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Research Article

Differential Distribution and Evolution of HIV-1 RNA Variants in the Gastrointestinal Tract of Antiretroviral Naïve African AIDS Patients with Diarrhea and (or) Weight Loss -

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ABSTRACT

Background: Obtaining samples of the gastrointestinal tract (GIT) is difficult. As a result, little is known about the diversification and genetic evolution of Human Immunodeficiency Virus-1 (HIV-1) in the GIT. Here, we evaluated the diversity and phylogenetic relationships among HIV-1 RNA quasispecies derived from the duodenum, jejunum, ileum, right colon, left colon and plasma of antiretroviral therapy (ART)-naïve South African AIDS patients infected with HIV-1 subtype C. A total of 45 *pol* and 350 cloned *gag* and *env* Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) fragments were sequenced and subjected to phylogenetic analysis.

Results: Significant differences were observed in the diversity, evolution and trafficking of HIV-1 RNA quasispecies in the small vs. large intestine. *Env* Ribonucleic acid (RNA) variants were the most diverse with the small intestine exhibiting the highest level of diversity. *Gag* RNA variants exhibited low levels of diversity in all tissues. Migration and exchange of HIV-1 RNA variants between plasma and different regions of the GIT was more evident in *gag* than in *env* trees. Some *env* variants formed tight monophyletic clusters of closely related viral quasispecies suggesting there was localized replication and adaptive evolution of the *env* gene, primarily in the colon, but also in the small intestine of some patients. Analysis of inferred amino acid sequences indicated that some *pol* sequences contained substitutions in protein domains associated with altered susceptibility to ART. A small number of differentially distributed *env* variants had substitutions that are known to alter the neutralization and co-receptor binding properties of the V3 loop.

Conclusions: Diversification of HIV-1 in the GIT of ART-naïve AIDS patients is a highly dynamic process involving localized, tissue-specific evolution in addition to extensive exchange of HIV-1 RNA variants between plasma and different gut compartments.

KEYWORDS: Gastrointestinal tract (GIT); HIV-1; RNA quasispecies; Genetic diversity

ABBREVIATIONS

ART: AntiRetroviral Therapy; GALT: Gut Associated Lymphoid Tissue; GIT: Gastrointestinal Tract; HAART: Highly Active Anti Retroviral Therapy

INTRODUCTION

The gastrointestinal tract (GIT) is a complex organ composed of multiple tissue compartments with different structural and functional elements and diverse cell types. Due to its continuous exposure to a wide range of microbes and food antigens, the GIT is in a constant state of “physiological” inflammation and contains a large number of activated CCR5⁺ memory CD4⁺ T cells, the primary target of HIV-1. As a result, the GIT is a site of intense viral replication and severe CD4⁺ T cell depletion (Veazey et al., 1998; Guadalupe et al., 2003; Mehandru et al., 2004; Brenchley et al., 2004; Mattapallil et al., 2005; Guadalupe et al., 2006; Centlivre et al., 2007), beginning during primary infection (Guadalupe et al., 2003; Mehandru et al., 2004; Centlivre et al., 2007) and continuing at a lesser pace throughout the chronic phase of infection (Brenchley et al., 2004). This is accompanied by progressive immunological and structural damage to the gut associated lymphoid tissue (GALT), a loss of epithelial integrity, increased microbial translocation and both local and systemic immune activation (Brenchley et al., 2006; Paiardini et al., 2008; Brenchley et al., 2008). Antiretroviral therapy (ART) is only partially effective in restoring the immunological and structural integrity of the intestinal mucosa (Mehandru et al., 2006; Poles et al., 2006; Cassol et al., 2010), even when initiated during primary infection (Mehandru et al., 2006).

Despite the importance of the GIT in the pathogenesis of HIV-1 infection, and as a reservoir of treatment refractory virus, little is known about the diversity and genetic evolution of HIV-1 variants in different compartments of the intestine, or whether these diverse variants are associated with differing pathologies. There is also a paucity of information relating to the dissemination and genetic relationships between intestinal variants and those found in other tissues including the circulation. This is due, in large measure, to difficulties and ethical considerations inherent in sampling the GIT

and to the small size of the samples obtained. Most studies have been confined to one or two anatomical sites (Poles et al., 2001; Avettand-Fenoel et al., 2011; Imamichi et al., 2011; Lerner et al., 2011; Evering et al., 2012) and have involved the sequencing of proviral DNA isolated from patients on suppressive ART (Lerner et al., 2011; Evering et al., 2012). DNA contains archived and defective genetic material and may not accurately reflect the full spectrum of replication competent viral strains while studies of patients on highly active ART (HAART) with low levels of viral replication may underestimate HIV-1 diversification. A study by Avettand-Fenoel *et al.*, found that prior to the introduction of HAART, HIV-1 DNA variants in the rectum were more diverse than those in blood. However, in patients on HAART, there was an “equalization” of viral dynamics in the rectum and blood. As a result, in HAART-treated patients, there was no difference in the degree of viral diversity between the rectum and peripheral blood (Avettand-Fenoel et al., 2011).

We recently reported that there are marked differences, not only in the level of CD4⁺ T cell depletion and viral replication, but also in immune activation and ART-induced CD4⁺ T cell restoration in the small versus large intestine of South African AIDS patients with chronic diarrhea and (or) unexplained weight loss (Cassol et al., 2013). The aim of the current study was to determine whether these different intestinal environments have an impact on HIV-1 genetic diversification. To address this issue, we assessed the diversity and investigated the phylogenetic relationships among HIV-1 RNA variants in the plasma, duodenum, jejunum, ileum, right and left colon of a subset of our ART-naïve ADIS cohort. An understanding of HIV-1 diversification in different regions of the GIT is important, not only for the design of strategies aimed at interrupting disease progression, but also for the optimization of ART and the assessment of therapeutic efficacy.

MATERIALS AND METHODS

Patients: Ten ART-naïve patients with a complete GIT sample set were selected for genetic studies. Three patients, HIV019, HIV021 and HIV029, who had sufficient sample were selected for extensive phylogenetic analysis of HIV-1 RNA quasispecies. These patients were recruited at the Comprehensive Care, Management and Treatment Clinic in Pretoria as part of a previously described study



of ART-induced CD4⁺ T cell restoration in South African patients with advanced HIV-1 infection (WHO stage III or IV), chronic diarrhea (duration of >4 weeks) and (or) unintentional weight loss (>10% from baseline) of unknown etiology. Patients were eligible for study if their sputum tested negative for *Mycobacterium tuberculosis* and their stool samples were culture negative and stained negative for fungal and parasitic organisms (*Cryptosporidium*, *Microsporidia* and *Isospora*) as determined by hematoxylin-eosin, periodic acid-Schiff diastase, Ziehl-Neelsen and Giemsa staining. The study protocol was reviewed and approved by the Faculty of Health Sciences Research Ethics Committee of the University of Pretoria and written informed consent was obtained from all study participants.

Specimen Collection

“Pinch biopsies” were collected from 5 different sites in the small and large intestine. The duodenum and jejunum were sampled using a Fujinon double balloon enteroscope and Radial III biopsy forceps (Boston Scientific). Endoscopic biopsies of the terminal ileum, right and left colon was obtained using a standard colonoscope. Biopsies were placed on ice in RPMI 1640/10% fetal calf serum (FCS) and immediately processed for flow cytometry or snap frozen in liquid nitrogen and stored at -80°C for viral load (VL) and sequence analysis.

