# DNA Methylation of CpG in the Promoter Regions of HIV Coreceptors Related Genes (CCR5 and CXCR4): Original Research

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**Abstract:** The persistence of human immunodeficiency virus (HIV) in viral reservoirs is a topical issue. There are many factors that can potentially influence this process. Epigenetic alterations according to the literature could be such factors. In this study, we present a detailed description of gene promoters research and development techniques for determining the methylation level of CpG loci in these promoters based on Sanger sequencing and pyrosequencing. The presented methods could be used for measuring the methylation levels in other research.
A total of 51 samples of biological material from 17 patients at three visits were used in this study. Applying the developed techniques, we found that the CpG loci of the *CXCR4* gene promoter were not methylated. For CpG loci of the *CCR5* gene promoter no change in methylation levels was observed visit to visit. CpG 1 methylation level ranged from 33% to 53%, CpG 2 - from 4.4% to 11%, CpG 3 - from 0 to 42% and CpG 4 - from 1% to 21%. These values were derived from measurements of C-peaks in sequences.

**Keywords:** Epigenetic; HIV Infections; CpG; Methylation; HIV coreceptors; *CCR5*; *CXCR4*; Biomarkers; ART

**Key Contribution:** The breakthroughs or highlights of the manuscript. Authors can write one or two sentences to describe the most important part of the paper.

## 1. Introduction

Lifelong persistence of human immunodeficiency virus (HIV) in infected organisms is a global public health problem. Antiretroviral therapy (ART) significantly reduces mortality and risk of transmission [1],[2] in people living with HIV (PLHIV) [3] through suppression of viral replication [4].
Despite these significant successes, current ART does not cure HIV-infection, and the virus continues to persist in reservoirs.

In ART recipients with sustained virologic response, HIV RNA counts are reduced to undetectable values (<50 copies/mL plasma). However, the provirus activates within a few weeks after the interruption of ART by an increase in viral load (VL) [5],[6].
HIV persists as a provirus in long-lived CD4-Positive T-Lymphocytes. Recent data indicates that resting CD4-Positive T-Lymphocytes are a basic reservoir of the virus and a primary barrier to HIV-1 elimination, despite ART [7].

The resting CD4-Positive T-Lymphocytes can be roughly divided into two types: naive ones and HLA-activated (memory) cells. Naive CD4-Positive T-Lymphocytes are characterized by higher expression of the *CXCR4* gene, while memory cells feature high expression of the *CCR5* gene, which could contribute to HIV replication [8]. Gene expression could be affected by various factors, such as epigenetic.

Epigenetic modifications such as methylation are not linked to DNA sequence changes and play an important role in normal cell activities. HIV-1 latency and reactivation could be regulated by affecting the chromatin condition next to the viral promoter, which is located at the 5' long terminal repeat (LTR) [9].

The main related forms of epigenetic inheritance are genomic imprinting, DNA methylation (CpG dinucleotides) and histone modification [10].

HIV-1 enters into CD4-Positive T-Lymphocytes by chemokine coreceptors. Gene methylation regulates its function, leading to a decrease in coreceptor's gene expression on the cell surface, and this could prevent HIV-1 entry into the cell.

C methylation as 5mC in CpG dinucleotides in the promoter region of genes is associated with transcriptional silencing and plays a central role in epigenetics [11], [12],[13], [14], [15].

The correlation between decreased expression level and methylation is observed more frequently in the proximity of the transcription initiation site.

In this study, we consider the chemokine receptors CXCR4 and CCR5 as major coreceptors for HIV-1 entry into CD4+ cells. During the early stages of clinical HIV-1 infection predominantly R5-tropic viruses are transmitted. On the contrary, X4-tropic viruses are commonly detected in humans who have received multiple ART regimens and are in the later stages of infection with rapid disease progression [16], [17], [18], [19]. Additionally, there is also some correlation with HIV-1 genetic variants. R5 viruses are more often detected in subtype A, regardless of the stage of the disease [20], [21], [22].

The *CCR5* gene encodes a CCR5 coreceptor for HIV entry into the cell [23] and also influences disease pathogenesis [24].

Deletion of 32 base pairs of the *CCR5* gene results in a truncated form of the protein that does not fulfill its function. Such deletion contributes to blocking the entry of R5 tropic viruses, forming resistance against nearly 50% of HIV-1 variants [23], [25].

High expression of the *CCR5* gene affects disease progression and reduces the rate of immune recovery during the course of ART [26]. Reduced expression of the *CCR5* gene correlates with decreased HIV-1 concentration in the cell [27].

The *CCR5* expression level on the surface of activated CD4-Positive T-Lymphocytes [8], [28],[29] is increased in HIV-positive individuals [30]. Increased DNA methylation in cis-regulatory modules reduces *CCR5* gene activity. There is a significant correlation between the methylation level of the gene and the expression of the *CCR5* on the surface of T cells [31].

The increased expression level is associated with demethylation of the cis-modules of the *CCR5* gene. Naive cells are activated by HLA receptors in response to infection. It has been shown that increased *CCR5* expression level can directly support T cell activation. Hypermethylation is associated with low VL levels and increased CD4+ T-cell counts; however, cis-modules in HIV-positive individuals remain demethylated compared to normalized CD4+ T-cell counts [32].

