant difference in the median CD4 increase between participants who had been infected with HBV in the past (127 cells/µl/year) and those who had never been exposed (113 cells/µl/year). Among those who had initiated ART, 69% of participants with active HBV or past HBV exposure at baseline achieved full HIV viral load suppression (defined as HIV RNA levels below 40 copies/ml) after 12 months of ART compared to 83% of the participants who had never been exposed to HBV ($p = 0.11$). In a multivariable analysis controlled for baseline CD4 count and baseline HIV viral load, participants with active HBV infection or past exposure to HBV at baseline had 2.40 increased odds of virological failure compared to non-HBV exposed participants (OR 2.40; 95% CI 1.04−5.51).

Participants with anti-HCV at baseline had a slower CD4 count recovery rate of 58 cells/µl/year compared to participants without anti-HCV (126 CD4 cells/µl/year; $p = 0.07$). HIV virological failure did not differ significantly between participants with anti-HCV (60%) and those without (76%; $p = 0.26$).

Discussion

The prevalence rates of active HBV infection and anti-HCV in this cohort of HIV patients seeking care in an urban HIV clinic in Rwanda were high at 5.2% and 5.7%, respectively. Almost half of all patients (42.9%) had ever been exposed to HBV. These rates are slightly higher than those reported in 2007 for HIV-positive pregnant women in Rwanda (2.4% for active HBV and 4.9% for anti-HCV)[15]. High rates have also been found in other African settings[17-24]. In fact, several African studies have found even higher rates for HBsAg prevalence in HIV-positive patients than we have[22-24].

The major limitation of our study is that we could not identify occult HBV infections in those who were HBsAg-negative but anti-HBc-positive by HBV DNA PCR testing, and that we could not identify active HCV infections among those positive for anti-HCV by HCV RNA PCR testing. Occult active HBV infections are thought to be common among HIV patients, with reported prevalence rates ranging from 10−14%[25-27] to 89% in one study in South Africa[20]. Given the fact that 42.9% of HIV patients in Rwanda had isolated anti-HBc, further research is needed to determine the prevalence of occult HBV in this group. Similarly, further

Table 3 | Liver enzymes in HBV and HCV infected patients initiating ART1

	Active HBV		Past HBV		Never Exposed to HBV		HCV
	Baseline n = 16	M12 n = 4	Baseline n = 81	M12 n = 52	Baseline N = 81	M12 n = 62	Baseline n = 11
Median ALT (IQR)	23.5(16.8–40.9)	15.6(10.8–24.7)	19.3(13.0–24.7)	23.2(16.3–32.6)	22.0(15.5–32.9)	21.2(15.4–31.0)	31.6(20.6–45.6)
Grade 12	1(6.3)	0	0	1(1.9)	2(2.5)	1(1.61)	0
Grade 2	0	0	0	0	1(1.2)	0	0
Grade 3	0	0	1(1.2)	0	0	0	0
Grade 4	0	0	0	0	0	0	0
Median AST (IQR)	32.1(27.4–52.2)	21.4(18.7–25)	26(21.2–34.7)	26.2(21.1–32)	27.9(22.8–36.4)	27.1(21–32.1)	28.8(23.3–59.4)
Grade 12	1(6.3)	0	1(1.3)	1(2.3)	1(1.3)	1(1.83)	0
Grade 2	0	0	0	0	1(1.3)	0	0
Grade 3	0	0	1 (1.3)	0	0	0	0
Grade 4	0	0	0	0	0	0	0

[1] ALT and AST only collected for patients initiating ART (n = 210). [2] According to the DAIDS Table for Grading the Severity of Adult and Pediatric Adverse Events (version December 2004): Grade 1 (1.25−2.5 ULN), Grade 2 (2.51−5 ULN), Grade 3 (5.1−10 ULN), Grade 4 (>10 ULN).

research is needed to determine the prevalence of active HCV infection among those with anti-HCV. About 25% of HCV infections clear spontaneously (resulting in a positive anti-HCV test but a negative HCV RNA PCR test)[28]. Furthermore, while anti-HCV testing has been used extensively to estimate HCV prevalence and incidence, recent studies comparing antibody tests with HCV RNA PCR suggest that false-positive antibody test results are common in African settings[29]. Unfortunately, routine confirmation of positive anti-HCV test results with HCV RNA PCR is expensive and not feasible in many African settings. Larger studies designed to determine the sensitivity and specificity of HCV assays, and to evaluate affordable diagnostic algorithms, in African settings are urgently needed.

Other limitations of our study included the fact that ALT and AST were only evaluated in patients after they had initiated ART and the fact that 37% of the patients had less than 12 months follow-up due to study closure. A strength of our study is that we worked within a public HIV care setting and are therefore reporting results that are relevant to local policymakers.

In our study, older age was associated with past exposure to HBV infection and with exposure to HCV infection. This most likely reflects ongoing exposure over time, as has been reported by others[17,21]. Gender was, however, not associated with active HBV, past HBV or anti-HCV in our study, which is in contrast with several other studies that have shown strong associations with male gender[17,19,20,23]. This could be explained by the fact that our study oversampled women by design and did not include any injecting drug users. We did not find any associations between HBV and sexual risk behaviors, which is also in contrast with other studies[1,2,18]. In our cohort, sexual risk taking was modest, with 8.2% of the participants reporting multiple partners and 47.0% reporting to have used a condom during the last sex act. We did not ask the male participants whether they have had sex with other men.

WHO clinical stage 3 or 4 at baseline was associated with a higher likelihood of having an active HBV or anti-HCV at baseline, but while the median baseline CD4 count was lower among those with active HBV (but not among those with anti-HCV), this was not statistically significant. However, having active HBV or anti-HCV at baseline were both associated with a slower CD4 count recovery rate during follow-up. This has also been found in a study in Ghana [30], but not in studies in South Africa, Nigeria, and Denmark[31-33]. Surprisingly, having a detectable HIV RNA viral load at baseline was less likely in those who had HCV antibodies at baseline. This has not been confirmed by other studies[34], and should be interpreted with caution because we only conducted anti-HCV testing and did not confirm infections with HCV RNA PCR. HIV virological failure after 12 months of ART was more likely in those with active HBV at baseline but not in those with anti-HCV. Reports in the literature about virological failure rates in those with and without active HBV are conflicting[31-33]. Finally, we did not find any statistical differences in median ALT and AST levels between the different HBV and HCV outcome groups, but the comparison groups were small. Reports in the literature in this area are also conflicting[20-22,30,31]. Additional carefully designed studies investigating the impact of HBV and HCV co-infection on ART response and on hepatotoxicity are therefore needed.

During the first 12 months of the study, almost all participants on ART were using a regimen containing lamivudine but not tenofovir, which was in accordance with the Rwanda antiretroviral treatment guidelines for HIV infection prior to the revision in 2011. Of the participants with HIV and active HBV co-infection who were on ART, 88% were receiving lamivudine monotherapy for their HBV infection. Studies have shown that treatment with two anti-HBV-active drugs simultaneously reduces the emergence of drug resistance[11-13].

In conclusion, active HBV infection and anti-HCV are both common in HIV-infected patients in Rwanda and are associated with a slower rate of CD4 recovery in the context of HIV-suppressive ART. Our study confirms the importance of screening HIV patients in Rwanda for HBsAg to ensure that HIV-HBV co-infected patients receive an HBV-active ART regimen. The prevalence of occult HBV infection should be determined and improved access to HBV vaccination considered. Active HCV prevalence and incidence should be investigated further to determine whether HCV RNA PCR testing should be introduced in Rwanda.

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CHAPTER 6

Hepatitis B virus prevalence and vaccine response in HIV-infected children and adolescents on combination antiretroviral therapy in Kigali, Rwanda

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Abstract

Objective: The aim of this study was to determine the prevalence of hepatitis B virus (HBV) infection in a cohort of HIV-infected Rwandan children and adolescents on combination antiretroviral therapy (cART), and the success rate of HBV vaccination in those children found to be HBV negative.

Methods: HIV-infected children and adolescents (age 8–17 years) receiving cART with CD4 T-cells count >=200 cells/mm^3 and/or >=15% and without prior HBV vaccination (by history, vaccination cards and clinic records) underwent serologic testing for past (negative HBV surface antigen [HBsAg] with positive antibody to HBV core antigen [cAb] and to HBsAg [anti-HBs]) or active HBV infection (positive HBsAg). Children with any positive HBV serologic tests were excluded from further vaccination; all others completed 3 HBV immunizations with 10 µg of ENGERIX-B. Anti-HBs titer was measured 4–6 weeks after the last immunization.

Results: Of 88 children, 6 (7%) children had active HBV infection and 8 (9%) had past HBV infection. The median (interquartile range) age, CD4 T-cell count and cART duration were 12.3 (10.1–13.9) years, 626 (503 to 942) cells/mm^3 and 1.9 (1.5–2.7) years, respectively. Seventeen children had detectable plasma HIV-1 RNA. Seventy-3 children completed 3 immunizations with median (interquartile range) postimmunization anti-HBs concentration of 151 mIU/mL (1.03–650). Overall, 52 children (71%, 95% confidence interval: 61–82) developed a protective anti-HBs response. HIV-1 RNA and CD4 T-cell count were independent predictors of a protective anti-HBs response. Protective anti-HBs response was achieved in 82% of children with undetectable HIV-1 RNA and 77% with CD4 T cells >=350/mm^3.

