

Shorter granulocyte telomeres among children and adolescents with perinatally-acquired HIV infection and chronic lung disease in Zimbabwe

Abhinav AJAYKUMAR^{1,2}, Glenn C. WONG³, Louis-Marie YINDOM³, Grace McHUGH⁴, Ethel DAUYA⁴, Edith MAJONGA^{4,5}, Hilda MUJURU⁶, Rashida A. FERRAND^{4,5}, Sarah L. ROWLAND-JONES³, H el ene C.F. C OT E^{1,2}

¹Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver, BC, Canada

²Centre for Blood Research, University of British Columbia, Vancouver, BC, Canada

³Nuffield Department of Medicine, University of Oxford, Oxford, UK

⁴Biomedical Research and Training Institute, Harare, Zimbabwe

⁵Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, UK

⁶University of Zimbabwe, Harare, Zimbabwe

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Corresponding Author/Address for reprints: Dr. H el ene C.F. C ot e, Department of Pathology and Laboratory Medicine, University of British Columbia, G227-2211 Wesbrook Mall, Vancouver, BC, Canada, V6T 2B5; E-mail: helene.cote@ubc.ca; Tel: +1-604-822-9777; Fax: 604-822-7635

Summary: Children with perinatally-acquired HIV infection (C-PHIV) and children presenting with reduced forced vital capacity (FVC) exhibit shorter granulocyte telomeres. Treatment-inexperienced C-PHIV, especially those with reduced FVC, have the shortest telomeres, and initiation of antiretroviral therapy improved their telomere length.

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ABSTRACT

Background: Chronic lung disease (CLD) has been reported among African children with perinatally-acquired HIV infection (C-PHIV), despite combination antiretroviral therapy (cART). In adults, shorter telomere length (TL) has been reported in association with both CLD and HIV. As little is known in children, our objective was to compare TL in HIV+ (cART-naïve or treated) and HIV-negative children with and without CLD.

Methods: Participants included Zimbabwean C-PHIV, aged 6-16, who were either newly diagnosed and cART-naïve, or on cART for >6 months, and HIV-negative controls of similar age and sex. Packed blood cell (granulocyte) TL from 621 children were compared cross-sectionally between groups. For a subset of newly diagnosed C-PHIV, changes in TL following cART initiation was evaluated.

Results: C-PHIV had shorter granulocyte TL compared to uninfected peers, regardless of cART. Among 255 C-PHIV without CLD, TL was shorter in cART-naïve participants. In multivariable analyses adjusted for age, sex, CLD, and HIV/cART status, shorter TL was independently associated with older age, being HIV+, and having reduced forced vital capacity (FVC). Lastly, cART initiation increased TL.

Conclusions: In this cohort, C-PHIV and those with reduced FVC have shorter granulocyte TL, possibly the result of increased immune activation and cellular turnover due to long-standing HIV infection with delayed cART initiation.

Keywords: Granulocyte telomere length, chronic lung disease, lung function, perinatal HIV infection, combination antiretroviral therapy

INTRODUCTION

Despite the success of combination antiretroviral therapy (cART) to prevent perinatal HIV transmission, the most recent estimates from UNAIDS suggest that 1.7M children under the age of 15 years are living with HIV-1 infection, 90% in sub-Saharan Africa (SSA) [1]. In 2018, an estimated 160,000 children acquired HIV-1 [1]. While in the pre-cART era, a majority of children with perinatally-acquired HIV-1 infection (C-PHIV) died in early childhood, something access to cART has dramatically improved, it has also been observed that approximately one third of C-PHIV survive to late childhood and adolescence in the absence of cART [2]. Thus, pediatric HIV programs are now seeing substantial and increasing numbers of older children and adolescents with HIV-1 infection. These young people often experience significant comorbidities [3,4], affecting multiple systems, which may not improve especially if cART is initiated later in childhood [5].

The most common comorbidity described in C-PHIV in SSA is a form of chronic lung disease (CLD) [6,7], typically presenting with a dry cough, reduced exercise tolerance, tachypnoea, hypoxia (at rest or following exercise) and progressive impairment of lung function, leading ultimately to pulmonary hypertension and respiratory failure. The first accounts of this condition in C-PHIV came from Zimbabwe [3,6], but similar observations were subsequently reported in Malawi [7], Kenya [8] and South Africa [9]. In the Zimbabwean ZENITH cohort of 385 children aged 6 – 15 years newly-diagnosed with PHIV (and hence cART-naïve), 54% of C-PHIV presented with a chronic cough and 28% had abnormal lung function [4]. Although CLD is more common in untreated older C-PHIV, there remains a substantial burden of disease even among cART-treated C-PHIV, as shown in the INHALE cohort in Zimbabwe, where 25% of C-PHIV on cART had CLD [10].

Chronic immune activation leads to the accumulation of exhausted and senescent cells, which characteristically feature shortened telomere length (TL). Telomeres consist of nucleoprotein complexes that protect the ends of chromosomes. In most somatic cells, TL shortens with each cellular division until a critical point, beyond which cells enter a stage of replicative senescence. In non-HIV adult populations, shorter leukocyte TL has been associated with reduced lung function [11,12], airflow limitation in non-smokers [13], chronic obstructive pulmonary disease (COPD) [14], and asthma [15]. In the context of HIV, the infection itself is associated with shorter leukocyte TL in adults without and with respiratory diseases [16–19], and in the latter, shorter TL was also measured in small airway epithelial cells [20]. In C-PHIV, two studies have reported shorter TL in HIV-infected compared to uninfected children [21,22], particularly in those not treated with cART [21]. A third study detected no difference but noted shorter leukocyte TL among C-PHIV with a detectable viral load, and TL attrition rate appeared higher among C-PHIV who received cART for <15% of their lifetime [23].