HIV-1 RNA Quantification, cDNA synthesis and PCR Amplification

RNA was extracted from plasma and biopsy samples using the NucliSENS easyMAG extraction kit from BioMerieux and quantified using the Nuclisens Easy HIV-1 QT v.1.2 kit. Results were expressed as the number of HIV-1 RNA copies per gm of tissue or mL of plasma. RNA extracted from gut biopsy samples was reverse transcribed using Superscript III and RNaseOUT (Invitrogen, San Diego, CA) and oligodT or KVL065 (Van Laethem et al., 2006) as primers for the synthesis of *env* and *gag* cDNA, respectively. Reverse transcription of the *pol* gene was performed using the MMLV Reverse Transcriptase 1st Strand cDNA Synthesis Kit (Epicentre Biotechnologies, Madison, Wisconsin) and the reverse primer, IN3 (Pillay, 2006). Following digestion with RNase H, cDNAs were purified using the Invitrogen (*env*) or the GeneJET RNA (*gag*, *pol*) Purification Kits (Fermentas Life Sciences, Burlington, Ontario). Nested PCR of *pol* (1856), *env* (667 bp) and *gag* (1997 bp) encoding regions was performed on 0.2 µg of cDNA using either Platinum Taq High Fidelity DNA polymerase (Invitrogen, San Diego, CA) or Excel Taq DNA polymerase (ExSel, JMR Holdings Inc., London, UK) and the following primer sets: MK605/CD4R2 and ES7/ES8 for *env* (Gordon et al., 2003); KVL064/KVL065 and KVL66/KVL67 for *gag* (Van Laethem et al., 2006); and G25Rev/IN3 and AV150/POLM4 for *pol* (Pillay, 2006). RT-PCR products were purified using the GeneJET™ Gel Extraction (Fermentas Life Sciences) or Purelink PCR Purification (Invitrogen) kits. *Pol* RT PCR products were sequenced directly using published primers: AV150, POLM4, SQ5F, SQ12R, SQ6F, and 13R(2)C(2) (Pillay, 2006; Rousseau et al., 2006).

Cloning and Sequencing

Gag and *env* RT-PCR products were cloned into the pCU⁴ TOPO TA vector (Invitrogen) and transformed into XL 10-Gold Ultra-competent cells (Agilent Technologies, Santa Clara, CA). Plasmids containing the expected inserts, as confirmed by restriction digestion with FastDigest EcoRI (Fermentas Life Sciences), were purified using the Wizard[®] SV 96 Plasmid DNA Purification System (Promega Corporation, Madison, WI). Plasmid DNA was quantified

and sequenced on an automated ABI 3100 DNA analyzer using the Big-Dye terminator v3.1 sequencing kit (ABI, Foster City, CA) and the following primers: T7 and M13R-ES8 for *env* (Gordon et al., 2003) and KVL066, KVL067, KVL078, KVL079, KVL080, GA1, AV103 and AV159 for *gag* (Van Laethem et al., 2006).

Sequence Alignment, Editing and Subtype Analysis

Chromatograms were imported and edited in CLC DNA workbench 5 () or Geneious version 5.3.6 (<http://www.geneious.com>). To rule out contamination, each new sequence was compared to other sequences amplified at the same time and to HIV-1 sequences previously amplified in our laboratory. Sequences were aligned using CLUSTAL W and manually edited using Bioedit version 7.0.4.1 (Thompson et al., 1994) and/or Se-AL (<http://tree.bio.ed.ac.uk>). Gap-stripped sequences were subtyped using the Rega version 3.0 (<http://www.bioafrica.net/rega-genotype/html/subtypinghiv.html>) and COMET (<http://comet.retrovirology.lu/index.php>) subtyping tools and compared to other subtype C strains from South Africa (Los Alamos subtype database,).

Genetic diversity

Pairwise distances among RNA quasispecies in the plasma, duodenum, ileum, jejunum, right and left colon were calculated using the Kimura 2 parametric model of nucleotide substitution implemented in MEGA (Kimura, 1980; Tamura et al., 2011). The gamma distribution model was used to estimate the rate of variation among sites and a bootstrap of 1000 replications was used to calculate the mean distance and standard error. The means of all pairwise genetic distances for each quasispecies relative to other *env* or *gag* quasispecies within and between different tissue compartments was also calculated.

Phylogenetic analyses

Nucleic acid sequences were subjected to extensive phylogenetic analysis. Trees were constructed using Maximum Likelihood (ML) (Guindon et al., 2010) and Bayesian tree inference methods (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) in PhyML and BEAST software packages, respectively. Both methods were performed using the Gamma heterogeneity alpha parameter. The SPRs method and a BioNJ starting tree implemented in PhyML were used to optimize and confirm ML tree topology. The reliability of branching orders was confirmed by performing 1,000 bootstrapping re-samplings. Values >70% were considered significant. The reproducibility of Bayesian trees was assessed using four MCMC chains of 10,000,000 generations sampled every 1,000 generations. Results were visualized in Tracer (Drummond and Rambaut, 2011) and the first 10% of sampled trees and their estimated parameters were excluded from the final analysis (i.e. burning). All the parameters had effective sample sizes greater than 300, suggesting good mixing and sampling of trees. BEAST version 1.6 (Drummond et al., 2012) was used to investigate migration and estimate the most recent common ancestor (MRCA). The initial tree was generated using a simple, constant population size coalescent model. The rate of nucleotide substitution was estimated using a general time reversible (GTR) substitution model with gamma distribution. An uncorrected relaxed clock with gamma distribution and an initial chain of 10⁸ was used to determine the time of divergence of each compartment. A burning of 10% was applied with the initial 10% of data points being excluded from the analysis. The log file output was viewed in Tracer and was deemed suitable for analysis if the estimated sample



sizes were greater than 100. Posterior probability support for the evaluation of the internal branches was estimated using FIGTREE. TreeAnnotator [] was used to generate and visualize the maximum clade credibility (mcc) trees.

Amino acid sequence analysis

Nucleic acid sequences were translated, aligned and used to obtain an inferred amino acid sequence for each of the 350 cloned HIV-1 quasispecies amplified from the plasma, duodenum, jejunum, ileum and colon of patients HIV019, HIV021 and HIV029. Each amino acid sequence was screened for integrity of the coding sequence (deletions, insertions, stop codons) and compared to the consensus sequence for that tissue compartment and to the subtype C consensus sequence for southern Africa. Three different methods were used to predict the tropism and co-receptor usage (CCR5 vs CXCR4) of the V3 loop: the Web Position Specific Scoring Matrix (PSSM) (Jensen et al., 2003), the geno2pheno (Lengauer et al., 2007) and the CoRSeqv3C (Cashin et al., 2013) prediction algorithms. Pol amino acid sequences were screened for substitutions at positions associated with primary and accessory drug resistance to nucleoside reverse transcriptase (RT) and non-nucleoside RT inhibitors (NRTIs, NNRTIs) and protease inhibitors (PIs) as determined using the HIV Drug Resistance Database from Stanford University site (/hiv/).

Nucleotide sequence accession numbers

Genbank accession numbers for the sequences obtained in this study are: KF905653- KF905865, for *env* gene sequences; KF905866- KF906024, for *gag* gene sequences; KF906025- KF906069, for *pol* gene sequences.

RESULTS

Patient

All ten patients were ART-naïve and had advanced HIV-1 infection (WHO stage III or IV). Symptoms leading to endoscopy were diarrhea in 8 and unexplained weight loss of >10% in 2 patients. The mean peripheral blood CD4⁺ T cell count (\pm SD) at the time of presentation was 102 ± 65 cells/ μ L; the mean plasma VL (\pm SD) was 5.41 ± 5.57 log₁₀ copies of HIV-1 RNA/mL and the mean age (\pm SD) was 37 ± 10.6 years. The mean % of CD4⁺ T cells (\pm SD) in the GIT ranged from $3.2 (\pm 2.28)$ in the duodenum to $3.8 (\pm 4.28)$ in the jejunum, $8.9 (\pm 9.92)$ in the ileum and $9.8 (\pm 7.66)$ in the right colon. VLs ranged from $3.68 (\pm 1.32)$ log₁₀ HIV-1 RNA copies/gm of tissue in the duodenum to $3.30 (\pm 1.29)$ in the jejunum, $4.27 (\pm 0.80)$ in the ileum and $4.39 (\pm 1.31)$ in the right colon. Non-specific mild-to-moderate enteritis or enterocolitis was detected in at least one site in 70% of patients, at a similar frequency in all four intestinal sites (70%, 70%, 80% and 70% in the duodenum, jejunum, ileum and colon, respectively).

