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Title:
Cell-Associated Human Immunodeficiency Virus (HIV) Ribonucleic Acid Has a Circadian Cycle in Males With HIV on Antiretroviral Therapy

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Title: Cell-associated HIV RNA has a Circadian Cycle in Males Living with HIV on Antiretroviral Therapy

Short Title: Circadian Rhythmicity of HIV Transcription

Brief summary: Despite suppressive antiretroviral therapy, males living with HIV display a circadian cycling in cell-associated HIV RNA, having implications on the design of cure trials such as sampling and intervention times. Circadian cycles offer a novel target for latency reversal.

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Abstract

Background: Circadian transcription factors that regulate cell-autonomous circadian clocks can also increase HIV transcription in vitro. We aimed to determine if circadian variation in HIV transcription exists in people living with HIV (PLHIV) on antiretroviral therapy (ART).

Methods: We performed a prospective observational study of male PLHIV on ART, sampling blood every four hours for 24 hours. Using qPCR, we quantified expression of circadian associated genes, HIV DNA and cell-associated unspliced (CA-US) RNA in peripheral blood CD4+ T-cells. Plasma sex hormones were quantified alongside plasma and salivary cortisol. The primary outcome was to identify temporal variations in CA-US HIV RNA using a linear mixed effect regression framework and maximum likelihood estimation.

Results: Salivary and plasma cortisol, and circadian genes including Clock, Bmal1, and Per3 varied with a circadian rhythm. CA-US HIV RNA and the ratio of CA-US HIV RNA-to-DNA in CD4+ T-cells also demonstrated circadian variations, with no variation in HIV DNA. Circulating oestradiol was highly predictive of CA-US HIV RNA variation in vivo.

Conclusion: CA-US HIV RNA in PLHIV on ART varies temporally with a circadian rhythm. These findings have implications for the design of clinical trials and biomarkers to assess HIV cure interventions.

Clinical Trial Registration: NCT02895087, https://clinicaltrials.gov/ct2/show/NCT02895087

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Introduction

Antiretroviral therapy (ART) for people living with HIV (PLHIV) suppresses viral replication and significantly reduces morbidity and mortality, however, lifelong treatment is required. ART is not curative due to the persistence of long-lived and proliferating latently infected CD4+ T-cells. These infected cells contain a single copy of integrated virus and in a truly latent cell there is no virus expression, allowing the cell to persist and avoid immune-mediated clearance[1-3]. However, in nearly all PLHIV on ART, there is persistent detection of cell-associated unspliced (CA-US) in blood and lymphoid tissue[4, 5] and low-level plasma HIV RNA[6], consistent with ongoing HIV transcription on ART. We sought to determine whether the detection of CA-US HIV RNA in PLHIV on ART was associated with time and had circadian variation.

Most organisms, including humans, have overarching day-night behavioural and physiological changes which are responsive to external light. Light cues are relayed from the retina to the suprachiasmatic nucleus, commonly referred to as the “master clock”, which then relays these circadian cues to entrain the clocks of peripheral tissues, allowing for free-running cell-autonomous circadian cycles[7, 8]. Such molecular clocks exert control over 10% of gene expression and consist of a canonical negative feedback loop and stabilising loop[9]. In the primary loop, the major transcription factors, Circadian-locomotor-output-cycles-kaput (CLOCK) and Brain-and-muscle-ARNT-like-1 (BMAL1), heterodimerise, binding to palindromic six-nucleotide motifs within gene promoters, known as E-boxes[7, 8]. The putative circadian cycling exists due to two clock-controlled genes, *Cryptochrome (Cry)* and *Period (Cry)* which

71 repress CLOCK:BMAL1 function. We recently demonstrated CLOCK and BMAL1 together
72 upregulate HIV transcription *in vitro* through binding to the second of four E-box elements
73 within the HIV long terminal repeat (LTR)[10]. Additionally, circadian rhythms have been
74 shown by others to be important in the entry, replication, and disease outcomes of other viral
75 infections such as herpes simplex virus, respiratory syncytial virus, parainfluenza, influenza,
76 severe acute respiratory syndrome coronavirus 2, hepatitis B and C viruses, dengue, and Zika
77 virus[11-14].
78
79 Multiple other processes display circadian rhythms, some that could impact on HIV
80 replication. For example, CD4 T-cell trafficking, gut microbial translocation and extracellular
81 vesicle distribution all demonstrate daily variation[15-17], as does the sex hormone
82 oestradiol[18]. Although classically regarded as a female sex hormone, oestradiol is found in
83 males and has been shown to be important in reproductive function and bone development
84 and function[19]. Das et al. have also shown oestradiol's impact oestradiol on HIV
85 transcription is applicable in both males and females[20].
86
87 In a prior prospective study of PLHIV on ART, we identified a significant relationship
88 between *Bmal1* expression in CD4+ T-cells isolated from PLHIV, time, and CA-US HIV RNA *in*
89 *vivo[10]*. However, these samples were obtained within a short window of time,
90 approximately two hours, precluding any predictions of circadian rhythmicity. In this study,
91 we performed a prospective observational study of male PLHIV on suppressive ART closely
92 observed as inpatients over 24 hours with repeated regular blood draws. Plasma and salivary
93 hormones with known circadian rhythms or influence on HIV transcription were measured,
94 along with cell-autonomous circadian genes, CA-US HIV RNA and HIV DNA in CD4+ T-cells[20,

21]. We observed clear and significant circadian variations in CA-US HIV RNA and the ratio of CA-US HIV RNA:DNA without any changes in HIV DNA in purified CD4+ T-cells. Finally, in a predictive model, circulating oestradiol, together with time, was highly predictive of the variation in CA-US HIV RNA occurring *in vivo*.

