CCR5 promoter variants among Ugandan HIV-1 elite and viremic controllers: a laboratory-based cross-sectional study

Brian Nyiro¹, Sharon Bright Amany², Rose Nabatanzi¹, Alice Bayiyana¹, Linda Igumba Kalazane⁴, Francis Waswa¹, Eva Nabulime¹,³, Daniel Karara¹, Joel Kabali³,⁶, Gerald Mboowa¹,⁷, Alex Kayongo⁵, Immaculate Nankya³, David Patrick Kateete¹ and Obondo James Sande¹

Author Institutions

¹Department of Immunology and Molecular Biology, Makerere University College of Health Sciences, Kampala, Uganda.
²Faculty of Health Sciences, Lira University, Lira, Uganda.
³Centre for AIDS Research Laboratory, Joint Clinical Research Centre, 10005, Wakiso, Uganda.
⁴Uganda Christian University, Plot 67-173, Bishop Tucker Rd, Mukono, Uganda.
⁵Makerere University Lung Institute, Kampala, Uganda.
⁶Medical Research Council, Uganda Virus Research Institute.
⁷The African Center of Excellence in Bioinformatics and Data Intensive Sciences, the Infectious Diseases Institute, McKinnell Knowledge Centre, Makerere University, Kampala, Uganda

*Corresponding author: Obondo James Sande (ojsande@gmail.com)

²Department of Immunology and Molecular Biology, Makerere University
College of Health Sciences, Kampala, Uganda
Abstract

Background

Mechanisms for HIV control among HIV-1 elite and viremic-controllers are not fully understood. In Uganda, studies have reported individuals who without Antiretroviral therapy have the inherent ability to control HIV progression to AIDS for a period of greater than 5 years. However, reasons for this phenotype are not understood. The study objective was to determine the distribution of CCR5 co-receptor on CD4+ T-cells and its associated promoter variants among HIV-1 elite and viremic-controllers.

Methods

We isolated CD4+T-cells from PBMCs using EasySep CD4+ T-cell negative selection kit, and stimulated them with anti-CD3 and anti-CD28 for 48 hours. To quantify CCR5 expression, we performed immune-phenotyping using flow cytometry. CCR5 promoter polymorphisms were determined through sanger sequencing. The Kruskal–Wallis and the Mann-Whitney test were used to compare differences in the percentages of CCR5+ CD4+ T-cells and the differences in CCR5 densities on CD4+ T-cells respectively. \( p \) values < 0.05 were considered significant.

Results

The percentage of CCR5+CD4+ T-cells was higher among the non-controllers compared to the controllers although, the difference was not statistically significant; elite and viremic-controllers (\( p=0.9173 \)), viremic and non-controllers (0.0702), elite and non-controllers (0.6010). Of significance was the CCR5 densities on CD4+ T-cells, which were significantly higher among non-controllers relative to the controllers; elite and viremic-controllers (\( p=3048 \)), viremic and non-controllers (\( P=0.0312 \)), elite and non-controllers (\( P=0.0210 \))
From the sequence analysis, the rs1799987A>G mutation was found among elite (71%) and viremic-controllers (61%), while the -2459A/A and rs41469351C>T mutation were among the non-controllers (57%). This study also identified two novel mutations 1070T>G and 785A>G among the elite controllers (14.3%).

Conclusion

Rs1799987 SNP highly detected among the elite and viremic controllers may be associated with reduced CCR5 densities on CD4+ T-cells while higher frequency of -2459 A/A and rs41469351 SNP among non-controllers may be associated with increased CCR5 densities on CD4+ T-cells. Thus Rs1799987 SNP may be responsible for the delayed HIV progression among elite and viremic controllers, while -2459A/A and rs41469351 SNP may be responsible for the rapid progression of HIV among non-controllers. In vitro studies are needed to study the effect of the two novel mutations 1070T>G and 785A>G among elite-controllers.

Key words

CCR5, HIV, Elite controllers, Viremic controllers and non-controllers

1. Background

HIV has claimed lives of more than 35 million people globally since its discovery, particularly in the WHO African region, home to 70% of the 36.7 million people currently living with HIV (1). Though no documented cure yet exists for HIV, those infected with the virus are enrolled on Antiretroviral Treatment (ART), which enables them to live long healthy lives.