In pediatric populations where tobacco smoking is a less likely confounder, little is known regarding CLD and TL (both HIV and non-HIV). A large study of non-HIV participants suggested a moderate association between shorter leukocyte TL and reduced lung function in adults, but not in children [24]. Knowing that uncontrolled HIV is associated with shorter leukocyte TL and that HIV causes chronic inflammation, which could negatively impact lung health, we hypothesized that C-PHIV with CLD would show shorter TL than non-CLD or non-HIV peers.

METHODS

Study population

Our study participants included C-PHIV aged 6-16 years enrolled between 2013 and 2016 in two previously described cohorts - ZENITH and INHALE [4,10]. Figure 1 and Table S1 provide more information on the study design, the study sample, and the two cohort studies, including inclusion/exclusion criteria. Briefly, children and adolescents were recruited following HIV diagnosis from seven primary health-care clinics (PHCs) in Harare, and were either cART naïve (ZENITH), or on cART for >6 months (with a median duration of 4.7 years and approximately 80% viral suppression (INHALE)). HIV-uninfected children of similar age and sex were also recruited as controls from the seven PHCs in Harare [10]. Written informed consent was obtained from the parents/guardians of all study participants. This study was approved by the Medical Research Council of Zimbabwe, the Harare Hospital Ethics Committee, the Biomedical Research and Training Institute Institutional Review Board and the London School of Hygiene and Tropical Medicine Ethics Committee. Secondary analysis of INHALE and ZENITH participants with available lung function data and blood DNA specimen was undertaken. Demographic and clinical data were obtained from the cohort databases. HIV plasma viral load (pVL) >50 copies/ml was considered detectable. Cytomegalovirus (CMV) pVL was determined by qPCR (Altona Real Star) and >1 copy/ml was considered detectable.

Chronic lung disease classification and Telomere Length measurement

Lung function spirometry measurements of the highest forced expiratory volume in 1 second (FEV1) and forced vital capacity (FVC) were determined for each participant. Measures were

categorized as reduced if below the 10th percentile according to the Global Lung Initiative 2012 reference ranges, accounting for height, sex, age, and ethnicity. CLD was as described previously [10], namely: (a) obstruction, defined by reduced FEV1: FVC ratio or (b) reduced FVC, defined as reduced FVC with a normal FEV1: FVC ratio.

As PBMCs were not available for most participants, packed blood cells consisting mostly of granulocytes were obtained during blood Ficoll Paque separation (detailed in Supplementary data). TL in this fraction were measured by monochrome multiplex qPCR as previously described [25]. Relative TL was expressed as a ratio between telomeric DNA (T) and single-copy nuclear gene (S) copies, yielding the T/S ratio. Specimens that did not meet quality control criteria of <15% difference between replicates over two assay attempts were excluded from analyses [25].

Statistical Analyses

Relative granulocyte TL data were log₁₀-transformed for all analyses. Correlations and comparisons of demographic and clinical characteristics as well TL for the three groups were done using Spearman's correlations, Mann-Whitney U, Kruskal-Wallis, χ^2 , and Fisher's exact tests. Dunn's test was used to adjust for multiple pairwise comparisons as appropriate. Factors important univariately ($p < 0.10$) were considered for inclusion in multivariable ANCOVAs. In sensitivity analyses, extreme TL values that fell outside the 1.5x interquartile range (IQR) for each group and considered biologically implausible were excluded. For a subset of cART-naïve C-PHIV who also had a longitudinal specimens available post cART initiation, intra-individual change in TL was compared using the paired Student's t-test.

RESULTS

Participant characteristics

A total of 237 newly diagnosed cART-naïve C-PHIV, 182 cART-treated C-PHIV, and 205 HIV-uninfected children were included in this study. Demographic and clinical characteristics of all children are presented in Table 1. There were no differences in age and sex between children in each group. Consistent with a previous report in these cohorts [26], a greater proportion of cART-naïve C-PHIV had detectable CMV compared to cART-treated C-PHIV and HIV-uninfected children ($p < 0.001$, Table 1). Twenty one percent (38/182) of cART-treated C-PHIV had a HIV pVL > 400 copies/ml at study enrolment, and among the cART-naïve C-PHIV, despite no treatment, 19% (45/237) remained undetectable.

Among cART-treated C-PHIV, the median (range) duration of cART was 4.4 (0.5-12.4) years, and the percent of lifetime on cART was 44% (4-90%). The majority (77%) received non-nucleoside reverse transcriptase inhibitor (NNRTI)-based cART (Table 1, Supplementary Table S2) and there were no differences between the average % of lifetime treated with NNRTI-based cART (46%) vs. protease inhibitor (PI)-based cART (41%).

