Effect of HIV infection and antiretroviral therapy initiation on genome-wide DNA methylation patterns

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eBioMedicine
2023;88: 104434
Published Online xxx
https://doi.org/10.1016/j.ebiom.2022.104434

Summary

Background Previous epigenome-wide association studies have shown that HIV infection can disrupt the host DNA methylation landscape. However, it remains unclear how antiretroviral therapy (ART) affects the HIV-induced epigenetic modifications.

Methods 184 individuals with HIV from the NEAT001/ANRS143 clinical trial (with pre-ART and post-ART samples [96 weeks of follow-up]) and 44 age-and-sex matched individuals without HIV were included. We compared genome-wide DNA methylation profiles in whole blood between groups adjusting for age, sex, batch effects, and DNA methylation-based estimates of leucocyte composition.

Findings We identified 430 differentially methylated positions (DMPs) between HIV+ pre-ART individuals and HIV-uninfected controls. In participants with HIV, ART initiation modified the DNA methylation levels at 845 CpG positions and restored 49.3% of the changes found between HIV+ pre-ART and HIV-uninfected individuals. We only found 15 DMPs when comparing DNA methylation profiles between HIV+ post-ART individuals and participants without HIV. The Gene Ontology enrichment analysis of DMPs associated with untreated HIV infection revealed an enrichment in biological processes regulating the immune system and antiviral responses. In participants with untreated HIV infection, DNA methylation levels at top HIV-related DMPs were associated with CD4/CD8 ratios and viral loads. Changes in DNA methylation levels after ART initiation were weakly correlated with changes in CD4+ cell counts and the CD4/CD8 ratio.

Interpretation Control of HIV viraemia after 96 weeks of ART initiation partly restores the host DNA methylation changes that occurred before antiretroviral treatment of HIV infection.

Funding NEAT-ID Foundation and Instituto de Salud Carlos III, co-funded by European Union.

Keywords: HIV infection; Epigenetics; Genome-wide DNA methylation; Antiretroviral therapy

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Research in context

Evidence before this study

DNA methylation is one of the epigenetic modifications that regulates gene expression, and could play a critical role in disease pathogenesis. In the context of HIV infection, previous cross-sectional studies have reported differences in DNA methylation profiles between individuals with and without HIV, suggesting an epigenetic modulation of host genes during HIV disease. However, longitudinal data on the effect of antiretroviral therapy (ART) initiation on DNA methylation dynamics are limited. A recent study has found that ART minimally restores the DNA methylation changes induced during acute HIV infection in monocytes and $CD4^+$ lymphocytes. Nevertheless, the small sample size (n = 21) and the absence of other studies do not allow to clarify whether the alterations in DNA methylation persist after the control of HIV viraemia by ART.

Added value of this study

Ours is the most extensive study evaluating the changes in genome-wide DNA methylation patterns after ART initiation in participants with HIV enrolled in a clinical trial (NEAT001/ANRS143). Our results prove that HIV infection disrupts the host DNA methylation landscape, primarily in genes involved in immune and antiviral responses. We report that 96 weeks of ART restores about half of the differentially methylated positions (DMPs) found in individuals with untreated HIV infection. Additionally, we identify that the DNA methylation changes induced by HIV are more pronounced in individuals with severe immunodeficiency and higher viraemia. To our knowledge, this is the first longitudinal study showing a benefit of starting ART in the epigenetic alterations induced during HIV infection.

Implications of all the available evidence

Our findings, together with those previously reported, support that the modulation of the host DNA methylation patterns by HIV might contribute to disease progression. The partial reversal of the HIV-induced DNA methylation changes after ART initiation is an additional beneficial effect of antiretroviral treatment. Our data also highlight the possible role of epigenetic regulation in controlling HIV. Further investigation is needed to elucidate the underlying mechanisms of the HIV-related DNA methylation alterations and their possible consideration for epigenome editing strategies for HIV control.

Introduction

DNA methylation is one of the epigenetic modifications that guide the transcriptional program of genes, which typically occurs at the carbon-5 position of cytosine residues within a cytosine-guanine dinucleotide (CpG site).[1,2] Alterations in DNA methylation can be caused by normal biological processes or in response to diseases, including infections.[3,4] Thus, DNA methylation is a reliable and accessible epigenetic marker for comprehensive analyses of gene expression and disease.[5]

During HIV infection, the virus–host interaction induces changes in the DNA methylation landscape that affect both the virus and host cells. On the one hand, the hypermethylation of 5′ long terminal repeats (5′-LTR) regions of HIV proviral DNA may inhibit viral replication contributing to maintenance of latency.[6,7] On the other hand, several genome-wide DNA methylation profiling studies have shown differences in blood DNA methylation among individuals with and without HIV, mainly in host genes involved in innate and adaptive immune responses.[8–11] As DNA methylation is considered an essential transcriptional regulator of the immune system,[12] these studies suggest that HIV infection might induce an epigenetic modulation with consequences in immune responses and HIV disease progression. However, many of these studies are cross-sectional; therefore, it remains unclear whether ART can reverse the observed alterations, and if so, to what extent.

Here, we report a comprehensive study of blood DNA methylation changes associated with HIV infection and ART initiation in a two-stage design. Firstly, in a cross-sectional analysis we assessed the effect of HIV infection by comparing DNA methylation patterns between a group of persons with HIV (PWH; both pre-ART and post-ART samples) and a group of HIV-negative persons. Secondly, longitudinal DNA methylation changes following ART initiation were investigated in PWH.

Methods

Study design and participants

We included participants from the NEAT001/ANRS143 clinical trial: a randomised open-label, non-inferiority trial conducted between August 2010 and October 2013 in 78 clinical sites in 15 European countries. Briefly, this trial showed, in ART-naïve adults with HIV, non-inferiority over 96 weeks of ritonavir-boosted darunavir plus raltegravir versus ritonavir-boosted darunavir combined with tenofovir disoproxil fumarate and emtricitabine.[13] For the present study, we selected 184 participants from the NEAT001/ANRS143 blood telomere length substudy with available whole blood samples both at study entry (pre-ART) and 96 weeks after ART initiation (post-ART).[14] Additionally, we included 44 samples from adults without HIV collected

at a single time point from an anonymised sample's collection of healthy volunteers, for whom only age and sex data were available. Thus, we used frequency matching to attain an HIV-uninfected group with a similar age and sex distribution.

Ethics

This study was approved by the Ethics Committee of La Paz University Hospital (reference number PI-3014), and all participants gave written informed consent.

Genome-wide DNA methylation profiling

Genomic DNA from frozen whole blood samples was purified using the MagPurix Blood DNA Extraction Kit 200 (Zinexts Life Science, New Taipei, Taiwan) and bisulphite-converted using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA), according to the manufacturers' instructions. Following the manufacturer's instructions, we performed the genome-wide DNA methylation profiling using the Illumina's Infinium MethylationEPIC BeadChip Kit (Illumina, San Diego, CA, USA). Samples were randomly distributed across plates and chips, and the two time points per participant with HIV (pre-ART and post-ART) were assessed in the same chip. The raw image intensities of the hybridised microarrays were scanned using an Illumina iScan SQ instrument. All experiments were performed in the same core facility (Institute of Medical and Molecular Genetics, La Paz University Hospital, Madrid, Spain).

