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Longitudinal sequencing of HIV-1 infected patients with low-level viremia for years while on ART shows no indications for genetic evolution of the virus.

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ABSTRACT

HIV-infected patients on antiretroviral therapy (ART) may present low-level viremia (LLV) above the detection level of current viral load assays. In many cases LLV is persistent but does not result in overt treatment failure or selection of drug resistant viral variants. To elucidate whether LLV reflects active virus replication, we extensively sequenced *pol* and *env* genes of the viral populations present before and during LLV in 18 patients and searched for indications of genetic evolution. Maximum likelihood phylogenetic trees were inspected for temporal structure both visually and by linear regression analysis of root-to-tip and pairwise distances. Viral coreceptor tropism was assessed at different time points before and during LLV. In none of the patients consistent indications for genetic evolution were found over a median period of 4.8 years of LLV. As such these findings could not provide evidence that active virus replication is the main driver of LLV.

KEYWORDS

HIV-1, low-level viremia, antiretroviral therapy, HIV evolution, HIV persistence, residual viremia

INTRODUCTION

Patients on combined antiretroviral therapy (ART) may continue to express small traces of virus in their plasma, in some cases exceeding the detection level of commercially available viral load (VL) assays. This so called low-level viremia (LLV) may persist for longer periods of time. From our previous work we learned that persistent LLV, in most cases, does not result in treatment failure or selection of drug resistance [1]. The absence of drug resistance mutations (DRM) is clinically reassuring, but it cannot exclude a role of active viral replication in LLV. A recent study by Lorenzo-Redondo *et al.* (2016) again emphasized the potential role of anatomic sites in the tissues (e.g. lymph nodes) that are insufficiently reached by ART and may act as viral replication hubs [2]. Pressure for selection of DRM may be limited at these sites, due to the low drug concentrations.

LLV in patients on ART has been associated with elevated levels of activated CD8+ T-cells and with chronic immune activation. This immune activation may induce extended release of virus from infected cells or *vice versa*, ongoing virus replication may induce and maintain immune activation [3, 4]. Knowing whether ongoing replication takes place under ART is also important for HIV cure research [5, 6].

Hypothesizing that active replication will ultimately present as ongoing genetic evolution of individual viruses, phylogenetic analysis is the most frequently used tool to prove or reject virus replication [7-17]. Already in 1999, Günthard *et al.* used phylogenetic analysis to demonstrate HIV-1 evolution, and thus active replication, in some patients with residual viremia on successful ART [11]. This finding was later supported by the results of Frenkel *et al.* and Zhang *et al*. [10, 17]. Using the same phylogenetic approach other studies, however, failed to demonstrate signs of evolution in comparable patient populations [7-9, 12-16]. In a recent publication, Lorenzo-Redondo *et al.* present results obtained after next generation sequencing (NGS) of HIV-1 DNA from blood and lymph node cells of 3 patients on ART for 6 months [2]. Time-calibrated phylogenetic analysis showed ongoing replication in lymphoid tissue despite undetectable VL in plasma. In a later study in children with a very homogeneous virus population at initiation of ART, thereby providing a low background to detect genetic evolution, no indications for evolution were seen in 9 of the 10 infants over a follow-up period of 7 years [18]. It was therefore hypothesized that the time-calibrated method of Lorenzo-Redondo *et al.* had skewed the phylogenetic analysis, as the input time point of the samples was considered in generating the phylogenetic tree, giving the appearance of evolution even if it is not present. These observations stress the importance of including distance based methods to identify genetic evolution.

In addition to phylogenetic analyses, some studies focused on the quantification of 2-LTR circles as markers of active virus replication [19-22]. In 2010, the observation that intensification of an ART regimen with the integrase inhibitor raltegravir led to an increase in number of 2-LTR circles fueled the hypothesis that seemingly suppressive ART failed to completely block virus replication [19]. In the

randomized, double-blind, placebo-controlled study of Hatano *et al.* in 2013 [21], addition of raltegravir to a stable ART regimen resulted in a transient increase in 2-LTR circles in 9 of 16 patients. Other studies, however, failed to confirm this effect of treatment intensification [20, 22]. Today, the hypothesis that the most important sources of residual viremia are long-lived cells that continuously express virus or latently infected cells that produce virus when they become activated while the presence of ART prevents further replication of this virus is gaining more and more acceptance [12, 13].

While a multitude of studies have investigated the role of virus replication in patients on ART with undetectable VL, studies on the sources of LLV above the detection limit of VL assays are limited and the conclusions from these studies even more contradicting [7, 15, 23]. Anderson *et al.* and Mens *et al.* failed to provide proof of ongoing replication, while Tobin *et al.* did report evidence of genetic evolution of plasma virus in a minority of the studied patients (3 of 11). Two studies suffered from a low sample number with respectively 1 and 11 LLV individuals included [7, 23]. The extent of patient to patient differences in causes of LLV is unknown and it is also unclear whether virus production on its own can result in plasma virus concentrations above the cut-off levels of currently applied routine VL assays.

In the current study, we searched for signs of viral replication and evolution in 18 patients with persisting LLV. Important added values of this work are the fact that both the plasma and cellular compartments of the blood were investigated and that samples were collected over a long period of time at multiple time points during LLV as well as before initiation of the ART regimen under which LLV manifested. The results of extensive phylogenetic analysis could not provide consistent proof of virus replication.