Lung function data was available for a total of 514 children of whom 54 (11%) presented with reduced FVC, and 21 (4%) with an obstructive spirometry (hereafter referred to as an “obstruction”). Prevalence of both reduced FVC and obstruction were significantly higher in the cART-naïve C-PHIV group compared to the HIV-uninfected group, however there were no differences in CLD status between cART-treated C-PHIV and HIV-uninfected children (Table 1).

Telomere length

Granulocyte TL data that passed quality control were obtained for 237 cART-naïve C-PHIV, 181 cART-treated C-PHIV, and 203 HIV-uninfected children. In univariate analyses, compared to HIV-uninfected children (median [IQR] \log_{10} TL: 1.01 [0.94 – 1.12]), both cART-naïve (0.98 [0.91 – 1.07]) and cART-treated (0.98 [0.93 – 1.06]) C-PHIV had shorter TL (Figure 2A), and among the latter, children on NNRTI-based regimens had significantly shorter TL compared to HIV-uninfected children (Supplementary Figure S1). Sex, age and number of household smokers showed no univariate association with TL (Supplementary Table S3). Although TL tended to be shorter in children with detectable CMV compared to those with undetectable CMV ($p=0.06$, Supplementary Figure S2), there was no relationship with CMV pVL (Supplementary Table S3). Overall, children with reduced FVC had significantly shorter TL (median [IQR] \log_{10} TL: 0.96 [0.87 – 1.01]) compared to both children with an obstruction (1.02 [0.95 – 1.08], $p=0.03$) and children without CLD (0.99 [0.93 – 1.08], $p=0.004$, Figure 2B). Based on this observation, in addition to our *a priori* definition of CLD, we also performed analyses restricted to CLD presenting as reduced FVC (Figure 3A, B). cART-naïve C-PHIV with CLD (median [IQR] \log_{10} TL: 0.95 [0.86 – 1.02]) and without CLD (0.95 [0.89 – 1.03]) had significantly shorter TL than HIV-uninfected children without CLD who had the longest TL (1.02 [0.95 – 1.13], Figure 3A). Among children without CLD, cART-naïve C-PHIV had shorter TL (0.95 [0.89 – 1.03]) than both cART-treated (0.98 [0.94 – 1.06], $p=0.03$) and HIV-uninfected children (1.02 [0.95 – 1.13], $p<0.001$), but there were no differences between cART-treated C-PHIV and the HIV-uninfected group (Figure 3A). Similar results were obtained when the analysis was restricted to children who presented with reduced FVC (Figure 3B). Our mosaic plot (Figure 3C) illustrates that the prevalence of CLD was ~25% (33/133) for cART-naïve C-PHIV, almost 2 times higher than the

CLD prevalence within cART-treated C-PHIV (14%, 26/181), and more than 3 times that of HIV-uninfected children (8%, 15/197). Further, only 35% (47/133) of cART-naïve C-PHIV TL were above the median TL of the overall study sample (median [IQR] \log_{10} TL: 0.99 [0.93 – 1.08]), compared to 45% (81/181) of cART-treated and 57% (113/197) of HIV-uninfected children. Likewise, the prevalence of reduced FVC, as well as the proportion of children with TL above the study median TL, was very similar to the overall model for CLD (Figure 3D).

In a multivariable model adjusted for age, HIV/cART status, and CLD status, only being C-PHIV was independently associated with shorter granulocyte TL (Figure 4A). In a sensitivity analysis, 21 cART-naïve, 12 cART-treated and 19 HIV-uninfected participants with implausibly high outlying TL values were excluded. In this model (Figure 4B), shorter TL was independently associated with older age, being HIV+ and having reduced FVC. In secondary models (Supplementary Figure 3A-B), we explored the effect of cART type and observed a modest association between NNRTI-based cART and shorter TL; the effect size was similar for PI-based cART but the 95% confidence interval was wider due to the smaller number of participants treated with PI. This effect was not related to age, nor the percent of life on cART.

Lastly, we did not observe any associations between TL and HIV pVL (Figure 5A, C) or CD4 count (Figure 5B, D) obtained from plasma collected at the same time point as DNA collection among C-PHIV, irrespective of cART status. However, for a subset of cART-naïve C-PHIV (n=21) who had a second specimen available post-cART initiation, a longitudinal increase in TL (p=0.013) was observed (Figure 6).

DISCUSSION

As reported previously [10], a high prevalence of CLD is observed among Zimbabwean C-PHIV, despite cART. Given the known relationships between HIV, TL and lung disease in adults, we tested the hypothesis that CLD among C-PHIV would be associated with shorter TL, compared to non-CLD or non-HIV peers. We report shorter granulocyte TL among C-PHIV, regardless of cART status, as well as among children presenting with CLD defined by reduced FVC. TL was shortest in cART-naïve C-PHIV with CLD, and appeared to improve following cART initiation in a subset of children.

Our findings are in contrast with the only other study of leukocyte TL and lung function in children which reported no association between TL and any spirometry index of lung function among 11-year old children [24]. However, that study took place in a high-income setting (Australia), measured TL in all blood cells, and included only HIV-uninfected children, making a comparison between the two studies difficult.