Materials and Methods

Study Outline

A prospective observational study, *Circadian HIV RNA Oscillations and Outcomes of Stress (CHRONOS)* was conducted. We recruited 17 adult males (25 years to 55 years old) living with HIV who were on suppressive ART for >3 years with documented plasma HIV RNA <50 copies/mL within three months of recruitment. Inclusion criteria included having regular sleeping habits and not having undertaken shift work, or international travel within one month of recruitment. Exclusion criteria included documented sleep disorders, Addison's disease, diabetes, thyroid/pituitary/adrenal/splenic disorders, psychiatric conditions or their pharmacological treatment, steroid use, or use of medications affecting study outcomes. We limited our study to males to prevent sex-based differences in hormones confounding analyses. Participants provided informed consent. PLHIV were recruited between January 4th., 2016 and January 7th., 2017 from the Infectious Disease clinic at the Froedtert Hospital and the AIDS Resource Center of Wisconsin Medical Clinic. Flyers were placed in the clinics and providers also let patients know about the study. Participants were paid $200 upon completion of the study. The study is registered at ClinicalTrials.gov (NCT02895087). Participants were admitted as inpatients on the morning of the study and supervised for 24 hours. Blood and salivary samples were taken four-hourly at 08:00, 12:00, 16:00, 20:00, 00:00 and 04:00.

Study endpoints

The primary study objective was to identify circadian variation in CA-US HIV RNA in PLHIV on suppressive ART. Secondary endpoints were to identify circadian variation in circulating hormones, cell-associated HIV DNA, and circadian genes in CD4+ T-cells.

Measurement of circulating hormones

Plasma sex hormone-binding globulin (SHBG) and thyroid stimulating hormone (TSH) were quantified by the Brigham Research Assay Core (Brigham and Women's Hospital, Boston, MA) using the Access Chemluminescent Immunoassay (Beckmam Coulter, Fullerton, CA). Oestradiol, total testosterone, and plasma cortisol were quantified by the Brigham Research Assay Core using liquid chromatography-tandem mass spectrometry, and free testosterone quantified using equilibrium dialysis.

Salivary cortisol concentrations were measured using a commercially available chemiluminescence immunoassay with high sensitivity (IBL International, Hamburg, Germany) and intra and interassay coefficients below 9%.

Circadian Gene Expression, CA-US HIV RNA and HIV DNA in CD4+ T-cells

CD4+ T-cells were isolated from whole blood using a CD4+ T-cell isolation kit and magnetic-activated cell sorting columns (Miltenyi Biotec, Teterow, Germany) and RNA and DNA extracted (Allprep isolation kit, Qiagen). CA-US HIV RNA and total HIV DNA were quantified as previously described[22, 23]. Eight samples (of 102 total) with low input DNA (less than 10^4 cell equivalents) were excluded from analysis. Wells with positive CA-US HIV RNA or total HIV DNA amplification but <1 copy were included and their value directly extrapolated from the standard curve. Undetected HIV DNA replicates were assigned a value of zero and included in all analyses, to avoid bias for larger numbers, as previously described[24-26]. For analyses of the HIV RNA:DNA ratio, samples with HIV DNA measured as zero were excluded.

Circadian genes, *Clock, Bmal1, Per1-3, and Cry1-2* and the housekeeping gene, *Gapdh,* were quantified using real-time quantitative PCR as previously described[10]. To standardise circadian gene expression, the relative standard curve method of quantification was utilised, using the Jurkat T-cell line and PBMCs from a healthy donor as the standard and calibrator samples, respectively. Circadian gene expression was normalised to *Gapdh*. Samples with low input, as indicated by high *Gapdh* Ct, were excluded from analysis.

Statistical Analysis

To identify temporal changes in measured parameters, three models of fit were created. First, we fitted a single mean value across all timepoints. Secondly, time was treated as a categorical variable and we fitted a model to test whether the levels were different at different times. Finally, in the third model the data were fitted to a cosinor curve with a fixed period of 24-hours (treating time as a continuous variable), later referred to as the "circadian cycle model". These models have the equations:

$$Y(t) = M \quad (1)$$

$$Y(t) = M + \alpha TIME_{(Categorical)} \quad (2)$$

$$Y(t) = M + \alpha x(t) + \beta y(t); \quad (3)$$

where $\alpha = A\cos\emptyset;\ \beta = -B\sin\emptyset; x(t) = \cos\left(\frac{2\pi t}{24}\right); y(t) = \sin\left(\frac{2\pi t}{24}\right)$.

The models were fitted using a linear mixed effect regression framework using a maximum likelihood estimation. For both models, within subject variability was represented by using a random intercept for each participant, using *lme* function from *nlme* library in *R* (v3.63).

A nested F-test was used to compare each models' fit to the mean of all timepoints (i.e. a straight line, comparing model (1) vs model (2); and model (1) vs model (3)), providing statistical evidence for the presence of temporal variations (categorical model) or specifically circadian rhythmicity (circadian cycle model). P-values below 0.05 support the acceptance of the time-varying models, with smaller p-values indicating stronger support of the model tested compared to the constant model.

Assessment of correlates with HIV parameters (circadian genes, hormones, and HIV virological markers – total of 56; Table 3) was performed by including hormone or circadian gene variables into the circadian cycle models of HIV parameters and statistical significance obtained by comparing each model's fit to the previous model lacking the additional variables. Model 3 above was modified by adding an extra parameter,

$$Y(t) = M + \alpha x(t) + \beta y(t) + \gamma V(t)\ ; \qquad (4)$$

where $V(t) =$ Hormone or circadian gene variables.

A nested F-test was again used to compare model (3) vs model (4). This was done using the *anova* function in R (v3.63). A Bonferroni adjustment was not performed due to the hypothesis-generating nature of these analyses.

