ORIGINAL PAPER

Impact of clinical status at the time of HIV diagnosis on intensity of epigenetic ageing in HIV-infected men treated with integrase inhibitors

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Abstract

Introduction: We have recently published a study indicating that, given a wide range of confounding factors, human immunodeficiency virus (HIV) infection remains an independent factor accelerating epigenetic ageing. In this research, a detailed analysis of medical history of ten (n = 10) participants from our previous study was conducted, for whom extreme results of global DNA methylation, a key marker of epigenetic ageing, were obtained.

Material and methods: The current study included five (n = 5) patients with the highest levels of global DNA methylation, and five (n = 5) with the lowest levels of global DNA methylation. The current analysis focused particularly on clinical status at the time of HIV diagnosis and at the start of combined antiretroviral therapy.

Results: In the analysis of continuous variables, no significant differences between groups were reported, even without multi-test corrections. This was attributable to small group sizes. The strongest (most negative) association was observed between global DNA methylation and years since HIV diagnosis (ρ = –0.400, permutation p = 0.249), followed by HIV RNA at diagnosis (ρ = –0.309, p = 0.386).

Conclusions: No common, distinct feature distinguishing HIV-infected individuals with low versus high global DNA methylation levels was identified. However, the results suggest a negative correlation between the global DNA methylation level and the time since HIV diagnosis as well as viral load at diagnosis.

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Introduction

Since the introduction of combined antiretroviral therapy (cART) in the 1990s, human immunodeficiency virus (HIV) infection has ceased to be a life-threatening disease leading inevitably to the development of acquired immunodeficiency syndrome (AIDS). Properly treated HIV infection is now considered a chronic disease, and life expectancy of people living with HIV (PLWH) has become similar to that of the general population [1, 2]. At the same time, a number of observational studies have suggested that PLWH demonstrate a shorter overall and disease-free survival compared with uninfected individuals [3, 4]. PLWH are also at higher risk of cardiovascular, respiratory, liver, and kidney diseases. However, some of these observations may be explained by accelerated epigenetic ageing [4].

A decrease in global DNA methylation is a well-studied marker of epigenetic ageing [5, 6]. A number of epigenetic clocks, which are algorithms used to estimate biological age based on site-specific DNA methylation, have also been developed [7]. The results of a recently published systematic review of studies investigating the impact of HIV infection on the intensity of epigenetic ageing [8] indicated an acceleration of epigenetic ageing in PLWH compared with uninfected individuals. However, it remains an open question whether successfully treated HIV infection causes these epigenetic changes, or whether they are related to the progression of immune system dysfunction prior to achieving treatment efficacy, or the presence of factors beyond the infection itself may accelerate epigenetic ageing in PLWH [8].

Recently, we have published the results of a case-control study examining the impact of HIV infection on the intensity of epigenetic ageing [9], considering a wide range of potential confounding factors. This study included 48 men living with HIV who were effectively treated with integrase inhibitor (INSTI)-based cART and 50 healthy controls. The results of analyses using machine learning techniques indicated that with confounding factors considered, HIV infection remains an independent feature accelerating epigenetic ageing. However, it was not possible to include participants' complete medical records and their immune status at both the time of HIV diagnosis and the initiation of cAR due to incomplete data of those diagnosed years back [9]. However, this was achieved in the current study with a detailed analysis of the medical history of ten (n = 10) study participants (viral load and immunological parameters at the time of both HIV diagnosis and onset of cART), for whom extreme results of global DNA methylation, a key marker of epigenetic ageing, were obtained. We wanted to determine whether any significant differences could be identified between participants with the most and the least intense epigenetic ageing that could explain the differences in biological age.