The underlying pathogenesis of CLD in older children and adolescents with PHIV is unknown and this study was not designed to examine potential mechanisms. However, in this sample, our observations of shorter granulocyte TL only among children presenting with reduced FVC may indicate a relationship with restrictive rather than obstructive lung disorder [27]. TL shortening in this case could be a result of increased immune activation and cellular turnover in response to HIV and/or CLD disease, something that has been reported in studies of HIV adults [18,19]. In contrast to the pre-ART era, where the majority of CLD was a result of lymphocytic interstitial pneumonitis, HRCT studies of adolescents with PHIV and CLD in Zimbabwe and South Africa have revealed radiological features of mosaic attenuation and air trapping consistent with obliterative bronchiolitis (OB), with or without bronchiectasis [28–30]. While OB is most

commonly seen following lung or hematopoietic stem cell transplantation in developed countries, the pathogenic mechanisms underlying this condition in African children with PHIV remain unclear, with persistent immune activation and chronic inflammation the most likely driving factors. In fact, outside the transplant population, OB has principally been described following severe lower respiratory tract infections in young children, often with adenovirus, and appears to be more common in the southern hemisphere [31].

CMV co-infection represents an important cofactor implicated in the development of HIV-associated comorbidities [32] and may play a more important role in African children, most of whom become infected with CMV in early life [33]. For children diagnosed late with PHIV, it is likely that primary CMV infection occurred in infancy at a time of uncontrolled HIV replication, hence could substantially contribute to comorbidities in this group. In keeping with this hypothesis, we recently described unexpectedly high levels of CMV viremia in older children and adolescents with PHIV in Zimbabwe, even in those who were treated with cART [26]. Furthermore, detection of CMV viremia at levels above 1000 copies/ml was associated with reduced lung function in cART-naïve children. In the current study, where the vast majority of children would be expected to be CMV seropositive, we observed no relationship between CMV pVL and granulocyte TL. It is likely that the frequency of CMV reactivations may modulate TL in these children, but longitudinal CMV viremia data were not available.

Within C-PHIV, TL was longer and the prevalence of CLD was lower among cART-treated children compared to cART-naïve children, suggesting that treatment may be beneficial for both lung health and cellular aging. Compared to two other studies of TL in pediatric PHIV [21,23] that included younger children, our study participants had slightly lesser cART exposure in terms of percentage of life on cART. It is possible that the observations of shorter TL among both

cART-naïve and cART treated C-PHIV, although modest, may become more pronounced later in life when the cumulative burden of HIV/cART and other environmental factors such as exposures to household smoke and other particulate pollutants have taken effect. A recent study from South Africa reported persistently lower lung function over two years among cART-treated adolescents living with HIV compared to HIV-negative controls [34]. Here, longitudinal CLD data was not available but we observed an improvement in TL following cART initiation in cART-naïve children, though it remained lower than in HIV-uninfected controls. As such, while cART may not necessarily improve lung function in older C-PHIV, longitudinal studies extending into adult life would help ascertain TL dynamics in the context of CLD severity/disease progression after an extended period of viremic control.

Our study has several strengths and some limitations. The ZENITH and INHALE cohorts included a well-characterized group of older children with PHIV, both treated and untreated, as well as HIV-negative control children of similar age and sex from the same population. This enabled us to delineate the effects of uncontrolled/ untreated HIV on CLD severity and cellular aging. Furthermore, in adult studies reporting TL in lung disease, tobacco smoking, a factor well-established to be associated with shorter TL [35], is often prevalent and an important confounder. Our investigation among children, where smoking is uncommon, allows a more robust analysis of the relationship between TL and lung disease. A major limitation of our study is the fraction of blood cells from which TL was quantified, which mostly consisted of neutrophils, an uncommon cell subset for telomere studies. Although we cannot ascertain that the TL measured here reflect overall PBMC or leukocyte TL for these children, several studies suggest a strong correlation between TL measured in neutrophils and either PBMCs, T lymphocytes, or leukocytes [36–38]. While it is possible that our finding is limited to granulocytes, immune

activation is heightened systemically during chronic HIV and increased stimulation of granulocytes by microbial translocation occurs even in the absence of viremia [39]. Furthermore, neutrophilic inflammation has been implicated in HIV and chronic lung diseases such as asthma, COPD, cystic fibrosis, and bronchiectasis [40], and therefore telomere dynamics in these cells may be relevant, potentially reflecting shorter telomeres in progenitor cells. Lastly, we did not have access to lung tissue from participants with CLD and could not measure TL in lung cells. Future studies investigating TL in lung tissue and leukocytes could help corroborate our findings in granulocyte TL, and ascertain the systemic nature of CLD.

In conclusion, treatment-inexperienced older African C-PHIV exhibited the shortest granulocyte TL and a higher prevalence of CLD in this cohort. Among children with CLD, only those presenting with reduced FVC had shorter TL, suggestive of increased cellular aging in relation to a restrictive lung disorder in these children. Lastly, cART-initiation in treatment-naïve children appears to improve TL. Taken together, our results suggest that cART treatment is protective against lung disease and cell aging in C-PHIV.

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FIGURE LEGENDS

Figure 1. Flow chart of study participants. Telomere length assay did not pass quality control for one cART-exposed and two HIV-uninfected participants. The numbers of participants with interpretable spirometry test data (lung function test) at enrolment were 133 (HIV+ cART-naïve), 182 (HIV+ cART-exposed) and 199 (HIV negative).