Sample size: Based on our previous studies of PLHIV on stable ART prior to any intervention, the coefficient of deviation of CA-US HIV RNA is 0.3 log copies per million 18S RNA copies[23, 26]. Having at least 15 participants with blood drawn every four hours but at staggered intervals, we anticipated the pooled data would form a sinusoidal curve of CA-US HIV RNA in association with time of blood collection. In a previous cross sectional study, the ratio of the curve's amplitude to the mean of CA-US HIV RNA was 0.5[10]. Recruitment of 15 participants

gave us a power of >90% to detect a sinusoidal curve as is expected in circadian expression. Given the potential of 10-20% of participants not completing the study, we recruited 17 participants.

Role of the Funding Source

Sources of funding had no involvement in study design, collection, analysis and interpretation of data, or in the writing of the report.

Study Approval

Written informed consent was received from participants prior study inclusion. Ethics approval was obtained from review boards of the University of Melbourne (ID 1544739), the University of San Francisco California (IRB 15-17652), and the Medical College of Wisconsin (ID PRO00024359).

Results

Significant variation in circadian genes, proteins and HIV parameters in PLHIV over a 24-hour period

17 male PLHIV on suppressive ART enrolled in a prospective observational study at the Madison Medical College in Milwaukee, WI had blood and saliva collected four-hourly for 24 hours. Numbers of participants screened, eligible and enrolled are summarised in supplementary figure 1.

The median age of the participants was 50 (interquartile range, IQR 32-53) years and baseline CD4+ T-cell count was 734 (IQR 574-945) cells/uL (Table 1). All hormones, circadian genes, and CA-US HIV RNA were quantifiable in all participants at most timepoints. In this

cohort, HIV DNA levels were low with a median of 39.63 copies/10^6 cell equivalents (range 0-349.5 copies/10^6 cell equivalents). As expected, we observed fluctuations in hormone levels (Supplementary Figure 2A) and circadian gene expression (Supplementary Figure 2B), but also observed clear variation in levels of CA-US HIV RNA in CD4+ T-cells from blood (Supplementary Figure 2C).

Table 1 Participant Demographics

Participant ID	Race	Age (years)	Current CD4 (cells/µL)	Nadir CD4 (Cells/µL)	Antiretroviral Regimen
1009	African American	61	458	191	TDF/FTC/DRVc
1063	African American	53	937	287	ABC/3TC/DTG
1075	White	50	945		TAF/FTC/DTG
1081	White	32	837		ABC/3TC/DTG
1099	African American	31	858	354	TDF/FTC/RPV
1241	African American	27	729		ABC/3TC/DTG
1294	African American	53	1226	186	TAF/FTC/DTG
1312	African American	47	416	47	TAF/FTC/EVG
1393	African American	54	1142		TAF/FTC/EVG
1422	White	44	705	390	TAF/FTC/EVG
1481	White	26	554	39	TAF/FTC/DTG
1691	White	51	574	8	ABC/3TC/DTG
1727	African American	50	707		TAF/FTC/EVG
1741	White	40	734		TAF/FTC/EVG
1779	African American	32	1078	634	TDF/FTC/EVG
1786	White	50	550		TDF/FTC/EFV
1837	African American	71	1000	301	TAF/FTC/EVG
Summary Median (IQR)	**10 African American 7 White**	**50 (32-53)**	**734 (574-945)**	**239 (81.75-340.75)**	

ABC; abacavir, DRVc; darunavir + cobicistat, DTG; dolutegravir, EFV; efavirenz, EVG; elvitegravir, FTC; emtricitabine, RPV; rilvipirine, TDF; tenofovir disoproxil fumarate, TAF; tenofovir alafenamide, 3TC; Lamivudine

Summary data represent the median and IQR, except for race which is summarised as count data

PLHIV on suppressive ART have intact circadian rhythms

To identify temporal variations in measured parameters, data from all participants were fitted using two models; 1) time was treated as a categorical variable, or; 2) a cosinor curve with a fixed period of 24-hours was superimposed (Figure 1). The former model demonstrates whether parameters fluctuate over the observation period, whilst the latter reveals circadian patterns of expression. Both models revealed that most variables varied over time, and those that did vary, cycled in a circadian manner (*Table 2*). As the circadian cycle model more rigorously assesses 24-hour cycling, this was used as the primary model for subsequent analyses.

As such, all hormone levels peaked in the morning, between 04:25 (oestradiol and free testosterone) and 10:20 (plasma cortisol) – except sex hormone binding globulin (SHBG) levels which peaked at 14:55 (Figure 1A). The early-morning peak in cortisol was consistent with literature in humans and other diurnal animals [27, 28] and the greatest variation was observed in these parameters, with amplitudes of 63.5% and 62.6% variation from the mean, for saliva and plasma respectively ($p<0.0001$).

***Table 2* – Circulating hormones, circadian gene expression, and HIV transcription varies over time** Two models of temporal change were fitted to data collected over a 24-hour period for the study participants. Changes in each parameter were assessed by treating time as a categorical variable (categorical model) or data were fitted to a cosinor curve with a period of 24 hours (circadian cycle model) using a linear mixed effect regression framework and a maximum likelihood estimation. A nested F-test was used to compare each models' fit to the mean of all timepoints to provide statistical evidence for the presence of temporal variations (categorical model) or specifically circadian rhythmicity (circadian cycle model) of measured parameters.

The p-values of the fit of the models are shown, wherein p-values below 0.05 are evidence for temporal variations (categorical model) or circadian rhythmicity (circadian cycle model) in the measured parameters against a model with no temporal variation. The peak and nadir times for each parameter from the circadian cycle models is reported, as well as the amplitude of the peak circadian cycles, represented as the percentage of variation from the mean.