Material and methods

For the currents research, participants were selected from our previous study [9], and consisted of 48 cisgender men living with HIV and receiving cART in Wrocław, Poland. At the time of enrolment, all subjects were aged 24-40 years, and had been treated with INSTI-based cART for at least two years. Only individuals who were successfully treated (who at the time of inclusion had an HIV viral load < 40 copies per milliliter and a CD4+ lymphocyte count of at least 330 cells per microliter) were included in the study. Exclusion criteria were active neoplastic disease, clinically significant cardiovascular disorders, congenital metabolic conditions, active autoimmune diseases, diabetes, active infections other than HIV (i.e., HBV infection with detectable HBs antigen, HCV replication, and untreated or inadequately treated syphilis), alcohol abuse, current intravenous drug use, and current AIDS-defining disease. All subjects signed informed consent to participate in the research and completed a questionnaire on lifestyle factors, which may potentially impact epigenetic ageing. All of them underwent basic blood tests, including complete blood count, lipid profiles, such as total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglyceride levels, and creatinine, glucose and C-reactive protein (CRP) levels, current HIV viral load, and CD3+, CD4+, and CD8+ lymphocyte counts. Global DNA methylation was also assessed in all patients based on the ratio of DNA immunoprecipitated with 5-methylcytosine antibodies (meC, Diagenode) to the total amount of input DNA, secured before precipitation. More detailed information on that group and analyses performed can be found in our previous publication [9].

In this study, from 48 subjects, ten (n = 10) with extreme global DNA methylation results were divided into two subgroups: five (n = 5) with the highest methylation rates (indicating the most advanced epigenetic ageing) and five (n = 5) with the lowest methylation rates. This number of subjects in both sub-groups was chosen due to the availability of complete medical records for all subjects selected in these subgroups. Distribution analysis of global DNA methylation in the full cohort revealed no outliers [9]. Box-Cox transformation normalized data (Shapiro-Wilk: p = 0.756; Figure 1), with no observations exceeding Tukey's 3 × IQR threshold or $|Z| > 3$. Thus, the five lowest and five highest values constituted low- and high-methylation groups, while the remaining subjects were included in other groups. A detailed analysis of medical history was performed for all subjects from both low and high sub-groups using data on adherence and possible interruptions in cART use, such as years since diagnosis of HIV infection, HIV viral load at diagnosis of HIV infection, CD4+ lymphocyte count at diagnosis of HIV infection, years since cART onset, HIV viral load at the onset of cART, CD4+ lymphocyte count at the onset of cART, CD4+ lymphocyte nadir, cART regimens used and duration of use, AIDS-defining diseases and time of their occurrence, and other relevant medical events. These data constituted extended variables set.

Continuous variables in both low- and high-methylation groups were analyzed statistically. They were summarized using means, medians, and standard deviations, reported separately for each group. Differences in variable distribu-

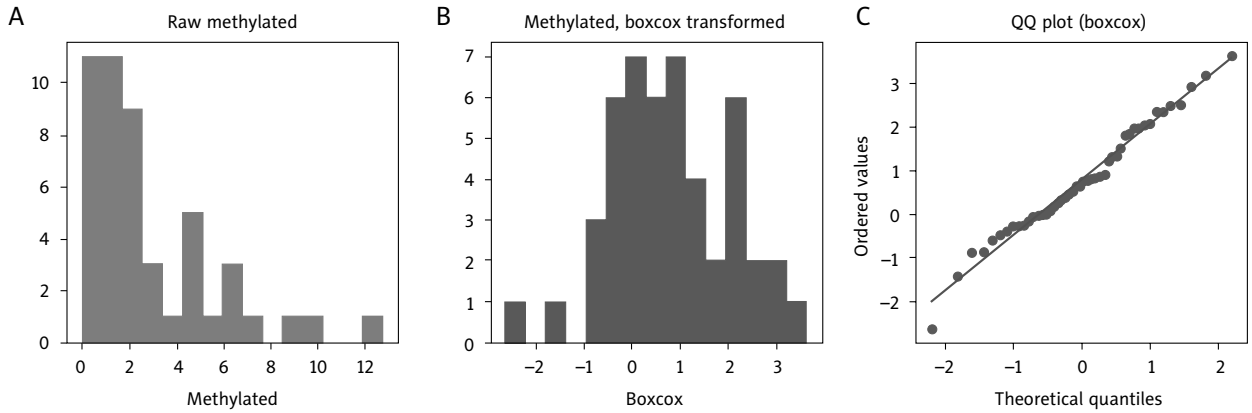

Figure 1. Distribution of methylation among 48 subjects from Bożejko *et al.* study [9]

tions between two groups were assessed using the exact Mann-Whitney test. Spearman's rank correlation coefficients were calculated to evaluate the associations between variables from extended dataset and global DNA methylation levels. *P*-values for these correlations were determined using a permutation test with 20,000 re-samples. Bootstrap re-sampling method (20,000 iterations) was employed to estimate 95% confidence intervals for correlation coefficients. To identify potential outliers within low- and high-methylation groups, considering both the extended set of variables and global DNA methylation, copula-based outlier detection (COPOD) ranks were calculated. Additionally, scatter plots of individual extended variables against global DNA methylation were generated for visual inspection. Due to very small size of groups, all statistical reasoning limited power, and the analysis was strictly exploratory.