Figure 2. Univariate comparisons of log-transformed relative TL: **A** –cART-naïve C-PHIV *vs.* cART-treated C-PHIV *vs.* HIV-uninfected (Mann-Whitney U tests). **B** – Children with reduced FVC *vs.* obstruction *vs.* normal lung function (Mann-Whitney U tests). For all panels, whiskers of the box plots represent the 5-95 percentiles.

Figure 3. A–B: Relative TL comparisons between HIV/cART status groups separated by **A** – CLD+ *vs.* CLD-, and **B** – Reduced FVC *vs.* CLD-. For both panels, Dunn’s adjusted p values (adjusted for multiple comparisons, Kruskal-Wallis $p < 0.001$) are shown. Whiskers of the box plots represent the 5-95 percentiles. **C–D: Mosaic plots showing the relationship between HIV/cART status, above *vs.* below median TL of the whole cohort (TL=9.83), and CLD+ *vs.* CLD- status (C) or reduced FVC *vs.* CLD- status (D).** The width of each box shows the proportion of participants for each group relative to the total number of participants for whom both TL and lung disease data was available ($n=511$). The height of each box shows the proportion of participants within each group who fall above or below the median TL of the entire study sample. The number within each box represents the actual number of participants for each of these sub-groups.

Figure 4. Multivariable regression analyses of the association between possible predictors and log-transformed relative TL: **A** – all participants ($n=511$, $R^2 = 0.05$). **B** – sensitivity

analysis excluding participants with implausibly high outlying TL values ($n=475$, $R^2 = 0.08$).

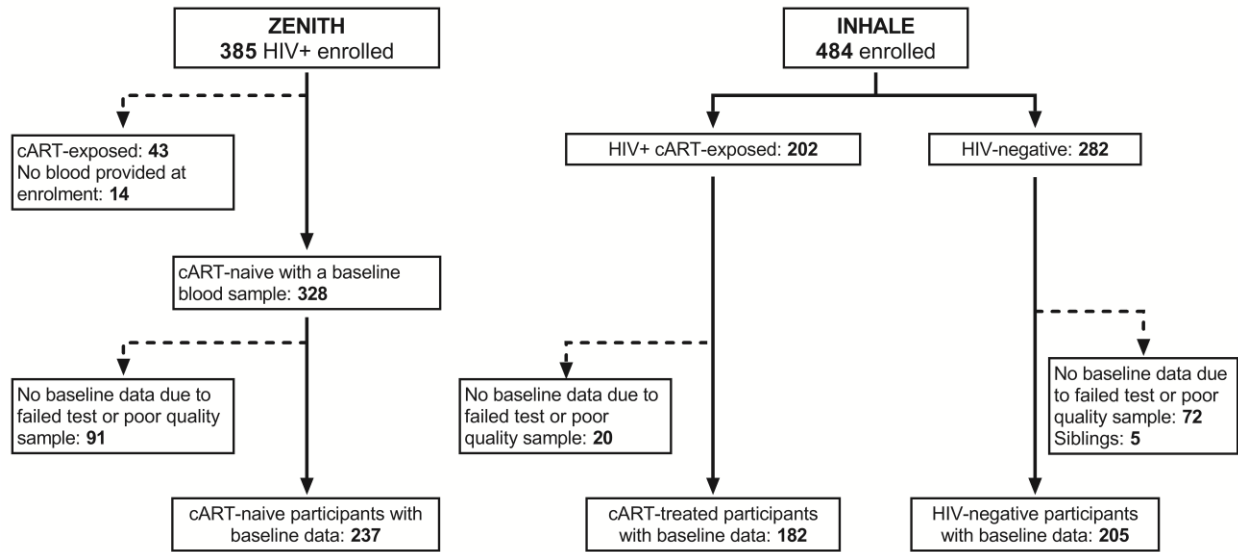
Other variables that were considered but were not included in the final model include: CMV VL (detectable *vs.* undetectable) and number of household smokers (any *vs.* none).

Figure 5. Comparisons showing that viral load and CD4 cell count are not univariately associated with relative TL among: cART-naïve C-PHIV (A and B), and cART-treated C-PHIV (C and D). Univariate comparisons were done using Mann-Whitney U tests and whiskers of the box plots represent the 5-95 percentiles. Similar results were obtained after adjusting for age and % of lifetime on cART (for cART-treated C-PHIV) through multivariable models (data not shown).

Figure 6. TL increases following cART-initiation in cART-naïve C-PHIV. Solid black lines indicate participants for whom TL increased and dashed grey lines indicate participants for whom TL decreased post-cART initiation. Comparisons were done using paired Student's t-test.