Raw DNA methylation data were pre-processed through the "*minfi*" R package.[15] Background correction and dye-bias normalization were performed using the *Noob* method implemented in *minfi*. Then, we removed the probes with low signal detection ($p > 0.01$), probes with less than three beads in at least 5% of samples, and probes within SNPs loci or located on sex chromosomes. After filtering, 835,260 probes were selected for downstream analyses. All samples satisfied the quality control criteria established in the *minfi* package.

Differentially methylation analysis

After data preprocessing, DNA methylation levels (β-values) at each probe were generated as the ratio of methylated signals to the sum of both methylated and unmethylated signals, ranging from 0 (completely unmethylated) to 1 (completely methylated). Logit transformed β-values (M-values) were used for downstream statistical analyses as previously recommended.[16] To identify differentially methylated positions (DMPs) among groups (pre-ART HIV+ against post-ART HIV+; pre-ART HIV against HIV−; and post-ART HIV+ against HIV−), we used linear models implemented in the *limma* R package, adjusting for age, sex, DNA methylation-based estimates of blood cell composition, and batch effects (barcode and sample position within the microarray). To account for blood cell heterogeneity, proportions of $CD4^+$ and $CD8^+$ T lymphocytes, B cells, natural killers, monocytes and granulocytes were estimated using the *estimateCellCounts()* function employed in the *minfi* package, based on the Houseman's reference method.[17] Benjamini-Hochberg corrected false discovery rate (FDR) was applied to adjust raw *p*-values for multiple testing. We considered as differentially methylated positions between groups those with an FDR-adjusted $p < 0.01$. Additionally, a cut-off of absolute difference in average DNA methylation greater than 5% ($\Delta\beta > |0.05|$) was applied to filter DMPs with a higher biological relevance.

Gene ontology enrichment analysis

Enrichment analysis of Gene Ontology (GO) biological processes of differentially methylated positions were performed using the *gometh* function employed in the *missMethyl* R package, which considers the number of probes per gene and those probes that are annotated to multiple genes.[18] GO terms with *p*-values <0.01 were considered statistically significant. We used the *Go-Module* web-accessible tool (version 1.3: https://lussiergroup.org/cgi-bin/GO-Module/GOModule.cgi) to reduce redundancy and simplify the interpretation of GO terms.[19]

Gene expression analysis

We randomly selected a sample of 23 participants with HIV to assess the effect of HIV infection and ART initiation on the transcriptional levels of four candidate differentially methylated genes (*PARP9*, *IFI44L*, *NLRC5* and *CD44*). Total RNA was purified using the QIAamp RNA blood Mini Kit (Qiagen, Hilden, Germany) and reversed-transcribed in five separate reactions per sample using the GoScriptTM Reverse Transcription Mix including random primers (Promega Corporation, Madison, WI, USA), following the manufactures' instructions. The resulting cDNAs were analysed by RT-qPCR using the Taqman Gene Expression Assays (Thermo Fisher Scientific, Waltham, MA, USA) for the relative quantification of *PARP9* (Hs00967084_m1), *IFI44L* (Hs00915292_m1), *NLRC5* (Hs01072148_m1) and *CD44* (Hs01075862_m1). *GAPDH* (Hs02786624_g1) was used as an endogenous gene control as previously recommended for molecular assays in the context of viral infections.[20] Reactions were performed using the QuantStudio 1 Real-Time PCR system (Thermo Fisher Scientific), and all samples were tested in duplicated. Fold change expression levels of the candidate genes were calculated, relative to pre-ART samples, as $2^{-\Delta\Delta Ct}$ ($\Delta Ct_{post\text{-}ART} - \Delta Ct_{pre\text{-}ART}$).

Statistics

Participants' characteristics were described using absolute and relative frequencies for categorical variables,

and medians and IQR for continuous variables. The analysis to identify differentially methylated positions between groups was done as described in the methods' section "*Differentially methylation analysis*". We used the Wilcoxon rank-sum test to assess differences in DNA methylation levels between participants with HIV according to their CD4/CD8 ratios and viral load levels. We established a CD4/CD8 ratio cutoff of 0.4 (median value in the whole population) to categorise the immune status of participants with HIV. Correlations between changes in DNA methylation levels and dynamics of $CD4^+$ cell counts and CD4/CD8 were performed using the Spearman's rank correlation test. The one-sample t-test was used to evaluate the fold change in gene expression. *p*-values <0.01 were considered statistically significant.

Role of the funding source

The study's funders had no role in study design; in the collection, analysis and interpretation of data; in the writing of the manuscript; and in the submission decision.

Results

Characteristics of participants

Characteristics of participants are detailed in Table 1. Persons with HIV were predominantly male (89.1%), with a median time since HIV diagnosis of 1.25 years (IQR: 0.35–2.58) at study entry (pre-ART). Of them, 51.1% started ART with a dual therapy regimen (ritonavir-boosted darunavir plus raltegravir), and 48.9% with triple therapy (ritonavir-boosted darunavir combined with tenofovir disoproxil fumarate and emtricitabine). After 96 weeks of ART initiation, 6.5% of participants (n = 12) were not virologically suppressed (HIV RNA > 50 copies/mL), of whom only 3 had viral loads over 1000 copies/mL (Supplementary Table S1). Notwithstanding, we performed the DNA methylation analyses including the whole population of individuals with HIV. Participants with and without HIV infection were well-balanced for age ($p = 0.914$) and sex ($p = 0.606$) [using the Wilcoxon rank-sum test and Chi-square test, respectively].

Characteristic	HIV+ participants (n = 184)	HIV− participants (n = 44)
Age, years[a]	38.1 (30.8–46.6)	39 (29.5–47.8)
Female sex	20 (10.9%)	7 (16%)
Ethnicity		–
Caucasian	153 (83.1%)	
Black	22 (12%)	
Asian	2 (1.1%)	
Other	7 (3.8%)	
Time since HIV diagnosis, years[a]	1.25 (0.35–2.58)	–
CD4 counts (cells/μl)		–
Pre-ART	344 (246–408)	
Post-ART	560 (432–687)	
CD8 counts (cells/μl)		–
Pre-ART	863 (637–1156)	
Post-ART	775 (526–1002)	
CD4/CD8 ratio		–
Pre-ART	0.37 (0.24–0.51)	
Post-ART	0.74 (0.55–1.06)	
Nadir CD4 (cells/μl)	310 (235–373)	–
Pre-ART HIV viral load (log copies/mL)	4.7 (4.3–5.2)	–
HIV RNA ≤ 50 copies/mL (post-ART)	172 (93.5%)	–