Conclusions: The substantial HBV prevalence in this cohort suggests that HIV-infected Rwandan children should be screened for HBV before cART initiation. HIV viral suppression and CD4 T cells >=350/mm^3 favored the likelihood of a protective response after HBV vaccination.

Introduction

HIV and hepatitis B virus (HBV) are both prevalent in sub-Saharan Africa, with between 1% and 19% of HIV-infected children reported to be coinfected with HBV[1,2]. Perinatal transmission is a major route of transmission for both HIV and HBV in children, and maternal HIV coinfection may increase the risk of perinatal HBV transmission[3,4]. HBV infection can also be acquired horizontally through close contacts and through blood products or sexual contact. The majority of children who acquire HBV early in life develop chronic HBV infection. Although the virus may be partially cleared during subsequent years, around 25% of these children will develop serious liver diseases such as cirrhosis or hepatocellular carcinoma if no treatment is given[3]. Available data in adult populations have identified HIV infection to modify the natural history of HBV infection with HIV/HBV coinfection being associated with higher HBV viral replication, a more rapid progression to chronic liver disease and a high liver-related mortality[5-9].

HBV infection in children can be prevented by HBV immunization in infancy. For infants of mothers with HBV, administration of the HBV immunization series started within 12 hours after birth and HBV immunoglobulin (HBIG) is recommended[10]. It is estimated that around 85% of HBV perinatal infections can be prevented using this strategy[5]. The high cost of HBV testing precludes routine testing of all pregnant women at all health facility levels in Rwanda. Moreover, most families often cannot afford HBIG administration.

HBV immunization was included in the Rwandan nationwide program in 2002 for all children, regardless of maternal HIV or HBV status, with the first HBV vaccination administered at 6 weeks of age. Hence most children born before 2002, including HIV-infected children, were not immunized against HBV in infancy as part of the standard program[11].

The World Health Organization recommends routine immunization of HIV-infected children with 3 doses of HBV vaccine and assumes that this standard immunization schedule confers protection[12]. However, recent data indicate that HBV immunization at infancy may be less effective in infants born to HIV/HBV coinfected mothers[13]. In addition, the response to HBV immunization in HIV-infected children and adults, especially those with severe immunosuppression, has been reported to be less effective[14-16]. Combination antiretroviral therapy (cART) might restore vaccine-induced immunity and lead to better responses to HBV immunization[15,17-20]. In sub-Saharan countries, studies evaluating the response to HBV vaccine among HIV-infected children receiving cART are limited, and hence it is currently unclear to what extent HIV-infected children receiving cART are protected by the vaccination and what vaccination schemes will be optimal.

The aim of this study was to determine the prevalence of HBV infection in a cohort of HIV-infected Rwandan children and adolescents receiving cART; and to determine the immunogenicity of the HBV vaccine series for those without HBV infection.

Methods

Setting

The study took place at the outpatient unit of the Treatment and Research AIDS Center (TRAC plus) clinic, a large research and treatment center for HIV, tuberculosis and malaria located in Kigali, Rwanda.

Study Population and Study Design

This was a prospective cohort study, carried out between February 2010 and October 2010. Inclusion criteria for the HBV prevalence portion of the study was age 8–17 years (children under 8 were excluded because as per national immunization program HBV vaccination has been introduced in 2002), and receiving cART for at least 3 months, CD4 T cells >= 200 cells/mm^3 and/or >=15%. Exclusion criteria included evidence or history of HBV vaccination and signs or symptoms for any severe illness as per clinical judgment. Previous HBV immunization status was determined by interviewing caregivers using a structured questionnaire and by checking the child's immunization card (when available), and reviewing patient files for immunization records. First line cART regimen consisted of 2 nucleoside reverse transcriptase inhibitors plus 1 non-nucleoside reverse transcriptase inhibitor. Generally abacavir or zidovudine and lamivudine were combined with nevirapine or efavirenz (children age >3 years). Protease inhibitors were reserved for second-line treatment unless the child had been exposed to single-dose nevirapine for prevention of HIV mother-to-child transmission[21].

For the HBV vaccine response portion of the study, lack of previous HBV immunization or exposure was an additional inclusion criterion. To determine previous exposure to HBV (vaccine-related or otherwise), all children underwent 3 hepatitis B tests: HBV surface antigen (HBsAg), antibody to HBV core antigen (anti-HBc) and antibody to HBsAg (anti-HBs).

Positive HBsAg was considered evidence of active infection (acute or chronic), negative HBsAg but presence of anti-HBc and anti-HBs as evidence of past infection, and presence of anti-HBs only as evidence of previous HBV immunization.

Children with active or past HBV infection or HBV vaccination were excluded from the HBV vaccine immunogenicity portion of the study (Figure, Supplemental Digital Content 1, http://links.lww.com/INF/B344). All children underwent a clinical examination and a blood sample was drawn for HBV serologic markers, CD4 T-cell count and plasma HIV-1-RNA concentration. Children with no history of previous HBV immunization received the first HBV vaccination. Once the results of the HBV markers were known, children with no evidence of active or past HBV infection and no history of previous HBV immunization were scheduled for the second and third immunization. Children who were positive for HBsAg, anti-HBc or anti-HBs were excluded from further immunization and analysis (Figure, Supplemental Digital Content 1, http://links.lww.com/INF/B344).

Children who were positive for any HBV marker underwent confirmatory HBV serologic testing; a negative result on the confirmatory test was used to classify the initial result as a potential false positive; these patients continued to be excluded from the vaccine response portion of the study.

A blood sample was drawn 26–48 days after the third immunization for determination of anti-HBs antibodies, CD4 T-cell count and plasma HIV-RNA concentration.

Patients with potential false-positive initial results (as their HBV status was not certain) as well as those children who did not show adequate response to the initial 3 vaccinations were contacted by the clinic to receive additional HBV vaccination dose(s).

Medical records (electronic and patients files) were used to extract patient demographics and baseline data, which included date of birth, weight and height at cART initiation, CD4 counts and percentage at cART initiation, date of cART initiation and regimen. Among children with positive HBV markers, blood for the determination of liver transaminases (alanine aminotransferase [ALT], aspartate aminotransferase [AST]) was collected at the time point of confirmatory testing.

Ethical Approval

Ethical approval was obtained from the Rwandan National Ethical Committee. Written informed consent was obtained from the child's parent or guardian before any interview, clinical evaluation or laboratory test; as advised by Rwandan National Ethical Committee, assent was obtained from children aged 12 years and above.

Data Collection and Analysis

HBV Vaccination and Safety Monitoring

A 10 µg dose of ENGERIX-B was given intramuscularly in the deltoid region at baseline, and after approximately 1 month and 6 months. Children were asked to come to the clinic when HBV vaccine-related adverse events were suspected within 3 days from the date of immunization. The following complaints, if present, were recorded: irritation, swelling, itching, pain, redness, warmth and bruising at site of injection, weakness, flu-like symptoms, nausea, fever, sore throat and dizziness.

Laboratory Methods

Blood samples were collected into ethylenediaminetetraacetic acid tubes for HBsAg, anti-HBc and anti-HBs antibodies. Aliquots of serum were separated and frozen at -20°C for future HBV marker analysis. Serological markers for HBV infection (HBsAg, anti-HBc and anti-HBs) were determined using commercial enzyme immunoassays (Axsym AUSAB, Abbott Laboratories, Abbott Park, IL). An anti-HBs titer >=10 mIU/mL was considered as fully protective, and <10 mIU/mL as nonprotective. All HBV assays were performed in the Rwanda Biomedical Center Laboratory, Kigali, Rwand.

CD4 T lymphocyte counts and percentages were performed by flow cytometric measurement using FACSFlow (Becton Dickinson FACS calibur, San Jose, CA); plasma HIV-1 RNA concentration

was quantified using the Roche Cobas AmpliPrep/Cobas TaqMan HIV-1 (CTM; Roche Diagnostics, Mannheim, Germany) with a lower limit of detection of 40 (1.6 log10) copies/mL.

The absolute CD4 T-cell count was used as the standard immunological assessment. Successful viral suppression was defined as an HIV-1 RNA concentration <40 copies/mL. CD4 T-cell count, plasma HIV-1 concentration and liver transaminases were performed at the National Reference Laboratory in Kigali, Rwanda. Transaminases (ALT and AST) were measured using Cobas Integra 400 Chemistry Analyzer machine.

Statistical Analysis

Baseline characteristics were reported as percentages for categorical data, mean with standard deviation for normally distributed data and median with interquartile range (IQR) when appropriate for non-normally distributed data.

Prevalence of active and past HBV infection was presented as percentage with 95% confidence interval (CI); [chi]2 was used to determine whether there were differences in prevalence of HBV infection by gender (male versus female), CD4 count (<350 cells/mL versus >=350 cells/mL), HIV-1 RNA load (detectable versus undetectable) or nutrition indexes (weight-for-age Z score <–2 versus >=-2 and height-for-age Z score <-2 versus >=-2). Weight and height were converted to World Health Organization Z scores using the Epinfo version 3.5.1 (CDC, Atlanta, GA).