Genetic diversity

A total of 395 sequences [45 *pol* RT PCR and 350 cloned *env* and *gag* HIV-1 RNA quasispecies obtained from the duodenum ($n = 61$), jejunum ($n = 72$), ileum ($n = 73$), right colon ($n = 80$) and left colon ($n = 64$) of patients HIV019, HIV021 and HIV029 were analyzed. All of these sequences were identified as subtype C when analyzed by REGA and COMET (bootstrap values of >95%). Diversity divergence, estimated in Mega [28, 29], differed significantly among different patients and between the *env* and *gag* gene regions. Mean overall pairwise distances among *env* HIV-1 RNA variants ranged

from 2.27% (S.E. 0.35%) in patient HIV019 to 4.25% (S.E. 0.54%) in patient HIV-29 and 5.99% (S.E. 0.74%) in patient HIV021 [$p < 0.001$]. Overall pairwise distances among *gag* HIV-1 RNA variants ranged from 1.20% (S.E., 0.14%) in patients HIV019 to 1.22% (S.E., 0.14%) in patient HIV029 and 1.96% (S.E., 0.16%) in patient HIV021 [$p < 0.01$]. For each patient, the diversity among *env* RNA quasispecies was significantly greater than among *gag* RNA quasispecies ($p < 0.05$ for patient HIV019; $p < 0.001$ for patients HIV021 and HIV029) **Figure 1**.

Next, we calculated the pairwise distances for each individual clone obtained from patients HIV019 (72 *env*, 59 *gag*), HIV021 (66 *env*, 44 *gag*) and HIV029 (65 *env*, 44 *gag*) compared to every other cloned quasispecies obtained from the same tissue of that particular patient. The number of clones analyzed for each individual compartment is shown in **Table 1**. In general, *env* variants in the small intestine were more diverse than those in the colon **Figure 2** and with few exceptions, most notably the jejunum of patient HIV021 and the right and left colon of patient HIV029, the diversity of *env* was greater than that of *gag* variants in the same tissue. Depending on the patient, inter-compartmental distances ranged from 0.20% to 5.80% among *env* and from 0.94% to 2.12% among *gag* HIV-1 RNA variants **Table 2**. As described below, the lower than expected diversity among *env* sequences in the jejunum of patient HIV021 and the colon of patient HIV029 was due to the presence of large monophyletic clusters of closely related quasispecies.

Phylogenetic analysis of *pol* RT PCR products reveals extensive heterogeneity in the consensus sequences derived from different regions of the GIT

As shown in **Figure 3**, the nucleotide sequences obtained from all 45 *pol* RT PCR products were unique and clustered by individual patient and tissue compartment with no cross-contamination of sequences (bootstrap values >70%). This pattern was observed irrespective of whether ML or Bayesian methods were used for tree construction. As expected, no primary drug resistance mutations were detected in the GIT, or in the plasma of the ten patients studied. However, at the amino acid level, we did detect a number of differentially distributed polymorphisms at positions that are known to cause resistance to RT inhibitors. One substitution, RT D67G, was detected in the colon, but not in the plasma or small intestine of patient HIV010. A second highly unusual substitution, RT L210V, was detected in the ileum of patient HIV011. Although D67N and L210W are well-known resistance mutations, the impact of D67N and L210V substitutions on drug susceptibility are not known. The mean number of substitutions in the RT gene relative to HXB₂ was 23.2 ± 2.3 (range 18-37) with 86.0% of sequences having 21 or more substitutions relative to HXB₂. Some substitutions, such as D123D/G/N/S and T286A, exhibited extensive inter-patient and inter-compartmental variation while others, including V35T, V245Q, T200A and I293V, were highly conserved among different patients and different tissues of the GIT **Table 3**. Polymorphisms associated with altered susceptibility to protease inhibitors were also differentially distributed. Two of these substitutions, T74S and V77I, were detected in the plasma, duodenum, jejunum, ileum and right colon of patient HIV011. PR T74S is present in up to 10% of ART-naïve patients infected with HIV-1 subtype C and is associated with decreased susceptibility to nelfinavir (NFV). PR V77I, is a common to compensatory mutation that is selected by NFV and saquinavir (SQV). Other protease polymorphisms that are common in subtype C viruses, M36I/R41I/ H69K/L89M and T12S/I15V/L19I were present in most, but not all, patients and compartments of the GIT **Table 3**.

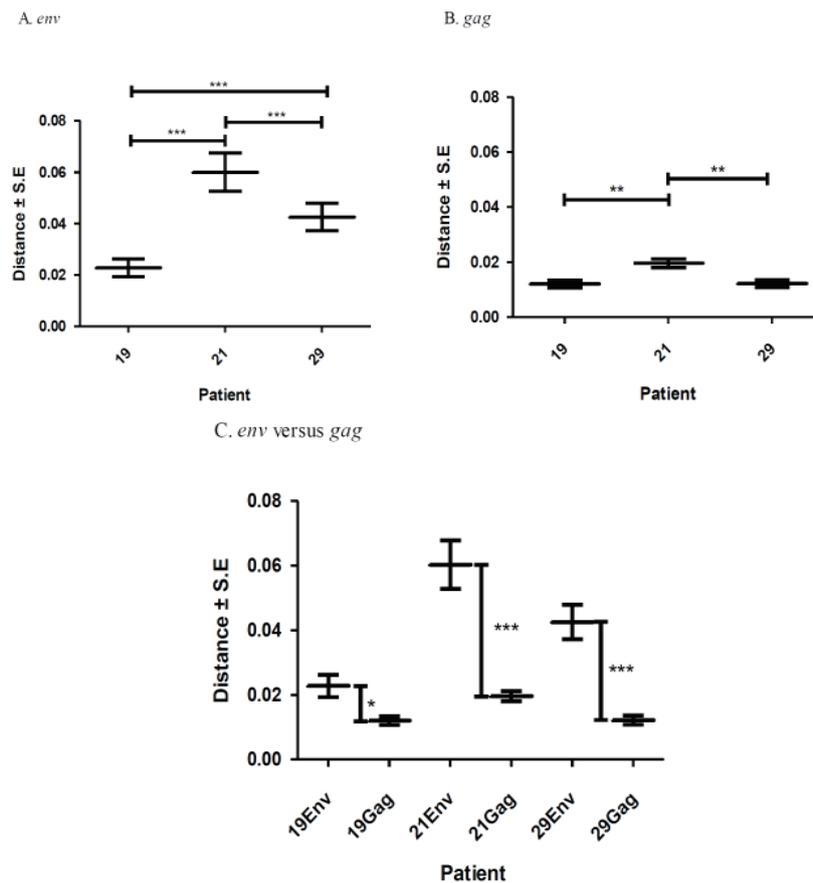


Figure 1: Mean overall pairwise distances among HIV-1 RNA variants in the GIT of ART-naïve AIDS patients. *Env* (A) and *gag* (B) HIV-1 RNA variants were more diverse in the GIT of patient HIV021 compared to patients HIV019 and HIV029. (C). For each of the 3 patients studied, the mean pairwise distances between *env* HIV-1 RNA quasispecies was greater than the mean distances calculated for the *gag* encoding region. Genetic distances between viral clones were computed using the Kimura model. Mean values (horizontal line) and statistical significance are indicated (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

The first series of substitutions is associated with increased catalytic activity of the protease enzyme (Velazquez-Campoy et al., 2001); the second series, when present in combination with I93L, has been linked to increased sensitivity to lopinavir (Gonzalez et al., 2003).

