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Quantification of total HIV-1 DNA in buffy coat cells, feasibility and potential added value for clinical follow-up of HIV-1 infected patients on ART

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ABSTRACT

Background: Successfully treated HIV-1 infected patients have a sustained undetectable viral RNA load. In these cases the total HIV-1 DNA load may constitute a valuable tool to further follow the overall viral burden. The value of this marker outside of cure research has been rarely studied.
Objectives: To develop a quantitative (q)PCR for total HIV-1 DNA quantification in buffy coat cells and to evaluate the value of this parameter in clinical follow-up.
Study design: A qPCR using primers and a probe in the conserved HIV-1 LTR region was adapted for use on DNA extracted from buffy coat cells. Sensitivity, accuracy and reproducibility were evaluated using 8E5 cells and samples from naive and treatment experienced patients. The clinical value of DNA load analysis was assessed by testing 119 longitudinal samples from 9 patients before and after ART initiation and 249 cross sectional samples from therapy-experienced patients.
Results: Inter- and intra-assay coefficients of variability were 5.56 and 5.94 (%CV). HIV-1 DNA was detected in 249 of the 263 (94.7%) patients on ART for at least 5 months (median: 53 months; IQR: 28–84 months). The HIV-1 DNA load varied between 0.60 and 3.37 copies/10^6 blood cells and showed significant correlation with the pre-ART $CD4^+$ T-cell count nadir and peak viral RNA load. ART initiation resulted in a slow and limited decline of the total HIV-1 DNA concentration.
Conclusions: Quantification of total HIV-1 DNA from buffy coat cells is feasible, sensitive and reliable. Although determination of the on-therapy HIV-1 DNA load may be informative, regular testing has limited clinical value because of the very slow evolution.

1. Background

Today, antiretroviral therapy (ART) is initiated immediately after HIV-1 diagnosis. As a result, most patients have an undetectable HIV-1 RNA load for prolonged periods of time. Quantification of total HIV-1 DNA in peripheral blood mononuclear cells (PBMC) is applied as a surrogate marker for the size of the HIV reservoir in several HIV cure studies [1,2]. In patients on ART, the HIV-1 DNA load may reflect the overall viral burden and therefore constitute a potentially interesting marker for clinical follow-up. A number of studies suggest that the HIV-1 DNA load may guide the selection of patients eligible for ART de-escalation. In the VISCONTI cohort, low HIV-1 DNA levels were correlated with better control of the virus after therapy cessation [3] and in other studies a correlation between HIV-1 DNA levels and post-treatment control was demonstrated [4,5]. But there are also reports of immediate viral rebound upon treatment interruption even in patients with undetectable HIV-1 DNA levels [6,7].

In untreated patients, association of the HIV-1 DNA load with the HIV-1 RNA load and with the number of $CD4^+$ T-cells, has been clearly demonstrated [6,8–14]. Others have shown that the total HIV-1 DNA load in the first 24 months after infection can predict progression to AIDS independent of the HIV-1 RNA load or $CD4^+$ T-cell count [5,15–17].

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2. Objectives

The purpose of this study was to evaluate the feasibility and added value of total HIV-1 DNA load measurement in routine laboratory monitoring of HIV-1 infected patients. To avoid cumbersome PBMC isolation, the possibility to use stored EDTA blood buffy coat cells for HIV-1 DNA quantification was examined. HIV-1 DNA quantification was performed using the real-time qPCR developed by Avettand-Fènoël et al. [18]. After ensuring the feasibility and reliability of the method, it was validated using large series of samples from patients on ART. The DNA load range and evolution over time, and the parameters associated with a high or low total HIV-1 DNA load were assessed.

3. Study design

3.1. Sample series used for assay validation

DNA extracted from HIV-1 LAV infected 8E5 cells [20] was spiked in 600 ng/ml HIV negative human DNA (Roche, Basel, Switzerland) to construct a titration curve. Test variability was defined after triplicate testing, both inter- and intra-assay, of 15 samples collected from therapy-experienced patients and selected to cover a broad HIV-1 DNA load range (0.60 and 3.20 c/10^6 cells).