Results

According to the available medical documentation, none of the ten participants showed any signs of incomplete adherence or interruptions in cART use. Nine subjects had been treated with integrase inhibitor-based regimens since the onset of cART. One patient from the high-methylation group was initially (for one year) treated with protease inhibitors, but subsequently (for eight years) also received an integrase inhibitor-based regimen. One subject in the low-methylation group had a history of Pneumocystis pneumonia that was successfully resolved; no AIDS-defining diseases were diagnosed in the other nine subjects. Moreover, another patient in the low-methylation group had a history of psychoactive substance abuse, mental disorders requiring hospitalization, and the presence of oligoclonal bands of unclear etiology in the cerebrospinal fluid. Among significant medical events, four patients have been diagnosed with syphilis, all of whom belonged to the high-methylation group.

Table 1 and Figure 2 report the characteristics of continuous variables (from the extended set of variables) for the study population. No significant ($p < 0.05$) differences between the groups were observed (without using multi-test corrections) due to small group sizes (both $n = 5$). In Figure 2, both the spread metrics (IQR, SD) and visual inspection

Table 1. Comparison of continuous extended variable set between the low-methylation and high-methylation groups

Variable	Low-methylation group (n = 5), median (IQR)/mean ± SD	High-methylation group (n = 5), median (IQR)/mean ± SD	p-value
CD4+ nadir (cells/µl)	374 (228-461)/315.4 ± 186.7	282 (211-339)/265.6 ± 81.5	0.421
Years since HIV diagnosis	7.0 (6.0-7.0)/7.4 ± 2.7	5.0 (5.0-8.0)/6.6 ± 2.9	0.548
CD4+ at cART initiation (cells/µl)	374 (228-481)/349.6 ± 230.6	339 (211-341)/316.6 ± 147.4	0.690
Years since cART initiation	7.0 (6.0-7.0)/6.4 ± 0.9	5.0 (5.0-8.0)/6.2 ± 2.2	0.841
CD4+ at HIV diagnosis (cells/µl)	374 (228-458)/345.0 ± 227.5	341 (339-537)/399.2 ± 149.9	1.000
HIV RNA at cART initiation (copies/ml)	14,300 (9,741-78,600)/116,961 ± 204,997	32,595 (12,400–39,100)/53,042 ± 71,365	1.000
HIV RNA at HIV diagnosis (copies/ml)	14,300 (9,741-78,600)/117,143 ± 204,871	32,595 (12,400–178,000)/94,822 ± 111,196	1.000