To determine the univariate association between anti-HBs responses (negative <10 IU/mL versus positive >=10 IU/mL) and other factors (age, gender, time since cART initiated, CD4 T-cell counts [<350 vs >=350 cells/mm^3] at cART initiation, CD4 T-cell counts at 1st immunization, viral load [<40 vs >= 40 copies/mL]), [chi]^2, Student's t test and Kruskal-Wallis or Mann-Whitney U tests were used, as appropriate.

Factors that were statistically significant in univariate analysis or clinically relevant as determined by the literature (eg, age, sex)[22] were used for multivariate logistic analysis in 2 statistical models. All continuous exposure variables were plotted by logit with the outcome (HBV vaccination response) to determine linear relationships (data determined to be nonlinear were transformed or categorized); additionally, we conducted correlation coefficients to determine collinearity among predictors.

Two variables, CD4 and viral load at baseline, as expected, showed collinearity and could not be added to 1 statistical model. Therefore, 2 models were conducted, 1 with viral load (<40 vs >40 copies/mL) and 1 with CD4 count (<350 versus >=350 cells/mm^3) as a predictor of HBV vaccine response. We aimed to determine which clinical factor was a better predictor of HBV vaccine response. The correlation between hepatitis B immune response and various factors was assessed by including them into the models, one by one to determine potential confounding; a change in odds ratio of >10% was considered confounding. Measures of associations were presented as odds

ratio together with 95% CIs and were considered to be significant if the corresponding 95% CI did not include one. Analyses were conducted with Stata version 10 (StataCorp LP, College Station, TX).

Results

General Characteristics and Hepatitis B Prevalence

Eighty-eight children receiving cART were included in the study; they represent respectively 15%, 31% and 56% of all children followed at the clinic (n = 600), all children receiving cART (n = 286) and all children >8 years receiving cART (n = 156) during the study period. The majority (97%; 86/88) had proven or suspected perinatal HIV infection; the mode of transmission in 2 other children, both adolescents, was unknown.

Males and females were equally represented in the study population. Eight-three (94%) were receiving a cART regimen consisting of 2 nucleoside reverse transcriptase inhibitors and 1 non-nucleoside reverse transcriptase inhibitor (62 on nevirapine versus 21 on efavirenz) whereas 5 children were receiving a regimen with 2 nucleoside reverse transcriptase inhibitors and a protease inhibitor (lopinavir/ritonavir). The median (IQR) age at study entry was 12.3 (10.1–13.9) years and the median (IQR) CD4 T-cell count at study entry was 626 (503–942) cells/mm^3. The median (IQR) CD4 T-cell count at initiation of cART was 328 (199–473) cells/mm3, and the median (IQR) duration of cART was 2.4 (1.6–4.6) years. Plasma HIV-RNA concentration was <40 copies/mL in 76% (67/84) of children. Fourteen (16%, 95% CI 10–25) of 88 children had evidence of active or past HBV infection. Six (7%, 95% CI 2–12) were HBsAg positive and 8 (9%, 95% CI 4–17) had evidence of past infection before the first vaccination.

The median (range) ALT and AST concentrations from children with active HBV infection were 64.5 (34.2–135.3) and 34.1(18.9–91.9) IU/mL, respectively. Among those children with active HBV infection, 3 of 6 (50%) had increased ALT levels >60 IU/L (upper limit: 60 IU/L) and 2 of 6 (33%) had increased AST levels >43 IU/L (upper limit: 43 IU/L) (Table, Supplemental Digital Content 2, http://links.lww.com/INF/B345). The remaining 74 children tested negative for all HBV markers before vaccination. The median (IQR) age of this group at study visit was 12.5 (10.0–13.9) years, the median (IQR) CD4 T-cell count at initiation of cART was 336 cells/mm3 (210–473 cells/mm^3) and the median CD4 T-cell count at study entry was 638 (500–955 cells/mm3). The median (IQR) time on cART for this group was 2.4 (1.6–4.6) years. Seventy-seven percent (57/74) of children had a plasma HIV-RNA concentration <40 copies/mL. When comparing children with an active HBV infection to HBV-negative children and those with past HBV infection, we did not find any significant difference in characteristics, including gender, CD4 T-cell count at cART initiation and CD4 T-cell count at study visit, plasma HIV-1 concentration or height and weight Z scores(Table, Supplemental Digital Content 2, http://links.lww.com/INF/B345).

Table 1 | Characteristics of Children According to Anti-HBs Antibody Levels After Immunization

Characteristics	Anti-HBs <10 mIU/mL (n = 21)	Anti-HBs ≥10 mIU/mL (n = 52)	P*
Age at 1st immunization (yr), median (IQR)	12.8(9.3–14.2)	11.7(10.0–13.6)	0.4
Duration of cART (yr), median (IQR)	1.9(1.5–2.7)	2.5(1.8–4.9)	0.06
Weight and height for age Z scores at 1st immunization			
Mean height for age (SD)	-1.2(0.3)	–1.5(0.1)	0.5
Mean weight for age (SD)	-1.3(0.3)	-1.5(0.2)	0.4
Immunological status, CD4 T cells/mm³; median (IQR)			
At cART initiation	298(201–401)	354(204–508)	0.2
At 1st immunization	580(346–688)	820(596–1011)	0.001
Proportion with ≥350 cells/mm³ at 1st immunization	15(23%)	50(77%)	0.001
Proportion with <350 cells/mm³ at 1st immunization	6(67%)	2(33%)	
HIV-RNA at 1st immunization, n (%)			
Proportion with HIV-1 RNA <40 copies/mL	10(18%)	46(82%)	0.001
Proportion with HIV-1 RNA >40 copies/mL	11(65%)	6(35%)	

*These analyses were completed by univariate logistic regression.
Anti-HBs indicates hepatitis B virus surface antibody; SD = standard deviation.

Table 2 | Predictors of Anti-HBs >10 mIU/mL, After Three Hepatitis B Virus Immunizations

Variables	Univariate		Multivariate model [1*]		Multivariate model [2†]	
	Odds Ratio (95% CI)	P	Adjusted Odds Ratio (95% CI)	P	Adjusted Odds Ratio (95% CI)	P
Age at 1st immunization	0.9 (0.7–1.1)	0.4	0.9 (0.7–1.2)	0.6	0.9 (0.7–1.1)	0.4
Weight-for-age Z-score	0.7 (0.2–1.9)	0.5	1.3 (0.6–2.7)	0.7	1.3 (0.6–2.7)	0.5
Height-for-age Z-score	0.6 (0.2–1.8)	0.4	0.6 (0.3–1.4)	0.2	0.6 (0.6–1.3)	0.2
CD4 T-cells ≥350 /mm³ at cART initiation	2 (0.7–5.8)	0.2	NE	–	NE	–
CD4 T-cells ≥350 /mm³ at 1st immunization	6.5 (1.5 – 29.30)	0.01	NE	–	6.0 (1.2 – 31.1)	0.03
HIV-RNA <40 copies/mL at 1st immunization	8.4 (2.5–28.2)	0.001	7.5 (2.9–28.4)	0.003	NE	–
Duration of cART‡	1.4 (0.9–1.1)	0.06	1.2 (0.8–1.8)	0.3	1.3 (0.9–1.9)	0.1

* In model 1, HIV viral load was included and CD4 excluded, adjusted for age, nutrition indexes (height-for-age and weight-for-age at 1st study visit) and cART duration.
† In model 2, CD4 was included and viral load excluded, adjusted for age, nutrition indexes (height-for-age and weight-for-age at 1st study visit) and cART duration.
‡ Duration of cART per yr increase.
NE indicates not examined.

Hepatitis B Vaccine Immunogenicity

Seventy-three children with negative HBV markers at testing completed 3 immunizations and were assessed for post immunization anti-HBs titers (Table 1). No immunization-related side effects were reported. The median (range) post immunization anti-HBs titer was 151 mIU/mL (0–1250). For this part of the study, children were subdivided in responders (anti-HBs >=10 mIU/mL, protected) and nonresponders (anti-HBs <10 mIU/mL, not protected) according to antibody levels attained after 3 doses of vaccination. Fifty-two (71%, 95% CI 61–82) children developed a protective anti-HBs response (>=10 mIU/mL) after 3 vaccination doses.

Three (33%) of 9 children with a CD4 T-cell count <350 cells before their first HBV vaccination responded adequately to the HBV vaccine as compared with 52 (77%) of the 67 children with a baseline CD4 T-cell count >=350 cells/mm^3 (P = 0.001, Table 1).

Of 17 children with a detectable viral load, 35% responded to the HBV vaccine adequately compared with 82% of the 56 children with a suppressed viral load (P = 0.001, Table 1). There was a trend for longer duration of cART for responders compared with nonresponders (2.5 versus 1.9 years, P = 0.06).