HIV-1 entry activates the human immune system by acting on HLA-receptor of naive cells. The cis-modules of the *CCR5* gene are demethylated and gene expression level rises, which increases the amount of protein on a cell membrane. HIV-1 enters uninfected CD4-Positive T-Lymphocytes through CCR5 coreceptor [33]. Thus, the human immune system promotes the replication of HIV-1.

Suppression of the virus replication by ART has been associated with increased methylation level of *CCR5* [31]: methylation status is reduced in untreated individuals.

High CCR5 levels could activate resting T-cells, causing apoptosis and their destruction. Therefore, therapy that blocks *CCR5* expression could be associated with slower depletion of the CD4-Positive T-Lymphocytes cell pool without an antiviral effect [31].

HIV-1 mainly use CXCR4 to enter cell as coreceptor but 5% of virus strains use it as main receptor. [23], [34], [35]. Naive cells, in contrary to activated cells, predominantly express *CXCR4* [29].
The X4 strain of HIV-1, which uses CXCR4 as a coreceptor for cell entry, is mostly diagnosed in the late stages of the disease [30]. Reduced *CXCR4* expression affects the HIV-1 life cycle, which is a hallmark of naive cells that have not passed the activation stage.

Despite the lack of studies reporting the effect of *CXCR4* gene methylation on HIV-1 replication, there is data that show a strong direct correlation between expression level and the potential entry of HIV-1 into the cell [36]. Activation of CD4-Positive T-Lymphocytes partly reduces the number of CXCR4 receptors that limit the spread of infection [34]. Reduced levels of the *CXCR4* gene expression correlate with disease progression [30].

A typical procedure for methylation research would include a gene expression study, the identification of genes which are downregulated, and NCBI search to locate the promoter sequence and transcription start site of the gene. The selected genomic sequence is then used to aid in the selection of primers.

In this study, techniques were developed to assess the impact of the methylation status of the promoters of the human *CCR5* and *CXCR4* genes.

## 2. Materials and Methods

### *2.1. Study Population*

Anonymized samples from 17 treatment-naive patients with HIV-1 infection were collected in Infectious disease clinics of Central Research Institute of Epidemiology (CRIE) during 2021-2023 were used in this study. Inclusion criteria: age over 18 years, viral load <500,000 copies/mL, CD4 T-cell count >200 cells/mm$^3$, less than 3 visits, written informed consent. The first line ART regimen prescribed was lamivudine/dolutegravir (3TC/DTG). Whole blood samples from patients were obtained at baseline (Week 0) and after ART (Weeks 24 and 48). In summary, the study included 51 samples from 17 patients at three visits. Samples obtained at baseline “Week 0” were naïve, had no ART before the study started and used as control group.

Viral load for this patients vary from 1,371 to 208,538 (copies/mL). All the patients had viral suppression (HIV plasma RNA< 50 copies/mL) at Weeks 24 and 48. Information about viral load, size of the viral reservoir measured in copies of HIV DNA per million cells, CD4 T-cell count (cells/mm$^3$) and age for all patients collected in [52] *https://figshare.com/s/2fce38ebe705a8501770.*
Analyses were performed retrospectively on frozen samples of whole blood. Clinical, demographic, and epidemiological data for the study were collected retrospectively from patients’ medical records, anonymized and coded prior to entry into the database. The samples were archived and anonymized at the moment of this study.

This study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of the Central Research Institute of Epidemiology, Federal Service for Surveillance on Consumer Rights Protection and Human Wellbeing, Russian Federation (protocol code No 117, 28 September 2021). Participants provided written informed consent prior to the sampling and collection of clinical, demographic, and epidemiological data.

### *2.2. Biological Samples*

Whole human blood samples were examined for the presence of HIV DNA using the AmpliSens® “DNA-HIV-FL” reagent kit (Amplisens, CRIE, Moscow, Russia). The reagent kit includes a plasmid calibrator with a known concentration of human *β-globin* gene and HIV *pol* gene.

Samples were purified with “Hemolytic“ reagents (Amplisens, CRIE, Moscow, Russia) is used for selective lysis of blood erythrocytes during pre-processing of clinical material of whole peripheral blood for prioring to DNA extraction.

DNA isolation was carried out by using the “RIBO-prep” kit (Amplisens, CRIE, Moscow, Russia) according to the instructions. The samples under study treated with lysis solution, resulting in the destruction of cell membranes, viral envelopes and other biopolymeric complexes and the release of nucleic acids and cellular components. After addition of the precipitation solution and centrifugation, the dissolved RNA/DNA precipitates, while other components of the lysed clinical material remain in solution and are removed by subsequent washes. In the final extraction step, the precipitate dissolved in elution buffer and the purified RNA/DNA is transferred into solution.

Bisulfite conversion was performed on samples using the “EpiTect Bisulfite KIT” (QIAGEN, Hilden, Germany). DNA samples were kept under — 20°C after bisulfite conversion and eluated 10x in the TE-buffer (AmpliSens, CRIE, Moscow, Russia) for PCR analysis.

It is necessary to note that incomplete bisulfite conversion of DNA results in the interpretation of unconverted cytosines as methylated in assays [37]. Large amounts of DNA (> 10 µg/ml) could decrease the conversion rate by depleting the available bisulfite in the reaction mix and by increasing the pH due to the formation of NH4+ as a by-product of the reaction.