One subset of individuals living with HIV, referred to as long term non progressors (LTNPs), are able to maintain their CD4+ T-cell count above 500 cells/µl for a period greater than five years prior to ART initiation (2). Among these, elite controllers have an undetectable viral load (HIV
plasma viral load < 50 viral RNA (vRNA) copies ml⁻¹) while others, viremic controllers, maintain their viral load between 50 and 2000 copies/ml (3). The existence of this population in Uganda has been reported in two previous prevalence studies. Laeyendecker reported a prevalence of 9.1% of LTNPs in Rakai district (2) while Kayongo, reported a prevalence of 0.26% among elite controllers in an Urban HIV ambulatory center in Kampala (2, 4). Reasons for intrinsic resistance to HIV in these controllers are not fully understood, especially in Uganda and other African populations with high genetic diversity (4).

Studies have identified mutations in CCR5, a major co-receptor for HIV infection that has been linked to delayed disease progression and resistance to HIV (5-8). The Δ32 mutation with a 32-base pair (bp) deletion in the open reading frame (ORF) of the CCR5 gene confers resistance to HIV in homozygous individuals and retards disease progression in heterozygotes (9). While the CCR5Δ32 allele occurs at a variable frequency of 4–15% in Caucasian populations, with an average of 10% in Europe ((10, 11), it is rare to find this mutation among Asian or African populations (12, 13). However, previous investigations have reported single nucleotide polymorphisms (SNPs) within the CCR5 promoter region associated with altered CCR5 expression (14) thereby positively or negatively influencing an individual’s susceptibility and rate of disease progression to AIDS (15). Yet, despite the overwhelming evidence of CCR5 promoter polymorphisms’ influence on HIV susceptibility and disease progression, data on variations in the CCR5 promoter region among elite and viremic controllers in Uganda is not known. Among different populations where it is found, the distribution of these CCR5 promoter polymorphisms varies greatly (16-18). An example being HHC promoter haplotype that has been associated with faster disease progression among African Americans (16), although in the Thai population, this haplotype is reported with slower disease progression (19). The impact of these promoter variants on disease progression
are still unknown in Uganda. Exploring variations in this promoter region is essential to identify protective mutations among Ugandan HIV-1 elite and viremic controllers that can be associated with delayed HIV progression to AIDS. Therefore, this study determined the distribution of CCR5 and its associated promoter variants among the elite and viremic controllers in Uganda. Data generated provides insights into mechanisms that could be responsible for the different clinical HIV courses of disease seen among this study population.

2. Methods

2.1 The aim, design and setting of the study

The study aimed at identifying and characterizing genetic variations within the CCR5 promoter region that may be responsible for the delayed progression of HIV among elite and viremic controllers in Uganda. This was a laboratory-based cross-sectional study leveraging peripheral blood mononuclear cells (PBMC) samples collected by the Elite study between 2016 and 2018. The Elite study focused on the role of host genes in T-cell resistance among elite and viremic controllers in Uganda. Cryopreserved PBMC samples collected from ART naïve HIV infected individuals followed for a duration greater than 5 years were used in this study. Study participants were enrolled from Makerere University Joint AIDS Program (MJAP), Mulago ISS clinic.

The Immunology assays were conducted at Makerere University, College of Health Sciences Immunology Laboratories while the molecular Biology assays were conducted at the Center For AIDS Research (CFAR) laboratory, Joint Clinical Research Center in Kampala, Uganda.
2.2 Patient Characteristics

PBMCs were collected from three (3) patient groups, namely; a) elite controllers (undetectable viral load with > 5 years in care without ART), b) viremic controllers (viral load between 50 and 2000 viral RNA (vRNA) copies ml\(^{-1}\)) and c) non-controllers (HIV infected individuals well controlled on ART).

Elite and Viremic controllers were recruited basing on the following attributes; ART naïve, maintained their CD4 count ≥ 500 cells/µl, and the two controller phenotypes were differentiated by their viral loads with elite controllers having HIV plasma viral load of < 50 viral RNA (vRNA) copies ml\(^{-1}\) while viremic controllers maintained a viral load between 50 -2000 viral RNA (vRNA) copies ml\(^{-1}\) for a period, greater than 5 years. At enrollment, patients provided a peripheral blood sample, and HIV Viral Load was determined by qRT-PCR using Abbott Real Time HIV-1 assay (Abbott Molecular, USA). The time interval between initial viral load and enrollment viral load was determined and recorded in months. To confirm controller (elite/viremic controller) status, a follow-up VL was performed and the time interval between baseline and follow-up VL was also calculated. Any individuals with a hemoglobin of < 10g/dl and active opportunistic infection were excluded.