3.2. Sample series used to define the added clinical value of DNA load analysis

In total, 119 consecutive samples were retrospectively selected from 9 patients (between 7 and 17 samples per patient). They were collected pre- and post-ART over a period of 69–87 months. The median age of the patients at diagnosis was 34 years (IQR: 32–37 years), 7 of them were men of which at least 5 were MSM (men who have sex with men), 7 were Belgians and 2 were Africans. Median pre-ART $CD4^+$ T-cell count nadir and peak viral load were 327 cells/mm^3 (IQR: 283–434 cells/mm^3) and 4.99 log copies/ml (c/ml) (IQR: 4.58–5.56 log c/ml) respectively.

Cross-sectional samples were retrospectively selected from 249 patients on ART. For inclusion, patients needed to have an undetectable viral load (< 20 c/ml) for at least 3 months though isolated viral blips (IQR: 58–183 c/ml; 26.1% of the patients) were accepted. The median age of the selected patients was 43 years (IQR: 37–52 years), 80.3% were men of which 70.0% were MSM, 68.7% were Belgians and 19.7% were Africans. The median time between diagnosis and sample collection was 72 months (IQR: 42–116 months), the median on-ART time was 53 months (IQR: 28–84 months) and the median time of undetectable viral load was 47 months (IQR: 24–79 months). Median pre-ART $CD4^+$ T-cell count nadir and peak viral load were 254 cells/mm^3 (IQR: 166–341 cells/mm^3) and 4.9 log c/ml (IQR: 4.5–5.4 log c/ml) respectively.

All patients providing the included samples were followed at Ghent University Hospital, Ghent, Belgium.

3.3. Blood collection and sample preparation

Venous blood, collected in EDTA containing collection devices, was centrifuged at 1900 *g* for 10 min within 24 h of collection. After removal of the plasma for viral load analysis, about 750 µl of the upper cell layer (buffy coat) was transferred to a clean vial for storage at −80 °C.

At the time of initiating the qPCR analysis, the buffy coats were thawed and 200 µl was subjected to DNA extraction using the QIAamp Blood Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. This extraction can be fully automated using the QIAcube (Qiagen). The resulting DNA, eluted in 200 µl elution buffer, was then concentrated in 50 µl ultrapure water by ethanol precipitation.

3.4. HIV-1 DNA load determination

Part of the HIV-1 *LTR* was amplified using the primers and probe described by Avettand-Fènoël et al. [18], the QuantiTect multiplex PCR kit (Qiagen) and the LightCycler480 (Roche, Basel, Switzerland). For normalization, part of the human *albumin* (*ALB*) gene was amplified in parallel using primers and probe described by Gault et al. [19]. All qPCR reactions were performed in a 50 µl reaction volume containing 20 µl DNA extract, 200 nM of each primer and probe and 1x Quantitect multiplex mastermix for the HIV PCR and 2 µl DNA extract, 100 nM of each primer, 150 nM of the probe and 1x Quantitect multiplex mastermix for the human *ALB* PCR. Thermocycling conditions were 2 min at 50 °C and 10 min at 95 °C, followed by 45 cycles at 95 °C for 15 s and 60 °C for 1 min and a final cool down to 37 °C for 3 min. Each run included 3 replicates of a low positive control (LPC), containing 600 ng/µl human genomic DNA (Roche) spiked with DNA from 20 8E5 cells [20]. For all runs performed, the average ALB Cq value of the LPC replicates fell within the two standard deviation (SD) boundaries that were predefined based on 11 independent measurements of the LPC. Normalization of the HIV-1 DNA load to the white blood cell number was done using the Cq values of *ALB* and the $2^{-\Delta\Delta Ct}$ method developed by Livak and Schmittgen [21]. The LPC contained 20 copies HIV-1 DNA from 8E5 cells per reaction. The total DNA concentration per reaction measured by NanoDrop™(Thermo Fisher Scientific, Waltham, MA) was 4 µg. Assuming that 1 µg of DNA is equivalent to ~150,000 cells [18,22], the formula used to calculate the number of HIV-1 DNA c/10^6 cells was $(20*(2\hat{}(-((CqLTR_{sample}\text{-}CqALB_{sample})\text{-}(CqLTR_{LPC}\text{-}CqALB_{LPC})))*(10^6/(4*150{,}000))$.