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Figure 1



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Figure 2

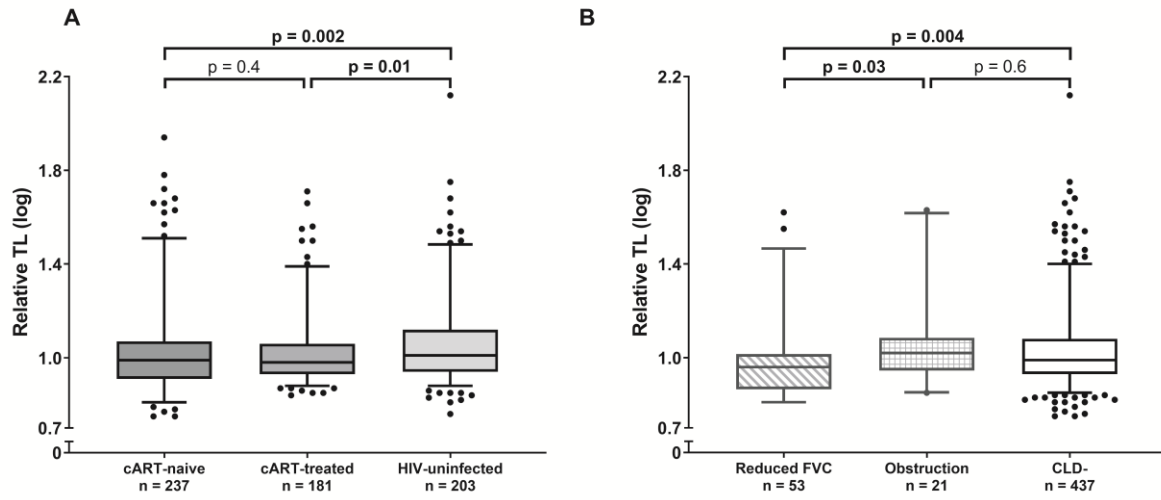
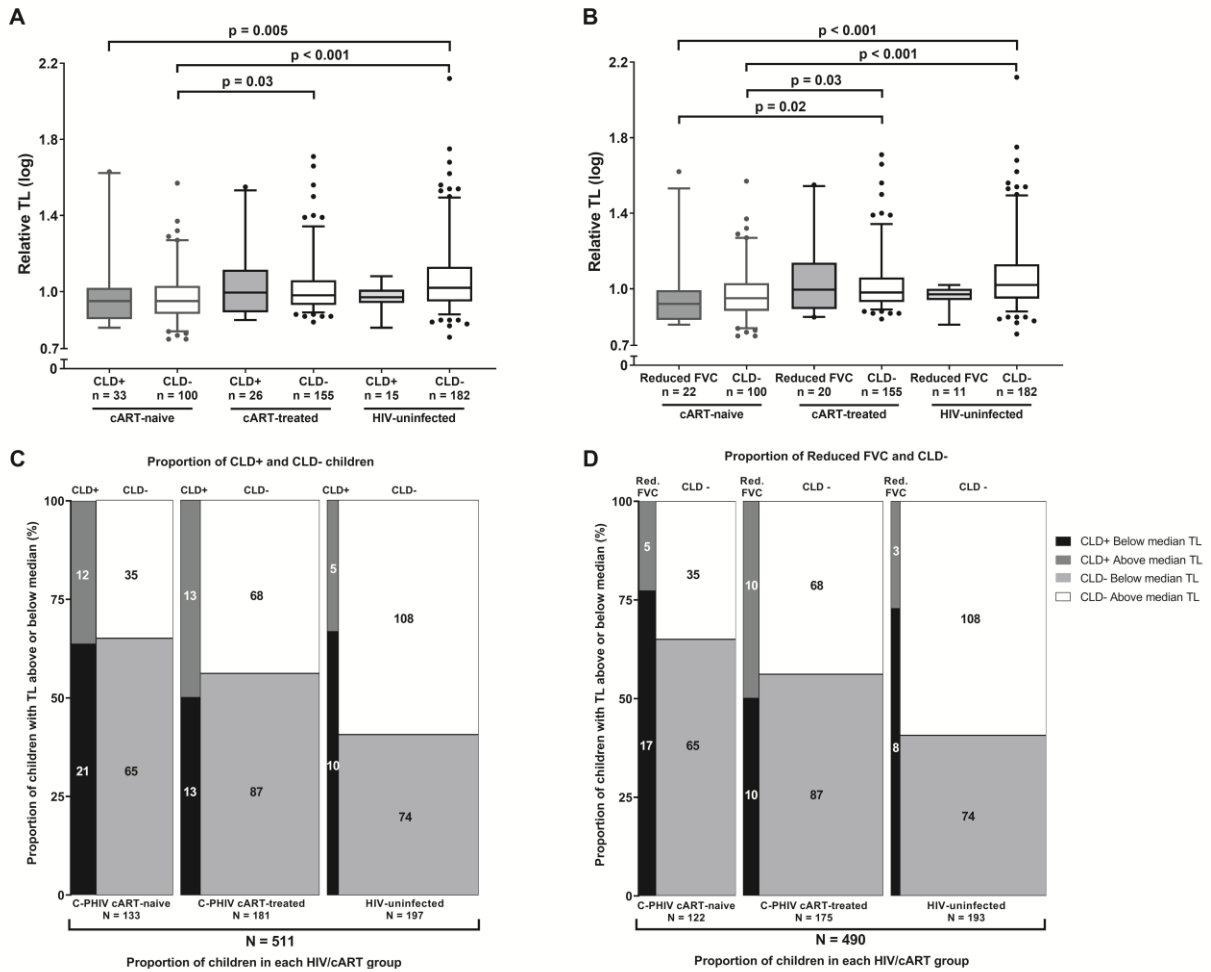
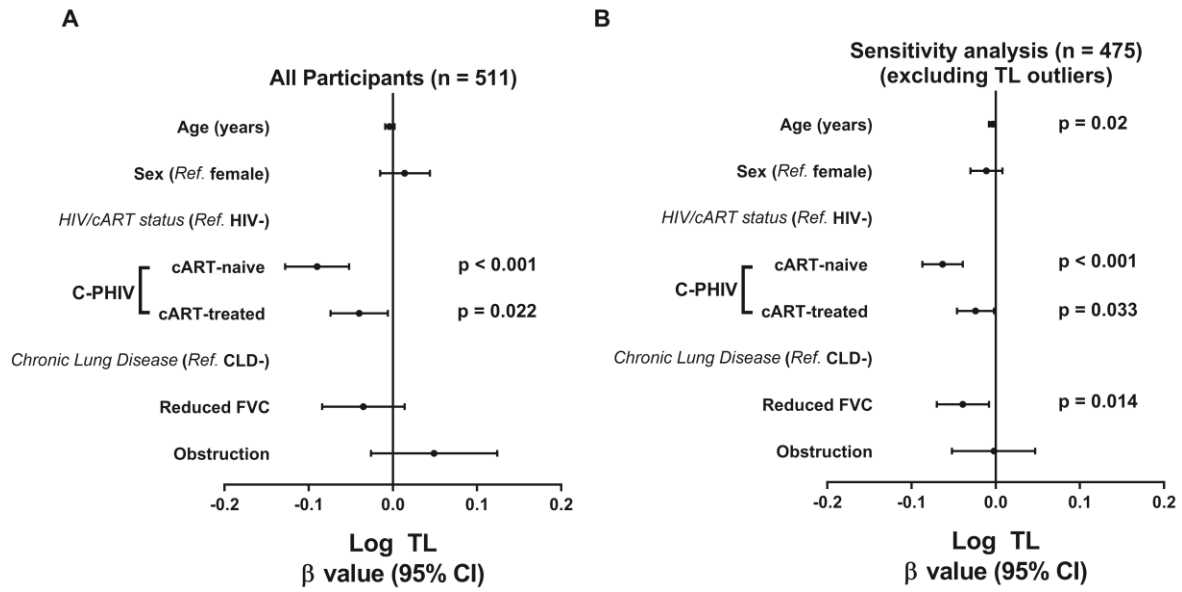