2.3 Laboratory Methods

2.3.1 Treatment of PBMCs before storage

PBMCs were isolated using the Ficoll gradient centrifugation method. The PBMCs were then washed with PBS and centrifuged at 1700 rpm for 5 min. The supernatant was discarded, and the pellet was re-suspended in 40 ml PBS. The cells were then washed twice before the cells were stained with trypan blue and counted using an automatic cell counter (Invitrogen, Carlsbad,
California, USA). The cells with viability ≥ 95% were prepared for storage by re-suspending them in 1 ml of freeze media, and each sample aliquoted and stored in 2 cryo-vials. The cryovials were immediately placed in Mr. Frosty storage container (Thermo Fisher Scientific, Waltham, Massachusetts, USA), and later stored overnight in a freezer at −80 °C. The cryovials were then transferred to liquid nitrogen for storage.

2.3.2 PBMC Thawing:
Cryopreserved PBMC samples were retrieved from liquid nitrogen at -196°C and immediately transferred to a preset 37°C water bath. Upon thawing, cells were washed with R10 media composed of RPMI 1640 medium (ThermoFisher Scientific, South America, catalogue no. 11875093), 1% Pen-Strep, 1% L-Glutamine, 1% Heps buffer and 10% Fetal Bovine Serum (ThermoFisher Scientific, South America, catalogue no. 10270106) in a 15 ml centrifuge tube. We then determined cell yield where viability testing was done using Trypan blue solution. Cells were stained using 0.4% trypan blue solution at 1:1 dilution ratio. Samples with at least 80% viability were considered for CD4+ T cell isolation. A portion of cells harvested off in R10 media were used for DNA extraction and the rest for CD4+ T cell isolation.

2.3.3 CD4+ T cells Isolation
Following thawing, CD4+ T cell were isolated using the EasySep™ Human Isolation Kit (Stem Cell Technologies, Catalogue no. 19052). The stem cell Isolation protocol was followed. But briefly, cells were centrifuged at 1500rpm for 10 minutes, decanted and the pellet re-suspended in 1ml of 2% FBS containing 0.5% EDTA. The samples were transferred into FACs tubes from where 50μl of the enrichment cocktail were added and then incubated at room temperature for 10 minutes. Thereafter, 100 μl of the magnetic beads were added and the sample incubated at room temperature for 5 minutes. The sample tube (lid removed) was then placed in the EasySep magnet and
incubated at room temperature for 5 minutes. In one continuous motion, the sample (isolated CD4+ T cells) was poured into a second tube after the 5 minutes’ incubation. The isolated CD4+ T cells were washed in 1ml PBS, centrifuged at 1500rpm for 10 minutes. These were re-suspended in 2ml R-10 media, stained for counting with trypan blue and then incubated at 37°C on a 24 well plate for 2 hours in a CO₂ incubator. The cells were also stained for purity using anti-CD3, and anti-CD4 and ran on a BD FACS Canto II (BD Biosciences, Franklin lakes, New Jersey, USA). Samples with an average purity of 98% and above determined after staining for flow cytometry were considered for stimulation. Prior to stimulation, the cells were rested in a 24-well-plate at 37°C in a CO₂ incubator.

2.3.4 CD4+ T cell Stimulation

We prepared stimulatory antibodies; Anti-CD3 (eBioscience Clone CD28.2) and anti-CD28 (eBioscience clone OKT3) at a concentration of 5µg/ml each. A clearly mapped out 96-well plate was used. The plate was coated by adding 100 µl of anti-CD3 at a concentration of 5µg/ml and incubated at 37°C in a CO₂ incubator for 2 hours. After incubation, the plate was blotted and 100,000 cells in 90 µl per sample were added. Using a pipette, 110 µl anti-CD28 was added to each well to make a total final volume of 200 µl at a concentration of 5µg/ml. For the negative control wells, 110µl of PBS was added to make volume of 200 µl per well. The plate was incubated for a total of 48 hours at 37°C in a CO₂ incubator. After incubation, cells were washed with 200 µL staining buffer per well and then transferred to the 5 mm round bottomed polystyrene FACS tubes.