	Variables	Categorical Model (p-value)	Circadian Cycle Model (p-value)	Peak Time* (Military Time)	Nadir Time* (Military Time)	Amplitude* (% variation from the mean)
Circadian Genes	*Clock*	0.0598	0.0052	13:10	01:10	14.0
	Bmal1	0.002	0.0181	01:20	13:20	15.7
	Per1	0.0051	0.0334	01:55	13:55	15.3
	Per2	0.0024	0.0029	00:20	12:20	14.5
	Per3	0.0671	0.0368	08:30	20:30	9.3
	Cry1	<0.0001	0.009	00:30	12:30	18.2
	Cry2	0.002	0.0256	00:20	12:20	18.6
Circulating Hormones	Cortisol (Saliva)	<0.0001	<0.0001	08:00	20:00	63.5
	Cortisol (Plasma)	<0.0001	<0.0001	10:20	22:20	62.5
	SHBG	0.0937	0.0283	14:55	02:55	7.8
	Oestradiol	0.2208	0.039	04:25	16:25	16.6
	TSH	0.0178	0.0006	05:30	17:30	16.2
	Total Testosterone	0.003	0.0175	04:50	16:50	6.5
	Free Testosterone	0.0108	0.0014	04:25	16:25	3.9
HIV	CA-US HIV RNA	0.002	0.0189	22:45	10:45	26.3
	HIV DNA	0.1749	0.1047	No variation	No variation	No variation
	HIV RNA:DNA Ratio	0.003	0.001	17:30	05:30	44.0

SHBG; sex hormone binding globulin, TSH; thyroid stimulating hormone, CA-US HIV RNA; cell-associated unspliced HIV RNA; * circadian model only

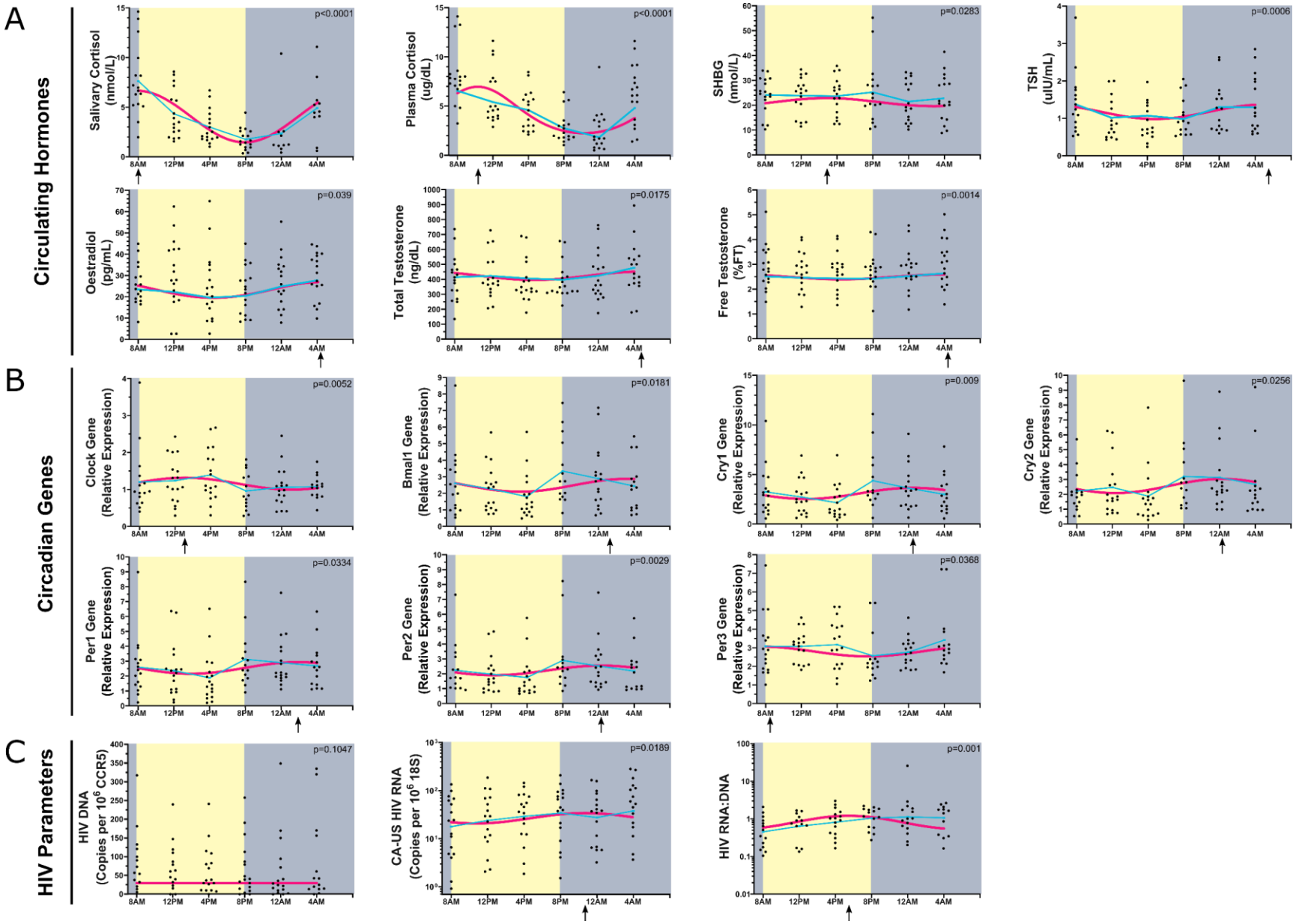

A
Circulating Hormones
Salivary Cortisol (nmol/L)
p<0.0001
Plasma Cortisol (ug/dL)
p<0.0001
SHBG (nmol/L)
p=0.0283
TSH (uIU/mL)
p=0.0006
Oestradiol (pg/mL)
p=0.039
Total Testosterone (ng/dL)
p=0.0175
Free Testosterone (%FT)
p=0.0014
B
Circadian Genes
Clock Gene (Relative Expression)
p=0.0052
Bmal1 Gene (Relative Expression)
p=0.0181
Cry1 Gene (Relative Expression)
p=0.009
Cry2 Gene (Relative Expression)
p=0.0256
Per1 Gene (Relative Expression)
p=0.0334
Per2 Gene (Relative Expression)
p=0.0029
Per3 Gene (Relative Expression)
p=0.0368
C
HIV Parameters
HIV DNA (Copies per 10^6 CCR5)
p=0.1047
CA-US HIV RNA (Copies per 10^6 18S)
p=0.0189
HIV RNA:DNA
p=0.001
8AM
12PM
4PM
8PM
12AM
4AM