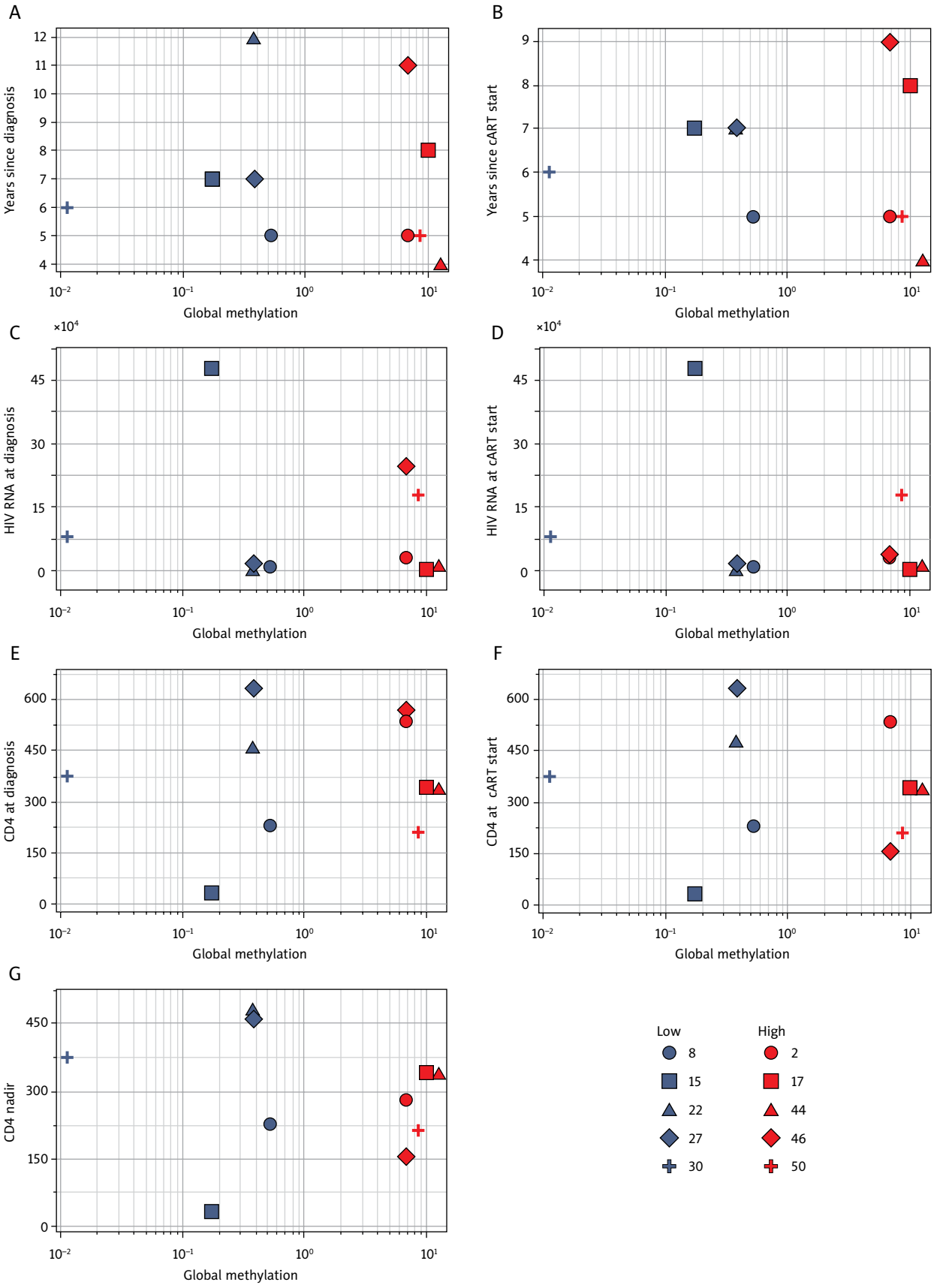

Figure 2. Continuous extended covariates against methylation for each subject, divided into groups

Table 2. Spearman's rank correlations between clinical variables and global DNA methylation in the extreme epigenetic aging sub-groups

Variable	Spearman's ρ	95% bootstrap CI	Permutation *p*-value
Years since HIV diagnosis	–0.400	(–0.906 to 0.372)	0.249
HIV RNA at diagnosis (copies/ml)	–0.309	(–0.805 to 0.475)	0.386
Years since cART initiation	–0.292	(–0.900 to 0.515)	0.413
HIV RNA at cART initiation (copies/ml)	–0.273	(–0.806 to 0.558)	0.445
CD4+ nadir (cells/µl)	–0.152	(–0.735 to 0.692)	0.682
CD4+ at HIV diagnosis (cells/µl)	–0.091	(–0.691 to 0.600)	0.814
CD4+ at cART initiation (cells/µl)	–0.079	(–0.648 to 0.640)	0.839

indicated that the low-methylation group demonstrated larger spread then high level of CD4+ and HIV RNA variables. However, these were mostly attributable to the subject No. 15 who also had the highest COPOD score (11.75). Spearman's rank correlations between global DNA methylation and key HIV-related clinical parameters were consistently weak-to-moderate and non-significant (Table 2). The strongest (most negative) association was observed between global DNA methylation and years since HIV diagnosis ($\rho = -0.400$, permutation $p = 0.249$), followed by HIV RNA at diagnosis ($\rho = -0.309$, $p = 0.386$). All other correlations were weaker ($|\rho| \leq 0.292$), with p-values ranging from 0.413 to 0.839. Notably, all 95% bootstrap confidence intervals included zero and were wide, reflecting the very small sample size and consequent high uncertainty in the estimates.