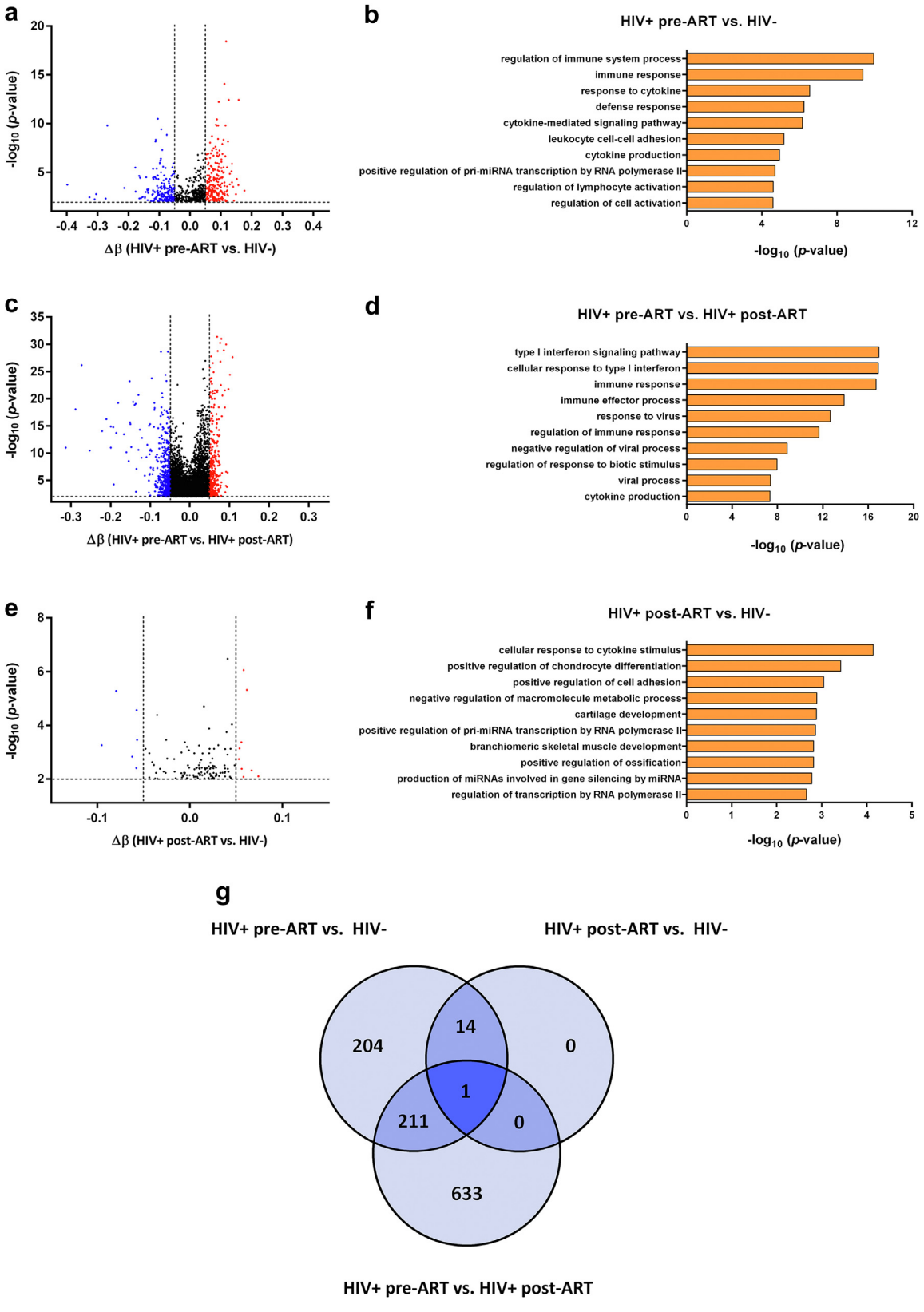
Data are presented as medians (IQRs) or n (%). [a]Pre-ART characteristics.

Table 1: **Characteristics of participants.**

ART initiation partly restores the DNA methylation disruption induced by HIV infection

To determine the effect of untreated HIV infection on genome-wide DNA methylation, we firstly compared DNA methylation patterns between HIV-uninfected participants and pre-ART samples from participants infected with HIV. In participants with untreated HIV infection we found 430 differentially methylated CpG positions (DMPs; FDR-adjusted $p < 0.01$ with an absolute mean difference in methylation greater than 5%: $\Delta\beta$-value > |0.05|), of which 231 were hypermethylated and 199 were hypomethylated (Fig. 1a and Supplementary Data 1). DMPs were distributed across the whole genome except for chromosome 21, with the highest number in chromosomes 1, 2, 3 and 6. A total of 127 (29.5%) of DMPs were annotated in promoter regions (considered as TSS1500, TSS200, 5′UTR and 1st Exon regions), and the majority (75.6%) were located outside the CpG island regions (open sea regions). The gene ontology enrichment analysis of the 430 DMPs revealed an enrichment in biological processes related to the regulation of the immune system responses (Fig. 1b and Supplementary Table S2).

Next, we compared the DNA methylation patterns between pre-ART and post-ART samples from participants with HIV to evaluate the effect of ART initiation on the DNA methylation disruption induced by HIV infection. We observed 845 DMPs between the two time points (FDR-adjusted $p < 0.01$ and a $\Delta\beta$-value > |0.05|), mainly being hypomethylated (n = 550, 66.3%) in pre-ART samples (Fig. 1c and Supplementary Data 2). In this case, DMPs were distributed across the whole genome (with the highest number in chromosomes 1, 6 and 2), 268 (31.7%) were annotated in promoter regions, and 545 (70.4%) were localised outside the CpG island regions. The gene ontology enrichment analysis of the 845 DMPs impacted by ART initiation showed an enrichment in biological processes involved in immune responses and defence responses to viral infections (Fig. 1d and Supplementary Table S3).


a
-log10 (p-value)
Δβ (HIV+ pre-ART vs. HIV-)
b
HIV+ pre-ART vs. HIV-
regulation of immune system process
immune response
response to cytokine
defense response
cytokine-mediated signaling pathway
leukocyte cell-cell adhesion
cytokine production
positive regulation of pri-miRNA transcription by RNA polymerase II
regulation of lymphocyte activation
regulation of cell activation
-log10 (p-value)
c
-log10 (p-value)
Δβ (HIV+ pre-ART vs. HIV+ post-ART)
d
HIV+ pre-ART vs. HIV+ post-ART
type I interferon signaling pathway
cellular response to type I interferon
immune response
immune effector process
response to virus
regulation of immune response
negative regulation of viral process
regulation of response to biotic stimulus
viral process
cytokine production
-log10 (p-value)
e
-log10 (p-value)
Δβ (HIV+ post-ART vs. HIV-)
f
HIV+ post-ART vs. HIV-
cellular response to cytokine stimulus
positive regulation of chondrocyte differentiation
positive regulation of cell adhesion
negative regulation of macromolecule metabolic process
cartilage development
positive regulation of pri-miRNA transcription by RNA polymerase II
branchiomeric skeletal muscle development
positive regulation of ossification
production of miRNAs involved in gene silencing by miRNA
regulation of transcription by RNA polymerase II
-log10 (p-value)
g
HIV+ pre-ART vs. HIV-
HIV+ post-ART vs. HIV-
204
14
0
1
211
0
633
HIV+ pre-ART vs. HIV+ post-ART

When comparing DNA methylation patterns between HIV-uninfected participants and post-ART samples from participants with HIV, we found only 15 DMPs (FDR-adjusted $p < 0.01$ and a Δβ-value > |0.05|): 9 hypermethylated and 6 hypomethylated in participants with HIV (Fig. 1e and Supplementary Data 3). The gene ontology enrichment analysis of these 15 CpG sites that remained differentially methylated after 2 years of ART revealed an enrichment in diverse biological processes, including the response to cytokine stimulus, metabolic and developmental processes, and the regulation of miRNAs (Fig. 1f and Supplementary Table S4). However, the biological interpretation of this functional analysis should be cautious due to the scarce number of resulting target genes.