Accordingly, before bisulfite conversion, samples should be measured for the amount of the starting DNA contents. The concentration was measured by real-time PCR for the β-globin gene using calibrated samples with concentrations of 10,000 and 100 copies per mL. The obtained values were then converted from "copies/µl '' to "ng" using the SciencePrimer tool- Copy number calculator for real-time PCR [38] (where 1 ng DNA is approximately 300 copies). The average concentration of starting DNA before the bisulfite conversion reaction was - 9000 copies per µl or 30 ng.

### *2.3. PCR Conditions*

All techniques were optimized for one protocol. Amplification system "Tercyc" (DNA-technology, Protvino, Russia) was used for PCR sample preparation before the sequencing operations. The amplification program was as follows: hold -95°C - 15 min, cycling 50 cycles: 95°C - 5 sec, 60°C - 20 sec, 72°C - 5 sec.

The protocol for PCR assays used the developed methodology as follows: the reaction mixture for one sample consisted of 5 µL of PCR-mix-1 (contains ultra-pure water, dNTPs and primers); the concentration of primers was experimentally matched — 0.7 µM), 10 µL of PCR-mix-2 (contains Tris-buffer and $MgCl_2$) and 0.5 µL of TaqF polymerase. All using reagents were from Amplisens, CRIE, Moscow, Russia. Finally, 10 µL of bisulfite-treated DNA samples were added to the prepared tubes.

Completely methylated DNA and fully unmethylated DNA, negative control (no matrix) were used as controls for amplification [39], [40] (QIAGEN, Hilden, Germany).

### *2.4. Pyrosequencing Conditions*

Sample preparation was carried out with the PyroMark Q24 vacuum station (QIAGEN, Hilden, Germany) and sample preparation kit "Amplisens® Pyroscreen" (Amplisens, CRIE, Moscow, Russia) according to manufacturers' recommendations.

The reaction was performed with 5 µl of the PCR products. Incubation with sequencing primer was carried out at 80°C for 2 min (at a concentration of 7.5 pmol/µL).

Pyrosequencing was performed using the "PyroMark Gold Q24 Reagents kit" (Qiagen, Hilden, Germany).

Methylation levels of CpG loci after pyrosequencing were calculated automatically using PyroMark Q24 software (Qiagen, Hilden, Germany). Quantitative bisulfite Pyrosequencing determines DNA methylation level by analyzing artificial “C/T” SNPs at CpG sites within a specific Pyrosequencing assay[41].

### *2.5. Sanger sequencing Conditions*

Sanger sequencing was performed using "AmpliSense® HIV-Resist-Seq" reagent kit (Amplisens®, CRIE, Moscow, Russia) and Applied Biosystems 3500 genetic analyser (LifeTechnologies, Waltham, USA).

The amplification was carried out on a "T100" device (BioRad Laboratories, Hercules, USA) and included the following steps: 96 °C - 1 min, 25 cycles: 96 °C - 10 sec, 50 °C - 5 sec, 60 °C - 4 min. The methylation levels after Sanger sequencing were calculated using the calculated average peak heights at the desired positions based on three reads of the same sample.

### *2.6. Genes promoter sequence search*

The database of the University of California, Santa Cruz (UCSC) was used to select the CXCR4 promoter region [42]. The UCSC database contains sequences from various databases related to the human genome.

Target loci were chosen by comparing promoter sequences from the EPD database, which only contains experimentally confirmed regions at the beginning of transcription, with sequences from CpG Islands, i.e., regions where CpG is present more frequently than in other regions. These compared loci were taken for further analysis and were described in this study.

Coordinate data and CpG identifiers were obtained from the Illumina GRCh37.p13 genome assembly methylation array (GEO Access Viewer) [43]. The previously published Illumina methylation array had no id for all CpG loci analyzed in this study. The coordinates for these loci were selected from the GRCh37.p13 genome assembly, which was published in the NCBI database [44]. The coordinates and symbols for the CpGs selected for this study are presented in **Table 1** and **Table 2**.

**Table 1.** CpG coordinates and their designations in the text for the *CCR5* gene.

| ***CCR5* (GRCh37.p13) Chr 3 - NC_000003.12** | | | | | |
|---|---|---|---|---|---|
| cg22984586* | CpG 1 | CpG 2 | CpG 3 | CpG 4 | cg07616471* |
| 46411541 | 46412248 | 46412333 | 46412454 | 46412478 | 46413711 |

**Table 2.** CpG coordinates and their designations in the text for the *CXCR4* gene.

| ***CXCR4* (GRCh37.p13) Chr 2 - NC_000002.12** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| cg25982140* | CpG 1 | CpG 2 | CpG 3 | CpG 4 | CpG 5 | CpG 6 | CpG 7 | CpG 8 | cg12311057* |
| 136875681 | 136875732 | 136875734 | 136875738 | 136875756 | 136875761 | 136875765 | 136875767 | 136875770 | 136875897 |

* Proximate CpGs that have a Cg ID.

CpGs presented in our study are indicated in blue colour. They are labelled accordingly further on in the text.

### *2.7. Methodology Design / Primers Design*

In this study, the CpG loci of the second promoter of *CCR5* and the CpG loci of the promoter region of the *CXCR4* gene were considered (**Table 3**). The design of PCR systems was prepared based on the length of the sequences.