2.3.5 Cell Surface Staining

Subsequently, cells were surface stained and incubated for 30 minutes with the following monoclonal antibodies; CD3^Percp cy5.5, CD4^APC, CCR5^PE, and Zombie Aqua (BD bioscience, San
Jose, CA, USA) (Table 1). The cells were acquired on an eight-color FACS CANTO II (BD Biosciences, San Jose, CA, USA). At least 50,000 events were recorded for analysis. Gating was standardized and set using fluorescence minus one controls (FMOS). Data obtained were analyzed using FlowJo version 10.1 (San Carlos, CA, USA) and GraphPad Prism 7.0 (GraphPad Software Inc., La Jolla, CA, USA).

**Table 1.** Cell surface markers used as parameters to define CCR5 expressing T cell phenotypes

| Cell Marker | Phenotype Function          |
|-------------|----------------------------|
| CD3         | T cell lineage marker      |
| CD4         | CD4+ T lineage             |
| CCR5        | Chemokine receptor         |

2.3.6 DNA Extraction

DNA was extracted using the QIAamp DNA mini Kit (Qiagen, Inc., Valencia, CA, USA) in accordance with the manufacturer's instructions as used in the previous studies (20). 200μl of sample containing 2x10^6 cells was added to micro-centrifuge tube together with 20μl of Qiagen protease. 200μl of buffer AL was added to the sample which was then mixed thoroughly to ensure efficient lysis and then incubated at 56^0C for 10 minutes. 200μl of ethanol was added to the sample and then mixed by pulse vortexing. After vortexing, the mixture was added to spin column (in a 2ml collection tube) and centrifuged at 8000rpm for 1 minute. The mini spin column was later placed into a clean 2 ml collection tube. The extracted DNA was washed using AW1 and AW2 and spun at 8000 rpm for 1 minute and 14000 for 3 minutes respectively. The empty column was spin to prevent possible buffer AW2 carry over and later DNA was eluted using AE buffer into a new 1.5ml micro-centrifuge tube.

2.3.7 PCR and Sequencing
PCR amplification of CCR5 promoter region was carried out using the following cycling conditions; Initial denaturation at 95°C for 3 minutes; 31 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 68°C for 2.40 minutes; followed by 68°C for 7 min. The PCR master mix contained High fidelity Super script III platinum Taq polymerase (Invitrogen, Carlsbad, CA, USA) in the presence of 2X reaction buffer, 5Mm MgCl2 with primers shown in table 2 developed using GenBank sequence with accession number U95626. as described in a similar study (21). The promote amplicon size was 2189 base pairs (21).

**Table 2. Primers used in the amplification of Promoter 1 region of the CCR5 gene**

| Primer | Binding position | Amplicon size | Annealing temperature |
|-------|-----------------|---------------|-----------------------|
| Forward | 5′CCAAGCACCAGCAATTAGC3′ | 58105 – 58122 | 2189 | 60°C |
| Reverse | 5′TGCCACCACAGATGAATGTC3′ | 60293 – 60274 | 60°C |

**2.3.8 PCR clean up**

From all samples that gave a single band after Gel electrophoresis, 10μl was aliquoted and added into a PCR tube followed by 2μl of ExoSAP IT. The samples were transferred into a thermocycler (Applied Biosystems, California, United States) and ran under the conditions: 37°C for 45 seconds, 800C for 45 seconds (inactivate ExoSAP-IT) and held at 4°C.

**2.3.9 Cycle sequencing**

Sequencing was performed using an ABI version 3.1 BigDye Kit (Applied Biosystems, Catalogue no. 4337456) and ABI3500xl Genetic Analyzer. Briefly, a master mix was prepared as follows; 0.5μl Big Dye terminator, 1.75μl 5X sequencing buffer, 2.5 μl primer as shown in the primer map (Fig 1) and primer sequences are shown in table 3 below (18) and 4.25μl water. 9μl of sequencing master mix was added into each well where 1μl of DNA was added.
Briefly, PCR amplifications was subjected to thermal cycling as follows: 96°C for 1 minute; 30 cycles of denaturation at 96°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 68°C for 2.40 minutes; followed by 68°C for 7 min.

Table 3 Primers used in sequencing of the CCR5 promoter 1 region

| Primer | Binding position |
|--------|------------------|
| Forward F 5’CCAAGCACCAGCAATTAGC3’ | 58105 – 58122 |
| Reverse R 5’TGCCACCACAGATGAATGTC3’ | 60293 – 60274 |
| Forward IFS 5’TTGCTGTGGGGTCT3’ | 58471 – 58486 |
| Forward F1 5’GAGTGGGAGAAAAGGGG3’ | 59013 – 59030 |
| Reverse R1 3’AGAATAGATCTCTGGTGAAAA5’ | 59375 – 59354 |

Fig 1. The CCR5 promoter primer map for the primers used in sanger sequencing.