METHODS

Patient selection

From January 1997 to December 2012, 1393 HIV-infected patients were followed at Ghent University Hospital and 621 were on ART during this period. Persisting LLV was retrospectively detected in a subpopulation of 71 patients. We defined PLLV as a VL between 20 and 250 copies (c)/mL for at least half of minimum 6 VL measurements from six months after the initiation of ART until study inclusion (January 2013) [1]. The VL was determined with the Roche Cobas AmpliPrep/Cobas TaqMan HIV-1 test v2 (Roche, Basel, Switzerland; limit of detection: 20 c/mL). Longitudinal sequence analysis during LLV was attempted for 28 patients randomly selected from the PLLV patients. Extensive phylogenetic and sequence analysis was then performed on the 18 patients for whom at least 3 env and pol sequences were obtained.

All analyses were performed retrospectively on stored plasma and buffy coat samples. The treating clinicians of the included patients were free to adapt the ART regimen during LLV. All patients received intensive individual counselling, including guidance on adherence, food restrictions and interference with other products, in order to exclude potential sources of reduced exposure to ART.

Study design

To be able to amplify and sequence two regions of the HIV genome (*pol* and *env*) from samples with very low viral load, an ultrasensitive Sanger sequencing protocol was developed and used for all plasma samples collected during LLV. For the samples collected pre-ART, standard population Sanger sequencing was performed. In-depth characterization of the virus population present at initiation of ART was done by next generation sequencing (NGS) of the last plasma sample collected before starting ART (baseline sample).

Because of expected low concentrations of viral DNA in blood cells collected on ART and because a longer sequence length allows better identification and elimination of sequences from defective viruses, limiting dilution sequencing was chosen over NGS for characterization of the HIV DNA

genetic variability. This limiting dilution DNA sequencing was only preformed for a selection of 8 patients with LLV of over at least 5 years and for whom buffy coat samples were available from at least 3 time points with an interval of 2 years.

Sequencing of HIV-1 *pol* and *env* gene fragments

Standard Sanger sequencing of viral RNA

HIV-1 RNA was extracted from EDTA plasma using the High Pure Viral RNA kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. For samples with a VL of <10,000 c/mL, 500 µL of plasma was ultracentrifuged for 1 hour (23,600 × *g* at 4 °C), after which the pellet was suspended in 200µL of phosphate buffered saline (PBS). For samples with a VL of >10,000 c/mL, RNA was extracted from 200 µl of plasma without ultracentrifugation.

Amplification of *pol* and *env* was performed using an in-house nested PCR protocol. Briefly, 10 µL of the 50 µL RNA extract was subjected to reverse transcription and amplification using the Titan One tube RT-PCR system (Roche, Basel, Switzerland) with a combination of 4 primers. The inner PCR reaction was performed using 2 µL of the outer PCR product and 4 nested primers. Sequencing of the amplicons was performed with the BigDye® Terminator Cycle Sequencing kit v. 3.1 (Life Technologies, Carlsbad, CA, USA) and sequencing products were analyzed on the ABI3130XL Genetic analyzer (Applied Biosystems, Foster City, CA, USA). Chromatograms were checked and edited manually using SmartGene (IDNS™) software (SmartGene GmbH, Zug, Switzerland). Further details on the amplification and sequencing procedures as well as the primer sequences can be obtained from the authors on request.

Ultrasensitive Sanger sequencing

To increase the sensitivity of the Sanger sequencing procedure, the input plasma volume was increased to 1.5 mL and the ultracentrifugation time to 2 hours. The elution volume of the extraction was reduced from 50 µL to 25 µL and 2 µL of MS2 RNA (Roche, Basel, Switzerland) was added as carrier. A 3-step nested amplification protocol was used instead of the standard 2-step reaction as

previously described [1]. A detailed description of the PCR conditions can be obtained from the authors on request. Briefly, 20 µL of RNA extract was subjected to 60 minutes of reverse transcription followed by 10 cycles of amplification in a multiplex reaction combining *env* and *pol* amplification. Ten µL of the obtained amplicon was then used as input material for two second round 40 cycle amplifications, one with *env* primers and one with *pol* primers. Finally, 2 µL of the second round reactions was used as input material for third round 40 cycle amplifications with nested primers.

Next generation sequencing of viral RNA

In-depth *env* sequencing of viral RNA isolated from the baseline samples was done using the Roche 454 GS Junior next generation sequencing system as described before [24]. Reads were trimmed to 333 nucleotides spanning the *env* HXB2 nucleotide positions 7,002 to 7,334. In-house software was used to correct errors in homopolymer stretches, using Sanger sequences from each patient as references. Identical reads were clustered and counted. All variants with at least 2 reads and comprising more than 0.2% of the total number of reads were included in phylogenetic analysis.

Single genome sequencing of viral DNA

In-depth *env* sequencing of PBMC-associated HIV-1 DNA was done using limiting dilution amplification. Briefly, DNA was extracted with the QIAamp Blood Mini kit (Qiagen, Hilden, Germany) from 200 µL of buffy coat and eluted in 200 µL elution buffer according to the manufacturer's instructions. For limiting dilution sequencing, the DNA extract was diluted until no more than 30 to 40% of the nested PCR reactions were positive [25]. Primers and PCR conditions were the same as for the standard Sanger sequencing protocol used to amplify *env* from plasma RNA, but the reverse transcription step was omitted.