**Figure 1** shows the general scheme of the techniques with the oligonucleotide positions indicated.

Red - primers for pyrosequencing, green - primer for sequencing in pyrosequencing. *NB!* CpGs at annealing sites should be avoided in oligonucleotide design. The length of the amplicon for pyrosequencing is in the range of 100-250 bp [41].

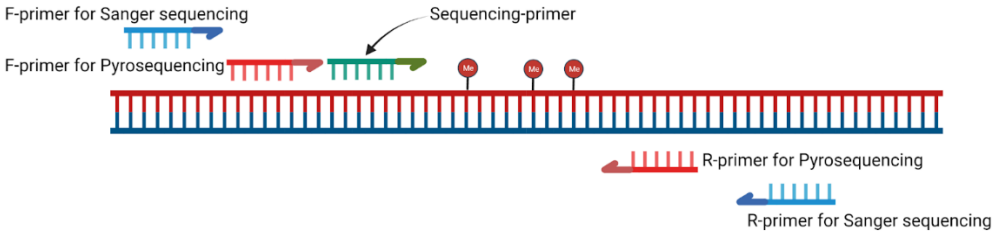


Figure 1. Scheme of techniques for determining the methylation level of CpG loci (created with BioRender [45]).

The Tm mean for designed primers calculated in the OligoAnalyzer™ Tool [46] was 60°C. The CG content is rather low because of the features of the converted DNA product and averages 33%. **Table 3** summarizes the sequences of the oligonucleotides used in this study.

The following primer combination was used to measure the methylation levels of CpG-dinucleotides located in the second promoter of *CCR5* by pyrosequencing: for **CpG 2 CCR5**—CCR5-F1p+CCR5-Rp, for **CpG 3 CCR5** and **CpG 4 CCR5**— CCR5-Fp +CCR5-Rp.
For pyrosequencing, CCR5-S2 was used for **CpG 2 CCR5** reads, and CCR5-S3 was used for **CpG 3 CCR5** and **CpG 4 CCR5** reads.

Amplicons for sequencing by Sanger sequencing were flanked with CCR5-Fs and CCR5-Rs, then the same primers were used for the second round as for pyrosequencing (CCR5-Fp and CCR5-Rp).
The following primer combination was used to measure the methylation level of eight CpG loci located in the *CXCR4* promoter: in pyrosequencing— CXCR4-Fp+CXCR4-Rp, reads were performed using the CXCR4-S primer; when sequencing by Sanger sequencing, combination CXCR4-Fs+CXCR4-Rs was used.

All of the corresponding Dispensation orders for the reads are also shown in **Table 3**.

**Table 3.** Oligonucleotides used in the study.

| Gene | Primers | P/S | 5`-sequence-3` | Amplicon size | Dispensation order for pyrosequencing |
|---|---|---|---|---|---|
| *CCR5* | CCR5-Fp | P | TATGATTGATTTGTATAGTTTATTTGGTTA | Fp+Rp 121 | - |
| | CCR5-Rp | P | biotin-CTCATCTCAAAAACTAACTAAC | | |
| | CCR5-F1p | P | GTAGTGGGATGAGTAGAGAATA | F1p+Rp 331 | - |
| | CCR5-S2 | P | GAAGAATTGTTTTTTGATTTTTTT | - | YGTTTTTAATATAT |
| | CCR5-S3 | P | GGTTAGAAGAGTTGAGATATT | - | YGTTTTTTTATAAGAAATT TTTTTYGGTAAG |
| | CCR5-Fs | S | GTGGGTTTTTGATTAGATGAATGTA | Fs+Rs 517 | - |
| | CCR5-Rs | S | CCAAACTATAACCCTTTCCTTATCTT | | |
| *CXCR4* | CXCR4-Fp | P | GTG GTT ATT GGA GTA TTT AGG T | Fp+Rp 168 | - |
| | CXCR4-Rp | P | biotin-TCT ACC CCT CTC CCC CA | | |

| Gene | Primers | P/S | 5`-sequence-3` | Amplicon size | Dispensation order for pyrosequencing |
|---|---|---|---|---|---|
| *CCR5* | CCR5-Fp | P | TATGATTGATTTGTATAGTTTATTTGGTTA | Fp+Rp 121 | - |
| | CCR5-Rp | P | biotin-CTCATCTCAAAAACTAACTAAC | | |
| | CCR5-F1p | P | GTAGTGGGATGAGTAGAGAATA | F1p+Rp 331 | - |
| | CCR5-S2 | P | GAAGAATTGTTTTTTGATTTTTTT | - | YGTTTTTAATATAT |
| | CCR5-S3 | P | GGTTAGAAGAGTTGAGATATT | - | YGTTTTTTTATAAGAAATT TTTTTYGGTAAG |
| | CCR5-Fs | S | GTGGGTTTTTGATTAGATGAATGTA | Fs+Rs 517 | - |
| | CCR5-Rs | S | CCAAACTATAACCCTTTCCTTATCTT | | |
| | CXCR4-S | P | GTTAATAAATTGAAGTTTTTGGT | - | YGYGGTYGGATTTTTATAA AAATAYGTTTYGAGYGYG GYGTAT |
| | CXCR4-Fs | S | ATGATAAAGTAGGTTGAAATTGGATTT | Fs+Rs 376 | - |
| | CXCR4-Rs | S | TCCCTCAAACTTAAAAAATACCTCTA | | |

**P/S** = Pyrosequencing /Sanger; **F**- forward primer; **R**-reverse primer; **S**- sequencing primer; **Y**- C/T.

### *2.8. Statistical Methods*

Methylation analysis of Cpg loci by Sanger sequencing was performed according to the protocol of Parrish et al. [47]. Comparison of patients' data from different visits was performed using one-way repeated measures ANOVA. If the null hypothesis was rejected, a pairwise post-hoc comparison with Bonferroni correction was carried out. All statistical calculations and data visualization were performed in the R environment (R foundation)[48], [49].