2.3.10 Data Analysis

Flow cytometry data were analyzed using FlowJo version 10.5.2 software. CD4+ T cells were distinguished by their surface expression of CD3 and CD4. CD4+ T cells, we identified CCR5+ T cells and determined both the percentage CD4+CCR5+ T cells and CCR5+ MFI (to ascertain the CCR5 density on CD4+ T cells). Statistical analysis was performed using GraphPad Prism 7. The Mann Whitney and Kruskal Wallis tests for non-parametric variables facilitated comparison of differences among groups. P values < 0.05 indicated a significant difference.

Sanger Sequence data analysis was performed using mutation surveyor Mutation version 5.5 (SoftGenetics; Pennsylvania, USA). U95626 and NT_022517 reference sequences were used in assembly as used in other studies (18). A search of the GenBank NCBI SNP database (dbSNP) determined whether polymorphisms detected in this study had been previously reported. The
CCR5 numbering system was used where the first nucleotide of the translational start site is designated as +1 and the nucleotide immediately upstream from that is −1 (22).

3. Results

3.1 Participant characteristics

This was a cross-sectional study conducted among 30 HIV-1 chronically infected individuals. These included 14 elite controllers [HIV plasma viral load < 50 viral RNA (vRNA) copies ml$^{-1}$], 9 viremic controllers [HIV plasma viral load between 50 -2000 viral RNA (vRNA) copies ml$^{-1}$] and 7 non-controllers (ART controlled) whose demographic characteristics are summarized in Table 4.
Table 4: Characteristics of elite, viremic and non-controllers derived per group (n=30)

| Participant | Age | Sex | CD4 count | Duration in Care (Years) | Viral Load | Months between VLs | ¹BMI |
|-------------|-----|-----|-----------|--------------------------|------------|---------------------|------|
| Elite Controllers |     |     |           |                          |            |                     |      |
| 1           | 53  | F   | 1245      | 10                       | UD         | 8                   | 33.9 |
| 3           | 32  | F   | 1008      | 5                        | UD         | 9                   | 31.8 |
| 4           | 38  | F   | 919       | 9                        | UD         | 12                  | 18.9 |
| 7           | 36  | F   | 1188      | 7                        | UD         | 8                   | 38.5 |
| 13          | 56  | M   | 833       | 7                        | UD         | 9                   | 17.2 |
| 36          | 41  | M   | 778       | 9                        | UD         | 12                  | 25.2 |
| 37          | 37  | F   | 1063      | 6                        | UD         | 8                   | 26.1 |
| 40          | 45  | F   | 1036      | 6                        | UD         | 11                  | 25.6 |
| 42          | 28  | F   | 653       | 8                        | UD         | 9                   | 23.5 |
| 15          | 42  | F   | 909       | 5                        | UD         | 12                  | 31.8 |
| 16          | 30  | F   | 1050      | 5                        | UD         | 10                  | 29.3 |
| 21          | 37  | F   | 728       | 6                        | UD         | 9                   | 23.9 |
| 6           | 38  | F   | 1162      | 6                        | UD         | 8                   | 25.3 |
| 22          | 39  | F   | 650       | 9                        | UD         | 5                   | 30.7 |
| Viremic Controllers |     |     |           |                          |            |                     |      |
| 24          | 32  | F   | 698       | 5                        | 280        | 14                  | 30   |
| 26          | 56  | F   | 652       | 8                        | 1380       | 8                   | 25.3 |
| 5           | 51  | F   | 895       | 10                       | 1299       | 10                  | 29.5 |
| 12          | 37  | F   | 805       | 5                        | 155        | 15                  | 24.5 |
| 38          | 40  | M   | 732       | 6                        | 388        | 9                   | 28.9 |
| 31          | 41  | F   | 852       | 10                       | 285        | 7                   | 30.2 |
| 20          | 56  | M   | 897       | 11                       | 1220       | 14                  | 25.6 |
| 32          | 37  | F   | 772       | 10                       | 243        | 9                   | 29   |
| 14          | 54  | F   | 669       | 8                        | 782        | 8                   | 25.3 |
| Non controllers |     |     |           |                          |            |                     |      |
| NC28        | 38  | F   | 589       | 5                        | 10500      | 6                   | 21.4 |
| NC30        | 42  | F   | 1021      | 8                        | 2840       | 10                  | 21.3 |
| NC 2        | 40  | M   | 920       | 6                        | 10800      | 15                  | 27.2 |
| NC 11       | 40  | F   | 940       | 5                        | 14800      | 8                   | 19.1 |
| NC 18       | 29  | F   | 747       | 5                        | 2310       | 8                   | 25.3 |
| NC025       | 43  | F   | 781       | 8                        | 5250       | 10                  | 32.7 |
| NC 8        | 41  | F   | 1192      | 6                        | 2840       | 6                   | 37.5 |