Phylogenetic analysis

All *env* and *pol* sequences, from plasma viral RNA and cellular DNA from the different collection points before ART initiation and during the course of LLV, were aligned using MUSCLE software

(v3.8.31) [26]. Alignments were manually edited and trimmed to 333 nucleotides for *env* and 573 nucleotides for *pol* using BioEdit software (v7.0.9) [27]. Shorter sequences and sequences with stop codons or gaps larger than a nucleotide triplet were removed from the alignments. The best-fitting nucleotide substitution model was selected with jModeltest software (v2.1.7) [28, 29], using the Akaike Information Criterion (AIC) and phylogenetic trees were inferred using PhyML software (v3.0) [30]. The trees were visualized and rooted using reference sequences from HIV-1 group M subtypes with Mega 6 software (v6.0) [31]. Bootstrap analysis was performed on 100 replicates using PhyML on the E-Biothon platform [32].

To assess temporal structure in the trees, root-to-tip (RTT) distances were calculated based on the ML phylogeny using TreeStat software (v1.2) and plotted against time [33]. In addition pairwise genetic distances were estimated from the sequence alignment with the Tamura-Nei model as implemented in MEGA 6. To visualize tree expansion over time, temporal tree graphs were constructed using the final maximum likelihood phylogenetic *env* tree as backbone. Onto this backbone, the sequences from different sampling points were added in a time-dependent order (Figure 1).

Coreceptor tropism analysis

The viral coreceptor tropism was determined using the individual *env* sequences and the geno2pheno [coreceptor] algorithm [34]. As recommended by the European Consensus Group on clinical management of HIV-1 tropism testing, a false positive rate cut-off of 10% was used for classification as CCR5- or CXCR4-using virus [35].

Statistical analysis

Linear regression analysis as implemented in SPSS v.22 (IBM, NY, USA) was used to determine if the slopes of the RTT distances and the pairwise distances in function of time significantly differed from zero.

Nucleotide sequence accession numbers

All HIV-1 *pol* and *env* sequences derived in this study have been submitted to GenBank and were assigned accession numbers # to # (submission pending).

RESULTS

Patient characteristics

Detailed information on the included patients can be found in Table 1. The median time of follow-up was 4.8 years (IQR 3.5 - 5.6). The median VL at the time of initiation of the ART regimen that resulted in LLV was 5.1 $\log_{10}$ c/mL (IQR 4.9 - 5.4) and the median VL during LLV was 51 c/mL (IQR 37 – 82; only for the measurements above the detection limit). The minimum duration of LLV at study inclusion (January 2013) was 2.5 years. All patients were infected with subtype B virus, 6 were treatment experienced but had interrupted all medication before initiation of the ART regimen resulting in LLV. There was no history of drug resistance development.

Amplification and sequencing

Pol and e*nv* sequencing was done on respectively 93 (median 5 per patient; IQR 3 – 7, median collection time 3.1 years) and 67 (median 4 per patient; IQR 2 – 5, median collection time 1.8 years) pre-ART plasma samples. *Env* and *pol* sequencing was successful for respectively 141 (55.7%) and 126 (49.8%) of the 253 plasma samples collected during LLV. The time interval between the successfully sequenced LLV samples was minimum 14 days and maximum 3.8 years. NGS of viral RNA collected at baseline was successful for all 18 patients and resulted in a total of 17,773 reads (median 596 per patient; IQR 526 – 896) representing 754 unique variants (median 38 per patient; IQR 31 – 48). For the 8 patients with an on-ART follow-up of at least 5 years, the limiting dilution sequencing of viral DNA samples resulted in a total of 349 DNA clones (median 45 per patient; IQR 39 – 48). All information on the number of sequences included in the phylogenetic analysis is listed in Table 1.

Phylogenetic analysis

Maximum likelihood *env* and *pol* phylogenetic trees were constructed with all available RNA and DNA sequences after removal of variants with gaps or stop codons. Visual examination of both trees revealed profound per patient clustering except for patients 03 and 17 (Supplemental figure S1). These two patients were known to be part of a larger MSM transmission cluster, explaining their close genetic homology. No indications for inter-patient contamination during the amplification or sequencing process could be inferred. Detailed phylogenetic trees of *env* sequences are provided in Supplemental figure S2.

If ongoing viral replication occurs during LLV, it is expected that viral variants isolated over time during LLV would show close genetic linkage. Phylogenetic clustering with bootstrap support higher than 70% is an indicator for genetic linkage [36], therefore clusters fulfilling this criterion were identified and examined for presence of sequences from samples collected during LLV (Table 2). Of the 46 and 7 clusters identified respectively in the *env* and *pol* trees, 11 and 6 contained at least 2 LLV sequences. For 2 patients (06 and 10) all *env* LLV sequences were present in a single cluster. Overall, clusters with 2 or more LLV sequences were detected in 11 patients. They contained LLV sequences sampled over a median period of 2.25 years for *env* (IQR 0.33 – 2.75 years) and 1.71 years for *pol* (IQR 0.61 – 3.82 years). Clusters were either composed uniquely of LLV sequences (patients 07, 15 and 23 for *env* and patients 01, 05, 14, 18 and 20 for *pol*) or contained a mixture of pre-ART, LLV RNA and LLV DNA sequences (patients 01, 05, 06, 10 and 11 for *env* and patient 06 for *pol*).