3.5. Statistical analyses

Groups were compared using χ^2 test for categorical variables and Mann-Whitney *U* non parametric test for continuous variables. The level of significance was set at $p \leq 0.05$. All data was analyzed using SPSS23.0 (SPSSInc., Chicago, IL).

4. Results

4.1. Method validation

Six dilutions of HIV-1 LAV infected 8E5 cells (0, 10, 20, 100, 500 and 2000 HIV DNA copies/reaction) spiked in 600 ng/µl negative human DNA, were tested in three independent runs. The average and standard deviation for each dilution was calculated and the titration curve constructed (Supplementary Fig. 1).

Fifteen cross-sectional samples, with an HIV-1 DNA load evenly distributed across a broad range (0.60 and 3.20 c/10^6 cells), were tested to define inter- and intra-assay reproducibility. The coefficients of variance for 3 independent measurements were 5.94 (intra-run) and 5.56 (inter-run) (Table 1). The Cq values for ALB varied between 16.17 and 20.89.

4.2. HIV-1 DNA load pre- and post-ART

HIV-1 DNA was quantifiable in 119 (96.0%) of 124 samples collected longitudinally from 9 patients before and after ART initiation (Fig. 1). The median ALB Cq value for these samples was 17.57 (IQR: 16.80–18.45). For two samples neither HIV-1 DNA nor ALB DNA were detected (Cq > 40). For three samples the HIV-1 DNA load did not reach the limit of quantification (Cq ≥ 35), likely due to the low amount of DNA present, with ALB Cq values of respectively 19.22, 21.53 and 24.06 reducing the detection limit for HIV-1 DNA to respectively 0.93, 1.63 and 2.39 log c/10^6 cells. The results of the two samples with undetectable and the three samples with unquantifiable HIV-1 DNA were not taken into account in Fig. 1.

The overall per patient fluctuations in pre-ART HIV-1 DNA load

Table 1
Calculation of the intra- and inter-assay coefficient of variation after triplicate testing of 15 cross-sectional samples, selected to cover a broad linear range of HIV-1 DNA loads. Differences in the Cq values for ALB reflect the variability in cell content of the buffy coats.

	Intra-assay variability				Inter-assay variability			
	ALB (Mean Cq)	HIV-1 DNA (Mean log c/10^6 cells)	SD	%CV	ALB (Mean Cq)	HIV-1 DNA (Mean log c/10^6 cells)	SD	%CV
sample 1	16.17	0.73	0.23	31.45	16.31	0.54	0.10	18.48
sample 2	17.23	1.40	0.03	1.94	17.36	1.43	0	0.32
sample 3	17.68	1.49	0.05	3.30	17.81	1.55	0.10	6.21
sample 4	17.90	1.42	0.34	24.36	17.92	1.39	0.31	22.48
sample 5	16.52	1.72	0.03	1.67	16.49	1.65	0.07	4.01
sample 6	18.26	1.73	0.10	5.97	18.24	1.74	0.05	2.61
sample 7	18.63	1.92	0.05	2.49	18.59	1.92	0.03	1.74
sample 8	18.21	2.02	0.06	2.91	18.01	1.96	0.16	8.03
sample 9	16.65	2.16	0.01	0.43	16.74	2.21	0.04	1.85
sample 10	17.17	2.05	0.02	0.74	16.95	1.95	0.10	5.29
sample 11	17.76	2.06	0.07	3.36	17.76	2.11	0.03	1.59
sample 12	16.92	2.24	0.08	3.56	16.95	2.22	0.03	1.41
sample 13	19.33	2.15	0.06	2.85	19.23	2.07	0.13	6.40
sample 14	18.49	2.56	0.05	2.06	18.26	2.57	0.03	1.33
sample 15	20.89	2.74	0.05	1.93	20.83	2.69	0.04	1.58
	intra-assay %CV: 5.94				**inter-assay %CV: 5.56**			

varied a maximum of 1.61 log c/10^6 cells (median: 0.50 log c/10^6 cells). After treatment initiation the per patient DNA load dropped from a median of 2.95 log c/10^6 cells to a median of 2.16 log c/10^6 cells. Overall, the median difference between the highest pre- and lowest on-ART DNA load was 1.16 log c/10^6 cells (minimum 0.67 log c/10^6 cells, maximum 1.64 log c/10^6 cells).