## 3. Results

### *3.1. Development of techniques*

We developed pyrosequencing and Sanger sequencing techniques to estimate CpG loci methylation in the promoters of *CCR5* and *CXCR4* genes.

The results of these techniques differed insignificantly. Pyrosequencing is undoubtedly more accurate in measuring the C/(C+T) ratio of peak heights, as this method is the "gold standard" for quantitative allele quantification at single base resolution [50].

The methylation levels of eight CpG loci located in the promoter of the *CXCR4* gene and four CpG loci located in the second promoter of the *CCR5* gene were determined by these techniques. The *CCR5* gene promoter was selected, relying on the study by *Gornalusse et al.,* when two promoter regions were analyzed for two spliced *CCR5* transcript isoforms.

Promoter region 1 follows the first exon and produces a truncated product that is primarily expressed in naive T-cells and in memory T-cells [51]. Promoter region 2 contributes to transcription of a product with exon 1 which is typical for T-cells.

We studied promoter region 2, since the results of *Gornalusse et al.* research show that hypermethylation of this DNA region correlates significantly with *CCR5* expression level [31].

**Figure 2** demonstrates an example of the *CXCR4* gene promoter sequence containing eight CpG sites. Pyrogram on PyroMarkQ24 in the upper part of the figure shows the same region by Sanger sequencing in the middle and expected sequence in the down part of the figure. Lines mark the appropriate CpG loci.

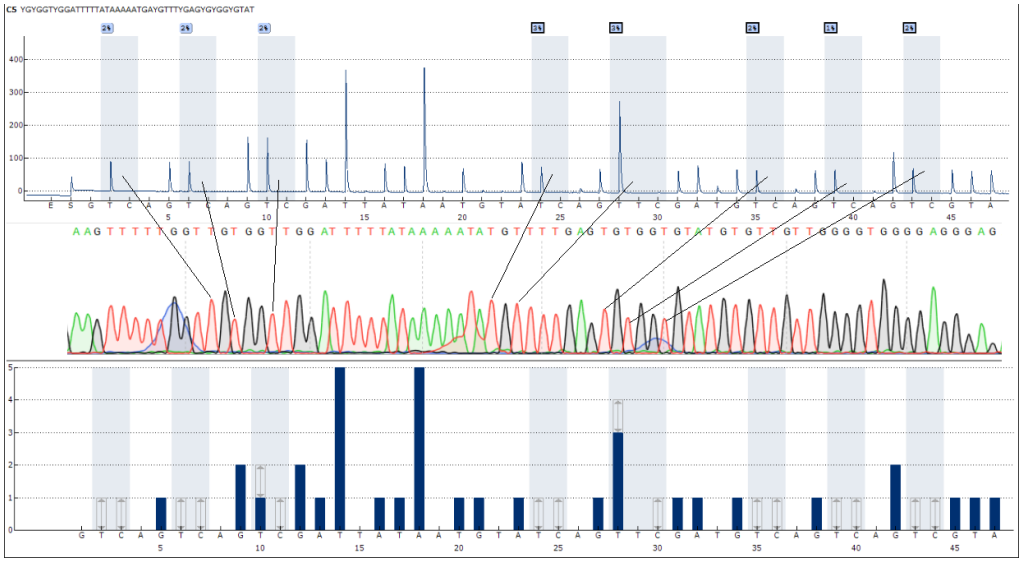

**Figure 2.** *CXCR4* promoter region sequencing results.

Considering the CpG loci location in the *CCR5* gene second promoter, several combinations of oligonucleotides were used to read 100-250 bp amplicon length sites. These combinations are presented in detail in section "*4.7. Methodology Design/Primers Design*".

First CpG loci (CpG1) in *CCR5* gene were detected only by the Sanger sequencing method because of the inconvenient location of primer sites.