Of the 430 DMPs found between individuals without HIV and pre-ART HIV+ participants, 212 (49.3%) overlapped with those affected by ART initiation (120 became hypomethylated and 92 hypermethylated after ART), indicating that ART partly restored the HIV-induced DNA methylation changes towards the state present at the HIV-uninfected group (Fig. 1g). Furthermore, the overlapping positions between groups might be underestimated as we applied a mean methylation difference cutoff of 5% (Δβ-value > |0.05|) to identify differentially methylated CpG sites. After removing this threshold, we found that 70.4% of differentially methylated positions associated with untreated HIV infection were significantly impacted by ART initiation. Lastly, the finding of only 15 DMPs between HIV-uninfected participants and post-ART samples from participants with HIV confirms that ART may revert most of the epigenetic changes induced by HIV infection.

Top HIV-induced DNA methylation changes occur in genes involved in immune responses and associate with the pre-ART immuno-virological status

The top DNA methylation positions (Δβ-value > |0.20|) associated with untreated HIV infection (those obtained from comparing both pre-ART-HIV+ vs. HIV− and pre-ART-HIV+ vs. post-ART-HIV+) were observed in genes involved in the regulation of antiviral and interferon-mediated immune responses: *PARP9/DTX3L* (cg00959259, cg22930808 and cg08122652), *B2M* (cg03425812, cg27537252, cg12828896 and cg05475649), *IFI44L* (cg13452062 and cg05696877), *NLRC5* (cg07839457), *VRK2* (cg09858955), and *EPSTI1* (cg12439472). All these CpG positions were hypomethylated in pre-ART HIV+ participants compared to individuals without HIV, and became significantly hypermethylated after 2 years of successful ART (Fig. 2). The rest of DMPs affected by HIV infection with a Δβ-value > |0.15| were located at the following genes: *DDX60, IFIT3, MX1, MSC, TAP1, FSB/RAD54B, LRBA, PARP11, BCL2L14, ATXN7L1, EIF2C2, ADAR, COTL1,* and *RARG* (Supplementary Data 1 and 2). Of them, only positions in *FSB/RAD54B*, *ATXN7L1*, *EIF2C2* and *RARG* were hypermethylated in ART-naïve HIV infection.

As the top DMPs associated with untreated HIV infection (Δβ-value > |0.20|) were annotated in genes with immunological functions, we further assessed the relationship between pre-ART DNA methylation levels and the immuno-virological status of ART-naïve participants determined by the CD4/CD8 ratio and viral load levels. Compared to participants with a CD4/CD8 ratio ≥0.4, those with a ratio below 0.4 had reduced levels of DNA methylation at CpG positions in the above-mentioned genes *PARP9/DTX3L* (cg00959259), *B2M* (cg03425812), *IFI44L* (cg13452062), *NLRC5* (cg07839457), *VRK2* (cg09858955) and *EPSTI1* (cg12439472) (Fig. 3). Furthermore, these positions were also significantly hypomethylated in participants with the highest pre-ART viral loads (HIV RNA ≥ 100,000 copies/mL) (Fig. 3). The association between these immuno-virological markers and pre-ART DNA methylation levels was also observed for the majority of the HIV-related DMPs with a Δβ-value > |0.15| (Supplementary Table S5), supporting that the epigenetic modulation of several genes might have an important role in HIV disease progression.

Longitudinal changes in DNA methylation levels after ART are weakly associated with the recovery of $CD4^+$ cell counts and CD4/CD8 ratio

To determine whether the longitudinal changes in DNA methylation following ART were associated with immune reconstitution, we correlated the dynamics of $CD4^+$ cell counts and CD4/CD8 ratio with the DNA methylation changes (Δβ: post-ART minus pre-ART) of the top DMPs affected by ART (mean Δβ-value > |0.15|).

Fig. 1: **Effect of HIV infection and ART initiation on genome-wide DNA methylation patterns.** Left panels show volcano plots of the differentially methylated positions (DMPs) resulting from comparing HIV+ pre-ART vs. HIV− (a), HIV+ pre-ART vs. HIV+ post-ART (c), and HIV+ post-ART vs. HIV− (e). The y-axis indicates transformed *p*-values ($-\log_{10}$ *p*-values) and the x-axis indicates the difference in DNA methylation between groups (Δβ). The horizontal dashed line shows the significance level (FDR-adjusted *p*-value <0.01), whereas vertical dashed lines show the cut-off of absolute difference in average DNA methylation greater than 5% (Δβ > |0.05|). Blue and red dots represent differentially methylated CpG sites (FDR-adjusted $p < 0.01$ and Δβ > |0.05|). Right panels show histogram plots of the top 10 most significant biological processes from the Gene Ontology enrichment analyses of the DMPs resulting from comparing HIV+ pre-ART vs-. HIV− (b), HIV+ pre-ART vs. HIV+ post-ART (d), and HIV+ post-ART vs. HIV− (f). Panel g is a Venn diagram representing the overlap of the DMPs identified between groups.

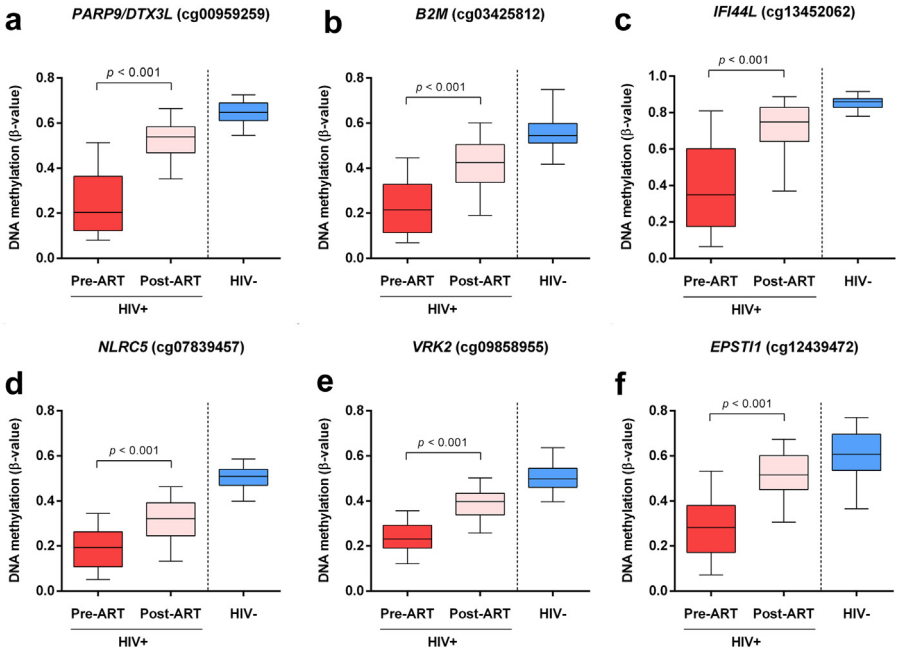


Fig. 2: **DNA methylation dynamics of top differentially methylated positions related to untreated HIV infection.** DNA methylation levels (β-values) at top differentially methylated positions associated with untreated HIV infection (Δβ-value > |0.20|), both in HIV-infected (pre-ART and post-ART samples) and HIV-uninfected participants: (a) *PARP9/DTX3L* (cg00959259); (b) *B2M* (cg03425812); (c) *IFI44L* (cg13452062); (d) *NLRC5* (cg07839457); (e) *VRK2* (cg09858955); (f) *EPSTI1* (cg12439472). Significance determined comparing HIV+ pre-ART and HIV+ post-ART samples and using an FDR-adjusted *p*-value <0.01. Boxes show medians and IQRs; whiskers correspond to 5th and 95th percentiles.