4.3. HIV-1 DNA load after long-term ART

HIV-1 DNA quantification was successful for 94.7% of the 263 cross-

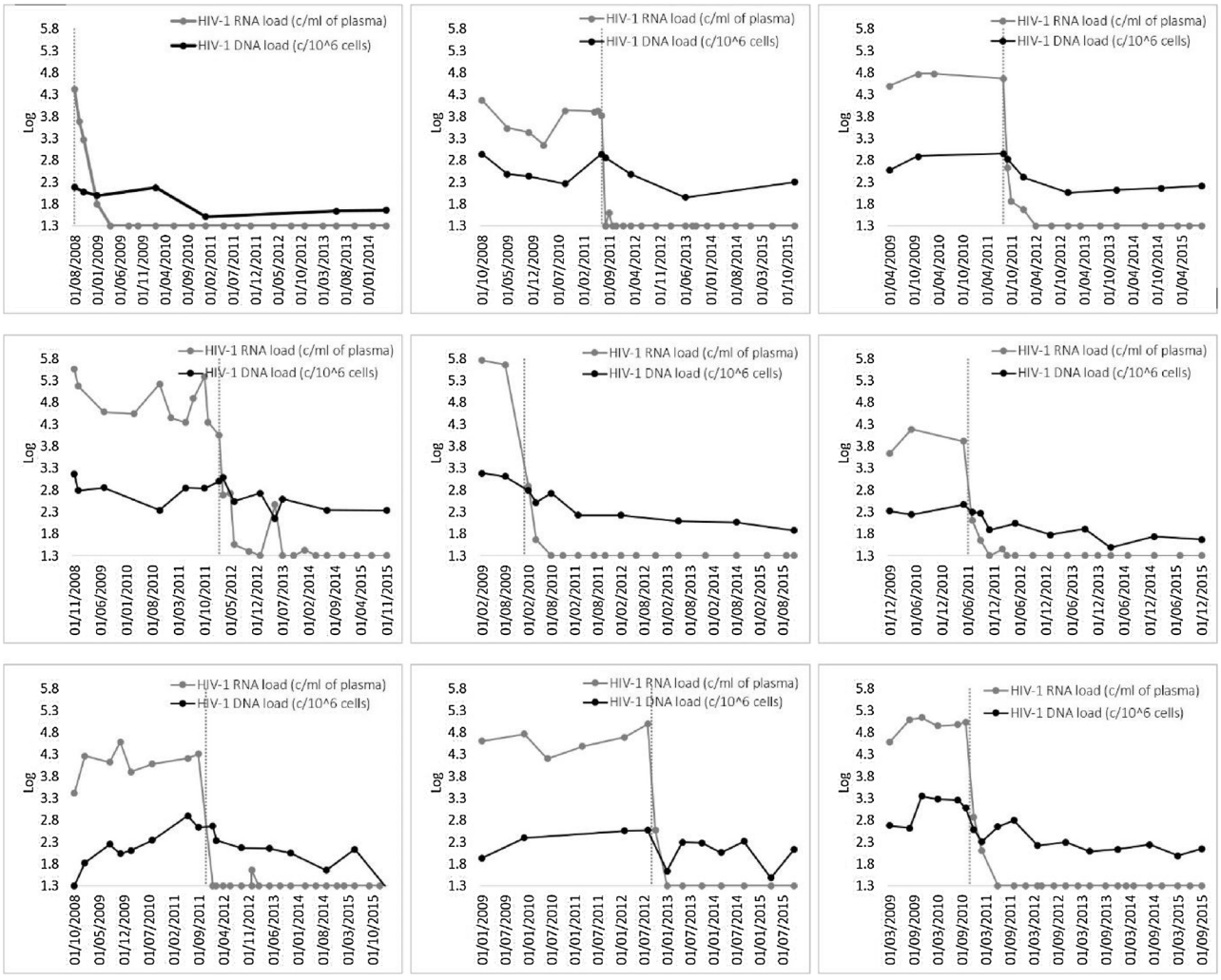

Fig. 1. Evolution of the HIV-1 DNA load (log c/10^6 cells) and HIV-1 RNA load (log c/ml plasma) over time in 9 patients. Gray dotted line: ART initiation.