**Figure 3** shows an example sequence part of the second promoter in *CCR5* gene containing CpG 2.

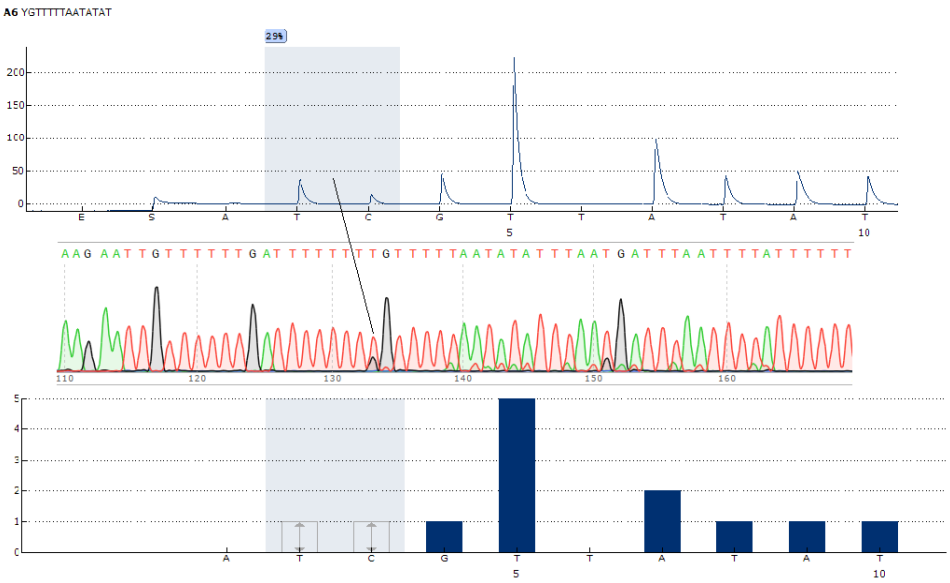

**Figure 3.** Pyrogram and spectrogram of the *CCR5* second promoter site containing CpG 2 of *CCR5.*

The range between CpG loci 3 and 4 in the promoter of the *CCR5* gene is 23 bp, which allows for straightforward sequencing by one pyrosequencing primer. **Figure 4** shows an example sequence part reading from the second promoter of *CCR5* gene containing CpG 3 and CpG 4.

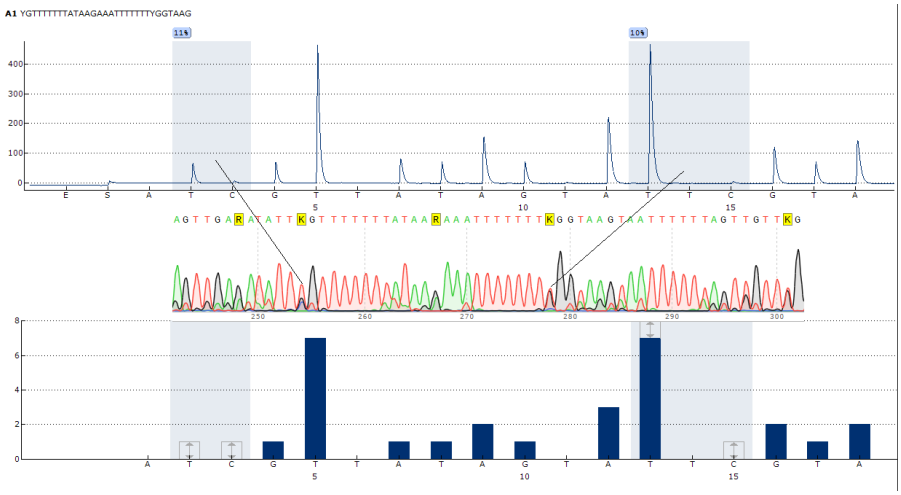

**Figure 4.** Sequence examples of the *CCR5* second promoter site containing CpG 3 *CCR5* and CpG 4 *CCR5.*

### *3.2. Application Results of the Developed Techniques*

Anonymized samples from 17 patients of Infectious disease clinics of Central Research Institute of Epidemiology (CRIE) were used in this study: 3 females and 14 males aged 19 to 52. People attended 3 visits to the physician (0 weeks- before ART, 24 weeks and48 weeks using ART).

A total number of 51 samples from HIV-infected individuals were assayed by both techniques (more details in section *2.1. Study population* and [52] *https://figshare.com/s/2fce38ebe705a8501770*).

There were no statistically significant differences in the level of methylation of the CpG dinucleotide when comparing patients from visit to visit (**Figure 5**).Applying the developed techniques, we determined that the CpG loci of the *CXCR4* gene promoter were unmethylated. A control containing bisulfite-treated DNA that is fully methylated showed an average of 93.5%. The completely unmethylated control containing bisulfite treated DNA showed an average of 4.8%. The maximum methylation value for all eight loci in the analysis of the studied samples obtained was 24.8% (in the analysis of C-peak chromatograms the result was considered "unmethylated").

We observed that the CpG 1 methylation level of the *CCR5* gene promoter ranged from 33% to 53%, CpG 2 - from 4.4% to 11%, CpG 3 - from 0 to 42% and CpG 4 - from 1% to 21%. C-peaks indicating the presence of methylated bases were marked on the chromatograms. In this case the completely methylated control containing bisulfite treated DNA showed an average of 88%. The completely unmethylated control containing bisulfite treated DNA showed an average of 1%. We also verified the resulting sequence and percentages by pyrosequencing (results in [52] *https://figshare.com/s/2fce38ebe705a8501770*).