Detecting time-dependent evolution

Close genetic linkage of the LLV viruses does not directly imply evolution. To evidence evolution, additional proof of genetic changes building up in a temporal manner must be demonstrated. Visual inspection of *env* temporal tree graphs clearly revealed absence of evolution in patient 10. For this patient, all obtained *env* LLV sequences were identical and an identical variant was also present in the baseline plasma sample. In 12 patients (01, 02, 03, 05, 07, 14, 15, 17, 18, 20, 21 and 23),

consecutive addition of LLV sequences resulted in an at random dispersal of these sequences over the tree, intermingled between pre-ART and baseline variants. In 5 patients (06, 09, 11, 19 and 22), the *env* LLV sequences showed evidence of clustering but there was no association between the position of the LLV sequences in the cluster and the sampling time. Figure 1 shows the expansion of the phylogenetic tree of *env* sequences through time for 2 representative patients.

Linear regression analysis of the RTT distances of LLV *env* RNA sequences in function of time revealed significantly increasing RTT distances in patients 07 ($p = 0.001$) and 15 ($p = 0.011$) but failed to indicate such a correlation in the other 16 patients. The same analysis performed on the LLV *pol* sequences showed a significant association between the RTT distances and time in 3 patients (10 ($p = 0.032$), 18 ($p = 0.028$) and 22 ($p = 0.010$)) (Table 3). For the *env* DNA sequences RTT distance analysis failed to detect any sign of evolution but linear regression analysis of the pairwise distances showed a significant positive slope in 3 patients (05, 14 and 18 ($p < 0.001$)) and a significant negative slope, suggesting loss of diversity, in 4 patients (03 ($p < 0.001$), 15 ($p < 0.001$), 17 ($p = 0.002$) and 20 ($p = 0.034$)). For one patient (21) the slope was not significantly different from zero.

Searching for sequence homology

Repetitive identification of identical *pol* and/or *env* sequences at different time points during LLV was observed in 11 patients. The time span separating the collection of the first and last of these identical sequences ranged from 0.2 to 7.5 years (median 1.0 years, IQR: 0.8 – 3.8 years) when considering the results of *pol* sequencing and from 0.1 to 2.8 years (median 1.3 years, IQR: 0.3 – 2.4 years) for the *env* sequences. In 8 patients (01, 05, 11, 14, 15, 20, 21 and 22), detection of these repetitive variants alternated with detection of other variants. Only in patient 10, the *env* sequence remained identical over all 9 LLV samples. In the DNA samples collected during LLV, predominant clones were observed at different sampling points in 4 patients: 03 (43 of the 50 sequences), 05 (5 of 44 sequences), 15 (11 of 48 sequences) and 20 (5 of 45 sequences). The clonal DNA sequences however were not identical to the clonal RNA sequences observed in the same patient.

Apart from investigating indications of viral evolution we also tried to identify the potential source of virus isolated during LLV. This was done by examining genetic homology between LLV RNA sequences, baseline RNA sequences and LLV DNA sequences. For only 4 of the 18 patients (10, 11, 14 and 23) the *env* sequence of at least one time point during LLV was identical to one of the variants detected in plasma at baseline. For patient 14, the predominant *pol* LLV variant was also detected in pre-ART samples. For 7 of the 8 patients with DNA sequences available one of the *env* variants detected in DNA was identical to the sequence of at least one time point during LLV.

Coreceptor use during LLV

Consistent CCR5-use over the whole LLV period was observed in 12 of the 18 patients, consistent CXCR4-use in 1 patient (Table 4). In the remaining 5 patients, CCR5-using and CXCR4-using variants alternated during LLV. In one patient with alternating CCR5 and CXCR4-use during LLV, only CCR5-using variants were detected in the pre-ART and baseline population. Of the 6 patients with a mixed population of CCR5 and CXCR4-using viruses pre-ART and at baseline, alternating detection of CCR5 and CXCR4 variants during LLV was observed in 4.

An overview of the results of all markers of genetic evolution that were analyzed is presented in Table 5.

DISCUSSION

Patients on ART may present with small amounts of virus in their plasma over a longer period of time without evolution to therapy failure. The source of this virus remains uncertain. In this report we present 18 patients with long-term persisting LLV on ART for whom the virus population was characterized extensively using population sequencing and NGS of viral RNA, and limiting dilution sequencing of viral DNA. The viral RNA and DNA samples were extracted from longitudinally collected blood samples over a very long time span pre- and on-ART. The genetic linkage between the viral sequences was defined by phylogenetic analysis in the assumption that ongoing replication

would result in clustering of the sequences collected during LLV with a time-ordered evolutionary trend away from the most recent common ancestor. Additionally, increasing diversity of the proviral DNA was expected in case of evolution.