Figure 3



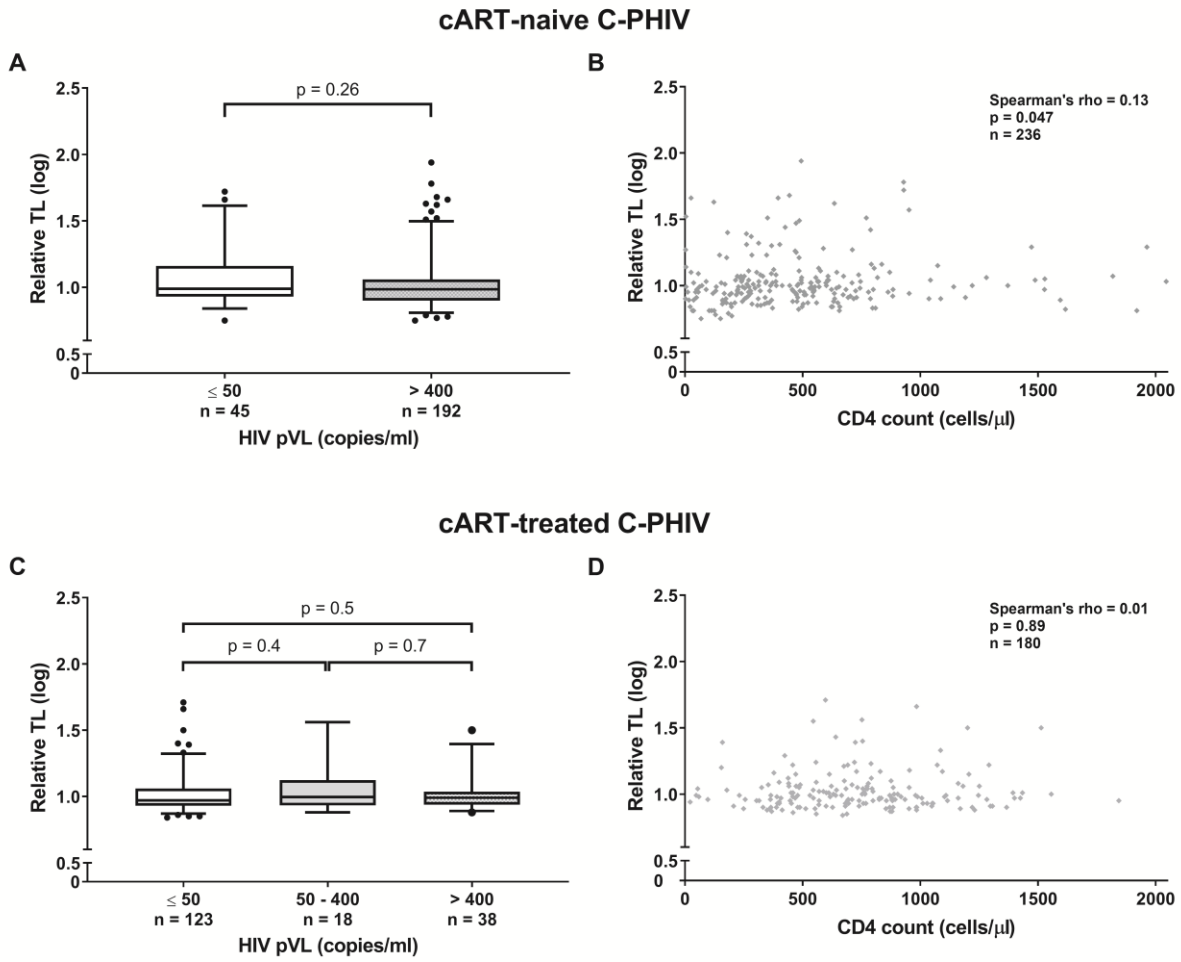
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Figure 4