Figure 1 Circadian cycling of HIV transcription exists in vivo. *The levels of A) circulating hormones, B) circadian gene expression in CD4+ T-cells from the blood and C) CA-US HIV RNA, HIV DNA and the HIV RNA:DNA ratio in CD4+ T-cells from the blood in all study participants (n=17). Each study participant at each timepoint is represented as a black circle. Two models of temporal changes are superimposed onto the cohort data where; 1) time was treated as a categorical variable (blue) and 2) data were fitted to a cosinor curve (pink). The peak time of the cosinor model is indicated by black arrows, and blue and yellow shaded rectangles represent approximate day and night times. P-values are denoted for the significance of the circadian cosinor models in the upper right corner for each parameter*

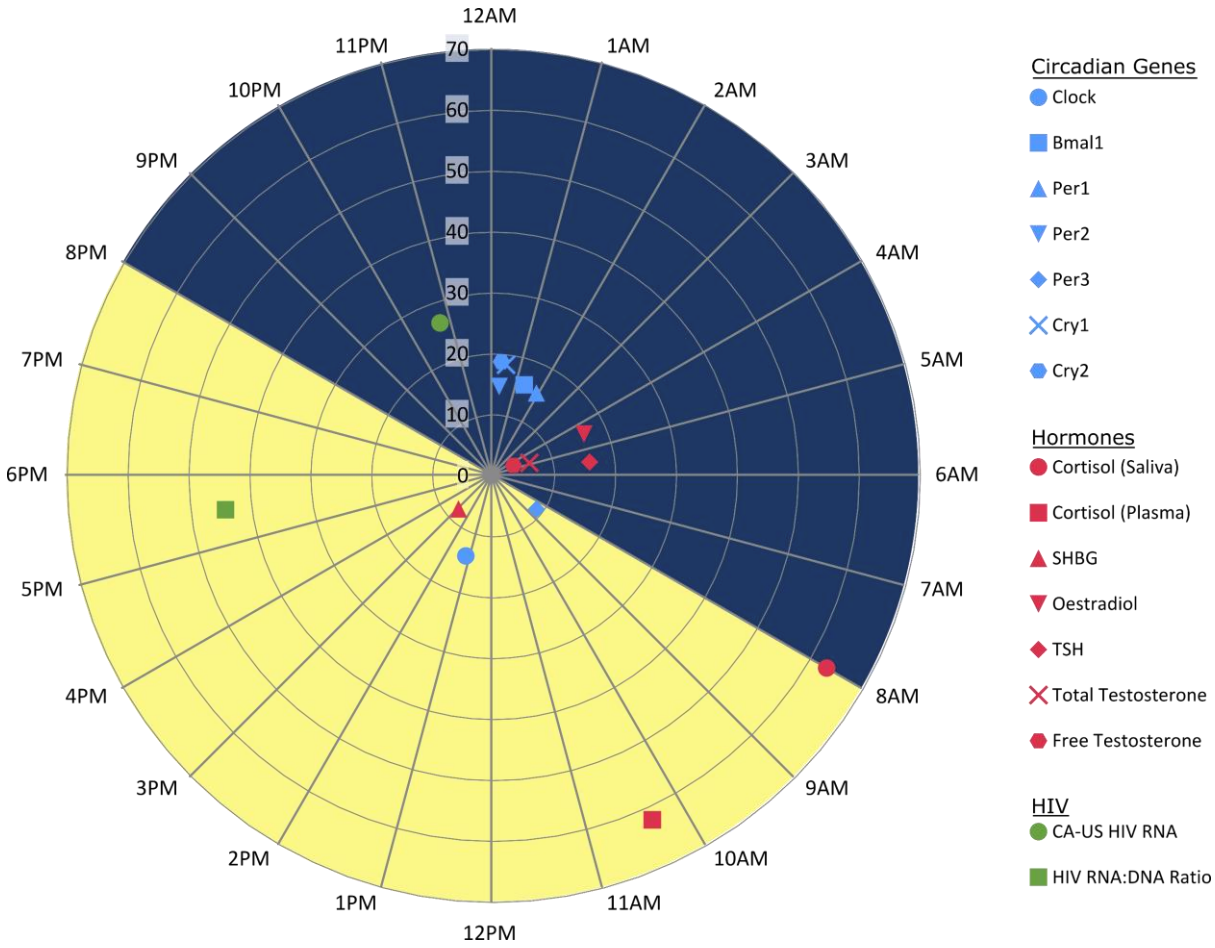
Circadian gene expression from blood CD4+ T cells varied, but in contrast to hormonal variation, the majority of genes peaked at approximately midnight e.g. *Bmal1* expression peaked at 01:20 with an amplitude of 15.7% from the mean (p=0.018) (Figure 1B). In contrast, expression of *Clock* and *Per3* peaked during the day at 13:10 (p=0.0052) and 08:30 (p=0.037) respectively. Of the circadian genes, expression of *Cry1* and *2* displayed the largest variations, whilst *Per3 showed the smallest,* with amplitudes of 18.2%, 18.6%, and 9.3% from the mean respectively (p=0.009, 0.026 and 0.0368 respectively). These data demonstrate that in PLHIV on ART, CD4+ T-cells have intact cell-autonomous circadian transcriptional machinery.