Phylogenetic analysis of HIV-1 RNA quasispecies reveals extensive trafficking and mixing of variants among different regions of the GIT and between the GIT and plasma

Partial or total inter-compartmental mixing of RNA quasispecies was observed among different compartments in the GIT of patients HIV019, HIV021 and HIV029 **Figure 4, A-F**. Mixing of HIV-1 RNA was more evident for the *gag* than the *env* gene with extensive exchange of *gag* quasispecies being observed among all 5 sites in the small and large intestine, and between the GIT and plasma of patient HIV029 **Figure 4, D-F**. Mixing of *env* HIV-1 RNA was less pronounced and observed primarily in the small intestine (duodenum, jejunum and ileum). In addition to mixed clusters, distinct monophyletic clusters of closely related *env* HIV-1 RNA variants were detected in the right and left colon of all 3 patients **Figure 4, A-C** and in the duodenum of patient HIV019, and the jejunum and ileum of patient HIV021. The presence of these tight clusters suggests there was localized tissue-specific evolution of the HIV-1 *env* gene, especially in the right and left colon, and in the small intestine of some patients. In contrast, distinct clustering of *gag* RNA quasispecies was not observed **Figure 4, D-F** suggesting that trafficking, rather than virus evolution, was

the main factor contributing to the heterogeneity of the *gag* encoding gene region in the GIT of AIDS patients with diarrhea and/or weight loss.

Clustering of *env* RNA quasispecies is most consistent in the colon

Clustering was most evident in patient HIV029 where 69% (9/13) of *env* HIV-1 RNA variants in the duodenum, 85% (11/13) of variants in the left colon and 100% (14/14) of variants in the right colon segregated as single monophyletic clusters (**Figure 4C**, clusters 1, 2 and 3, respectively) with posterior probability scores of 1.0, 0.48 and 0.35, respectively. Similarly, 93% (14/15) of *env* HIV-1 RNA sequences in the right colon of patient HIV019 fell into 3 distinct clusters (**Figure 4A**, clusters 3, 4 and 5) with posterior probabilities ranging from 0.47 to 0.99. A notable feature of patient HIV021 **Figure 4B**, was the relationship between *env* HIV-1 RNA sequences in the colon and the ileum. These sequences were found in 4 clusters that segregated together on a single branch of the tree: a basal cluster consisting of 6 sequences from the left colon (cluster 1); an external cluster (cluster 2) containing the majority (12/14, 86%) of *env* sequences from the right colon and two closely linked clusters of *env* RNA from the ileum (clusters 3 and 4), one of which was a mixed ileal/colonic cluster (cluster 4). The posterior probability for these clusters was 1.0, 1.0, 0.93 and 0.96, respectively. In contrast to the lower GIT, most *env* HIV-1 RNA variants in the small intestine were inter-



Table 1: Number of HIV-1 RNA quasiespecies analyzed per patient and per compartment.

Patient	No. <i>env</i> HIV-1 RNA quasiespecies analyzed per compartment		No. <i>gag</i> HIV-1 RNA quasiespecies analyzed per compartment		Total no. HIV-1 RNA clones obtained
HIV019	Duodenum	15	Duodenum	15	131
	Jejunum	10	Jejunum	9	
	Ileum	16	Ileum	10	
	Right Colon	15	Right Colon	15	
	Left Colon	16	Left Colon	10	
	Total :	72	Total:	59	
HIV021	Duodenum	6	Duodenum	1	110
	Jejunum	16	Jejunum	12	
	Ileum	15	Ileum	11	
	Right Colon	14	Right Colon	10	
	Left Colon	15	Left Colon	10	
	Total:	66	Total:	44	
HIV029	Duodenum	13	Duodenum	11	109
	Jejunum	14	Jejunum	11	
	Ileum	11	Ileum	10	
	Right Colon	14	Right Colon	12	
	Left Colon	13	Left Colon	0	
	Total:	65	Total:	44	
Total	203		147		350

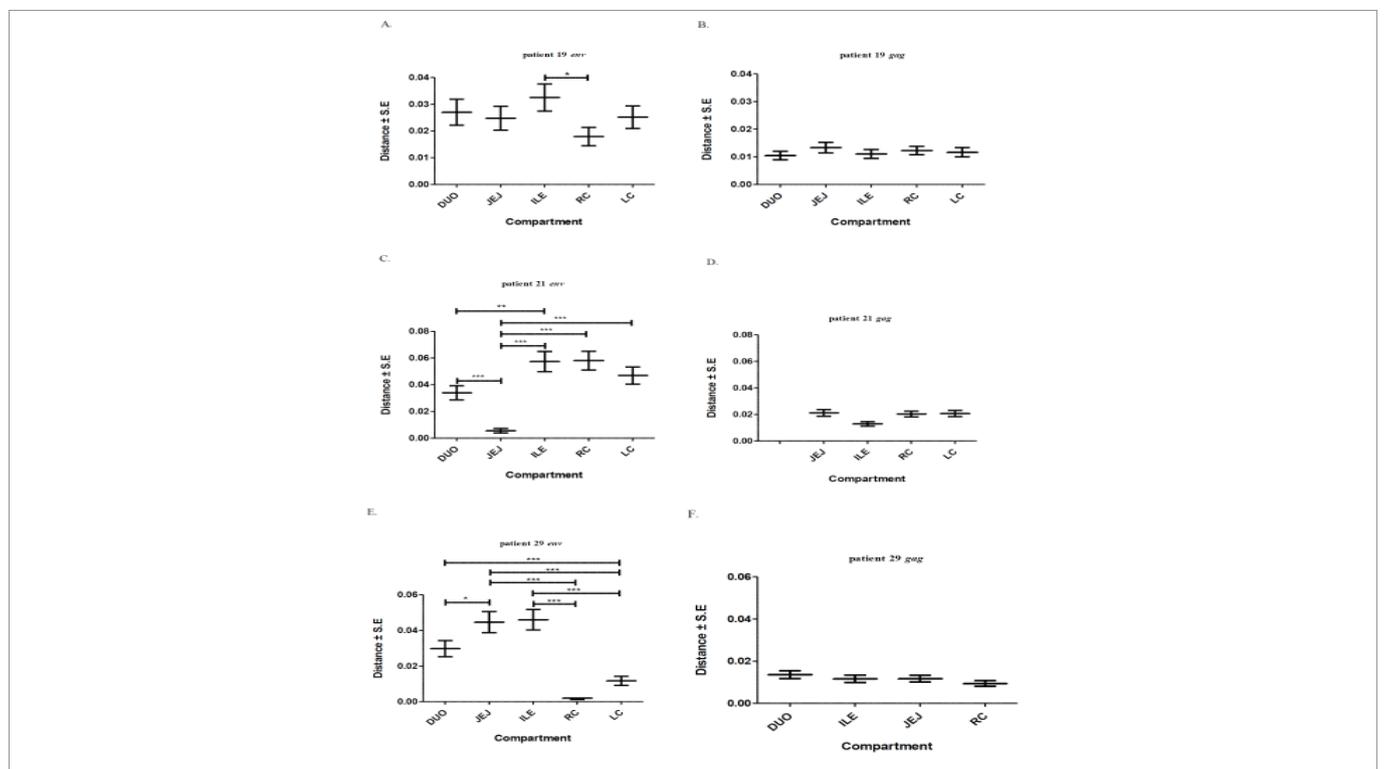


Figure 2: Mean pairwise distances between HIV-1 RNA variants in different compartments of the GIT. *Env* (A, C, and E) and *gag* (B, D, and F) RT-PCR products derived from the duodenum (DUO), jejunum (JEJ), ileum (ILE), right colon (RC) and left colon (LC) of patients HIV019, HIV021 and HIV029 were cloned, sequenced and analyzed for genetic diversity using the Kimura model. With few exceptions (the jejunum of patient HIV021 and the right colon of patient HIV029), *env* HIV-1 RNA sequences were more diverse than *gag* HIV-1 RNA variants derived from the same tissue compartment. Mean values (horizontal line) and statistical significance are indicated (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

dispersed among the duodenum, jejunum and ileum. Exceptions included two *env* RNA clusters in the duodenum of patient HIV019 (clusters 1 and 6) and a large cluster of 17 closely related *env* RNA sequences in patients HIV021, 16 from the jejunum and 1 from the duodenum. Many sequences from the ileum clustered with *env* HIV-1 RNA sequences in the jejunum (and plasma) and to a lesser extent the duodenum, suggesting that there was extensive exchange of viral variants between the ileum and other tissue compartments including blood.