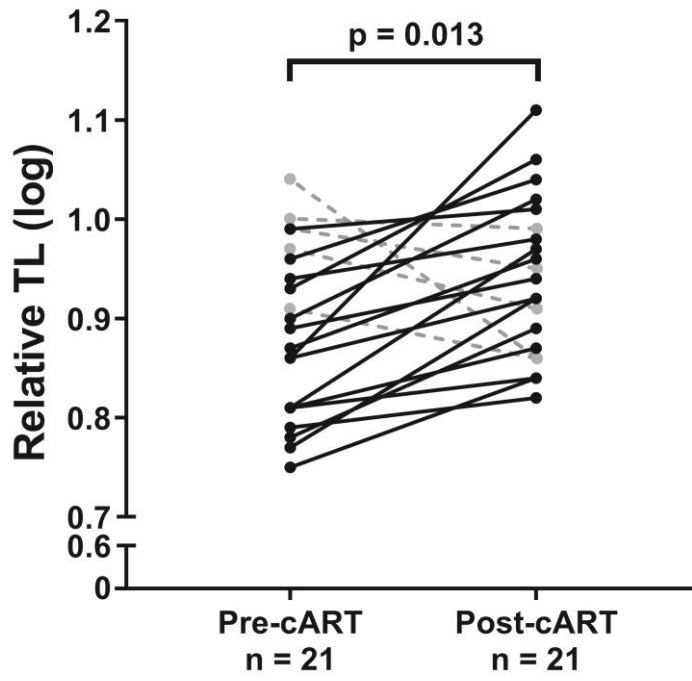
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Figure 5



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Figure 6



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Table 1. Demographic and clinical characteristics of study participants at baseline visit.

Characteristics	HIV+ cART-naïve	HIV+ cART-treated	HIV-uninfected	P value
	N = 237	N = 182	N = 205	
Male sex	115 (49)	94 (52)	98 (48)	p=0.73
Age, years	11 (6 - 15)	11 (6 - 16)	11 (6 - 16)	p=0.68
BMI	15.76 (11.49 - 24.96)	16.05 (10.73 - 24.96)	16.86 (8.97 - 31.37)	p<0.001
Detectable CMV	53 (40) (n=133)	39 (21)	17 (8)	p<0.001
CMV viral load (copies/ml)	1122 (42 - 38190)	1120 (175 - 106026)	1220 (402 - 2703)	p=0.98
Plasma HIV viral load		n=180		p<0.001
> 400 copies/ml	192 (81)	38 (21)	---	
50 - 400 copies/ml	0 (0)	18 (10)	---	
< 50 copies/ml (undetectable)	45 (19)	124 (69)	---	
HIV viral load (copies/ml)	38074 (3517 - 939350)	2254 (55 - 878301)	---	p<0.001
CD4 count (cells/ μ l)	420 (2 - 2045) (n=236)	710 (22 - 1844)	---	p<0.001
Chronic Lung Disease (CLD)	n=133	n=182	n=199	p=0.004
Reduced FVC	22 (17)	21 (12)	11 (6)	
Obstruction	11 (8)	6 (3)	4 (2)	
No reduced FVC or Obstruction (CLD-)	100 (75)	155 (85)	184 (92)	
# of household smokers				p>0.99

0	184 (78)	131 (72)	177 (86)	
1	44 (19)	34 (19)	27 (13)	
2 or 3	6 (3)	6 (3)	1 (1)	
Unknown	0 (0)	11 (6)	0 (0)	
Duration on cART, years	---	4.4 (0.5 - 12.4)	---	---
% of lifetime on cART	0 (0)	44 (4 - 90)	---	
Type of cART				---
<i>NNRTI-based</i>	---	140 (77)	---	
<i>PI-based</i>	---	36 (20)	---	
<i>Other^a</i>	---	6 (3)	---	
Relative TL		(n=181)	(n=203)	p=0.005
<i>Raw</i>	9.66 [8.09 - 11.69]	9.66 [8.56 - 11.46]	10.30 [8.80 - 13.21]	
<i>Log₁₀ transformed</i>	0.98 [0.91 - 1.07]	0.98 [0.93 - 1.06]	1.01 [0.94 - 1.12]	

Data are presented as n (%), median (range) or median [interquartile range, IQR]. Comparisons were done using Mann-Whitney U, Kruskal-Wallis, χ^2 , and Fisher's exact tests as appropriate. BMI – body mass index, CMV – cytomegalovirus, FVC – forced vital capacity, cART – combination antiretroviral therapy, NNRTI – non-nucleoside reverse transcriptase inhibitor, PI – protease inhibitor, TL – telomere length

^a List of "Other" cART regimens is detailed in Supplementary Table S2