Table 2
Viral and host factors associated with HIV-1 DNA load.

	All n = 249	Low HIV-DNA level ≤2.18 log c/10^6 cells n = 123	High HIV-DNA level > 2.18 log c/10^6 cells n = 126	*P*
Sex, n (%),	249 (100)	123	126	0.307
Man	200 (80.3)	102 (82.9)	98 (77.8)	
Woman	49 (19.7)	21 (17.1)	28 (22.2)	
Age, median (IQR), years, (n = 249)	43 (37–52)	43 (37–52)	44 (37–52)	0.693
Infection route, n (%)	197 (79.1)	104	93	0.940
MSM	140 (71.1)	75 (72.1)	65 (69.9)	
HE	43 (21.8)	22 (21.2)	21 (22.6)	
Other	14 (7.1)	7 (6.7)	7 (7.5)	
Time since diagnosis, median (IQR), months, (n = 249)	72 (42–116)	64 (42–103)	86 (40–127)	0.112
Time on ART, median (IQR), months, (n = 249)	53 (28–84)	53 (29–83)	53 (28–89)	0.807
$CD4^+$ cell count nadir, median (IQR), cells/mm^3, (n = 248)	254 (166–341)	294 (189–405)	213 (145–295)	< 0.001
$CD4^+$ cell count nadir, < 200 cells/mm^3, n (%)	248 (99.6)	123	125	0.011
Yes	90 (36.3)	35 (28.5)	55 (44.0)	
No	158 (63.7)	88 (71.5)	70 (56.0)	
Peak VL, median (IQR), log c/ml, (n = 249)	4.9 (4.5–5.4)	4.85 (4.4–5.2)	5.0 (4.7–5.4)	0.020
Peak VL, > 5 log c/ml, n (%)	249 (100)	123	126	0.034
Yes	114 (45.8)	48 (39.0)	66 (52.4)	
No	135 (54.2)	75 (61.0)	60 (47.6)	
Viral load rebound (c/ml), n (%)	249 (100)	123	126	0.054
< 50	180 (72.3)	96 (78.0)	84 (66.7)	
50-200	47 (18.9)	22 (17.9)	25 (19.8)	
201-1000	15 (6.0)	3 (2.4)	12 (9.5)	
> 1000	7 (2.8)	2 (1.6)	5 (4.0)	
Viral load rebound, n (%)	249 (100)	123	126	0.058
Yes	183 (73.5)	97 (78.9)	86 (68.3)	
No	66 (26.5)	26 (21.1)	40 (31.7)	
Duration of viral suppression, median (IQR), months, (n = 248)	47 (24–79)	47 (27–71)	47 (23–84)	0.748
Origin, n (%)	249 (100)	123	126	0.518
Belgium	171 (68.7)	91 (74)	80 (63.5)	
Europe	12 (4.8)	5 (4.1)	7 (5.6)	
Africa	49 (19.7)	20 (16.3)	29 (23.0)	
America	7 (2.8)	3 (2.4)	4 (3.2)	
Asia	10 (4.0)	4 (3.3)	6 (4.8)	

sectional samples collected from 263 patients on long-term ART. The median Cq value for ALB was 18.60 (IQR: 17.66–19.83). The median total HIV-1 DNA load registered was 2.18 log c/10^6 cells (IQR 1.84–2.40 log c/10^6 cells). The maximum range of inter-patient variation was 2.77 log c/10^6 cells.