Differential distribution of env variants with altered neutralization and co-receptor binding domains

The primary antibody neutralization domain of subtype C, a GPGQ tetramer located at the tip of the V3 loop, was conserved in 97.0% (197/203) of GIT and 100% (9/9) of plasma samples. Six tetramer motifs, four in patient HIV021 (GPGH in the duodenum; GPCR and 2 RPGQ in the right colon) and two in patient HIV029 (RPGQ in the duodenum; RPGY in the left colon) had a basic amino acid substitution in the first or last position of the tetramer, either



Patient	Compartment	Mean % distances (SE)	
		Envelope	Gag
HIV019	Duodenum	2.70 (0.48)	1.05 (0.15)
	Jejunum	2.48 (0.45)	1.34 (0.19)
	Ileum	3.25 (0.51)	1.12 (0.16)
	Right colon	1.79 (0.34)	1.23 (0.15)
	Left colon	2.52 (0.42)	1.17 (0.17)
HIV021	Duodenum	3.39 (0.53)	ND
	Jejunum	0.56 (0.17)	2.12 (0.25)
	Ileum	5.73 (0.76)	1.29 (0.17)
	Right colon	5.80 (0.71)	2.03 (0.22)
	Left colon	4.68 (0.65)	2.07 (0.23)
HIV029	Duodenum	2.97 (0.45)	1.36 (0.19)
	Jejunum	4.46 (0.59)	1.17 (0.16)
	Ileum	4.60 (0.57)	1.16 (0.18)
	Right colon	0.20 (0.08)	0.94 (0.14)
	Left colon	1.17 (0.26)	ND
	Plasma	5.68 (0.72)	1.16 (0.16)

Table 2: Diversity of *env* and *gag* HIV-1 RNA quasiespecies in the GIT of patients HIV019, HIV021 and HIV029. With the exception of the jejunum of patient 21 and right colon of patient 29, the *env* gene region was more diverse (higher mean total distance) than the *gag* encoding regions. *Env* sequences in the small intestine (duodenum, ileum) of patients 19 and 29 were also more diverse than those in the right and left colon. These observations suggest that different selection pressures (or selection factors) were acting on the *env* gene in different regions of the GIT. ND, not done.

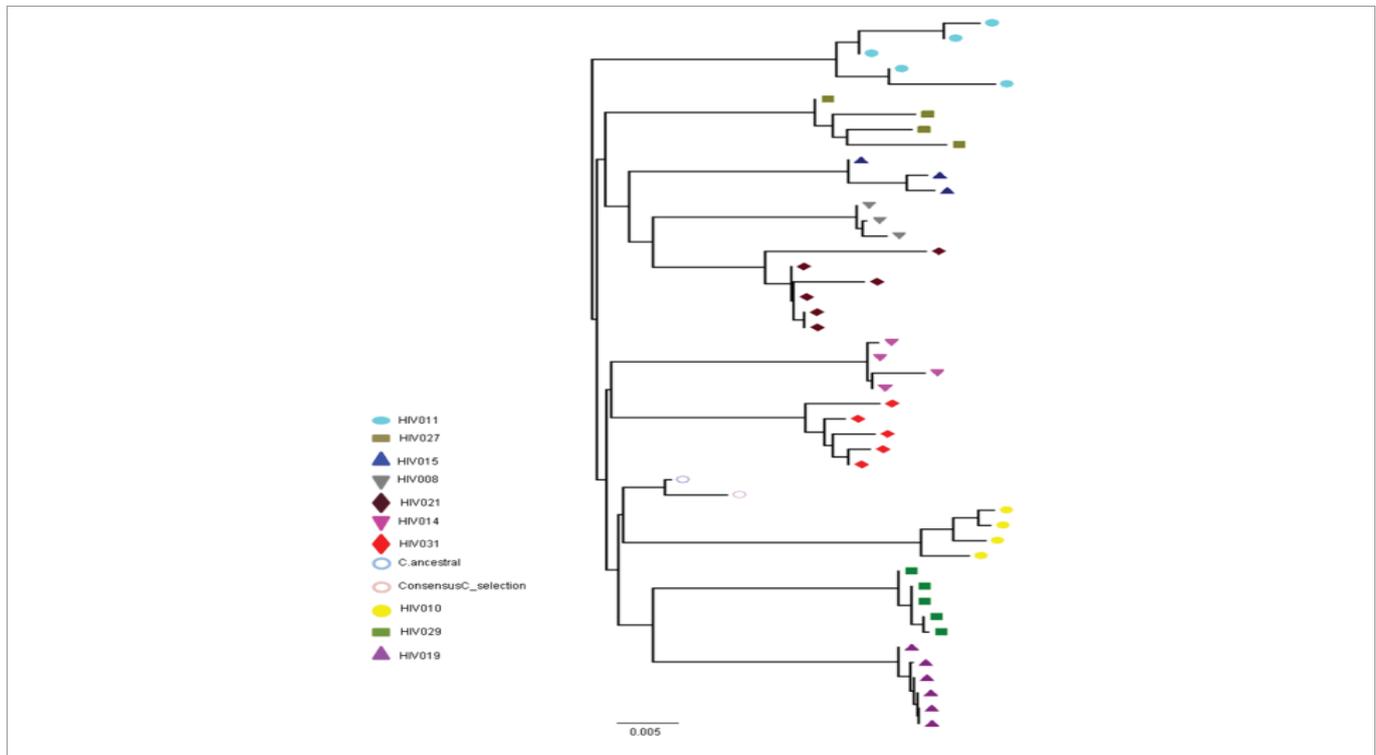


Figure 3: Representative Bayesian tree of HIV-1 *pol*/RT PCR sequences from different regions of the GIT. All of the clusters identified by the Bayesian approach were confirmed by the Maximum Likelihood method. Patient specific clusters and IDs (HIV008, HIV010, HIV011, HIV014, HIV015, HIV019, HIV021, HIV027, HIV029 and HIV031) are color coded and indicated by the brackets on the right side of the tree.

arginine (R) or histidine (H). An analysis of tropism using three different algorithms, the Web Position Specific Scoring Matrix (PSSM) tool (SINSI matrix), the Geno2pheno and the CoRSeqv3C co-receptor predictor algorithm indicated that, depending on the algorithm used, from 83.1% to 100% of all variants in the GIT of patients HIV019 (n = 72), HIV021 (n = 66) and HIV029 (n = 65), were CCR5-tropic and non-syncytium inducing (NSI). The most sensitive method, CoRSeqv3C, identified 5 CXCR4-tropic variants in the duodenum (n = 1), left colon (n = 1) and right colon (n = 3) of

patient HIV021. These variants included the four viral quasiespecies with a basic amino acid substitution in the tetrameric GPGQ tip of the V3 loop and a fifth variant with a basic (arginine) residue at position 1 of the V3 loop. A total of 11 CXCR4-tropic variants were identified in samples obtained from the duodenum (n=1), jejunum (n = 5), ileum (n =1), left colon (n=1) and plasma (n = 3) of patient HIV029 using the CoRSeqv3C method. All of these variants contained a basic substitution in the V3 loop, either a lysine residue at position 32 (n=9) or an arginine residue at position 15 (n=2). No CXCR4-using