In a multivariate analysis, after adjusting for age, weight-for-age and height-for-age Z scores and cART duration, having an undetectable HIV-1 plasma concentration (in 1 model) and a CD4 T-cell count >=350 cells/mm3 (in a separate model) at first vaccination remained independent predictors of a protective anti-HBs response (Table 2). Children with a CD4 T-cell count >=350 cells/mm^3 compared with children with CD4 <350 cells/mm^3 at the time of the HBV screening visit were 6 times more likely to develop an adequate HBV immune response; and children with an undetectable (<40 copies/mL) HIV RNA concentration at the time of the HBV screening visit were 7.5 times more likely to develop a protective HBV immune response than those with detectable HIV-RNA (Table 2). No other factors were significant.

Discussion

The prevalence of active HBV infection in this cohort of HIV-infected Rwandan children and adolescents who were receiving cART for at least 3 months was 7%, whereas 9% had evidence of past HBV infection.

Although the majority of HBV-negative children and adolescents developed a protective antibody response after 3 HBV immunizations, nearly one third failed to do so. A CD4 T-cell count <350 cells/mm^3 and having detectable plasma HIV RNA concentration at the time of vaccination were found to be the main predictors of failure to respond.

Studies of the prevalence of HIV/HBV coinfection among children and adults show large variations in different geographic regions as well as in different populations. A recent study in a Tanzanian cohort

of HIV-infected children indicated that only 1.2% were coinfected with HBV, whereas studies from West Africa have reported HBV coinfection in 12% of HIV-infected children from the Ivory Coast and 19% in a cohort of Nigerian children[1,2,23].

The risk of developing a chronic HBV infection is strongly related to the age at acquisition. More than 90% of infants who are infected perinatally and 25%–50% of children infected between 1 and 5 years of age develop chronic HBV infection[3]. HBV infection in our cohort is likely perinatal or horizontal, but information about maternal HBV status and longitudinal HBV testing to assess timing of HBV infection in the children is not available to confirm the relative contributions of these sources of pediatric HBV infection. The prevalence of active HBV infection in our cohort was significantly higher than the 2.4% found in Rwandan HIV-infected pregnant women in 2008 from the same geographical location area[24], but significantly lower than the prevalence reported from samples collected between 2001 and 2006 in Rwandan HIV-infected women, the majority of whom were pregnant, who showed a prevalence of HBsAg as high as 37%[25].

Knowledge of HBV co-infection in HIV-infected children and adolescents is important for clinicians to determine which antiretroviral treatment best be prescribed. Pediatric guidelines advise lamivudine as a component of first line cART[26]. Lamivudine is also active against HBV, but it is known to select for resistant HBV variants when used as monotherapy against HBV. Resistance to lamivudine increases with duration of treatment, with up to 80% of patients selecting for lamivudine-resistant HBV strains after 5 years of treatment[27]. With the combination of tenofovir plus emtricitabine (or lamivudine), both active against HIV and HBV, such resistance profiles do not occur, and this combination is therefore advised as first-line treatment in HBV/HIV-coinfected adults and adolescents. However, in prepubertal children, tenofovir may interfere with bone mineralization and is therefore less attractive[28].

HBV in children is mostly preventable by maternal HBsAg screening, HBV immunization (ideally together with HBIG) beginning immediately for infants of HBsAg-positive mother and routine HBV immunization for all other infants and for children not previously immunized. These public health measures are especially important in high-risk populations including those with HIV infection. In HIV-infected people who are not receiving ART, however, HBV vaccination elicits an impaired HBV immune response, leaving 22%–80% unprotected[16-17,29,30]. Effective HIV treatment results in an increase of CD4 T cells, which provide critical help to B cells in the production of antibodies against T-cell-dependent antigens and in the establishment of memory T cells. However, restoration of specific immune responses has been shown to be incomplete for various antigens[19-20,31].

We found a reduced effectiveness of HBV vaccination in children on cART with relatively low CD4 T cells. Other studies also reported an association between the level of CD4 T cells and the vaccination response, in both adults and children[15,20,30,32]. Our findings that children with current CD4 T cells >= 350/mm^3 had a significantly better response than children with lower CD4 T cells was in agreement with the findings by Fonseca et al. in adults[15]. Veiga et al. 30 advised to preferentially

vaccinate adult patients with CD4 T cells >450/mm^3. In contrast, Kim et al 33 reported the nadir CD4 T-cell count rather than the current CD4 T-cell count to be associated with an adequate response to HBV vaccination.

Our study also supports the association found between an adequate HIV suppression and a successful response to vaccination[17-19,30]. It is thought that with ongoing viral replication, increased levels of proinflammatory cytokines persist in lymphoid tissue and depletion of CD4 T cells and activation of CD8 T cells continues, inhibiting adequate responses to T-cell-dependent HBV vaccination[17,19,30].

The overall protective response rate in our study was over 71%, but as few as 35% of those with detectable plasma HIV RNA and 33% of those with CD4 T cells <350 cells/mm^3 before HBV vaccination developed HBV-protective antibodies. This is consistent with the findings from a study conducted in Tanzania by Pippi et al who evaluated the HBV immune response in 89 children with and without cART after a 3-dose HBV immunization schedule. They found an overall protective response rate of 60%, but the 47 children on cART did better with a response rate of 70.8%[20].

Lao-araya et al. 31 vaccinated 63 Thai children on cART and observed an increasing protective HBV immune response after each dose; the rate was 17.4% after the first, 82.5% after the second and 92.1% after the third immunization. In contrast to the children in our study and the Tanzanian cohort, the Thai children had been vaccinated in infancy as part of the HBV immunization program; only those with a nonprotective HBV antibody concentration received 3 booster vaccinations. Perhaps they had developed specific memory cells against HBV, and were able to mount an immune response comparable to that of non-HIV-infected children suggesting that HBV immunization at infancy may increase HBV vaccine responses in HIV-positive children after booster vaccinations later in life[20]. This phenomenon was not observed by Abzug et al who evaluated HBV revaccination in prevaccinated children on cART in the United States. Only a moderate response and no immunologic memory were documented in a significant proportion of children[19]. The percentage of children with a protective anti-HBs concentration increased from around 25% at entry to 46% after 2 booster vaccinations. The best response (64%) was observed in children who had never experienced low CD4 T-cell counts[19]. Pessoa et al. 15 revaccinated Brazilian adolescents who had previously received a schedule of 3 HBV vaccinations when they were younger and found a response rate of 66.7%. However, half of these adolescents only responded after additional and double HBV vaccine doses.

The mean anti-HBs concentration of 150 mIU/mL in our responder group was relatively low as compared with mean concentrations (9283 mIU/mL) in non-HIV-infected children[34]. This is consistent with the findings from Abzug et al. 19 who reported a mean concentration of 190 IU/mL. Several studies in adults and adolescents have evaluated whether additional vaccine doses, accelerated schedules or the use of higher doses may be effective measures to improve HBV vaccine responses to protective levels. Results, however, were conflicting[14-15,32,35-37].

Due to financial limitations, we were unable to perform HBV viral load testing to detect occult HBV infection in HBsAg-negative children, or further explore other factors that could impair vaccine responses, such as hepatitis C virus coinfection. We were also unable to follow children over time to assess the persistent of protective antibodies. Finally, we relied on passive rather than active surveillance for the data on immunization and adverse events; however, we do not feel this affected the study results.

One in 11 HIV children on antiretroviral treatment showed evidence of past HBV infection whereas 1 in 15 children were actively HIV/HBV coinfected. HIV-infected children in Rwanda should be screened for HBV before initiating cART. A cART regimen that included tenofovir and emtrictiabine (or lamivudine) would then be the first choice in postpubertal children. However, for prepubertal children choices are more complicated. Tenofovir has recently been approved for use in children between 2 and 12 years[38]. Virologic arguments would favor a tenofovir/lamivudine-based regimen in HIV/HBV coinfected children. However, long-term safety in young children has not yet been defined, and monitoring, in particular, for renal and bone complications would be required.

As HBV vaccination is an expensive intervention in many settings, it is important to choose the moment where an optimal response can be expected. A combination of CD4 T cell and HIV-1 RNA testing would be best to determine this timing but because the prohibitive cost of HIV-1 RNA testing would support use of CD4 T-cell counts. Whether the vaccine's efficacy in HIV-infected children can be further improved by administering extra vaccinations or higher doses warrants further study. The findings of this study are relevant for the Rwandan National ART program in advancing our understanding of the best timing and conditions for effective HBV immunization.

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CHAPTER 7

General discussion

Expanded global efforts towards universal access to combination antiretroviral therapy (cART) in recent years have dramatically increased the number of HIV patients on cART worldwide, particularly in resource limited settings1. Notwithstanding this great achievement, monitoring of laboratory parameters as well as other factors associated with treatment outcomes have been given less consideration. This could lead to high rates of virological failures and the emergence of high levels of HIV drug resistance (HIVDR)[2,3].

This thesis aimed to provide insights into the clinical, immunological, and virological (including HIVDR) outcomes of cART in previously treatment-naive HIV-positive children, adolescents and in adult HIV-1 infected patients eligible and not yet eligible for cART. It also portray the HIV subtypes circulating among adults recently versus chronically infected HIV patients in the country and their epidemiological connection to HIV strains from neighboring countries.

Briefly, the studies described in this thesis report on:

I. cART treatment outcomes, as well as determinants of virological failure and HIV drug resistance, one year after cART initiation in cohorts of Rwandan adult and paediatric HIV patients.