Discussion

The results of studies published to date clearly suggest that HIV infection accelerates epigenetic ageing, leading to global DNA hypomethylation [4, 8-10]. The most frequently indicated potential causes of this effect include accelerated immunosenescence, chronic inflammation, and side effects of ART occurring in PLWH [4, 8, 11-13]. A study by Seghal *et al.* [14] suggests that antiretroviral therapy is a particularly effective intervention in reversing the process of epigenetic ageing. All participants in our study were treated with integrase inhibitors that do not cause many side effects associated with older-generation drugs, which may have contributed to the acceleration of epigenetic ageing in some patients [4]. However, it is very important to note that epigenetic ageing can be influenced by a wide range of factors unrelated to HIV infection. Numerous studies link accelerated epigenetic ageing to, among others, cancer, alcohol consumption, substance abuse, infections, obesity, diabetes, and cardiorespiratory dysfunction [7, 15-19].

In our previous study, the obtained results suggested that HIV infection is a factor, independent of other parameters, that accelerates epigenetic ageing by increasing global DNA hypomethylation [9]. Now, we aimed to determine if there were any significant differences among the patients with extreme DNA methylation results, which could explain the differences in the intensity of epigenetic ageing. An in-depth review of medical records of the ten individuals included in the present analysis revealed no common, distinct feature that could differentiate the low-methylation from the high-methylation group. This is an interesting observation when combined with the fact that, in our previous study, HIV infection itself, independent of all confounding factors, was strongly associated with more intense epigenetic ageing compared with uninfected individuals [9]. However, it should be noted that two of the subjects in the low-methylation group had significant comorbidities: one had a history of an AIDS-defining disease (*Pneumocystis pneumonia*), while the other, among other things, had a history of substance abuse and mental disorders. In the context of results of the previously cited studies indicating the potential influence of many medical conditions on the acceleration of epigenetic ageing, it can be concluded that these comorbidities may have contributed to the low levels of global DNA methylation in these two individuals. Concurrently, a rather surprising observation is the fact that four individuals from the high-methylation group had a history of syphilis, whereas it was not observed in the low-methylation group. Presently, there is a lack of in-depth research investigating the impact of syphilis on epigenetic ageing in the literature. However, based on the results of studies published to date, syphilis as a chronic infection is more likely to accelerate epigenetic ageing and decrease global DNA methylation [7]. We believe that the absence of such a correlation in this study can be explained by the fact that all participants were regularly monitored at HIV outpatient clinic and were receiving cART; thus, syphilis was detected quickly and treated effectively at an early stage.

Due to the small sample sizes of both sub-groups of the study, the statistical analysis did not reveal any significant differences between them nor any statistically significant correlations between global DNA methylation and key HIV-related clinical parameters. However, the two strongest correlations found were quite expected, based on the postulated mechanisms of HIV infection's impact on epigenetic ageing. Lower levels of global methylation were

associated with a longer time since HIV diagnosis and higher viral load at diagnosis. With regard to the mechanisms discussed above, it seems likely that a longer period of infection and higher viral load may contribute to more intense epigenetic ageing. In Heany et al. study, accelerated epigenetic ageing showed a positive correlation with viral load and a negative correlation with CD4+ count throughout the observation period [20]. However, it should be noted that in at least two studies, a longer period of infection was associated with a lesser acceleration of epigenetic ageing [21, 22].

In the current study, global DNA methylation was negatively correlated with CD4+ lymphocyte count at the time of HIV diagnosis and initiation of cART and also with CD4+ nadir. These correlations are rather surprising and, at this stage, difficult to explain. However, due to the small size of both the analyzed sub-groups, any conclusions from this study are limited in power and strictly exploratory in nature.

Conclusions

In this study, we did not identify any common, distinct feature that could differentiate HIV-infected individuals with low versus high global DNA methylation level. This is an interesting observation in the context of a strong association between HIV infection itself and global DNA hypomethylation with acceleration of the epigenetic ageing. The results of statistical analysis indicate a negative correlation between global DNA methylation and time since HIV diagnosis as well as viral load at diagnosis. Further studies involving larger cohorts, with detailed clinical parameters at the time of HIV diagnosis and at initiation of cART, with sufficiently long follow-up periods, are needed.

Disclosures

1. Institutional review board statement: The study was conducted in accordance with the Declaration of Helsinki, with the consent of the Bioethics Committee of Wroclaw Medical University, dated March 9, 2023 (approval No. 251/2023).
2. Assistance with the article: None.
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4. Conflicts of interest: None.

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