Protein	Amino acid	% of Samples					
		DUO	JEJ	ILE	RC	LC	PLA
Protease (PR)	H69K	100	100	100	100	100	100
	M36I ^a	100	88	100	100	100	100
	L89M ^b	100	100	100	83	80	75
	R41K ^c	86	75	75	83	80	88
	L19I/T ^d	100	88	88	100	80	88
	I93L	86	88	88	83	100	88
	I15V ^e	86	75	75	67	60	88
T12S ^f	70	63	63	67	60	75	
Reverse Transcriptase (RT)	V35T	100	100	100	100	100	100
	T39D/E/K/T	100	100	100	100	100	100
	K43D	100	100	100	100	100	100
	K122E	100	100	100	100	80	100
	K173A/T	100	100	100	100	100	100
	T200A	100	100	100	100	100	100
	Q207E/D/E/G/K/R/T	100	100	100	100	100	100
	R211A/E/G/K/N	86	100	100	100	100	100
	I293V	100	100	88	100	80	88
	E36A/E	100	75	50	67	60	63
	V292I	86	88	100	100	100	88
	V245E/K/Q/V	86	88	100	100	100	88
	A272P	86	88	88	83	100	88
	E291A/D/E	71	88	88	83	100	100
	DL23D/G/N/S	57	63	50	83	20	50
	DL77E	57	63	63	83	40	50
	T286A	43	38	38	50	40	25
K277K/R/S	38	50	63	67	63	50	

Table 3: Frequency of Protease and Reverse Transcriptase Amino Acid Substitutions Detected in Different Regions of the GIT

^aPresent as a mixed M36I/M residue in the duodenum of patient 11. ^bPresent as a mixed L89L/M residue in patient 31; present in the duodenum but not in the right colon or plasma of patient 31; not detected in any GIT or plasma samples from patient 8. ^cDetected in the plasma but not in the jejunum, ileum or left colon of patient 31; not detected in samples from patient 27. ^dL19I/T was present in 5.9% of GIT and 12.5% of plasma samples. ^ePresent as a mixed I15I/V residue in the duodenum of patient 10 and the ileum of patient 31; detected in the right colon and plasma but not in the duodenum or right colon of patient 31; not detected in samples from patient 14, or in the jejunum, left colon or plasma of patient 10. ^fPresent as a mixed T12S/T residue in the ileum of patient 15.

variants were detected in the GIT of patient HIV019 using any of the 3 testing algorithms.

DISCUSSION

There are still many unanswered questions relating to the role of the GIT in the genetic evolution and dynamics of HIV-1 infection. It has also been difficult to compare the data generated in different studies due to inter-study differences in sampling sites and the stage of HIV-1 disease, as well as the confounding effects of different treatment regimens. In addition, most studies have been performed on HIV-1 proviral DNA and thus, may not be representative of the full spectrum of replication competent viral quasispecies. In this study, we examined the diversity of HIV-1 RNA quasispecies in the intestine of a well-characterized cohort of African patients with AIDS, chronic diarrhea and (or) unexplained weight loss. To our knowledge this is the first study of HIV-1 intestinal diversity in patients infected with subtype C, a viral subtype that may be particularly well adapted to replication in the GIT (Sala et al., 2006; Centlivre et al., 2006). It is also the first study to be conducted in Africa, a region of the world where gastrointestinal symptoms and wasting (Slim⁷ disease) are common. In our study, a major effort was made to exclude patients with enteric co-infections (stool cultures, histological staining for acid-fast bacilli, fungal and parasitic infections). Despite these efforts, we cannot conclusively rule out the possibility that other co-infectious may have affected our results.

Overall, our results are in agreement with a pre-HAART study of proviral DNA in patients infected with subtype B (van Marle et al., 2007). Of interest, was the higher overall diversity of *env* compared to *gag* HIV-1 RNA variants and the finding that *env* variants were, in general, more heterogeneous in the small intestine than the colon.

These findings suggest that the *gag* and *env* genes are under selection pressures and that these selection pressures vary in different parts of the GIT. Factors contributing to the HIV-1 diversity in different regions of intestine have not been defined but may, among others, include site-specific differences in inflammation and immune activation and in the magnitude and specificity of the anti-HIV-1 CD8⁺ CTL response, as well as factors related to differences in CD4⁺ T cell levels, target cell tropism and viral entry, all of which would be expected to have a greater impact on the *env* than the *gag* gene.

Phylogenetic analysis indicated that there were also distinct differences in the distribution and clustering of *gag* and *env* RNA variants. The extensive migration and mixing of *gag* HIV-1 RNA variants between plasma and different regions of the GIT is consistent with the view that, in the absence of ART, there is widespread dissemination of newly produced viral particles and (or) infected cells. *Env* RNA variants exhibited a more complex pattern. In addition to mixed phylogenetic clusters, *env* trees contained large monophyletic clusters of closely related RNA variants. The presence of these clusters, especially in the colon, suggests the presence of distinct foci of HIV-1 replication that could contribute to the emergence of new viral variants with altered pathogenicity. Additional longitudinal studies will be required to further confirm that there is isolated generation of pathogenic variants of HIV-1 in the GIT of ART-naïve patients. To be informative, these studies will require sequencing of a highly variable gene region that is under strong selection pressure. The reason for the consistent and preferential evolution of *env* RNA variants in the colon is not known but may be related to the higher levels of viral replication and target cell availability in the lower vs. upper GIT (Cassol et al., 2013; Schneider et al., 1995; Yukl et al., 2010). Alternatively, it may be more closed related to microbial translocation and high levels of non-

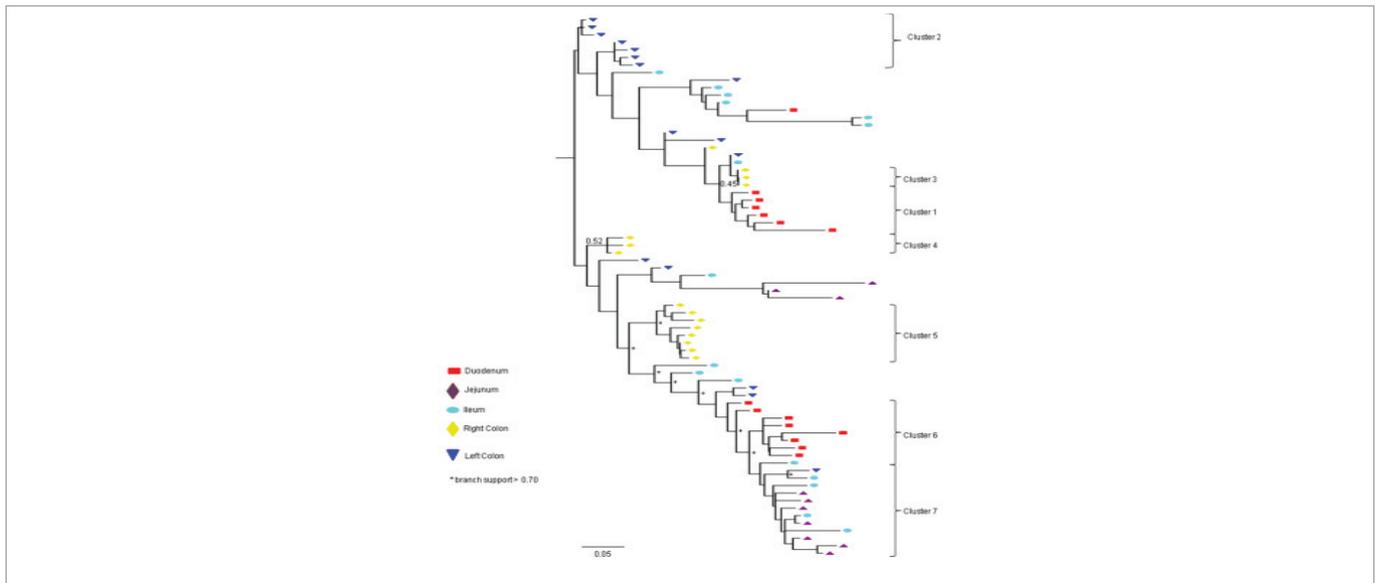


Figure 4a: Phylogenetic relationships among *env* (A-C) and *gag* (D-F) RNA quasiespecies in different compartments in the GIT. Envelope trees were constructed using a general time reversible (GTR) with likelihood and γ -distribution α values of >3281.4 and 5929.5 for patient HIV019 (A), >5103 and 3411.5 for patient HIV021 (B) and >2704.6 and 1392.3 for patient HIV029 (C). The corresponding values for the construction of *gag* trees were >336 and 3420.2 for patient HIV019 (D); >3666.3 and 4217.6 for patient HIV021 (E) and >5473.2 and 3037.3 for patient HIV029 (F). In addition to extensive trafficking and exchange of HIV-1 RNA variants among different sites in the GIT, especially in the small intestine, clustering of *env* sequences by tissue compartment was observed in the right and left colon of all 3 patients, and in the duodenum of patients HIV019 and HIV029 and the jejunum and ileum of patient HIV021, a finding that is suggestive of localized tissue-associated evolution of HIV-1 RNA quasiespecies. The trees are color coded as follows: duodenum, red; jejunum, purple; ileum, light blue; right colon, yellow; left colon, dark blue.