II. The prevalence of hepatitis B virus (HBV) and/or Hepatitis C virus (HCV) infections in two cohorts of HIV-infected Rwandan adults and children and the success rate of HBV vaccination in children receiving cART.

III. Baseline HIV drug resistance and genetic subtypes of strains derived from chronically and recently infected adults as well as clusters of HIV transmission within Rwanda and across its five neighboring countries.

Based on the results of these studies, the utility and implications of using clinical-immunological criteria versus viral load monitoring to identify virological failure in adults and children are discussed, as well as inequalities in treatment outcomes between adults and children receiving cART and future challenges of the cART program in Rwanda.

Treatment outcomes

Despite the impressive treatment coverage achieved in Rwanda (93% in 2012) and the substantial decline of HIV-related patient morbidity and mortality[4], our studies identified several concerns that should be addressed to achieve and maintain WHO-recommended targets for HIVDR prevention. These targets include the monitoring of cART prescribing practices, patient retention in care, continuity of drug supply as well as optimization of adherence to ensure improvement of the quality of patient care[5]. In **Chapters 2** and **3** we describe treatment outcomes amongst adults and children receiving cART. Although retention in care and virological suppression rates were in line with WHO-suggested targets for HIVDR prevention[5], a significant number of adult patients in our

studies initiated therapy at advanced stage of the disease: a total of 25% were already at WHO stage 3 or 4 when treatment was started and 44% of patients had a CD4 count lower than 200 cells/ml. Our findings corroborate with the study of Kayigamba and colleagues who reported poor linkage between HIV diagnostic and HIV care services6. In our pediatric study, early cART initiation was positively associated with clinical improvement. These findings are in line with previous experiences in HIV infected children from resource-limited settings where early cART initiation was reported to significantly improve patient outcomes including immunological responses[7], weight and height for age z scores (WAZ&HAZ).

As of July 2014, Rwanda has adopted the new WHO guidelines recommending to initiate cART treatment in adults living with HIV when their CD4 cell count falls to below 500 cells/mm^3. Successful implementation of these new guidelines for standard of care will require the intensification of efforts such as to increase patient awareness, to increase immunological and virological monitoring, to improve the linkage between HIV testing and cART initiation to increase actual early initiation of cART hence improving the outcomes of cART[8,9].

Lack of adherence to the treatment seems to play an important role in virological failure in adult as no evidence of HIVDR mutations was observed in 22% of patients failing cART. None of the treatment adherence measures collected were identified as predictors for treatment failure, suggesting that reliable measurements of drug adherence and appropriate adherence support strategies are much needed[10,11].

Treatment failure and drug resistance are major concerns in both pediatric and adult treatment programs. In our cohort, one out of three children had virological failure, a rate substantially below the WHO recommended target[3]. These findings are in line with previous studies[12,13]. Together with the profile of HIVDR showing the accumulation of both NRTIs and NNRTIs resistance mutations, our results provide a worrisome picture of poor virological and HIVDR outcomes in the pediatric population. In the vast majority of children failing cART who were tested for HIVDR genotypes, at least one major NRTI and/or NNRTI mutations was detected (91% and 95%, respectively). This observation corroborates with previous reports of major mutations related to NRTI and NNRTI resistance occurring in high proportions of patients due to the extensive use of these agents in first line combined regimens, the low genetic barrier described for these agents toward resistance as well as the relative fitness of NNRTI resistant strains[14].

In addition, comprehensive reports have highlighted the link between single dose nevirapine in the context of prevention of mother-to-child transmission (PMTCT) and a high level of NNRTI resistance[15,16,17]. In our pediatric study, data on PMTCT exposure were available from only 60% of children, of whom 27% reported to have been exposed to single dose nevirapine. Due to the retrospective and cross-sectional nature of the study in the pediatric population, we were also unable to systematically determine the impact of factors such as poor adherence, baseline HIVDR mutations and social characteristics on the occurrence of treatment failure and viral resistance. Attrition rates

were not defined either. Interventions are needed to assess factors associated with poor treatment outcomes in Rwandan children and adolescents. Systematic information on exposure to PMTCT and updated data on the prevalence of transmitted HIVDR in children born from HIV infected mothers are also warranted. These data would inform strategies aiming at improving patient retention, adherence to treatment and choice of optimal first and second-line empirical cART regimen in this particular population.

Interplay between hepatitis and HIV infection

Chapters 5 and **6** generated evidence that the prevalence of HBV and/or HCV is high in Rwandan children and adults infected by HIV.

In **chapter 5**, we report evidence of active HBV infection, as indicated by the presence of hepatitis B surface antigen (HbsAg) in blood, in 5.2% of adult HIV patients, while 42.9% had evidence of past exposure to HBV. Prevalence of anti-HCV antibodies was 5.7%. While these prevalences are in line with previous reports from other African settings20,21one of the major health priorities, accounts approximately for 350 million chronic cases and a global total of 33 million people were living with human immunodeficiency virus (HIV, they are slightly higher than those reported in 2007 in HIV-positive pregnant women in Rwanda (2.4% for active HBV and 4.9% for anti-HCV)[22].

Active HBV infection and HCV seropositivity were associated with poor CD4 recovery during suppressive cART. Whether this negative impact of HBV or HCV co-infection also occurs in children was not investigated but is likely.

In **Chapter 6**, we report a 7% prevalence of HBsAg-positivity in HIV-infected children, while 9% had evidence of past HBV exposure. Studies of the prevalence of HIV/HBV co-infection among children show large variations in different geographic regions as well as in different populations. A study in a Tanzanian cohort of HIV infected children indicated that only 1.2% were co-infected with HBV, whereas studies from west Africa have reported HBV co-infection in 12% of HIV infected children from Ivory coast and 19% in Nigerian children[18,19].

A number of anti-HIV drugs are also active against HBV[23] with large differences according to geographical region. Lamivudine, a component of first-line cART with activity against HBV, is known to select for resistant HBV variants when used as HBV monotherapy[24]. Studies have reported HBV resistance to lamivudine to increase with the duration of treatment, with up to 80% of patients selecting for lamivudine-resistant HBV strains after 5 years of treatment[25,26]. Such rapid development of resistance does not occur when using a combination of tenofovir plus emtricitabine (or lamivudine), both active against HIV and HBV[27]. Therefore, the Rwandan cART program recommends this combination as first-line treatment in HBV/HIV co-infected adults combined with virological monitoring hence adequately addressing the issue of controlling HBV infection.

HBV control in HIV infected children is more complicated as tenofovir may interfere with bone mineralization and is therefore less attractive for paediatric treatment[28]. However, in most clinical settings, children with HIV/HBV co-infection do receive tenofovir based regimens despite the risk of decrease in bone mineral density[29].

At the time of our studies, tenofovir had not yet been introduced in the Rwandan cART program. This may have led to a substantial rate of HBV resistance among adults and children co-infected with HBV and receiving a lamivudine-containing first line treatment, with consequently a negative impact on both HBV and HIV disease progression[30]. However, this has not been investigated in the scope of this thesis due to logistical and financial constraints.

In adult and children with no previous exposure, HBV infection can be prevented by HBV immunization[31]. Our results indicate that suppressive cART and a robust immunological response was associated with an adequate response to HBV vaccine in the pediatric cohort: 71% of children (95% CI: 61–82) with CD4 T cells ≥350/mm^3 and viral load <20 copies/ml developed a protective anti-HBs response (≥10 mIU/mL). In contrast, after three vaccinations, the vaccine response rate was only 33% in those with CD4 T <350 cells/mm^3. These results underscore that providing suppressive cART at early stages of HIV disease is not only important for the control of HIV and the reduction of opportunistic infections, but also for the optimal prevention of other serious infectious diseases like HBV.

There is no vaccine available against HCV but the efficacy of HCV therapy is rapidly improving. Current regimens have nearly 90% sustained viral response rates, and therapies in development are achieving over 95% rates of viral eradication, even in patients traditionally considered difficult to treat[32]. However, the costs of these effective HCV treatment regimens are prohibitively high for most resource-limited settings. Therefore, similar to what has been achieved for HIV, affordable prices need to be negotiated for resource limited regions of the world where HCV is as a major cause of morbidity and mortality, with or without HIV co-infection. The relatively significant rate of exposure to HCV among adult patients raises the question of the risk of mother-to child transmission of HCV during birth. Research is needed to address this question and inform appropriate HCV infection prevention and management strategies among adults and children in Rwanda.

Collectively, our data indicate that systematic screening for HBV and HCV infections among all patients initiating cART is important. The relatively high prevalence of HBV and HCV has several important implications for HIV disease progression, as well as for therapeutic and prophylactic approaches. However, routine identification of both HBV and HCV remains a serious bottleneck in the continuum of care in Rwanda. Expanding access to HBV and HCV diagnostics and reinforcing the linkage between diagnostics and care are highly needed priorities to support the clinicians' decisions on appropriate care and treatment. In Rwanda, screening for viral hepatitis is currently done at the discretion of individual clinicians due to the fact that there is no policy for screening of these infections.