We found that pre-ART levels of $CD4^+$ cell counts and CD4/CD8 ratio were negatively correlated with the changes in DNA methylation levels of the selected DMPs (all became hypermethylated after starting ART), indicating that participants with more severe immunodeficiency at baseline experienced more extensive changes in DNA methylation (Table 2). However, longitudinal variations in DNA methylation were weakly associated with the recovery of $CD4^+$ cell counts and CD4/CD8 ratio. Thus, DNA methylation changes at the majority of the analysed CpG positions had a weak correlation with $CD4^+$ changes (Spearman's ρ values < 0.35), whereas the methylation variation of only 4 positions correlated weakly with the longitudinal changes in CD4/CD8 ratio (Table 2). We also found no association between the time since HIV diagnosis and the longitudinal changes in DNA methylation after ART.

Effect of ART on mRNA levels of candidate differentially methylated genes associated with HIV infection

To investigate whether the epigenetic disruption associated with HIV infection implies changes in gene transcription, we determined the mRNA levels of four candidate differentially methylated genes (whose CpG positions mapped to promoter regions) in a subpopulation of 23 randomly-selected participants with HIV (pre-ART and post-ART samples). We selected two genes (*PARP9* and *IFI44L*) that were hypomethylated in pre-ART HIV+ participants and became significantly hypermethylated after ART initiation reaching normalization; one gene (*NLRC5*) that remained differentially hypomethylated after ART despite increasing methylation levels following virological suppression; and one gene (*CD44*) that was significantly hypomethylated in ART-naïve participants but was not affected by ART. The DNA methylation levels of these genes in the selected 23 participants were similar to those observed for the whole population (Supplementary Fig. S1). For *PARP9* and *IFI44L*, we observed a significant down-regulation of gene expression after 96 weeks of ART initiation (Fig. 4), indicating that the hypomethylation of these genes during untreated HIV infection might lead to an increase in transcription. However, there were no significant differences in transcription levels of *NLRC5* despite being significantly hypermethylated after ART. Lastly, we did not observe changes in *CD44* mRNA levels between pre-ART and post-ART samples (Fig. 4), similarly to DNA methylation levels. When we explored the association between the fold change expression of these genes and their DNA methylation change after ART (Δβ) we found weak-moderate negative correlations for *NLRC5* (cg07839457: $\rho = -0.42$, $p = 0.048$) and *IFI44L* (cg13452062: $\rho = -0.40$, $p = 0.061$;

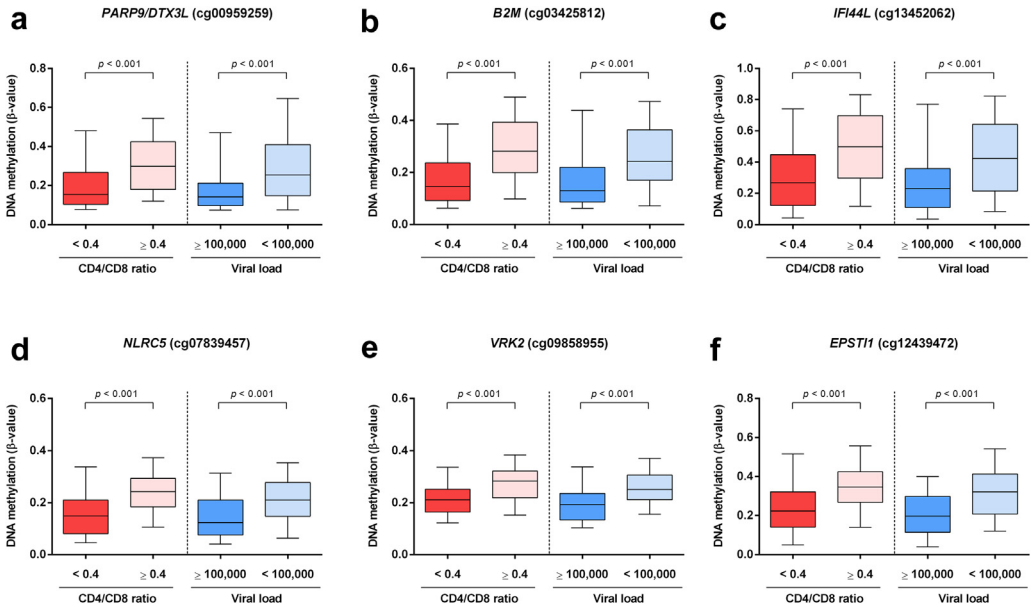


***Fig.* 3: Associations of DNA methylation levels at top differentially methylated positions related to untreated HIV infection with pre-ART CD4/CD8 ratio and viral load.** DNA methylation levels (β-values) at top differentially methylated positions associated with untreated HIV infection (Δβ-value > |0.20|) in HIV+ pre-ART individuals according to their CD4/CD8 ratio (<0.4 [n = 106] or ≥ 0.4 [n = 78]) and viral loads (≥100,000 copies/mL [n = 59] or < 100,000 copies/mL [n = 125]): (a) *PARP9/DTX3L* (cg00959259); (b) *B2M* (cg03425812); (c) *IFI44L* (cg13452062); (d) *NLRC5* (cg07839457); (e) *VRK2* (cg09858955); (f) *EPSTI1* (cg12439472). Differences between groups were assessed by the Wilcoxon Rank Sum test. Boxes show medians and IQRs; whiskers correspond to 5th and 95th percentiles.

cg05696877: ρ = –0.51, $p = 0.012$), but no correlations for *PARP9* (cg00959259: ρ = 0.05, $p = 0.81$; cg22930808: ρ = –0.01, $p = 0.99$; cg08122652: ρ = –0.02, $p = 0.92$) and *CD44* (cg20971158: ρ = –0.15, $p = 0.48$) [using the Spearman's rank correlation test].

Discussion

This study assessed how HIV infection and ART initiation impact the host DNA methylation landscape. Our data showed that untreated HIV infection causes profound alterations in the host DNA methylation profiles, especially in individuals with a more severe immunodeficiency, and being the most relevant in genes coding for proteins involved in immune responses. Our findings also reveal that the HIV-induced DNA methylation disruption is partly restored after two years of successful ART and virological suppression.

Here, differentially methylated CpG positions associated with untreated HIV infection were enriched in biological processes related to innate and adaptive immune responses. In particular, most of the top differentially methylated positions mapped to interferon-stimulated genes (ISGs), such as *PARP9/DTX3L*, *IFI44L*, *DDX60*, *IFIT3* and *MX1*, which have been shown to participate in interferon-mediated antiviral defences.[21–25] These genes were hypomethylated in pre-ART samples, suggesting their transcriptional up-regulation during untreated HIV infection. Supporting this hypothesis, we observed higher mRNA levels of *PARP9* and *IFI44L* genes in a subpopulation of 23 individuals before starting ART. The hypomethylation of the ISGs mentioned above has been previously reported in other recent HIV epigenome-wide association studies.[26–28]

Among the most prominent differentially methylated positions we found two CpGs in the genes *VRK2* and *EPSTI1*. Both genes were hypomethylated in pre-ART HIV+ individuals, and their roles in the context of HIV infection are still unknown. *VRK2* encodes for a serine/threonine kinase with several biological functions, and recently it has been identified playing a critical role in mitochondrial DNA-triggered innate immune responses upon viral infections.[29] On the other hand, *EPSTI1* is an interferon-stimulated gene whose expression can promote an antiviral effect during HCV infection,[30] and influences the macrophage polarization contributing to inflammation.[31] Interestingly, a recent study has reported that *EPSTI1* remains differentially methylated in individuals with SARS-CoV-2 infection,[32] supporting that its epigenetic regulation might influence the progression of viral diseases.