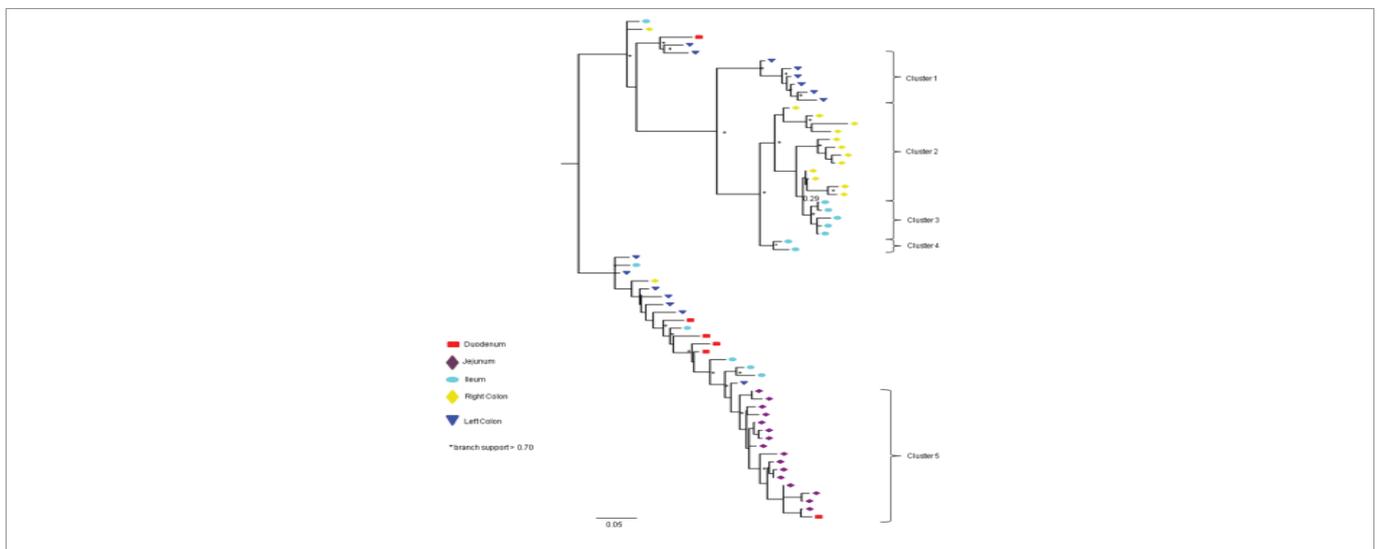


Figure 4b: Phylogenetic relationships among *env* (A-C) and *gag* (D-F) RNA quasiespecies in different compartments in the GIT. Envelope trees were constructed using a general time reversible (GTR) with likelihood and γ -distribution α values of >3281.4 and 5929.5 for patient HIV019 (A), >5103 and 3411.5 for patient HIV021 (B) and >2704.6 and 1392.3 for patient HIV029 (C). The corresponding values for the construction of *gag* trees were >336 and 3420.2 for patient HIV019 (D); >3666.3 and 4217.6 for patient HIV021 (E) and >5473.2 and 3037.3 for patient HIV029 (F). In addition to extensive trafficking and exchange of HIV-1 RNA variants among different sites in the GIT, especially in the small intestine, clustering of *env* sequences by tissue compartment was observed in the right and left colon of all 3 patients, and in the duodenum of patients HIV019 and HIV029 and the jejunum and ileum of patient HIV021, a finding that is suggestive of localized tissue-associated evolution of HIV-1 RNA quasiespecies. The trees are color coded as follows: duodenum, red; jejunum, purple; ileum, light blue; right colon, yellow; left colon, dark blue.

specific inflammation and immune activation in the colon.

An analysis of inferred amino acid sequences indicated that some nucleotide substitutions in the *env* gene involved the replacement of neutral or acidic amino acids with positively charged residues that are known to play a role in the switch from CCR5 to CXCR4 co-receptor usage (Jensen et al., 2003). These CXCR4-tropic variants

were detected in 2 of 3 patients and were present in some, but not all, parts of the GIT. Although HIV-1 subtype C viruses generally use the CCR5 chemokine receptor (along with CD4) for virus entry into susceptible target cells, genotypic changes can occur that allow C viruses to use CXCR4 as an entry receptor. The switch to CXCR4 usage is associated with accelerated CD4⁺ T cell depletion

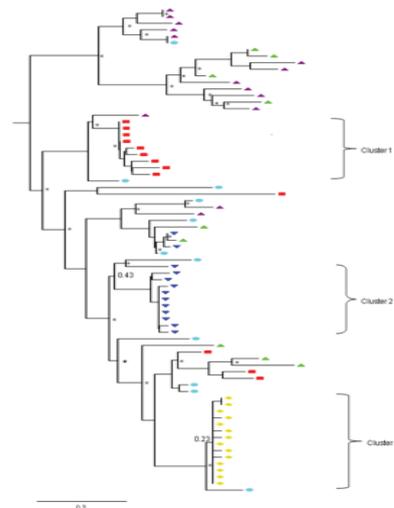


Figure 4c: Phylogenetic relationships among *env* (A-C) and *gag* (D-F) RNA quasiespecies in different compartments in the GIT. Envelope trees were constructed using a general time reversible (GTR) with likelihood and γ -distribution α values of >3281.4 and 5929.5 for patient HIV019 (A), >5103 and 3411.5 for patient HIV021 (B) and >2704.6 and 1392.3 for patient HIV029 (C). The corresponding values for the construction of *gag* trees were >336 and 3420.2 for patient HIV019 (D); >3666.3 and 4217.6 for patient HIV021 (E) and >5473.2 and 3037.3 for patient HIV029 (F). In addition to extensive trafficking and exchange of HIV-1 RNA variants among different sites in the GIT, especially in the small intestine, clustering of *env* sequences by tissue compartment was observed in the right and left colon of all 3 patients, and in the duodenum of patients HIV019 and HIV029 and the jejunum and ileum of patient HIV021, a finding that is suggestive of localized tissue-associated evolution of HIV-1 RNA quasiespecies. The trees are color coded as follows: duodenum, red; jejunum, purple; ileum, light blue; right colon, yellow; left colon, dark blue.