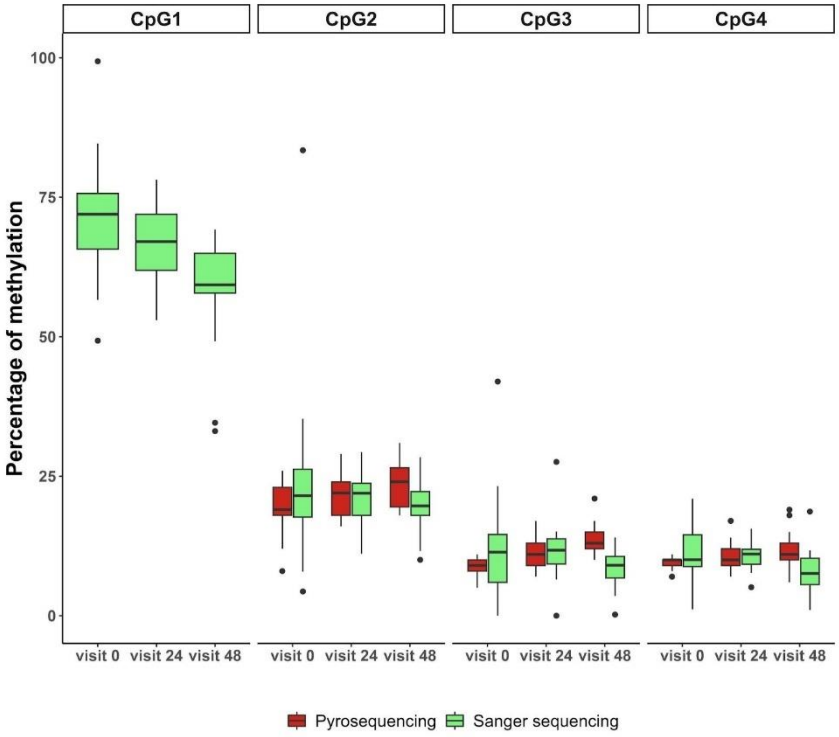


**Figure 5.** CpG dinucleotide methylation level in the *CCR5* gene promoter of HIV-1-infected individuals (1st visit - 0 weeks, 2nd visit - 24 weeks and 3rd visit - 48 weeks from 1st visit) using Pyrosequencing and Sanger techniques.

The median of viral load at the first visit for patients was 38,181 copies/mL. At the second visit, through ART treatment, the viral load decreased and was below 50 copies/mL.

HIV-1 viral reservoir volume decreased systematically over the course, however there was no statistically significant reduction (**Figure 6**). In contrast, the number of CD4+T cells per mm^3 increased, but also did not reach a statistically significant increase (**Figure 7**).

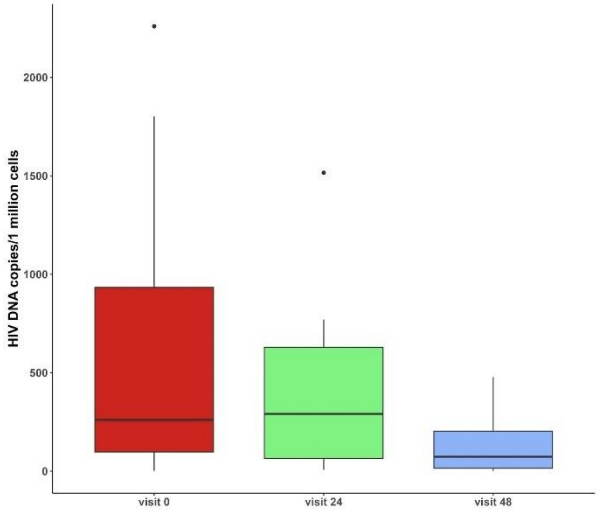


**Figure 6.** The results of the study on the size of the viral reservoir measured in copies of HIV DNA per million cells in a sample of individuals living with HIV-1 at visit 0 weeks, 24 weeks and 48 weeks.

There were no treatment-related adverse effects requiring regimen changes. There was no clinical progression of infection in the sense of HIV-associated diseases. All patients reached the virological efficacy of ART (HIV DNA < 50 copies/µl) by 24 weeks of treatment and remained at week 48.

Positive immunological dynamics was observed in the form of an increase in the number of CD4 lymphocytes (**Figure 7**).

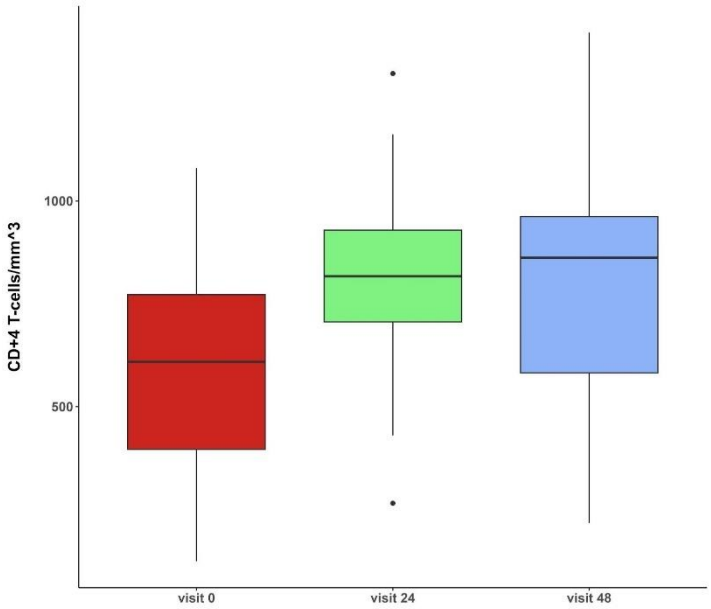


**Figure 7.** Number of CD+4 T-cells per mm3 in a sample of individuals living with HIV-1 at visit 0 weeks, 24 weeks and 48 weeks.