The activation of interferon-mediated pathways in the early stages of HIV infection leads to the expression of restriction factors that limit virus replication.[33] However, exacerbated interferon responses during

Change in DNA methylation (Δβ)		Pre-ART CD4⁺ cell counts		Change in CD4⁺ cell counts[a]		Pre-ART CD4/CD8		Change in CD4/CD8[a]	
CpG position[b]	Gene	ρ	p	ρ	p	ρ	p	ρ	p
cg13452062	*IFI44L*	−0.33	<0.001	0.27	<0.001	−0.17	0.024	0.10	0.194
cg22930808	*PARP9/DTX3L*	−0.50	<0.001	0.30	<0.001	−0.41	<0.001	0.06	0.463
cg00959259	*PARP9/DTX3L*	−0.43	<0.001	0.27	<0.001	−0.36	<0.001	0.093	0.228
cg05696877	*IFI44L*	−0.30	<0.001	0.28	<0.001	−0.21	0.007	0.04	0.628
cg12439472	*EPSTI1*	−0.47	<0.001	0.30	<0.001	−0.37	<0.001	0.09	0.254
cg08122652	*PARP9/DTX3L*	−0.52	<0.001	0.28	<0.001	−0.48	<0.001	0.04	0.565
cg07815522	*PARP9/DTX3L*	−0.49	<0.001	0.27	<0.001	−0.49	<0.001	0.09	0.226
cg24678928	*DDX60*	−0.54	<0.001	0.33	<0.001	−0.48	<0.001	−0.02	0.771
cg06188083	*IFIT3*	−0.51	<0.001	0.21	0.005	−0.51	<0.001	0.04	0.580
cg21549285	*MX1*	−0.45	<0.001	0.30	<0.001	−0.40	<0.001	0.06	0.469
cg03425812	*B2M*	−0.27	<0.001	0.08	0.276	−0.30	<0.001	0.17	0.024
cg22012079	*IFI44L*	−0.38	<0.001	0.23	0.002	−0.27	<0.001	−0.03	0.678
cg12828896	*B2M*	−0.25	<0.001	0.06	0.420	−0.28	<0.001	0.11	0.143
cg14943355	*PARP11*	−0.44	<0.001	0.21	0.005	−0.46	<0.001	0.08	0.276
cg04268125	*ADAR*	−0.43	<0.001	0.20	0.007	−0.51	<0.001	0.09	0.026
cg09858955	*VRK2*	−0.42	<0.001	0.31	<0.001	−0.28	<0.001	0.27	<0.001
cg13155430	*MX1*	−0.53	<0.001	0.27	<0.001	−0.48	<0.001	−0.01	0.911
cg27537252	*B2M*	−0.31	<0.001	0.19	0.012	−0.24	0.002	0.28	<0.001

ρ: indicative of the Spearman's rank correlation test. [a]Changes in CD4 counts and CD4/CD8 ratios: Post-ART minus Pre-ART. [b]Top differentially methylated positions affected by ART: those with a Δβ > |0.15| (post-ART minus pre-ART).

Table 2: **Correlations between longitudinal changes in DNA methylation levels at top differentially methylated positions affected by ART and CD4 and CD4/CD8 dynamics.**

chronic infection may also contribute to disease progression by increasing CD4⁺ T lymphocytes depletion and immune exhaustion.[34] In a study that compared DNA methylation profiles between ART-naïve individuals with high or low viral loads (HIV RNA >50,000 copies/mL or <10,000 copies/mL), a cluster of hypomethylated genes was found in participants with higher viraemias that included ISGs like *PARP9/DTX3L*, *IFI44L*, *DDX60*, *IFIT3* and *MX1*.[26] Here, we also observed that DNA methylation levels at ISGs and other top differentially methylated genes related to HIV infection were associated with pre-ART CD4/CD8 ratio and viral loads. Furthermore, differences in the host epigenetic landscape exist between elite controllers and viraemic individuals,[35,36] and a recent study have observed that DNA methylation patterns are predictive for viral rebound after a kick-and-kill therapeutic intervention.[37] Additionally, DNA methylation levels of some ISGs such as *PARP9*, *IFI44L* and *EPSTI1* have been identified to be associated with mortality risk in participants of the Veterans Aging Cohort Study (VACS).[38] All these data suggest that the epigenetic modulation of ISGs and other host genes may play an essential role in HIV pathogenesis. However, further investigation is needed to determine the underlying mechanisms of these changes and clarify whether they contribute to or are a consequence of disease progression.

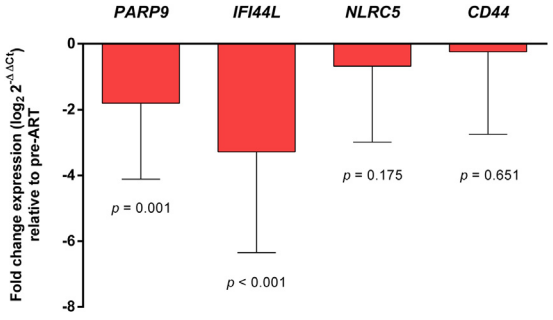


Fig. 4: **Effect of ART initiation on gene expression of four candidate genes differentially methylated during HIV infection.** Transcriptional levels of four candidate genes differentially methylated during HIV infection (*PARP9*, *IFI44L*, *NLRC5* and *CD44*) in a subpopulation of 23 randomly-selected participants with HIV. Gene expression is represented as the log_2 fold change of post-ART samples relative to pre-ART samples ($2^{-\Delta\Delta Ct}$). Data are presented as mean with standard deviation. Statistical analysis was performed by one sample t-test.

To our knowledge, ours is the largest longitudinal study assessing the impact of ART initiation on genome-wide DNA methylation profiles. Approximately half of the differentially methylated CpG positions found in untreated HIV infection were significantly impacted two years after ART initiation, reaching 70.4% if we do not establish a threshold for DNA methylation difference. The majority of the CpG sites affected by ART are involved in immune and antiviral functions, suggesting that the control of HIV replication by ART may induce a modulation of the DNA methylation landscape to regulate the immune responses against HIV infection. Furthermore,

longitudinal changes in DNA methylation after treatment were weakly associated with variations in $CD4^+$ cell counts and CD4/CD8 ratio, supporting that the control of HIV viraemia may be the main trigger for the epigenome stabilization. Nevertheless, it cannot be ruled out that other not evaluated variables such as inflammatory mediators or the HIV reservoir size might play an important role in the DNA methylation dynamics during HIV infection.

This beneficial effect of ART on DNA methylation dynamics differ from the results by Corley et al. study, that found, in a population of 22 individuals with acute HIV infection, that initiating ART restored less than 1% of the HIV-induced DNA methylation changes in monocytes and had no effect upon DNA methylation alterations in $CD4^+$ T lymphocytes.[39] However, this discordancy in the impact of ART on DNA methylation dynamics might be consequence of the difference in the duration of ART between both studies (approximately 8 months against 96 weeks in the present study), and in differences in the two populations (primary infection versus chronic infection in the present study). Thus, this could indicate that the trend towards normalizing DNA methylation changes induced by HIV does not occur following the immediate initiation of ART, although more longitudinal data are needed to confirm this hypothesis.