Monitoring of antiretroviral treatment response: the utility of clinico-immunological criteria

The present work confirms previous reports that clinical status and CD4-T cell level are not good predictors of virological failure among patients on cART[33,34]. One third of children and almost one quarter of adults receiving suppressive cART fulfilled the definition of immunological failure and would have been misclassified as experiencing treatment failure based on immunological criteria at 12 months. Conversely 77% of adults and 94% of children failing cART were categorized as good immunological responders, with the likelihood of being maintained on a failing regimen.

Acknowledging the unreliability of clinical and immunological criteria to identify virological failure and implementing the new WHO recommendations[35], Rwanda aims to scale up the use of virological monitoring countrywide. Theoretically, annual or targeted VL measurements should allow for the timely identification of patients failing therapy[36], reduce the emergence of acquired HIVDR and contribute to maintain the cost-effectiveness of cART in resource limited settings (RLS)[37]. In our study presented in chapter 2, we report that targeted VL monitoring was requested for only 0.2% (9 of 41) of the participants with suspicion of treatment failure at month[12]. This finding suggests that, despite the availability of laboratory-based monitoring tools to support clinical decision-making and reduce HIVDR, barriers exist at the level of clinicians that limit their utilization for appropriate clinical care. Various initiatives from WHO and CDC are currently taking place to develop and validate simple point-of care VL instruments[38,39]. In order to be successful, the roll-out of VL testing will need to be accompanied by appropriate training of clinicians, so that laboratory results are properly interpreted and taken into consideration for the reinforcement of adherence to the treatment or switch to second-line regimen.

Beyond the clear diagnostic challenged posed by the use of immunological criteria to define virological failure, poor CD4 recovery in the face of virological suppression may have some serious consequences on clinical outcomes[12,40]. Higher mortality and occurrence of clinical events are indeed observed in patients with suboptimal immunological recovery during suppressive cART[41]. More research is needed to better understand the magnitude and the cause of this phenomenon in Rwanda and other RLS. This would inform on the utility of strategies aimed at identifying virologically suppressed patient at risk of adverse clinical outcome as well as strategies to boost immunological responses among particular groups of patients. Altogether, these observations indicate that, whilst CD4 counts have little value for the timely identification of virological failure, they may carry important information on HIV disease progression, also during suppressive cART.

The profile of HIVDR mutations in both adults and children indicate that the vast majority of patients failing first line treatment do not develop resistance mutations in the protease gene. This suggest that an HIVDR genotyping assay targeting only the RT gene could be useful in a setting like Rwanda, as this would reduce costs of testing and simplify interpretation of test results. Such assays are currently being evaluated in several settings in Africa[42].

Drug resistance mutations and genetic subtypes of HIV-1 strains among cART-naïve adult HIV-infected patients in Rwanda

Our investigations in adults indicate that the prevalence of both pre-treatment and transmitted HIVDR in Rwanda are rather low, although our results may not be nationally representative. These results may reflect the rather short history of cART use in the country but also suggest that uncontrolled utilization of cART is not frequent despite the impressively high ART coverage in Rwanda[43]. The low prevalence of baseline HIVDR also suggests the appropriateness of the current empirical first-line regimen in the country.

In contrast, the high treatment failure rate in children might point to high pre-treatment HIVDR in this group and warrants further investigation to assess the expected efficacy of first and second-line pediatric cART. More specifically, a survey to assess transmitted HIVDR among infants born from HIV infected mothers as well as a survey for pre-treatment HIVDR in children initiating cART are urgently needed. The latter protocol is not yet available but is currently being developed by WHO HIVRes Net[44].

Keeping both transmitted and acquired HIVDR low in adult population is important. Our findings indicate that surveillance of acquired HIVDR among patients receiving first-line cART might be most needed. This survey would provide a nationally representative overview of acquired drug resistance in Rwanda and should be conducted in combination with the monitoring of early warning indicators such as on time pill pick-up, drug supply continuity, patient retention in care at 12 months, dispensing practices as well as viral load suppression at 12 months, as recommended by the WHO[45].

The geographical distribution of HIV-1 sub -types is reported to be a dynamic process. Furthermore, the intermixing of HIV-1 variants is unavoidable in regions where diverse strains of HIV-1 co-circulate[46,47]. In **chapter 4**, we detail the molecular characterization of HIV-1 subtypes circulating in Rwanda. The study highlighted the predominance of A1 subtype representing 71.7% of all viruses. This is in line with a previous study on the phylogenetic inference of HIV in East Africa[48]. However, our study noted a high proportion of pol A1/C recombinants (18.1%) and a discreet introduction of subtype D (2.9%). This may reflect a change in HIV subtype distribution over time or may be due to the complex sexual network between countries bordering Rwanda. In studies conducted in the region, subtype A1 and D subtype were equally distributed[49,50]. This shift from A1 to D subtypes in the epidemic has been reported to have an impact on the disease progression with subtype D virus known to have quicker progression to AIDS as well as a higher mortality rate as compared to patients infected with subtype A virus[51,52,53]. Other recent reports have shown the presence of subtypes C and G among different cohorts which has led to the emergence of AC recombinant forms in the region[54-56]. More studies are needed to disentangle the emergence of these novel strains that might be taking over previous subtypes, as well as to find the transmission networks and the potential implications these new strains might have for the Rwandan cART program.

Our study also highlighted the absence of evidence from the Rwandan strains for direct transmission events. This finding is in line with studies which documented that in populations where HIV transmission occurs mainly through heterosexual contacts like in Rwanda, evidence for transmission clusters is generally more limited. This is probably due to the complex sexual network in the country. It is also well documented that study populations recruited in areas of quite high HIV prevalence are frequently sparsely sampled[57], resulting in a reduced probability of including patients belonging to the same transmission network(s) and a bias toward the underreporting of infection clusters.

Given regular cross-border population movements in the Eastern African region[58], we assume that migration may have played and continues to play an important role in the past and present HIV epidemic in Rwanda. Although we did not find phylogenetic evidence of a connection with the HIV-1 epidemic in the five neighboring countries, irregular availability of up-to-date pol sequences from East and Central Africa in the Los Alamos database may have biased our results. Consequently, the presence of genetic links between HIV strains from Rwanda and neighboring countries cannot completely be excluded.

Although the clinical relevance of different HIV-1 genetic subtypes remains controversial[59,60], further studies could contribute to address the significance of emerging HIV-1 subtypes in Rwanda in terms of transmissibility, response to cART and overall clinical outcomes.

Inequalities between adults and children infected with HIV

This thesis highlights unacceptable inequalities in cART outcomes between adults and children. Although virological failure and acquired HIVDR were seen in both populations, the proportion of children with virologic failure and with multiple drug resistance was higher than in adults: virological failure after cART initiation was observed in 30% of children, compared to 11% in adults.

Sadly, too few initiatives are currently ongoing to improve the efficacy of pediatric cART and develop strategies aiming at increasing retention in care of children and adolescents and support their adherence to the treatment.

As of June 2013, 488 sites (97%) of all public health facilities in Rwanda were providing PMTCT services. However only 34% of children in need of cART had access to the treatment[61]. Identification of children in need of cART as well as access of treatment to these children should urgently be improved.

Limitations of the studies

While the results presented in this thesis have provided important insights into outcomes of cART in HIV-positive children and adults in Rwanda and have identified priorities for improvement, our studies have several limitations.

A major limitation is that the studies took place in one HIV clinic in Kigali, hence results may not be representative for the entire country of Rwanda. Another limitation is the limited follow-up period. As follow up was less than a year in a substantial number of patients of the adult cohort, possible clinical outcomes occurring later in the course of cART were not recorded. Finally, as studies in children were partly retrospective and cross-sectional in nature, important factors that may determine cART outcome, such as pre-treatment HIVDR and attrition could not be investigated.

As for the studies on viral hepatitis, occult HBV infections could not be excluded in patients who were HBsAg-negative but anti-HBc-positive since HBV DNA PCR testing was not performed. Similarly, we could not identify active HCV infections among those positive for anti- HCV due to lack of HCV RNA PCR testing. The lack of availability of molecular assays for viral hepatitis in our studies also presented a missed opportunity to measure viral load and the presence of HBV drug resistance mutations in our patients. Finally, many parameters such as Alanine transaminase (ALT) and aspartate transaminase (AST) were only evaluated in patients after they had initiated cART.

Opportunities for future research

This thesis identified several research questions that remain to be answered. In addition, it identified several public health interventions that need strengthening in the Rwandan context.

Research questions

What is the clinical relevance of HIV genetic variability in Rwanda and in its neighboring countries?
With respect to HIV subtypes circulating in Rwanda and in the region, further studies completing our molecular data are needed to assess the clinical relevance of HIV genetic variability. In addition to that, larger prospective studies are needed to assess the significance of co-circulating newly emerging strains, such as A1/C recombinants and subtype D strains, for transmission of infection, disease progression and response to cART.

What is the prevalence of occult HBV in HIV infected patients?
With a prevalence of 42.9% of anti-HBc in HIV patients reported in our study, further research is needed to determine the accurate prevalence of occult HBV in HIV infected patients by HBV DNA detection rather than relying on serology test results.

What are the impacts of HIV, HBV and HCV infections on their respective clinical courses in co-infected patients?
Limited information is available on the impact of HIV on the course of viral hepatitis and vice versa, hence larger studies in co-infected patients are urgently needed to assess these impacts. Moreover, studies investigating HBV status in mothers and children, including longitudinal HBV testing are urgently needed to assess the sources and timing of HBV infection in children.