HIV RNA – but not HIV DNA – displays circadian cycling in PLHIV on ART

In PLHIV on stable ART, nearly all HIV DNA is integrated [29], thus total HIV DNA is a surrogate for the frequency of infected cells. Using the circadian mathematical model, no changes were observed in total HIV DNA in purified CD4+ T-cells over time (p=0.10, *Table 2*).

Conversely, CA-US HIV RNA and the ratio of HIV RNA:DNA varied with circadian rhythmicity, peaking at 22:45 and 17:30 respectively. CA-US HIV RNA and the ratio of HIV RNA:DNA also had substantial variations, with amplitudes of 26% and 44% from the mean, respectively ($p=0.019$ and $p=0.001$, respectively; *Table 2*, Figure 1D). These data suggest that in PLHIV on ART, production and/or clearance of CA-US HIV RNA within CD4+ T-cells has an autonomous circadian cycle.

Phase alignments of each variable confirmed the presence of two distinct clusters of expression throughout the 24-hour day; a cluster of circadian genes (*Bmal1, Per1-2, Cry1-2*) that peaked between 00:20 and 01:55, followed by a cluster of peak in expression of hormones between 04:25 and 05:30 (*Figure 2*). This was then followed by peaks in cortisol levels in the saliva at 08:00 and plasma at 10:20. *Clock* and *Per3* expression, and circulating SHBG peaked in an antiphasic manner to the clusters of expression of circadian genes and hormones. The peak of CA-US HIV RNA in CD4+ T-cells most closely associated with the circadian gene cluster. In contrast, the HIV RNA:DNA ratio peaked in the late afternoon.



306

307 **Figure 2 – Phase alignments display main clusters of circadian gene and hormone expression**

308 *Peak times of cosinor models of circadian gene expression (blue), circulating hormones (red),*

309 *and CA-US HIV RNA and the HIV RNA:DNA ratio (green) are plotted radially starting at*

310 *midnight (0°). The amplitudes of each cosinor peak are represented as the distance from the*

311 *centre of the plot and plotted as percentage of variation from the mean. Yellow and blue*

312 *hemispheres represent the approximate day and night times.*

313

314 **Oestradiol levels improves the prediction of the variation in the HIV RNA:DNA ratio**

315 Next, we determined whether each circadian parameter – be it circadian gene

316 expression or hormone – could explain the cycling seen in HIV measurements. These

317 parameters were included as additional variables in the cosinor models of the three different

HIV parameters. A negative correlation between oestradiol levels and the HIV RNA:DNA ratio, when accounted for, statistically improved the fit of the model for the HIV RNA:DNA ratio (p=0.029, *Table 3*). These data indicate that circulating oestradiol and its own circadian rhythm are associated with the circadian variation in CA-US HIV RNA.

Table 3 – Associations between circulating hormones levels, circadian gene expression and virologic markers To identify associations between circulating hormones or circadian gene expression and virologic markers, circulating hormone levels, and circadian gene expression of CD4+ T-cells were included as additional variables in the circadian cycle models of CA-US HIV RNA, HIV DNA, and the HIV RNA:DNA ratio. If the addition of a variable statistically improved the virological models' fit (determined by nested F-tests comparing to the initial model), it was deemed that a significant association exists. This table summarises the p-values obtained from these F-tests, and those that are significant are in bold

Variables	CA-US HIV RNA (p-value)	HIV DNA (p-value)	HIV RNA:DNA Ratio (p-value)
Clock	0.4257	0.6829	0.9386
Bmal1	0.3306	0.4838	0.4845
Per1	0.9996	0.9816	0.6278
Per2	0.3094	0.4449	0.5348
Per3	0.4418	0.9364	0.5397
Cry1	0.7421	0.6211	0.4248
Cry2	0.6561	0.5525	0.4187
Cortisol (Saliva)	0.1313	0.2037	0.2636
Cortisol (Plasma)	0.3458	0.1217	0.1764
SHBG	0.7766	0.3963	0.6273
Oestradiol	0.2424	0.1624	**0.0293**
TSH	0.5056	0.3936	0.8629
Total Testosterone	0.845	0.888	0.8243
Free Testosterone	0.8516	0.4563	0.7675

Discussion

We show for the first time, that male PLHIV on suppressive ART have demonstrable circadian rhythms of hormones and circadian genes. Furthermore, CA-US HIV RNA, but not HIV DNA, in CD4+ T-cells from the blood displayed variation over a 24-hour period. Mathematical modelling displayed circadian rhythmicity in these variations, and the inclusion of oestradiol within this model improved the prediction of CA-US HIV RNA cycling, consistent with an impact of oestradiol expression on CA-US HIV RNA.

Although there has been previous interest in variation in plasma HIV RNA over a 24-hour period in PLHIV, most of this work was performed prior to ART's advent[30, 31]. In male PLHIV on suppressive ART, we detected significant circadian variation in CA-US HIV RNA without variation in HIV DNA, consistent with changes in either production or clearance of CA-US HIV RNA over a 24-hour period. The primers used to quantify CA-US HIV RNA were located in the LTR and *gag* gene and therefore detect initiation of HIV transcription. It is therefore possible that even if there is variation in transcription initiation, this may not lead to completion of virus production, given the well described blocks to completion of virion production, at least in resting CD4+ T-cells[32]. Variation in CA-US HIV RNA due to changes in transcription initiation is biologically plausible, given our prior work *in vitro* showing that CLOCK and BMAL1 can both bind to and activate the HIV LTR [10], however altered clearance of viral RNA is also possible.

The changes in CA-US HIV RNA *in vivo* cannot be explained by cell trafficking leading to alterations in the total number of CD4+ T-cells in blood, since all viral quantification was performed in sorted CD4+ T cells. Changes in CD4+ T-cell subsets over time as shown to occur

in mouse models [33, 34] are also unlikely to explain our findings since total HIV DNA levels remained stable over time and the frequency of HIV DNA differs amongst T-cell subsets[35, 36]. Furthermore, whilst the percentages of total CD4+ T-cells in peripheral blood display circadian cycles in humans, the percentage of each subset has not been shown to significant cycle *in vivo[17]*.