Although the majority of studies attempting to investigate viral replication in patients on ART rely on phylogenetic analysis [7-14, 16, 23], there is no standard approach on how to reliably evidence genetic evolution. Conclusions are often based on visual inspection of the phylogenetic trees for presence or absence of intermingling of sequences collected at different time points [7-9, 13, 16]. Alternatively, the most commonly used mathematical methods calculate the divergence (pairwise distances) from the sampled sequences to the inferred most recent common ancestor [10, 11, 23], the RTT distances from the inferred tree [12, 13], or the overall evolution of pairwise distances between sequences of different sampling points [9, 13, 16, 17]. The identification of genetic evolution following slow rates of virus replication in a background of large overall genetic variability is however challenging. We therefore used a combined approach applying different tools to assess genetic evolution and analyzed two regions of the HIV genome, the more conserved *pol* gene and the highly variable *env* gene.

For 8 of the 18 patients none of the assessed markers revealed evidence of temporal genetic evolution over a median period of 4.8 years of LLV. For 8 patients, the result of one marker pointed towards virus evolution but this was never confirmed for both the *env* and *pol* sequences and was not supported by the outcome of the other measurements. For 2 patients the results of 2 markers indicated genetic evolution. In case of patient 18, RTT analysis of *pol* and pairwise distance analysis of the DNA sequences suggested increasing diversity but the phylogenetic tree revealed profound intermingling of the sequences with the pre-ART sequences. In patient 05, bootstrap analysis revealed clustering of on-ART RNA sequences and pairwise distance analysis of the DNA sequences indicated increasing variability, but no significant temporal structure could be detected by RTT analysis of the RNA sequences.

Coreceptor use analysis of virus isolated during LLV has, to our knowledge, not been done before. We used the obtained *env* sequences to predict the coreceptor preference of the viral isolates. In 6 patients (02, 03, 06, 07, 10, 15) NGS revealed the presence of a mixture of CCR5- and CXCR4-using variants before ART initiation. It was surprising to see that in 3 of these 6 patients (02, 03 and 06) CCR5 and CXCR4-using variants were detected alternately over time during LLV. This finding pleads against viral evolution because in case of evolution and concomitant selective pressure, one would expect the consistent detection of descendants of the same strain with unchanged coreceptor use or possibly a single switch at a certain time point.

So overall we were unable to provide firm arguments for the existence of virus replication and resulting genetic evolution during longer episodes of persistent low viremia under ART. If not virus replication but virus production is considered to be the major contributor to LLV, potential sources of the plasma virus may be long-lived HIV-infected cells continuously releasing virus or latently infected cells that become activated and produce virus before dying [37]. In both cases one would expect considerable intermingling of the sequences from viruses isolated at different time points as well as intermingling of viral RNA and DNA sequences. This intermingling was indeed observed in all 8 patients with both RNA and DNA tested. A hypothesis formulated recently is that the process of stochastic virus production may be driven to a large extent by clonally expanded cells [8, 12, 13, 23]. Already in 2006, Bailey *et al.* described the persistence of a predominant plasma clone in patients on ART with a viral load <50 c/mL [8]. More recently, integration site analysis provided further compelling evidence for the presence of clonally expanded HIV-infected cells in vivo [38-40]. In 11 of the 18 patients we observed identical RNA sequences at different time points during LLV, often spread over a long time span, supporting the idea of production from clonally expanded cells. However, the origin of these predominant plasma sequences could be traced to the cellular compartment only in 2 patients, indicating that the producing cells do not constitute the majority of infected cells in the blood.

Whether clonally expanded cells are capable of producing replication competent virus remains a subject of debate [41, 42]. There are indications that a large majority of proviruses in these expanded cell clones is defective [12, 38, 39, 42]. But Simonetti *et al.* recently showed a highly expanded CD4+ T cell clone carrying replication competent provirus in 1 patient and Kearney *et al.* found that in 2 patients who underwent treatment interruption the origin of rebound plasma virus was an expanded cell clone [43, 44]. Whether patients with higher numbers of clonally expanded cells are more prone to LLV under ART is a hypothesis that needs to be investigated further.

A drawback of our study is that only bulk sequencing was performed on the plasma samples collected during LLV. As a result, it is likely that only the most represented variant is sequenced and this may have reduced the sensitivity to detect evolution over time. Ambiguous nucleotide positions were detected at 1 or more positions in 59 of the 143 *env* sequences, an indication that in these cases a mixture of variants was present. Another shortcoming is that the *env* phylogenetic analysis was performed on short sequences because of the limited read length of the NGS. Recently, Laskey *et al.* showed that sequence identity in subgenomic regions of the viral genome does not guarantee clonality across the full viral genome [45]. So although we sequenced a highly variable part of the *env* gene we cannot exclude missing some diversity.

The median sequencing depth of our NGS analysis was 596 reads per patient. This was considered sufficient for a global estimate of the viral variability and to characterize the most represented viral strains at initiation of ART, which was the goal of the analysis. Low-frequent viral variants will be missed, but this will not impact the conclusions regarding viral evolution during LLV as this conclusion is based on the phylogenetic relationship between the LLV sequences.