What are affordable sensitive and specific diagnostic algorithms of HCV infection in African settings?
Information on HCV infections in sub Saharan Africa is limited, hence affordable diagnostics are urgently needed.

What are reliable tools to monitor adherence?
Tools to monitor adherence are essential to maximize clinical effectiveness, to improve cART treatment outcomes and minimize the potential risks associated with the development of drug resistance.

What are the reasons behind the low utilization of available laboratory tools?
Although access to reliable lab testing remains challenging in many resource limited countries, we need to understand the reasons why existing lab facilities are underused in these settings. This is very crucial for the disease prevention, detection and control.

Public health interventions that need strengthening

Improved diagnosis and management of viral hepatitis in HIV-infected patients.
Policy makers should mobilize funds and capacity to allow for systematic screening of HBV infection in HIV infected children and adults. HBV in children is mostly preventable by maternal HBsAg screening, HBV immunization beginning immediately for infants of HBsAg-positive mother and routine HBV immunization for all other infants. Screening is also applicable to adults not previously immunized and eligible for the vaccine. This best practice should be part of government policies to ensure that all non-infected patients are vaccinated and infected patients receive an HBV-active ART regimen. In addition, rather than screening for HCV antibodies to estimate HCV prevalence and incidence in African settings, routine confirmation of positive anti-HCV test results with HCV RNA testing is needed.

HIV drug resistance prevention at a population level.
Although the rate of transmitted HIV drug resistance currently seems to be low, policy makers should plan regular systematic HIV drug resistance surveys at least each five years, as recommended by WHO for all countries that are scaling-up and maintaining populations on antiretroviral therapy. These surveillance activities can be embedded in demographic household surveys. These data would contribute to the rational use of cART and the revision of treatment guideline as needed.

Decentralization of HIV lab monitoring tools to lower levels of health care.
Given the minimal health infrastructures, policy makers should develop strategies for improved access to affordable laboratory monitoring tools, such as point of care tests. This is needed to implement the WHO global strategy for assessment and prevention of HIVDR at lower levels of health care delivery in order to improve the quality of care for the underserved population.

Improvement of linkage between HIV diagnostic and HIV care services.
This is essential for early identification of patients eligible for cART and for monitoring treatment efficacy and adherence, hence ultimately improving patient outcomes.

Donor funding for HIV programs has decreased in recent years, which limits the ability of HIV programs worldwide to achieve universal treatment access and sustain current progress. To date, 90% of the Rwanda Health financing system relies on external partners[62]. In order to tackle this challenge, Rwanda has fully implemented a community-based health insurance program (Mutuelles de santé) as a key component of the national health strategy to provide universal health care, universal coverage of medical services and financial risk protection[63,64]. The country has also been ranked among countries that achieved the Abuja Declaration target of allocating at least 15% of government expenditures to health[65]. However, to maintain a sustainable health system, more need to be done to replace donor HIV funding. The country is planning to mobilize internal sources of funding as the most important alternative. Nevertheless, successful plans and implementation of resource mobilization will require close collaboration with ministries other than the health sector, particularly the Ministry of Finance, to make sure that the upcoming domestic HIV financing strategies are fully implemented and coordinated.

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Summary

Information on patient outcomes of antiretroviral therapy (ART) is scarce in Rwanda. The aim of this work was to provide information on clinical, immunological, and virological outcomes and outcomes predictors of combined antiretroviral therapy (cART) in HIV -infected adults and children initiating or already under treatment in Rwanda. The burden of hepatitis B (HBV) and C (HCV) co-infections and their implication on treatment and immunization outcomes were examined. These data are intended to inform the improvement of HIV care guidelines in Rwanda. Chapter 1 provides an overview of the HIV epidemic worldwide and narrows down to the situation of the ART program in Rwanda.

1 | Treatment outcomes

In **Chapters 2** and **3**, we described the treatment outcomes as well as the potential determinants of virological failure and HIV drug resistance (HIVDR) in longitudinal cohorts of Rwandan adults and cross sectional cohorts of adolescent and children, respectively. The study reports a high patient retention rate among adults, with more than 70% remaining on first-line treatment after 12 months of follow-up. Adult participants initiated cART at advanced stage of the disease, with 25% of them presenting WHO disease stages 3 and 4 and 44% with a severe depletion of CD4 count <200 cells/ml.

There were marked differences in the virological failure rate between adults (11% at month 12) and children (1 in 3 children tested between year 1 and 4). Major drug mutations were found in 91% of the children as compared to 8 of the 12 adults with virological failure and tested for HIVDR genotyping. Sixty percent of the children reported previous exposure to Prevention of Mother to Child Transmission (PMTCT). One third of children and almost one quarter of adults receiving suppressive cART fulfilled the definition of immunological failure and would have been misclassified as experiencing treatment failure based on immunological criteria at 12 months. Seventy percents of adults and 94% of children failing cART were categorized as good immunological responders, with the likelihood of being maintained on a failing regimen. Moreover, viral load monitoring appeared to be underused by clinicians, as indicated by the lack of request for targeted viral load in participants with continuous immunological failure. Collectively, our data highlight the need to improve accessibility and use of viral load in order to timely detect virological failure and also to avoid unnecessary switch to second line in patients that are virologically suppressed with a poor CD4 recovery.

The differences in virological outcomes between adults and children suggest the following. First, better first-line regimen (protease inhibitor- based) options should be made available for children previously exposed to PMTCT and with a high risk of presenting pre-treatment drug mutations.

Second, strategies are needed to increase retention in care of children and adolescents and support their adherence to the treatment.

2 | Genetic subtypes of HIV-1 strains among cART-naïve HIV-infected adults

In **chapter 4**, we depicted the molecular characterization of HIV-1 subtypes circulating in Rwanda and the influence of HIV strains circulating in five countries bordering the country on the epidemic. An important observation was the low prevalence of baseline drug resistant mutations in both chronically and recently infected adult participants (<5%). This is in line with the relatively short history of ART roll-out in the country and suggests that current first-line regimen are likely to be efficient in the vast majority of patients initiating ART. The study found a large predominance of subtype A1 (71.7%), and increasing proportion of A1/C recombinants (18.1%) while the frequency of subtype C and D remained stable as compared to previous similar studies. This finding indicates that a change in HIV subtypes distribution is possibly taking place in Rwanda, with the need to assess the potential impact of the new A1/C recombinants strains on the Rwandan HIV epidemic.

We did not find any evidence of direct transmission clusters neither in recently nor in chronically HIV-infected patients. Equally there were no evidence of phylogenetic link between the HIV strains isolated from Rwandan patients and those characterized from the five neighboring countries. Our results underline the difficulty of collecting representative data to explore the existence of intra and cross border networks of HIV heterosexual transmission, in the context of a generalized HIV epidemic.

3 | Interplay between hepatitis and HIV Infection

In **Chapters 5** and **6**, we show worrisome prevalence of HBV with 7% of children and 5.2% of adults co-infected by HIV infection showing positive reaction to the HBsAg serology and thus carrying an active (acute or chronic) HBV infection. Evidence of past HBV infection was reported in 9% of children and in 42% of the adult cohort as indicated by a positive serology for anti-HBc in combination with a negative serology for HBsAg. 5.7% of adult patients with a positive test for anti-HCV antibodies were identified as carrying a past or ongoing HCV infection. Given the influence of hepatitis and HIV infections on respective disease course, as well as the activity of some antiretroviral drugs on HBV replication, we recommend that a systematic screening of hepatitis infections in all HIV infected patients, prior initiating cART for better clinical management. Affordable and more sensitive assays to differentiate between occult and past HBV and HCV infections are warranted.

The study reported that 71% of children without HBV infection, had protective anti-HBs response (≥10 mIU/mL) when their CD4 count ≥350 cells/ mm^3 and viral load <40 RNA copies/ml. In contrast,

protective response against HBs antigen was mounted only in 33% of children with CD4 count <350 cells/mm^3. This outcome highlights the comprehensive added value of antiretroviral therapy, not only in the control of HIV infection and others opportunistic infections but also in the prevention of HBV infection.

Key messages

– The level of baseline HIVDR mutations is low (<5%) in both recently and chronically infected adult patients, possibly suggesting the good performance of the cART program in Rwanda. However, the emergence of acquired HIV drug resistance mutations upon 12 month of ART suggests that although first-line regimen are anticipated to be efficient in most adults initiating therapy, strategies to prevent HIVDR needs to be reinforced at the national level. The high rate of treatment failure in children is particularly alarming. Improving access and utilization of viral load monitoring of ART and adapting first-line regimen for children previously exposed to PMTCT are two strategies that could contribute to prevent virological failure and reduce the risk of acquiring HIVDR in Rwandan patients initiating ART.

– The proportion of A1/C recombinant strains increases in Rwanda with the need to address the significance of these viruses in terms of transmissibility, response to cART and overall clinical outcomes.

– The high prevalence of hepatitis B and C in HIV infected children and adult patients, indicate the need to expand access to hepatitis diagnostic tools in order to support appropriate care and treatment. Policy on a systematic screening for viral hepatitis infection prior cART initiation should be available, supported by novel affordable and more sensitive diagnostic techniques.