When we compared DNA methylation profiles between participants with HIV after ART and HIV-uninfected participants we only found 15 differentially methylated CpG sites, supporting that viral replication control by ART has a significant impact on the host epigenome. Among the positions that remained differentially methylated, one located within the promoter region of *NLRC5* stands out. The hypomethylation of this gene was strongly associated with untreated HIV infection, and we did not observe significant changes in its transcriptional level between pre-ART and post-ART samples despite being significantly hypermethylated after ART initiation. Similarly, the hypomethylation in *NLRC5* promoter has been previously reported in adults and children with HIV on ART.[9,40] *NLRC5* encodes for a transcription factor that promotes the activation of genes related to the MHC class I pathway,[41] and also negatively regulates the NF-kb activation and type I interferon signalling, playing a regulatory role in inflammatory and innate immune responses.[42] The consistent identification of *NLRC5* in epigenome-wide studies suggests that this gene's epigenetic modulation might be relevant in HIV disease, but further research is needed to elucidate its impact on the clinical course of the well-controlled HIV infection.

Some limitations in the present study should be noted. Firstly, the sample size of the HIV-uninfected group was considerably smaller. Thus, we might be underestimating the number of differentially methylated positions between HIV-infected and HIV-uninfected individuals due to the lower statistical power for these comparisons. This would also explain why a large number of the DMPs affected by ART were not detected between pre-ART HIV+ and HIV– participants. Additionally, as chronological age and sex were the only available matching variables, comparisons between participants with and without HIV could not be controlled for potential confounders such as socioeconomic status and lifestyle factors (body mass index and tobacco consumption), which have been previously shown to be associated with changes in genome DNA methylation patterns.[43–45] Secondly, the cell type heterogeneity in whole blood samples might affect the DNA methylation variability in epigenome-wide association studies.[46] Since blood composition significantly differs between HIV-infected and HIV-uninfected individuals, we attempted to reduce this potential bias by adjusting models for the estimated proportions of the main leucocyte subpopulations calculated based on the Houseman's method.[17] However, this approach might be suppressing DNA methylation signatures related to HIV that may also be associated with changes in cell type proportions. Finally, in this study we could not assess the functional roles of the DNA methylation signatures associated with HIV infection.

In summary, our study provides clear evidence that, in HIV-infected patients virologically suppressed after 96 weeks of ART, the host DNA methylation disruption induced by chronic HIV infection is partly restored. These findings highlight the role of epigenetic regulation as a potential mechanism to modulate HIV progression and control.

Contributors

B.R. and J.R.A. designed and oversaw the study. A.E.C., J.R.C., P.B., G.S.M., J.N., B.M.G. and L.G.G. did the sample processing and the DNA methylation assays. J.C.S., F.S.C. and M.J.G. conducted the bioinformatics analysis of DNA methylation data. R.M., J.I.B., R.M., J.C., C.M., P.M.M., C.W. and F.R. acquired clinical data. A.E.C., J.R.C., B.R. and J.R.A. reviewed the data analyses and interpreted the results. A.E.C., B.R., J.R.C. and J.R.A. drafted the first version of the manuscript. A.E.C., J.R.C., B.R. and J.R.A. verified the underlying data. All authors revised and amended the draft report, and approved the final version of the manuscript.

Data sharing statement

DNA methylation data were uploaded in the GEO database under accession number GSE217633 and will be available with manuscript publication. Additional data will be made available upon reasonable request to the corresponding authors.

Declaration of interests

A.E.C. reports grants from Instituto de Salud Carlos III, during the conduct of the study. J.R.C. reports grants from Instituto de Salud Carlos III, during the conduct of the study. J.I.B. has received honoraries from Gilead, ViiV healthcare, Janssen and MSD for lectures, presentations and educational activities. C.W. reports grants from the European Commission and Inserm-ANRS, non-financial support from Gilead, and grants and non-financial support from Janssen and Merck, during the conduct of the study. F.R. received research funding or

honoraria from or consulted for Astra Zeneca, Gilead Sciences, MSD, Roche, ViiV Healthcare. B.R. declares personal fees from Gilead and non-financial support from ViiV Healthcare, outside the submitted work. J.R.A. reports advisory fees from ViiV, GSK, Janssen, Gilead, MSD and Aelix; speaker fees from ViiV, MSD and Janssen. Grants from ViiV and Gilead. All other authors declare no competing interests.

Acknowledgements

NEAT is a project funded through the Instituto Superiore di Sanita–Rome by the EU under the Sixth Framework Programme (project number: LSHPCT-2006-37570). The authors acknowledge the financial support for this study from the NEAT-ID Foundation (through a grant from Gilead Sciences) and Instituto de Salud Carlos III (ISCIII), co-funded by European Union (Grants numbers: FI17/00194, CM19/00059, CM21/00030 and PI18/00569). The NEAT001/ANRS143 trial was also supported by Gilead Sciences, Janssen Pharmaceuticals, and Merck Laboratories, and The French National Institute for Health and Medical Research–France Recherche Nord & Sud Sida-HIV Hepatites (Inserm-ANRS) is the sponsor and a founder of the trial. We are grateful to all study participants, their partners, families, caregivers, and local investigators and study coordinators from all the centres participating in the NEAT001/ANRS143 trial.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2022.104434.