CONCLUSIONS

Taken together, the results of this study failed to support presence of active virus replication and evolution in patients with LLV above the detection limits of commercial assays while being on ART. This finding is in line with our previous observations that in the majority of cases of LLV on ART we

could not document treatment failure or drug resistance [1]. Today, about 3 years after closure of sample inclusion, 10 of the 18 study patients still present recurrent LLV. Eight patients have evolved to a continuously undetectable viral load (<20 c/mL), 6 of them without ART changes and 2 after a treatment adaptation. None of the 18 patients has developed virologic failure or drug resistance.

All these observations support the hypothesis that residual viremia mainly results from a process of virus production. It is however important to emphasize that, although generally used for these purposes, the power of phylogenetic analysis to exclude very low levels of virus replication may be limited. Absence of evidence is therefore not necessarily evidence of absence and future research should concentrate on the development of alternative methods for the detection or exclusion of active replication in the presence of ART.

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FIGURES

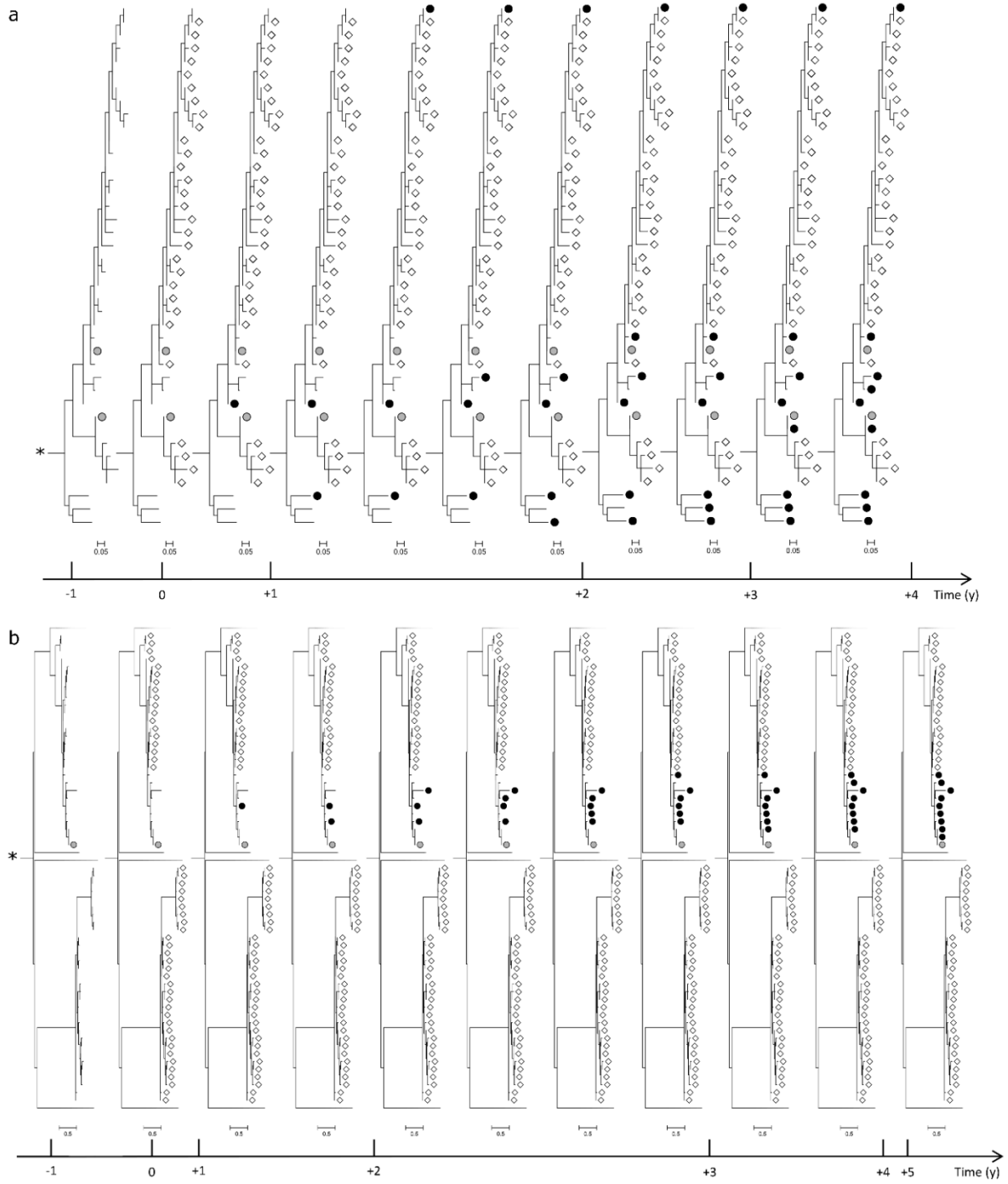

Figure 1. Temporal tree graphs. Expansion of the phylogenetic tree of *env* sequences through time shown for 2 representative patients: patient 23 (**a**) and patient 06 (**b**). ART initiation is depicted as time 0. The backbone of the phylogenetic tree with all pre-ART sequences, regardless of their sampling time, is copied and viral variants of each time point during LLV are consecutively added. Pre-ART sequences are shown as filled grey circles, baseline NGS variants as diamonds and LLV Sanger sequences as filled black circles.