It is generally regarded that the human immune response is most active during the day when people are most likely to be exposed to pathogens. Antigen-specific T-cells have been shown to vary in number and function with a circadian variation[34, 37]. In these studies using mouse models, enhanced proliferative responses of CD4+ and CD8+ T-cells to foreign antigen during the day compared to night time was observed. If the expression of CA-US HIV RNA in PLHIV on ART results in protein expression, then intermittent clearance of infected cells that express RNA, could also potentially explain our findings. However, we believe this is unlikely given the extremely low levels of HIV protein expression in PLHIV on ART, at least in CD4+ T-cells from blood[38].

Though an HIV-negative cohort was lacking in this study, comparing the patterns in cortisol cycling – the most studied parameter of circadian rhythmicity – in our cohort and others' published works reveals both highly comparable acrophases and amplitudes[27, 39]. Our data confirm the cell-autonomous circadian machinery in CD4+ T-cells remain intact, despite the chronically infected and exhausted state of many such cells in PLHIV[40, 41].

The surprising relationship between time, oestradiol and variation in CA-US HIV RNA suggests that a mechanism other than transcription initiation by CLOCK:BMAL1 could also explain

variations in CA-US HIV RNA. Oestrogen receptors have been shown to be key regulators of HIV latency and selective oestrogen receptor modulators can activate HIV transcription *ex vivo* using CD4+ T-cells from PLHIV on ART[20]. Our study was unable to differentiate between a direct effect of CLOCK and BMAL1 on the HIV LTR within infected cells, or an indirect effect of circadian oscillations in oestradiol or cortisol production which could mediate changes in CA-US HIV RNA[42]. Our study cohort was intentionally limited to males to prevent sex-based differences in hormones as confounders of our analyses. Recruiting only males may have allowed for this novel relationship between oestradiol levels, time, and CA-US HIV RNA to be uncovered. The circadian rhythms of HIV and their possible interactions with the hormonal cycle in females living with HIV should be further explored.

Our findings also have important implications on the design of clinical trials and interventions, most notably highlighting the need for consistency in sampling times across the entire study period and the requisite for multiple baseline measurements – particularly when assessing the effect of an intervention on HIV transcription, such as in the assessment of latency reversal agents[23]. CLOCK and BMAL1 are able to bind to the 5' HIV LTR[43] and we have shown a direct interaction between CLOCK and BMAL1 on increasing HIV transcription[10], whilst others have shown agonists of BMAL1 inhibit HIV replication[44]. As such, the cell-autonomous circadian machinery itself poses a specific target to reactivate latently infected cells, such as through the use of circadian-modulating compounds[45, 46]. Finally, the correlation between HIV transcription, time, and oestradiol implies that considerations of participants' sex and use of hormone replacement therapy is also important.

Our study had several limitations. First, as we only sampled the peripheral blood, we were unable to assess changes in infected cells in tissue, where there is a higher frequency of infected and transcriptionally active CD4+ T-cells[47, 48]. Second, our measurements of total HIV DNA include both defective and intact proviruses, precluding any conclusions specifically relating to intact replication-competent proviruses[49]. Third, given defective proviruses are known to be transcriptionally- and translationally-competent, it is possible that the CA-US HIV RNA we measured was from defective virus and would not result in virion formation[32, 50]. Additional investigation into variation of other viral species – downstream of transcription initiation (i.e. RNA splicing and degradation, protein production, and viral release) – are warranted.

In conclusion, in a prospective observational study of PLHIV, we showed for the first time a clear circadian variation in expression of CA-US HIV RNA over a 24-hour period. Our findings are consistent with either direct effects of CLOCK/BMAL1 on the HIV LTR, an indirect effect on HIV transcription through oestradiol, or enhanced clearance of CA-US HIV RNA or HIV RNA-expressing cells. We believe that these variations in CA-US HIV RNA should be considered when designing clinical trials that specifically focus on HIV transcription, such as trials of latency reversing agents. Additionally, drugs that perturb the circadian cycle could be explored as novel latency reversing agents.