## 4. Discussion

Epigenetic processes are key elements that regulate gene expression. The advancement of approaches focused on gene methylation research is undoubtedly relevant. In this study, we have provided an example of a possible approach to studying the subject through the development of systems based on PCR and sequencing (pyrosequencing and Sanger sequencing).

The gene methylation studies makes it possible to search for promising targets for therapies based on the use of genomic editing techniques.

Genomic editing technologies in HIV therapy could be used in conjunction with ART or completely replace existing treatment regimens [53]. For example, the CRISPR/Cas9 method would allow the delivery of methyltransferases and demethylases to CpG loci to change the methylation status and, consequently, the level of gene expression [54].

We considered the genes of the CXCR4 and CCR5 coreceptors. The activity of CXCR4 and CCR5 regulates the entry of HIV-1 into a cell in most cases. The methylation of the genes promoters reduces the concentration of non-integrated HIV-1 within cells and could contribute to the elimination of the virus equally with or in combination with ART [26].

The association of virus replication and the levels of *CXCR4* and *CCR5* expression allows one to hypothesize a direct effect of methylation of these genes on HIV-1 progression in human immune cells [30]. Further study of epigenetic modifications of the chemokine receptor group will reveal the molecular mechanisms of HIV-1 entry. The hypermethylation of *CCR5* and *CXCR4* promoters can reduce both the concentration of HIV-1 RNA inside cells and the risk of infecting new cells.

This study is more focused on developing and applying techniques, more individuals living with HIV are needed to obtain statistically valid results in future studies. In addition, the transcriptional activity of the chosen genes should be studied. It is also relevant to investigate the level of methylation of CpG loci in the first promoter of the *CCR5* gene. The limitation of our study is the small sample size. Despite this, the approaches we have developed allows to work with larger samples, which will be useful for the following studies. We will plan further studies in this direction using the techniques we have developed.

When DNA methylation affects the promoter region, transcription of this gene is usually suppressed. On the contrary, actively expressed genes have unmethylated promoters. Considering the sample size, we can observe essentially only a difference in the methylation level of *CCR5* and *CXCR4* genes and cannot conclude that the methylation level has a protective effect.
However, we can only assume that the *CCR5* gene activity in observed samples is reduced. It is possible that the R5 virus is tropic in these patients, but all of the above requires additional experiments and verification.

While planning this study, we aimed at reading the promoter sequences of the chosen genes with the use of several tools. Using the presented algorithms, it is possible to apply them in other studies related to methylation level measurement of specific loci.

Another direction of further research is to study HIV tropism in these patients and the level of methylation of CpG loci in the promoters of coreceptor genes. This could be important to improve understanding of what mechanisms HIV uses to keep itself in the cell. The eradication of HIV-1 in infected people will be impossible without solving the problem of the persistence of the virus in its reservoirs.

The developed techniques also made it possible to compare the methods, we noted that no significant difference was observed when assessing the level of CpG methylation using pyrosequencing and Sanger sequencing. A number of researchers prefer to use pyrosequencing for such tasks, stating that this is the "gold standard", but the Sanger method is also convenient [55]. In addition to standard bisulfite sequencing, another effective method for determining site-specific DNA methylation is pyrosequencing. This method also uses bisulfite-treated DNA and has been previously shown similar accuracy to bisulfite sequencing. However, one disadvantage of this method is that it has a small maximum reading length, forcing the use of multiple sets of primers for analyzing more than one CpG site. This limitation has been noted in previous studies by *Ronaghy* [56] and *Reed et al.* [55]. For example, when placing the necessary several targets at a distance of more than 60-70 nucleotides from each other, it will be more convenient to use the Sanger method, since a well-readable fragment for pyrosequencing is usually limited to about 50 nucleotides. In this case, if it is necessary to read the sequence with CpG at a distance from each other, it will be necessary to use several sequencing primers and carry out several reaction formulations, which is undoubtedly not very convenient. We have demonstrated combinations of primers for pyrosequencing in this study. In the case of Sanger sequencing, it will be sufficient to use either one pair of primers or two pairs to suppress the "noise" during reading.

## 5. Patents

Patent No. 2022135017(RU) dated 28 December 2022, Set of oligonucleotide primers for determining methylation of human *CCR5* gene promoter in samples of biological material that has undergone preliminary bisulfite conversion using pyrosequencing.
Patent No.2022135019 (RU) dated 28 December 2022, Set of oligonucleotide primers for determining methylation of human *CXCR4* gene promoter in samples of biological material that has undergone preliminary bisulfite conversion using pyrosequencing.

**Supplementary Materials:** The following supporting information can be downloaded at: https://figshare.com/s/2fce38ebe705a8501770, Table S1: DTG 3TC HIV EPI study.xlsx.

**Author Contributions:** Conceptualization, A.E., M.V., S.S. and D.F.; methodology, A.E. and M.V.; validation, S.S. and D.F.; resources, A.K. and A.P., writing—original draft preparation, A.E., S.S., M.V., D.F., A.K., writing—review and editing, A.P., visualisation, M.V., supervision, V.A., project administration, A.E., funding acquisition, A.E. All authors have read and agreed to the published version of the manuscript.

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