Table 1: Overview of patient characteristics

Patient	Follow-up (years)	Pre-ART VL (c/mL)	LLV VL (c/mL)[a]	Treatment experienced at baseline	LLV burden (%)[b]	Number of sequences included in phylogenetic analysis							
						env					*pol*		
						pre-ART	ART-start[c]	LLV	Interval (days)[d]	DNA	pre-ART	LLV	Interval (days)[d]
03	5.7	149,000	47.0 [26 – 200]	-	59	8	20 (219)	8	210 [158 - 304]	50	7	8	96 [84 – 203]
21	9.2	>100,000	43.0 [20 – 131]	yes	74	4	41 (317)	5	571 [363 – 805]	49	6	9	347 [245 – 410]
15	9.8	291,000	47.0 [26 – 211]	-	80	5	32 (530)	9	264 [137 – 447]	48	3	6	387 [266 – 1106]
17	5.3	219,000	34.0 [20 – 68]	yes	52	5	39 (525)	5	218 [115 – 318]	39	7	4	326 [234 – 541]
18	5.6	105,000	38.0 [34 – 93]	-	72	5	31 (455)	9	220 [157 – 304]	36	5	6	238 [53 – 461]
05	6.2	>100,000	43.0 [24 – 203]	-	52	1	16 (545)	5	261 [120 – 532]	44	4	6	171 [122 – 511]
20	5.5	35,800	59.0 [29 – 106]	-	68	6	36 (612)	8	231 [63 – 332]	45	8	5	63 [28 – 101]
14	5.7	104,000	80.5 [24 – 218]	yes	90	7	65 (970)	12	202 [121 – 233]	38	10	8	227 [185 – 271]
01	3.3	1,086,005	65.0 [21 – 114]	yes	89	3	17 (219)	6	106 [84 – 286]	NA	5	10	63 [14 – 91]
02	4.7	291,000	72.0 [24 – 220]	yes	88	5	42 (579)	11	118 [101 – 170]	NA	10	13	97 [72 – 114]
06	4.7	210,000	51.5 [29 – 158]	-	81	1	49 (1573)	9	123 [90 – 152]	NA	5	12	76 [53 – 136]
07	4.8	70,000	48.0 [21 – 250]	-	67	1	71 (1087)	8	91 [88 – 213]	NA	5	4	182 [109 – 221]
09	4.4	29,700	62.0 [23 – 210]	-	41	4	34 (840)	7	187 [105 – 341]	NA	4	6	127 [98 – 372]
10	3.3	67,799	68.0 [28 – 293]	-	53	2	88 (6284)	9	90 [57 – 125]	NA	3	7	71 [47 – 152]
11	4.1	152,170	41.0 [22 – 75]	-	44	0	45 (700)	7	121 [104 – 263]	NA	1	3	397 [295 – 500]
19	3.3	54,110	32.0 [23 – 71]	yes	91	6	35 (899)	10	125 [104 – 167]	NA	6	9	140 [119 – 176]
22	2.2	374,584	56.5 [22 – 190]	-	90	2	64 (886)	4	309 [206 – 349]	NA	3	6	104 [52 – 172]
23	3.2	1,905,647	45.5 [23 – 82]	-	70	2	29 (533)	9	110 [85 – 177]	NA	1	4	467 [289 – 512]

VL, viral load; c/mL, copies/mL; LLV, low-level viremia; ART, antiretroviral therapy

[a] Median [min – max]

[b] Percentage of VL measurements >20 c/mL compared to the total number of VL measurements performed during follow-up

[c] NGS clones (# corresponding reads).

[d] Median [IQR] time interval between sequenced LLV samples.

Table 2: Results of the phylogenetic cluster analysis

Patient	ENV					PRRT				
	Total # clusters	*# clusters containing ≥ 2 LLV*	*# clustered LLV sequences[a]*	*Bootstrap value*	*Time span (years)*	*Total # clusters*	*# clusters containing ≥ 2 LLV*	*# clustering LLV sequences[a]*	*Bootstrap value*	*Time span (years)*
03	2	-	-	-	-	-	-	-	-	-
21	3	-	-	-	-	-	-	-	-	-
15	2	1	6/9	100	2.25	-	-	-	-	-
17	1	-	-	-	-	-	-	-	-	-
18	5	-	-	-	-	1	1	2/6	70	2
05	2	1	4/5	87	4.25	1	1	4/6	75	4.25
20	5	1	2/8	91	1.25	1	1	4/5	76	0.67
14	-	-	-	-	-	1	1	5/8	74	3.67
01	2	1	4/6	75	2.5	1	1	3/10	95	0.42
02	5	-	-	-	-	-	-	-	-	-
06	2	1	9/9	75	3.83	1	1	2/12	78	1.42
07	5	3	2/8	87	0.08	-	-	-	-	-
			2/8	77	0.17					
			2/8	100	0.33					
09	4	-	-	-	-	-	-	-	-	-
10	2	1	9/9	100	2.5	1	-	-	-	-
11	2	1	6/7	96	2.75	-	-	-	-	-
19	3	-	-	-	-	-	-	-	-	-
22	-	-	-	-	-	-	-	-	-	-
23	1	1	2/9	75	2.08	-	-	-	-	-

[a] Depicted as number of LLV sequences in the cluster/total number of analyzed LLV sequences