Patients were then divided in two groups, patients with a DNA load above the overall median DNA load of 2.18 log c/10^6 cells (123 patients) and patients with a DNA load below or equalizing this median (126 patients) in order to define potential correlates for high or low HIV-1 DNA load (Table 2). A pre-ART $CD4^+$ T-cell count nadir below 200 cells/mm^3 and a pre-ART peak viral load above 5 log c/ml were significantly associated with a higher HIV-1 DNA load ($p = 0.011$ and $p = 0.034$ respectively). Patients experiencing viral blips were also more likely to have higher DNA loads but this association was not statistically significant (Table 2). The same analysis comparing patients with a HIV-1 DNA load in the upper quartile range with the patients with a HIV-1 DNA load in the lower quartile range resulted in a similar outcome (data not shown).

5. Discussion

Quantification of total HIV-1 DNA in blood cells or body compartments is frequently applied in HIV reservoir and cure research [2,23–26] but the potential of this marker for routine clinical monitoring remains virtually unexplored. The vast majority of HIV-1 infected patients are successfully treated, resulting in an undetectable viral load for extended periods of time. Thus, having a marker that permits to further monitor the overall viral burden looks interesting, especially in the light of increasing interest in the possibilities to downscale or even interrupt treatment [1,4,27–35]. Of all the reservoir markers proposed, the total HIV-1 DNA load is definitely the most easily accessible for broad scale screening and there are several indications for a potential value in routine practice. It has been demonstrated that patients with the ability to suppress virus replication naturally have lower HIV-1 DNA levels than control patients [36–38] and, more importantly, patients with a low HIV-1 DNA load are more likely to evolve to functional cure than patients with an extended reservoir [39,40]. Broad scale screening of the viral DNA load may therefore enable identification of patients with the highest chance of benefitting from treatment de-escalation or cessation.

One of the reasons why HIV-1 DNA load measurement remains largely restricted to research environments may be that the analysis is technically challenging and commercial kits specifically developed for this purpose are unavailable. The HIV-1 DNA quantification protocols described in literature generally use DNA extracted from purified PBMC and some even recommend isolation of the $CD4^+$ T-cell population [6,10,26,41]. These procedures are cumbersome and difficult to implement in routine laboratory practice. The aim of this study was to establish a method for HIV-1 DNA quantification that can easily be implemented in a routine clinical laboratory. The buffy coat cell fraction is the upper cell layer of EDTA blood after centrifugation and can easily be recuperated and stored. We used a manual DNA extraction procedure from the company Qiagen that can easily be automated, but alternative DNA extraction procedures can be applied as well, enabling the easy integration of both collection and processing in a routine clinical lab. An additional ethanol precipitation step was introduced to increase the sensitivity of the HIV-1 DNA quantification by concentration of the extracted DNA, but this step is optional.

To our knowledge the use of whole blood for HIV-1 DNA quantification was only mentioned by Casabianca et al. [42] and Avettand-Fènoël et al. [18]. Though Avettand-Fènoël et al. [18] used purified PBMCs for most of their work, they did compare DNA quantification in ficoll-hypaque separated PBMC with DNA quantification in whole blood for 48 samples and found a very good correlation (Spearman test $r = 0.900$, $p < 0.0001$). One of the difficulties when using whole blood or buffy coat cells is defining the number of white blood cells in the cell fraction, necessary for normalization of the results. This problem was solved by introducing the ALB qPCR described by Gault et al. [19]. The concentration of human ALB gene DNA is used as a measure for total genomic DNA content and the total genomic DNA content as a measure for cell count. An alternative is to quantify the extracted DNA by NanoDrop spectrophotometry prior to real-time PCR and to report the HIV-1 DNA load as c/μg DNA as proposed by Casabianca et al. [43].