References

1 Singal R, Ginder GD. DNA methylation. *Blood*. 1999;93:4059–4070.
2 Smith ZD, Meissner A. DNA methylation: roles in mammalian development. *Nat Rev Genet*. 2013;14:204–220.
3 Bergman Y, Cedar H. DNA methylation dynamics in health and disease. *Nat Struct Mol Biol*. 2013;20:274–281.
4 Bannister S, Messina NL, Novakovic B, Curtis N. The emerging role of epigenetics in the immune response to vaccination and infection: a systematic review. *Epigenetics*. 2020;15:555–593.
5 Berdasco M, Esteller M. Clinical epigenetics: seizing opportunities for translation. *Nat Rev Genet*. 2019;20:109–127.
6 Kauder SE, Bosque A, Lindqvist A, Planelles V, Verdin E. Epigenetic regulation of HIV-1 latency by cytosine methylation. *PLoS Pathog*. 2009;5:e1000495.
7 Palacios JA, Pérez-Piñar T, Toro C, et al. Long-term nonprogressor and elite controller patients who control viremia have a higher percentage of methylation in their HIV-1 proviral promoters than aviremic patients receiving highly active antiretroviral therapy. *J Virol*. 2012;86:13081–13084.
8 Zhang Y, Li SK, Tsui SKW. Genome-wide analysis of DNA methylation associated with HIV infection based on a pair of monozygotic twins. *Genom Data*. 2015;6:12–15.
9 Zhang X, Justice AC, Hu Y, et al. Epigenome-wide differential DNA methylation between HIV-infected and uninfected individuals. *Epigenetics*. 2016;11:750–760.
10 Gross AM, Jaeger PA, Kreisberg JF, et al. Methylome-wide analysis of chronic HIV infection reveals five-year increase in biological age and epigenetic targeting of HLA. *Mol Cell*. 2016;62:157–168.
11 Nelson KN, Hui Q, Rimland D, et al. Identification of HIV infection-related DNA methylation sites and advanced epigenetic aging in HIV-positive, treatment-naive U.S. veterans. *AIDS*. 2017;31:571–575.
12 Morales-Nebreda L, McLafferty FS, Singer BD. DNA methylation as a transcriptional regulator of the immune system. *Transl Res*. 2019;204:1–18.
13 Raffi F, Babiker AG, Richert L, et al. Ritonavir-boosted darunavir combined with raltegravir or tenofovir-emtricitabine in antiretroviral-naive adults infected with HIV-1: 96 week results from the NEAT001/ANRS143 randomised non-inferiority trial. *Lancet*. 2014;384:1942–1951.
14 Stella-Ascariz N, Montejano R, Rodriguez-Centeno J, et al. Blood telomere length changes after ritonavir-boosted darunavir combined with raltegravir or tenofovir-emtricitabine in antiretroviral-naive adults infected with HIV-1. *J Infect Dis*. 2018;218:1523–1530.
15 Aryee MJ, Jaffe AE, Corrada-Bravo H, et al. Minfi: a flexible and comprehensive Bioconductor package for the analysis of Infinium DNA methylation microarrays. *Bioinformatics*. 2014;30:1363–1369.
16 Du P, Zhang X, Huang CC, et al. Comparison of Beta-value and M-value methods for quantifying methylation levels by microarray analysis. *BMC Bioinformatics*. 2010;11:587.
17 Houseman EA, Accomando WP, Koestler DC, et al. DNA methylation arrays as surrogate measures of cell mixture distribution. *BMC Bioinformatics*. 2012;13:86.
18 Phipson B, Maksimovic J, Oshlack A. missMethyl: an R package for analyzing data from Illumina's HumanMethylation450 platform. *Bioinformatics*. 2016;32:286–288.
19 Yang X, Li J, Lee Y, Lussier YA. GO-Module: functional synthesis and improved interpretation of gene ontology patterns. *Bioinformatics*. 2011;27:1444–1446.
20 Watson S, Mercier S, Bye C, Wilkinson J, Cunningham AL, Harman AN. Determination of suitable housekeeping genes for normalisation of quantitative real time PCR analysis of cells infected with human immunodeficiency virus and herpes viruses. *Virol J*. 2007;4:130.
21 Zhang Y, Mao D, Roswit WT, et al. PARP9-DTX3L ubiquitin ligase targets host histone H2BJ and viral 3C protease to enhance interferon signaling and control viral infection. *Nat Immunol*. 2015;16:1215–1227.
22 DeDiego ML, Martinez-Sobrido L, Topham DJ. Novel functions of IFI44L as a feedback regulator of host antiviral responses. *J Virol*. 2019;93:e01159-19.
23 Miyashita M, Oshiumi H, Matsumoto M, Seya T. DDX60, a DEXD/H box helicase, is a novel antiviral factor promoting RIG-I-like receptor-mediated signaling. *Mol Cell Biol*. 2011;31:3802–3819.
24 Diamond MS, Farzan M. The broad-spectrum antiviral functions of IFIT and IFITM proteins. *Nat Rev Immunol*. 2013;13:46–57.
25 Haller O, Staeheli P, Schwemmle M, Kochs G. Mx GTPases: dynamin-like antiviral machines of innate immunity. *Trends Microbiol*. 2015;23:154–163.
26 Oriol-Tordera B, Berdasco M, Llano A, et al. Methylation regulation of antiviral host factors, interferon stimulated genes (ISGs) and T-cell responses associated with natural HIV control. *PLoS Pathog*. 2020;16:e1008678.
27 Shu C, Jaffe AE, Sabunciyan S, et al. Epigenome-wide association scan identifies methylation sites associated with HIV infection. *Epigenomics*. 2020;12:1917–1927.
28 Yang CX, Schon E, Obeidat M, et al. Occurrence of accelerated epigenetic aging and methylation disruptions in human immunodeficiency virus infection before antiretroviral therapy. *J Infect Dis*. 2021;223:1681–1689.
29 He WR, Cao LB, Yang YL, Hua D, Hu MM, Shu HB. VRK2 is involved in the innate antiviral response by promoting mitostress-induced mtDNA release. *Cell Mol Immunol*. 2021;18:1186–1196.
30 Meng X, Yang D, Yu R, Zhu H. EPSTI1 is involved in IL-28a-mediated inhibition of HCV infection. *Mediators Inflamm*. 2015;2015:716315.
31 Kim YH, Lee JR, Hahn MJ. Regulation of inflammatory gene expression in macrophages by epithelial-stromal interaction 1 (Epsti1). *Biochem Biophys Res Commun*. 2018;496:778–783.
32 Li Z, Mei Z, Ding S, et al. Identifying methylation signatures and rules for COVID-19 with machine learning methods. *Front Mol Biosci*. 2022;9:908080.
33 Soper A, Kimura I, Nagaoka S, et al. Type I interferon responses by HIV-1 infection: association with disease progression and control. *Front Immunol*. 2017;8:1823.
34 Cheng L, Ma J, Li J, et al. Blocking type I interferon signaling enhances T cell recovery and reduces HIV-1 reservoirs. *J Clin Invest*. 2017;127:269–279.
35 Moron-Lopez S, Urrea V, Dalmau J, et al. The genome-wide methylation profile of CD4+ T cells from individuals with human immunodeficiency virus (HIV) identifies distinct patterns associated with disease progression. *Clin Infect Dis*. 2021;72:e256–e264.
36 Mantovani N, Defelicibus A, da Silva IT, et al. Latency-associated DNA methylation patterns among HIV-1 infected individuals with distinct disease progression courses or antiretroviral virologic response. *Sci Rep*. 2021;11:22993.
37 Oriol-Tordera B, Esteve-Codina A, Berdasco M, et al. Epigenetic landscape in the kick-and-kill therapeutic vaccine BCN02 clinical

trial is associated with antiretroviral treatment interruption (ATI) outcome. *eBioMedicine*. 2022;78:103956.
38 Shu C, Justice AC, Zhang X, et al. DNA methylation biomarker selected by an ensemble machine learning approach predicts mortality risk in an HIV-positive veteran population. *Epigenetics*. 2021;16:741–753.
39 Corley MJ, Sacdalan C, Pang APS, et al. Abrupt and altered cell-type specific DNA methylation profiles in blood during acute HIV infection persists despite prompt initiation of ART. *PLoS Pathog*. 2021;17:e1009785.
40 Shiau S, Strehlau R, Wang S, et al. Distinct epigenetic profiles in children with perinatally-acquired HIV on antiretroviral therapy. *Sci Rep*. 2019;9:10495.
41 Kobayashi KS, van den Elsen PJ. NLRC5: a key regulator of MHC class I-dependent immune responses. *Nat Rev Immunol*. 2012;12:813–820.
42 Cui J, Zhu L, Xia X, et al. NLRC5 negatively regulates the NF-kappaB and type I interferon signaling pathways. *Cell*. 2010;141:483–496.
43 Ambatipudi S, Cuenin C, Hernandez-Vargas H, et al. Tobacco smoking-associated genome-wide DNA methylation changes in the EPIC study. *Epigenomics*. 2016;8:599–618.
44 Sayols-Baixeras S, Subirana I, Fernández-Sanlés A, et al. DNA methylation and obesity traits: an epigenome-wide association study. The REGICOR study. *Epigenetics*. 2017;12:909–916.
45 McDade TW, Ryan CP, Jones MJ, et al. Genome-wide analysis of DNA methylation in relation to socioeconomic status during development and early adulthood. *Am J Phys Anthropol*. 2019;169:3–11.
46 Jaffe AE, Irizarry RA. Accounting for cellular heterogeneity is critical in epigenome-wide association studies. *Genome Biol*. 2014;15:R31.