¹BMI denotes body mass index
UD denotes undetectable;
3.2 CCR5 expression on CD4+ T cells among elite, viremic and non-controllers

Because CCR5 is expressed only on activated CD4+ T cells (23, 24), we activated CD4+ T cells in vitro using Anti-CD3 (eBioscience Clone CD28.2) and anti-CD28 (eBioscience clone OKT3). We then performed flow cytometry to study CCR5 expression. Flow cytometry data was analyzed using Flow Jo and the gating strategy used to determine CCR5 expression (Fig. 2A).

3.3 Percentage CCR5+CD4+ T cells and their CCR5 densities among elite, viremic and non-controllers

Considerable evidence suggests that CCR5+CD4 T cells are needed during early stages of HIV infection (25, 26). To elucidate the distribution of CCR5+CD4 T cells among elite, viremic and non-controllers, we stimulated CD4 T cells from participants with Anti-CD3 (eBioscience Clone CD28.2) and anti-CD28 (eBioscience clone OKT3). We later carried out flow cytometry to ascertain the percentage of CCR5+CD4 T cells. The Kruskal–Wallis test was used to compare differences in the percentage of CCR5 expressing CD4+ T cells among elite, viremic and non-controllers. $p$ values $< 0.05$ were considered significant. We found that elite and viremic controllers had lower percentage of CCR5+CD4+ T cells compared to non-controllers, although the differences were not statistically significant; elite controllers and viremic controllers ($P=0.9173$), viremic controllers and non-controllers ($P=0.0702$), elite controllers and non-controllers ($p=0.6010$) (Fig 2B). These results suggest that the percentage of CCR5+CD4 T cells have no statistical contribution to progression to AIDS among elite and viremic controllers.

However, because CCR5 densities are independent of the percentage of CCR5+CD4 T cells and they have been associated with high viral loads (27-29), we carried out experiments to ascertain whether elite, viremic and non-controllers have differences in their CCR5 densities. Cells were stimulated and flow cytometry was carried out. The Mann Whitney test was used to compare
differences in MFI of CCR5-expressing CD4+ among elite, viremic and non-controllers. $p$ values < 0.05 were considered significant. We found significant variation in the Medium Fluorescent Intensity (MFI) of CCR5-expressing CD4+T cells between controllers (elite and viremic controllers) and the non-controllers; elite and non-controllers ($P=0.0210$), viremic and non-controllers ($P=0.0312$) (Fig 2C). However, there was no statistically significant difference in the MFI of CCR5-expressing CD4+ T-lymphocytes between viremic and elite controllers ($P=0.3048$) (Fig 2C).

**Fig 2. CCR5 expression on CD4+ T cells:** A) A sequential gating strategy used to analyze CCR5 expression on CD4+ T cells. We first gated on the lymphocytes using a forward scatter-area (FSC-A) against side scatter-area (SSC-A) gate. (2) We excluded doublets with a singlet gate by gating on forward scatter-area (FSC-A) against forward scatter-height (FSC-H). (3) Live cells were selected by gating on scatter-height (FSC-H) against ARM Cyan (Live-dead marker). (4) Conventional T cells were selected by gating on CD3+ cells from the total lymphocyte population, from which (5) CD4+ T cells were selected. (6) From CD4+ T cells, CCR5+ T cells were selected. B) **Percentage of CCR5+CD4+ T cells** is higher among elite controllers (n=14), viremic controllers (n=9) compared to non-controllers (n=7) although the difference is not statistically significant; EC and VC ($P=0.6010$), EC and NC ($P=0.9156$), VC and NC ($P=0.0702$). C) **Differences in CCR5 densities** among EC, VC and NC is statistically significant (between EC and VC; $P=0.3048$, EC and NC; $P=0.0210$, VC and NC; $P=0.0312$).