HIV-1 DNA was detectable and quantifiable in 94.7% of cross sectional buffy coat samples from 263 patients with undetectable viral load, confirming the high sensitivity of the assay. Moreover, the obtained HIV-1 DNA copy numbers closely resembled DNA loads reported for comparable populations [9,14,18]. Our selection of on-ART patients represented a very heterogeneous group with regard to the duration of infection before ART initiation, the on-ART period, pre-treatment viral load and CD4 count. Despite this heterogeneity, the range of HIV-1 DNA

concentrations remained limited, varying between 0.60 and 3.37 log c/10^6 cells (IQR of 1.84 to 2.40 log c/10^6 cells). Because the majority of these samples were collected more than a year after ART initiation, the measured viral DNA concentration most probably reflects the long-term persistent latent reservoir and it is known that this reservoir is small and very stable [44–46]. Interestingly, although these samples were collected a long time after ART initiation, pretreatment viral load and pretreatment $CD4^+$ T-cell count remained significantly predictive for the on-ART HIV-1 DNA load. Burgard et al. [9] and Fourati et al. [13] came to the same conclusions using PBMCs for DNA load analysis. Other groups only observed a correlation between the HIV-1 DNA load and the $CD4^+$ T-cell count nadir [10] or between the HIV-1 DNA load and pre-ART viral load [14].

The variation in HIV-1 DNA load over time is much lower than the variation of the viral RNA load, as illustrated for the 9 patients followed longitudinally. The overall difference in median pre-ART DNA load and median on-ART DNA load did not exceed 1 log c/10^6 cells. In comparison, the drop in RNA load for the same samples varied between 2.88 and 6.52 log c/ml. Re et al. [47] analyzed the evolution of the HIV-1 DNA load in PBMC cells of 19 ART-naive patients. They found no appreciable difference in DNA load between two samples collected with an interval of 12 months (median 2.86 log c/10^5 PBMC for the first sample versus 2.72 log c/10^5 PBMC for the second sample; $p > 0.1$). In treated patients, the HIV-1 DNA load decreased significantly but with limited magnitude, not exceeding 1 log c/10^5 PBMC [47]. A similar observation was made by Koelsch et al. [48] who reported median rates of decay between 0.25 and 1.03 log c/µg total DNA. All these observations question the value of frequent DNA load determination.

The results of the longitudinal follow-up samples revealed some unexpected fluctuations in HIV-1 DNA load probably reflecting the biological variability although technical errors due to founder effects during sample collection and DNA extraction that are inherent to this type of analyses on low input material cannot be excluded. Overall however, the inter- and intra-run coefficients of variance remained low indicating that the qPCR error rate is limited. High inter-laboratory reproducibility of the HIV-1 DNA load measurement has also been reported by Désiré et al. [49], De Rossi et al. [50], Avettand-Fènoël et al. [1] and Gantner et al. [51].

Apart from the fact that the inter-patient viral DNA load range is limited and that the evolution over time of the DNA load is slow, recent findings also revealed that the vast majority of HIV-1 DNA in blood cells is defective [52–54]. This may further impact the potential value of DNA load assessment. The likeliness that the ratio of defective over replication competent viral DNA is comparable between patients [52,53] however may somewhat refute the last criticism.

Our results show that the curve of the DNA load before and during ART overall follows a similar course in different patients and that small differences in this curve are largely determined by pre-ART conditions reflected in the CD4 nadir and peak viral load. This suggests that regular follow-up of the DNA load has limited added value. Determination of the baseline DNA load at treatment initiation and assessment of the set point HIV-1 DNA load at a specific time point afterwards, however, may be appropriate (as suggested by Maurizio Zazzi – oral presentation 'The clinical use of HIV-DNA quantity and resistance testing', 15th European Meeting on HIV & Hepatitis Treatment Strategies & Antiviral Drug Resistance, 7–9 June 2017, Rome, Italy).

Additionally, for patients for whom historic lab results are missing, the HIV DNA load measured during ART may provide some indications on the pre-ART stage.

Author contributions

Conceived and designed the experiments: VM CV. Performed the experiments: VM ED DS MS KD SK LH LV. Analyzed the data: VM CV. Wrote the manuscript: VM CV.

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Competing interests

None declared.

Ethical approval

The study was approved by the Ethics Committee of the University Hospital Ghent, EC number 2016/0610. All analyses were performed on rest fractions of stored samples and patients have provided written informed consent for the use of left over material. Patients were anonymized for selection, analysis and retrieval of information on gender, age, origin, HIV transmission route, CD4+ T-cell count, viral load, time of diagnosis and time of ART initiation.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jcv.2018.07.008.

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