Table 3: Results of linear regression analysis of root-to-tip and mean pairwise distance

Patient	RTT distance (Lin. Reg.)		RTT distance DNA (Lin. Reg.)[a]	PW distance DNA (Lin. Reg.)[a]
	env[a]	*pol*[a]		
03	-0.010 (0.350)	5.63E-11 (0.354)	-5.039E-11 (0.221)	**-0.004 (<0.001)**
21	0.002 (0.497)	1.05E-11 (0.311)	-2.687E-11 (0.616)	0.000 (0.730)
15	**0.003 (0.011)**	-1.09E-11 (0.055)	1.258E-10 (0.283)	**-0.002 (<0.001)**
17	-0.004 (0.648)	7.41E-11 (0.062)	7.882E-11 (0.399)	**-0.002 (0.002)**
18	0.001 (0.534)	**1.87E-10 (0.028)**	-6.498E-11 (0.140)	**0.003 (<0.001)**
05	-0.002 (0.387)	1.78E-11 (0.695)	2.321E-10 (0.123)	**0.003 (<0.001)**
20	0.006 (0.324)	-3.11E-12 (0.981)	-2.369E-11 (0.831)	**-0.001 (0.034)**
14	-0.004 (0.455)	5.32E-11 (0.700)	-4.793E-12 (0.932)	**0.004 (<0.001)**
01	0.044 (0.366)	-1.37E-10 (0.124)	NA	NA
02	-0.012 (0.565)	6.65E-11 (0.264)	NA	NA
06	-0.002 (0.958)	-3.63E-10 (0.313)	NA	NA
07	**0.076 (0.001)**	1.05E-13 (0.853)	NA	NA
09	0.030 (0.309)	1.59E-10 (0.117)	NA	NA
10	NA	**5.08E-10 (0.032)**	NA	NA
11	0.037 (0.066)	1.16E-10 (0.149)	NA	NA
19	**-0.010 (0.029)**	6.72E-11 (0.118)	NA	NA
22	-0.019 (0.403)	**5.44E-11 (0.010)**	NA	NA
23	-0.009 (0.796)	6.95E-11 (0.122)	NA	NA

Lin. Reg., Linear regression analysis; RTT, root-to-tip; LLV, low-level viremia; PRRT, protease and reverse transcriptase; ART, antiretroviral therapy; PW distance, pairwise distance; NA, not applicable

[a] Slope (p-value)

Significant values shown in bold

Table 4: Results of coreceptor tropism analysis pre-ART (RNA, DNA) and during LLV (RNA)

Patient	Pre-ART tropism	LLV 1	LLV 2	LLV 3	LLV 4	LLV 5	LLV 6	LLV 7	LLV 8	LLV 9	LLV 10	LLV 11	LLV 12
03	R/X	R	X	X	X	X	R	X	R	-	-	-	-
21	R	R	R	R	R	R	-	-	-	-	-	-	-
15	R/X	X	X	R	R	R	R	R	R	R	-	-	-
17	R	R	R	R	R	R	-	-	-	-	-	-	-
18	R	R	R	R	R	R	R	R	R	R	-	-	-
05	R	R	R	R	R	R	-	-	-	-	-	-	-
20	R	R	R	R	R	R	R	R	R	-	-	-	-
14	R	R	R	R	R	R	R	R	R	R	R	R	R
01	R	R	R	X	R	R	R	-	-	-	-	-	-
02	R/X	X	X	R	X	X	X	X	X	R	X	X	-
06	R/X	R	R	R	R	R	R	X	R	X	-	-	-
07	R/X	X	X	X	X	X	X	X	X	-	-	-	-
09	R	R	R	R	R	R	R	R	-	-	-	-	-
10	R/X	R	R	R	R	R	R	R	R	R	-	-	-
11	R	R	R	R	R	R	R	R	-	-	-	-	-
19	R	R	R	R	R	R	R	R	R	R	R	-	-
22	R	R	R	R	R		-	-	-	-	-	-	-
23	R	R	R	R	R	R	R	R	R	R	-	-	-

X, CXCR4-tropic virus; R, CCR5-tropic virus
LLV1 to 12: consecutive sampling, time interval differs between patients

Table 5: Interpretation of the results of the performed analyses: virus evolution indicated or not

Patient	Tree topology	RTT distances	DNA Pairwise distances	Viral tropism
03	×	×	×	Alternating ×
21	×	×	×	Constant[a]
15	×	✓	×	Alternating ×
17	×	×	×	Constant[a]
18	×	✓	✓	Constant[a]
05	✓	×	✓	Constant[a]
20	×	×	×	Constant[a]
14	×	×	✓	Constant[a]
01	✓	×	NA	Alternating ×
02	×	×	NA	Alternating ×
06	✓	×	NA	Alternating ×
07	×	✓	NA	Constant[a]
09	×	×	NA	Constant[a]
10	×	✓	NA	Constant[a]
11	✓	×	NA	Constant[a]
19	×	×	NA	Constant[a]
22	×	✓	NA	Constant[a]
23	×	×	NA	Constant[a]

×, absence of indications for virus evolution; ✓, presence of indications for virus evolution; NA, not applicable

[a] No indication against or in favor of virus evolution.