**3.4 SNPs identified within CCR5 promoter region in this cohort**

The rare occurrence of delta 32 bp deletion within Africa (12), has led to a number of studies to explore additional CCR5 regions for possible causes of the phenotypes seen among African ART naïve individuals who have the capacity to control HIV (13). Studies have reported several CCR5 promoter polymorphisms associated with either reduced or increased CCR5 expression among different cohorts in Africa (13, 15). Controversies have arisen where some mutations are protective in some regions and detrimental in others, thus this study was set out to explore which CCR5 promoter variants are associated with the different phenotypes in this study. We used previously
stored PBMCs which were thawed and then DNA extracted using Qiagen Blood Genomic DNA Kit (QIAamp DNA kit; Qiagen, Inc., Valencia, California, USA). The DNA was PCR amplified and then sequenced.

We found that rs1799987 single nucleotide polymorphisms (SNPs) were predominant among elite controllers and viremic controllers (71% and 61% respectively) while rs41469351 was more among non-controllers (68%). Furthermore, we also identified two Novel mutations; 1070 T>G (14.3%) and 785 A>G (14.3%) among elite controllers (Table 5).

**Table 5**: CCR5 promoter SNPs among elite, viremic and non-controllers

| SNP   | Chromosome position | dbNo.    | Percentage of Occurrence (%) |
|-------|---------------------|----------|------------------------------|
| Elite controllers (n=14) |                     |          |                              |
| -2459A>G | chr3:46370444 | rs1799987 | 71                            |
| 1017C>T | chr3:46370658 | rs142710698 | 7                             |
| 1070 T>G | Chr3:46370711 | Novel | 14.3                           |
| 785 A>G | Chr3:46370426 | Novel | 14.3                           |
| Viremic controllers (n=9) |                     |          |                              |
| -2459A>G | chr3:46370444 | rs1799987 | 61                            |
| -2554G>T | chr3:46370349 | rs2734648 | 56                            |
| Non controllers (n=7) |                     |          |                              |
| -2132C>T | chr3:46370771 | rs41469351 | 68                            |
| -1835C>T | chr3:46371068 | rs1800024 | 53                            |
| -2733A>G | chr3:46370170 | rs2856758 | 29                            |

4. **DISCUSSION**

Expression of CCR5 on CD4+ T cells has been shown to be highly variable between individuals (30). *In vitro* studies have shown that this variability affects HIV Infectivity in cell lines (31), macrophages (32), and lymphocytes (33). In our study, the number of CCR5+CD4 T cells and
CCR5 densities on the surface of CD4 T cells vary among elite, viremic and non-controllers and this could explain the different phenotypes among these populations. This is supported by Reynes et al. who demonstrated that CCR5 expression affects virus production and viral load, and individuals with a low viral load have reduced CCR5 densities on the CD4+ T cell surface. (27, 28)

In this study, we found no statistical difference in the reported percentage of CD4+CCR5+ T cells between elite, viremic and non-controllers. However, elite and viremic controllers had lower percentage of CD4+CCR5+ T cells compared to non-controllers. The reduction in CD4+CCR5+ T cells is in agreement with the findings by Potter et al who showed lower expression of CCR5 CD4+ T lymphocytes in HIV controllers (28, 34). This reduction may contribute to the low levels of infection in elite and viremic controllers since CCR5 expressing CD4+ T cells are required during initial stages of HIV infection by the R5 tropic virus (35).