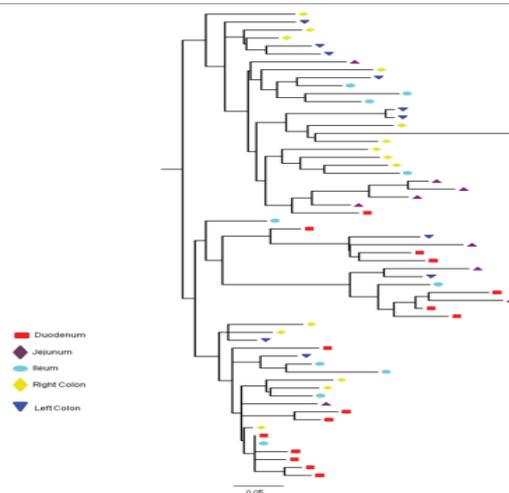


Figure 4d: Phylogenetic relationships among *env* (A-C) and *gag* (D-F) RNA quasiespecies in different compartments in the GIT. Envelope trees were constructed using a general time reversible (GTR) with likelihood and γ -distribution α values of >3281.4 and 5929.5 for patient HIV019 (A), >5103 and 3411.5 for patient HIV021 (B) and >2704.6 and 1392.3 for patient HIV029 (C). The corresponding values for the construction of *gag* trees were >336 and 3420.2 for patient HIV019 (D); >3666.3 and 4217.6 for patient HIV021 (E) and >5473.2 and 3037.3 for patient HIV029 (F). In addition to extensive trafficking and exchange of HIV-1 RNA variants among different sites in the GIT, especially in the small intestine, clustering of *env* sequences by tissue compartment was observed in the right and left colon of all 3 patients, and in the duodenum of patients HIV019 and HIV029 and the jejunum and ileum of patient HIV021, a finding that is suggestive of localized tissue-associated evolution of HIV-1 RNA quasiespecies. The trees are color coded as follows: duodenum, red; jejunum, purple; ileum, light blue; right colon, yellow; left colon, dark blue.

and progression to AIDS (Connor et al., 1997). Additional *in vitro* testing will be required to confirm the tropism of these CXCR4-encoding variants. Using population-based sequencing, a method that is commonly used to screen for HIV-1 resistance mutations in plasma RNA, we also detected a number of nucleotide substitutions and inferred amino acid polymorphisms that have been linked to altered susceptibility to ART. As observed for the *env* gene and for primary resistance mutations in patients on mono- or dual-therapy

(van Marle et al., 2010) and in ART-treated patients with ongoing viral replication ($>1,000$ HIV-1 RNA copies/mL of plasma) (Poles et al., 2001), these resistance-associated variants were not present in all patients, and when present, were differentially distributed throughout the GIT.

The above results are consistent with other studies conducted in pre-HAART patients (van Marle et al., 2007; van Marle et al., 2010) and in viremic patients on HAART (Poles et al., 2001). They

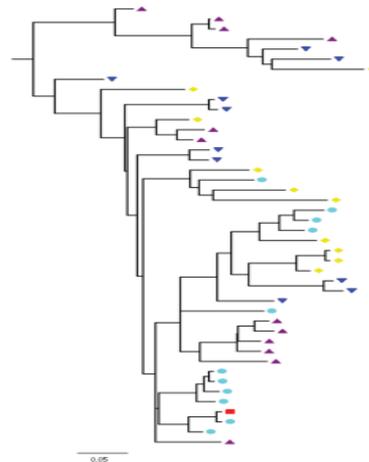


Figure 4e: Phylogenetic relationships among *env* (A-C) and *gag* (D-F) RNA quasiespecies in different compartments in the GIT. Envelope trees were constructed using a general time reversible (GTR) with likelihood and γ -distribution α values of >3281.4 and 5929.5 for patient HIV019 (A), >5103 and 3411.5 for patient HIV021 (B) and >2704.6 and 1392.3 for patient HIV029 (C). The corresponding values for the construction of *gag* trees were >336 and 3420.2 for patient HIV019 (D); >3666.3 and 4217.6 for patient HIV021 (E) and >5473.2 and 3037.3 for patient HIV029 (F). In addition to extensive trafficking and exchange of HIV-1 RNA variants among different sites in the GIT, especially in the small intestine, clustering of *env* sequences by tissue compartment was observed in the right and left colon of all 3 patients, and in the duodenum of patients HIV019 and HIV029 and the jejunum and ileum of patient HIV021, a finding that is suggestive of localized tissue-associated evolution of HIV-1 RNA quasiespecies. The trees are color coded as follows: duodenum, red; jejunum, purple; ileum, light blue; right colon, yellow; left colon, dark blue.

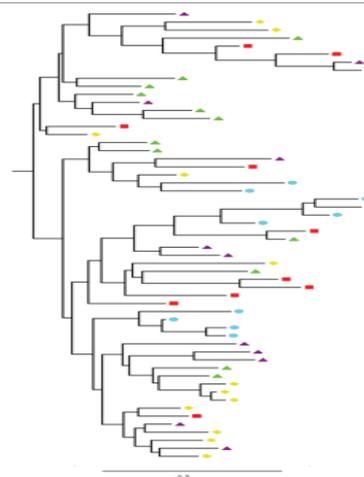


Figure 4f: Phylogenetic relationships among *env* (A-C) and *gag* (D-F) RNA quasiespecies in different compartments in the GIT. Envelope trees were constructed using a general time reversible (GTR) with likelihood and γ -distribution α values of >3281.4 and 5929.5 for patient HIV019 (A), >5103 and 3411.5 for patient HIV021 (B) and >2704.6 and 1392.3 for patient HIV029 (C). The corresponding values for the construction of *gag* trees were >336 and 3420.2 for patient HIV019 (D); >3666.3 and 4217.6 for patient HIV021 (E) and >5473.2 and 3037.3 for patient HIV029 (F). In addition to extensive trafficking and exchange of HIV-1 RNA variants among different sites in the GIT, especially in the small intestine, clustering of *env* sequences by tissue compartment was observed in the right and left colon of all 3 patients, and in the duodenum of patients HIV019 and HIV029 and the jejunum and ileum of patient HIV021, a finding that is suggestive of localized tissue-associated evolution of HIV-1 RNA quasiespecies. The trees are color coded as follows: duodenum, red; jejunum, purple; ileum, light blue; right colon, yellow; left colon, dark blue.

underscore the highly dynamic nature of HIV-1 infection and RNA diversification in the GIT of ART-naïve patients and suggest that diversification should be viewed both in terms of genetic evolution and migration into, and out of different compartments in the GIT. Strengths of our study relate to the homogeneity of our study cohort with respect to stage of disease, the focus on RNA rather than proviral DNA and the sampling of multiple tissue sites. Limitations relate to the small sample size and the potential biases introduced by population-based PCR cloning (Taq induced recombination, nucleotide misincorporation) (Meyerhan et al., 1990; Yang et al., 1996). However, this does not appear to be a problem when analyzing

samples with a high number of PCR templates, as in the current study (Jordan et al., 2010).

It is important to note that several recent studies conducted in HAART-treated patients with plasma viral loads <50 copies/mL have minimized the role of the GIT in the evolution and compartmentalization of HIV-1 and suggested that the GIT is not a source of rebound virus during therapeutic failure (Imamichi et al., 2011; Lerner et al., 2011; Evering et al., 2012). The reason(s) for the failure to detect HIV-1 diversification in patients on maximally suppressive HAART is not known but may be related to the marked



decreased viral replication and *de novo* infection of new target cells, or to reduced chemokine levels and decreased trafficking of HIV-1-infected cells. Alternatively, virus transmission in patients on highly suppressive ART may occur *via* a cell-cell route (Sigal et al., 2011) that does not involve exposure to strong diversifying selection pressure. Of interest in this regard, is a study by Avettand-Fenoel *et al.*, showing that, following the introduction of HAART, there is reduced viral replication and trafficking between the rectum and peripheral blood (Avettand-Fenoel et al., 2011).

In conclusion, this finding, together with our data, highlight the need for more systematic and comprehensive studies of HIV-1 diversification in different compartments of the upper and lower GIT, both as a function of disease progression and in response to therapy. In addition to serving as a baseline for improved therapeutic monitoring, such studies may provide new insights for preventing the trafficking, dissemination and evolution of more pathogenic and drug resistant strains of HIV-1. These studies will be challenging given the technical and ethical considerations associated with repeat sampling of multiple sites.

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