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Conflicts of Interest

There are no conflicts of interest to declare

Author Contributions

FMH, LC, and SRL designed the study. LC, SGD and SRL developed the clinical protocol. JS, AS, AD, JM, LC, and MR performed experiments. JS, AR, MPD, WH, MR, and SRL analysed the data. AR and MPD developed the mathematical models. JS wrote the first manuscript draft, and JS, AR, MPD, MR, and SRL contributed to subsequent revisions. All authors approved the final manuscript.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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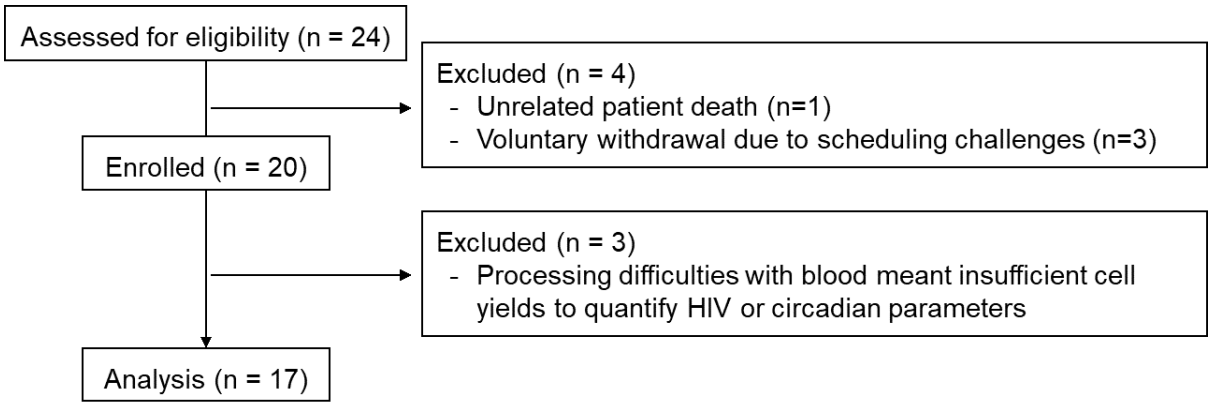
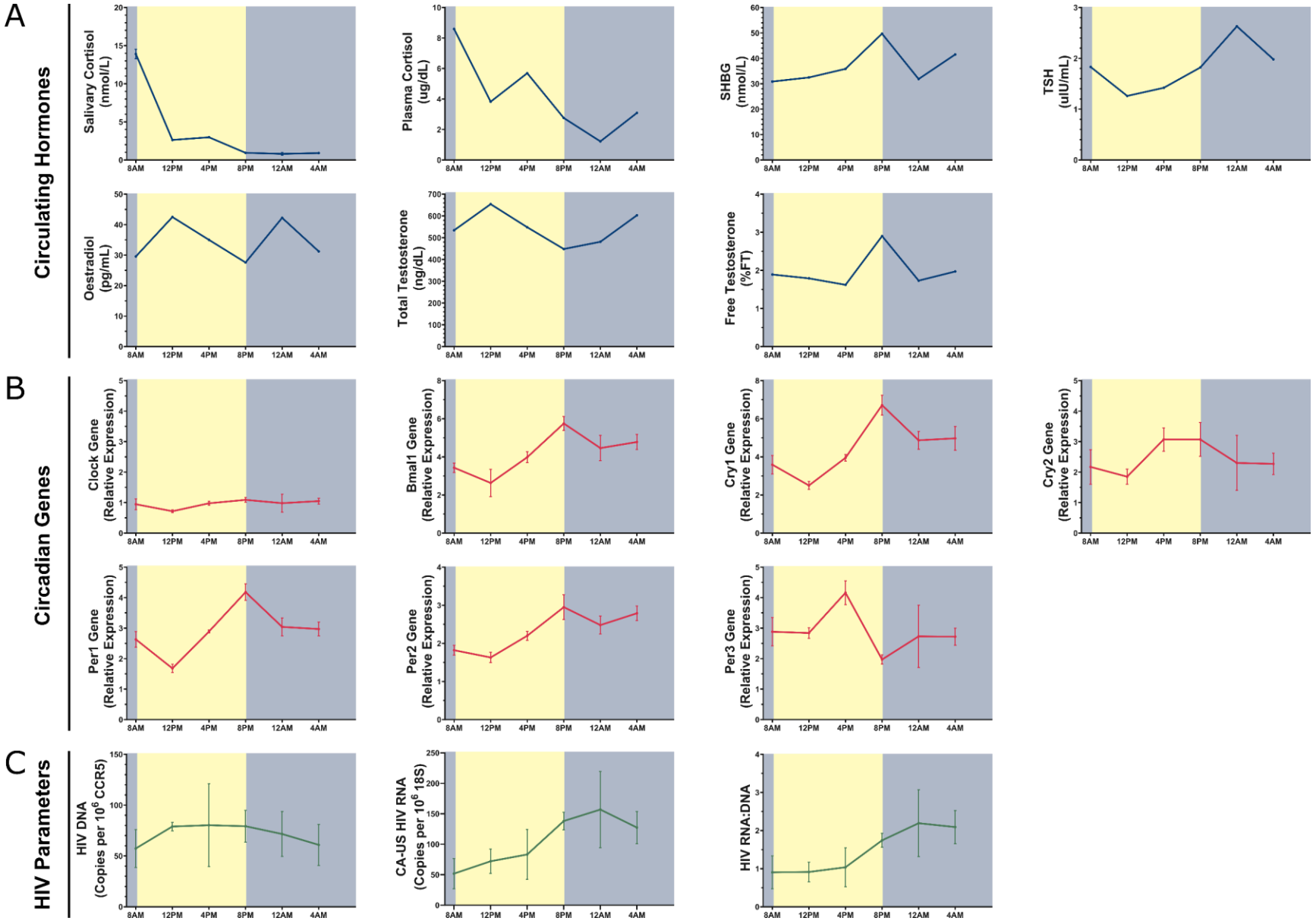
Supplementary Figures



Supplementary Figure 1: Consort Diagram summarising screening, enrolment and analysis


A
Circulating Hormones
Salivary Cortisol (nmol/L)
Plasma Cortisol (ug/dL)
SHBG (nmol/L)
TSH (uIU/mL)
Oestradiol (pg/mL)
Total Testosterone (ng/dL)
Free Testosterone (%FT)
B
Circadian Genes
Clock Gene (Relative Expression)
Bmal1 Gene (Relative Expression)
Cry1 Gene (Relative Expression)
Cry2 Gene (Relative Expression)
Per1 Gene (Relative Expression)
Per2 Gene (Relative Expression)
Per3 Gene (Relative Expression)
C
HIV Parameters
HIV DNA (Copies per 10^6 CCR5)
CA-US HIV RNA (Copies per 10^6 18S)
HIV RNA:DNA
8AM
12PM
4PM
8PM
12AM
4AM

463

Supplementary Figure 2. Representative example of the variation in circulating hormones, circadian gene expression, and HIV parameters *The levels of A) circulating hormones, B) circadian gene expression in CD4+ T-cells from the blood and C) CA-US HIV RNA, HIV DNA and the HIV RNA:DNA ratio in CD4+ T-cells from the blood. Plots represent the mean ± standard deviation of assay replicates at each timepoint. Blue and yellow shaded rectangles represent approximate day and night times*

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