Samenvatting

Informatie over HIV-1 geïnfecteerde patiënten die antiretrovirale therapie (ART) krijgen in Rwanda is zeer beperkt. Het doel van dit onderzoek was om meer inzicht te krijgen in de klinische, immunologische en virologische uitkomsten, en de voorspellers hiervan, in geïnfecteerde volwassenen en kinderen die net starten met een gecombineerde antiretrovirale therapie (cART) of al enige tijd op therapie staan. De virologische implicaties van een hepatitis B en C co-infectie op de therapie en immuunsysteem werden onderzocht. De verkregen gegevens zijn bedoeld om de richtlijnen aangaande HIV-behandeling in Rwanda te verbeteren. Hoofdstuk 1 geeft een overzicht weer over de globale HIV-epidemie en richt zich daarna specifiek op de situatie van het ART-programma in Rwanda.

Resultaten van de behandeling

In de hoofdstukken 2 en 3 beschrijven we de resultaten van de therapie tezamen met mogelijke potentiële determinanten van HIV falen en van HIV drugs resistentie in een longitudinaal cohort in Rwanda bestaande uit respectievelijk volwassenen en adolescenten/kinderen. De studie laat zien dat een groot deel van de patiënten trouw blijft aan het programma en dat na 12 maanden 70% van deze patiënten nog steeds op een eerstelijns therapie staat. De volwassenen begonnen hun ART-therapie in een laat stadium van hun ziekte, waarbij 25% zelfs in fase 3 en 4 zaten volgens de WHO richtlijnen. Deze laatste groep vertoonde extreem lage hoeveelheden CD4 cellen. Er waren verschillen in virologisch falen tussen volwassenen (11% na 12 maanden) en kinderen (1 op de 3 van alle kinderen die werden getest tussen 1 en 4 jaar). In 91% van de kinderen werden majeure mutaties gevonden, dit in vergelijking tot 67% bij de volwassenen. Zestig procent van de kinderen hadden in een PMTCT programma gezeten. Een derde van de kinderen en bijna 25% van de volwassenen die op cART therapie stonden, voldeden aan de definitie van immunologisch falen en zouden onterecht geclassificeerd worden als falend op therapie, gebaseerd op de immunologische criteria na 12 maanden.

Zeventig procent van de volwassenen en 94% van de kinderen die op cART stonden en faalden werden onterecht gecategoriseerd als goede immunologische responders, met het risico dat ze ondanks falen op dezelfde therapie gehouden zouden worden. Bovendien werd het gebruik van de meting van de virale load niet ten volle benut door de artsen, zoals te zien was aan het lage aantal aanvragen van deze test bij immunologisch falende patiënten. Onze gegevens laten zien dat er behoefte is aan verbeterde toegankelijkheid en het gebruik van de virale load test om op tijd het virologisch falen te kunnen detecteren. Hierdoor kan men ook voorkomen dat men onnodig naar een 2de lijn therapie omschakelt bij patiënten die virologisch gesupprimeerd zijn en een slechte CD4 recovery hebben.

De verschillen in de virologische resultaten tussen volwassenen en kinderen suggereren het volgende. Ten eerste zou er een betere eerstelijns therapie (met PI´s) beschikbaar moeten komen voor kinderen die al eerder blootgesteld zijn geweest aan PMTCT en dus een hoog risico hebben dragers te zijn van gemuteerde virussen. Ten tweede moet er meer aandacht besteed worden om deze kinderen op te volgen opdat ze beter begeleid kunnen worden tijdens hun behandeling.

Subtypen van HIV-1 in gART naive HIV geinfecteerde volwassenen

In hoofdstuk 4 wordt de moleculaire karakterisatie van HIV-1 subtypes voorkomend in Rwanda besproken en wordt besproken wat de invloed is van de HIV-stammen in de vijf omringende landen op de epidemie. Een belangrijke observatie hierin was de lage prevalentie van baseline drug gerelateerde mutaties in chronisch geïnfecteerde en recent besmette patiënten. Dit gegeven is in lijn met de relatief recente introductie van ART in dit land. Men zou veder kunnen afleiden dat eerstelijns therapie efficiënt genoeg is bij de meeste patiënten die op ART zijn gezet. De dominante subtypepopulatie is A1 (71.7%) gevolgd door een toenemende proportie van A1/C recombinanten (18.1%), terwijl vergeleken met vergelijkbare studies de frequentie van subtypen C en D vrij constant is. Dit suggereert dat er een mogelijke verschuiving in de subtypepopulatie aan het optreden is waardoor er aandacht besteed dient te worden aan de mogelijke implicaties van deze nieuwe A1/C recombinant in de Rwandese HIV epidemie.

Er zijn geen aanwijzingen gevonden voor clustervorming in recente en chronisch geïnfecteerde patiënten. Verder was er geen sprake van fylogenetische verwantschap tussen de virussen geïsoleerd in Rwanda en de vijf buurlanden. Onze resultaten benadrukken hoe lastig het is om gegevens te verzamelen die representatief genoeg zijn om intra- en inter-transmissienetwerken van heteroseksuelen te identificeren in de context van een HIV-epidemie.

Wisselwerking tussen hepatitis en HIV infectie

In de hoofdstukken 5 en 6 tonen we een onrustbarende prevalentie aan van kinderen (7,0%) en volwassenen (5,2%) met een positieve reactie op HbsAG serologie, wijzend op actieve (acute of chronische) HBV-infectie. In 9% van de kinderen en 42% van de volwassenen werd er anti-HBc in combinatie met een negatieve HBsAg serologisch aangetoond. 5,7% van de volwassenen met een positieve anti-Hbc-antilichamentest werd als drager van het HCV gediagnosticeerd. Gezien de invloed van een hepatitis- en HIV-1-co-infectie op het uiteindelijke ziekteverloop én de activiteit van sommige anti-retrovirale middelen op de replicatie van het HBV, raden wij aan om voor de initiatie van cART alle HIV geïnfecteerde patiënten systematisch te screenen om ze zo beter te kunnen opvolgen. Betaalbare en meer gevoelige testen zijn nodig om te kunnen differentiëren tussen HBV- en HCV-infecties.

Uit de resultaten blijkt dat 71% van de kinderen zonder een HBV-infectie een beschermende anti-HBs respons hadden (≥10 mIU/mL) terwijl de CD4 en de virale load respectievelijk ≥350 cellen/mm^3 en <40 RNA copies/mL bedroegen. In vergelijking, slechts 33% van de kinderen met een CD4 concentratie <350 cellen/mm^3 had een immunologische respons tegen het HBs antigeen. Dit gegeven duidt de omvangrijke toegevoegde waarde van antiretrovirale therapie aan, niet alleen tegen HIV maar ook ter preventie van andere opportunistische infecties en HBV-infectie.

Conclusies

De hoeveelheid HIVDR-mutaties bij recente en chronische infecties is vrij laag. Dit kan mogelijk verklaard worden door de goede uitvoering van het cART programma in Rwanda. Echter, het opduiken van verworven HIV drugs mutaties na 12 maanden ART suggereert dat ondanks dat de eerstelijns therapie effectief is in de meeste volwassenen, er gekeken dient te worden naar strategieën voor het voorkomen van HIVDR. Alarmerend is wel de hoge graad van therapeutisch falen bij kinderen. Verbeterde toegang tot en gebruik van de virale load om de ART te monitoren én het aanpassen van eerstelijns therapie voor kinderen die blootgesteld zijn geweest aan PMTCT, zijn twee strategieën die mogelijk kunnen bijdragen aan het voorkómen van virologisch falen bij patiënten die met ART starten in Rwanda en het risico op HIV drug resistentie kunnen verlagen.

De proportie van A1/C recombinanten in Rwanda is aan het toenemen. De significantie hiervan dient bestudeerd te worden met betrekking tot de spreiding, respons op cART en het algehele klinisch beeld.

De hoge prevalentie van HBV en HCV in HIV-geïnfecteerde kinderen en volwassenen geeft aan dat de toegang tot diagnostische middelen uitgebreid dient te worden om zo de zorg verder te verbeteren. Er dient een beleid geïntroduceerd te worden dat het mogelijk maakt om met goedkopere en gevoeligere technieken systematisch te screenen op virale hepatitis infecties voordat cART gestart wordt.

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Curriculum Vitae

John RUSINE was born in the Democratic Republic of Congo, former Zaïre in January 22nd, 1970 to Rwandese parents. He completed secondary and high school in 1991 in Zaïre. After the liberation of Rwanda, he studied Medicine at the National University of Rwanda and obtained his medical degree in 2001.

From 2001 to 2003, John worked at the Treatment and Research AIDS Centre (TRAC) as the Director of the HIV AIDS Laboratory Department.

From 2004 to 2005, John studied Biomedical Sciences at the Liege University in Belgium and obtained a Masters in Biomedical Sciences. Upon completing his Master degree, he returned to Kigali and since then, he is the Director of the Immuno-Virology Department at the National Reference Laboratory which is now a Division within the Rwanda Biomedical Centre (RBC). He started his PhD work in 2007.

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