Of significance was the finding that showed that elite and viremic controllers had statistically significant reduction in CCR5 densities compared to non-controllers. This shows that even though there was no statistical significance in the percentage of the CCR5+CD4 T cells, there were differences in the number of CCR5 expressed on the CD4+ T cell surfaces. These results agree with findings by Reynes et al. who reported data supporting the hypothesis that the rate of evolution of HIV-1 disease in an individual is influenced by the median number of CCR5 co-receptors at the surface of the CD4 T cells of the individual. They also demonstrated that CCR5 expression affects virus production and viral load, and individuals with a low viral load have CCR5 densities below the threshold value (27). The low expression of CCR5 on CD4+ T cells could explain why the elite and viremic controllers have the capacity to control the R5 tropic HIV virus since they have reduced CCR5 expression on the surface of their CD4+ T cells.
In the present study we report a high frequency of rs1799987 A>G mutation among elite and viremic controllers. Joshi et al, in an *in vitro* study demonstrated that rs1799987 A>G was associated with reduced expression of CCR5 on the 293 T cell lines - which were transfected with plasmid DNA containing this specific CCR5 gene promoter variant (36). These findings are in agreement with findings from studies by McDermott and Mehlotra who argued that this mutation was associated with reduced CCR5 expression (37, 38). Taken together, these findings could mean that rs1799987 SNP confers protection against HIV disease progression and may contribute to low HIV susceptibility, explaining its occurrence in the HIV controllers. Furthermore, -2459 A/A was noted to be enriched among HIV-1 non-controllers. Joshi et al in an individual SNP analysis *in vitro* study, also demonstrated that -2459 A/A was associated with increased CCR5 expression in individuals with the mutation compared to those without. These findings point to the potential role of -2459 A/A in CCR5 expression among Non-controllers. Still in the current study, we found rs41469351 C>T SNP in the promoter region of CCR5 gene which was highly concentrated among HIV-1 non-controllers (57%). Similar to our findings, Kostrikis LG et al who reported the -2132C/T SNP to be associated with higher viral loads and higher CCR5 densities on CD4 T cell in a cohort that was aimed at studying perinatal transmission (39). Our findings and those of previous researchers (40-42) could imply that -2132C/T increases HIV disease progression by facilitating increased CCR5 densities on CD4+ T cells among NCs thus increased viral replication. The roles of the other SNPs (1017C>T, -2554G>T, -1835C>T and -2733A>G) found in this present study in HIV disease progression have not been reported yet and as such require *in vitro* studies to elucidate their effect on CCR5 expression on the surface of CD4+ t cells.

This study’s limitations included a limited sample size due to the low occurrence of elite and viremic controllers in the general population. Furthermore, the Test and treat policy rolled out by
the World Health Organization in 2016, where all individuals that test positive for HIV are enrolled on HAART, made it impossible to identify and recruit more controllers.

Additionally, this study didn’t consider other factors for example the Human Leukocyte Antigen which might have a contributory protection role against HIV disease progression to AIDS among elite and viremic controllers (43, 44).

5. Conclusion

In summary, our study has confirmed the presence of SNPs which have been previously associated with either delaying or increasing HIV progression to AIDS. Rs1799987 A>G mutation identified among HIV-1 elite and viremic controllers was associated with reduced CCR5 density on CD4+ T cells as compared to non-controllers, while -2459 A/A and rs41469351 C>T SNP identified among non-controllers were associated with increased CCR5 density on CD4+ T cells as compared to controllers. This suggests that the high frequency of Rs1799987 A>G mutation among elite and viremic controllers may be associated with delayed HIV progression, while the high frequency of -2459 A/A and rs41469351 C>T SNP among non-controllers may facilitate HIV progression. Additionally, the study identified two novel mutations among elite controllers - 1070 T>G and 785 A>G, however, in vitro studies are needed to study their effect on CCR5 expression.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Abbreviations

ART: Antiretroviral Treatment; ORF: Open reading frame; CCR5: C-C chemokine receptor type 5; HIV: Human Immunodeficiency Virus; AIDS: Acquired Immunodeficiency Virus; SNPs: Single Nucleotide polymorphisms; MJAP: Makerere University Joint Aids Program; MFI: mean fluorescence intensity; DNA: Deoxyribonucleic acid; PCR: Polymerase Chain reaction; PBMCs:
Peripheral Blood Mononuclear cells; UNCST: Uganda National Council of Science and Technology; LTNPs : Long Term Non Progressors.

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Confidentiality

No patient identifiers were attached to patient data. All data was password protected, stored in fully encrypted databases and accessible only to research assistants and investigators responsible for analysis.

Contributions

Conceptualization, NB and OJS; Methodology, NB, SBA, AB, DK, JK, EN, JM, FW and OJS; Investigation, Data curation, Visualization and Project management were performed by NB; Writing—Original Draft, NB; Writing—Review and Editing, NB, SBA, OJS, DPK, RN, AK and
IN; Funding Acquisition, DPK, and OJS; Resources, IN, RN, OJS and SBA; Supervision, DPK, and OJS. All authors read and approved the final manuscript.

**Ethics declarations**

**Ethics approval and consent to participate**

The parent study was approved by the Makerere University School of Biomedical Sciences Higher Degree Research and Ethics Committee (SBS-HDREC, study no. SBS-372) and the Uganda National Council of Science and Technology (UNCST, study no. HS 2169).

For this study, we obtained a waiver of consent from the Institutional Review Board of the School of Biomedical Sciences, Makerere University to use the stored samples collected from the approved parent study (SBS-605).

**Consent for publication**

Not applicable.

**Conflict of interest:**

The authors declare no conflict of interest.
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