

CCR5 Promoter SNP Genotype in HIV Seropositive Infants on Combination Antiretroviral Therapy in Uganda: Association with Virological Failure

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Abstract

According to the UNAIDS 2022 report, 52% of the HIV positive children between 0-14 years were able to access treatment and of these 68% attain viral suppression. CCR5 promoter genotype specifically single nucleotide polymorphisms have been linked to modulate patient virological status. However, the few studies that have studied the association in infants have utilized allele-specific PCR a genotypic method limited to detecting already known SNPs. By using Sanger sequencing, we explored the association taking into account novel mutations.

Methods

Following ethical approvals, a cross sectional study was conducted using archived buffy coat samples from a pediatric HIV drug resistance study between May 2019 and January 2022 at the Joint Clinical Research Centre in Kampala, Uganda. 100 HIV seropositive infant buffy coat samples were sequenced for CCR5 SNP genotype following nucleic acid extraction, polymerase chain reaction and sanger sequencing. Odds ratios were used to determine the association between CCR5 SNPs and infant HIV plasma loads.

Results

10 SNPs were identified with frequencies as follows; 58227G/A (21.8%), 58636A/G (28.7%), 58934T/G (74.7%), 59029G/A (48.3%), 59353T/C (55.2%), 59356C/T (23.0%), 59402G/A (94.2%) 59653C/T (66.7%), 59802A/C (34.5%), and 60024A/G (34.5%). Notably, 58227G/A, 58636A/G, 59802A/C, and 60024A/G are novel SNPs. However, none of these SNPs was significantly associated with infant virological failure but SNPs 59029G/A and 59353T/C showed higher odds of occurring in non-suppressors whereas 59356C/T and 59402G/A appeared to correlate with reduced plasma HIV loads. Infant virological suppression remained at 59% and in agreement with the national data.

Conclusion

Our study augments previous studies that CCR5 promoter SNPs play a role in modulating patient virological status however, strong conclusions could be drawn from either utilizing in-vitro studies or large epidemiological studies.

Keywords: CCR5 promoter; Virological failure; HIV

List of Abbreviations

AIDS: Acquired Immune Deficiency Syndrome; ARMS-PCR: Amplification Refractory Mutation System Polymerase Chain Reaction; CART : Combination Antiretroviral therapy; CCR5: Chemokine receptor 5; CD4: Cluster of differentiation 4; CFAR: Centre for AIDS Research; DNA: Deoxyribonucleic acid; DRIBS: HIV Drug resistance babies' study; EID: Early Infant Diagnosis; EMTCT: Elimination of Mother to Child Transmission; HIV: Human Immunodeficiency Virus; HLA: Human Leukocyte Antigen; IRB: Institutional Review Board; JCRC: Joint Clinical Research Centre; MTCT: Mother-to-child transmission; MUAC: Mid-Upper Arm Circumference; NGS: Next-generation sequencing; PCR: Polymerase Chain Reaction; RNA: Ribonucleic acid; SNP: Single Nucleotide Polymorphism; UNCST: Uganda National Council for Science and Technology; WHO: World Health Organisation

Introduction

An estimated 37.7 million people were living with HIV worldwide in 2020 (1). Of these, 1.7 million were children aged 0-14 years. Mother-to-child transmission (MTCT) of HIV was estimated to have resulted in over 150,000 infant cases in 2015 with approximately 90% of the cases occurring in sub-Saharan Africa (2). In Uganda where 11% of the 1.6 million individuals living with HIV are children, despite the scale-up of early infant diagnosis (EID) and initiation of combination antiretroviral therapy (cART) programs to HIV infected children, approximately 40.7% of all children under 5 years still have detectable plasma HIV RNA loads greater than 1000 copies/mL after six months of cART initiation (3). Poor adherence to ART, HIV drug resistance, and host immunological and genetic make-up are the major factors cited in modulating patients' virological status (4-6). The impact of host genetic variation on HIV susceptibility and infection was first reported early in the pandemic with a major role attributed to genes encoding class I human leukocyte antigens (HLA) and the CCR5 (7, 8). The latter being the major co-receptor for HIV infection, several studies have reported that single nucleotide polymorphisms (SNPs) within its promoter region might influence susceptibility to HIV-1 infection and progression to AIDS in different hosts mainly by changing promoter activity and transcription factor binding thus modifying gene expression and consequently affecting cell surface expression of CCR5 (7). However, most studies have been conducted outside sub-Saharan Africa whereas the few conducted among paediatrics have used allele specific PCR or amplification refractory mutation system (ARMS-PCR) for SNP typing, a genotypic method limited to detect already known SNPs (9, 10). Using Sanger sequencing, we have sequenced the entire human CCR5 promoter region scanning for SNPs that could be associated with virological failure among infants on cART in Uganda.

Methods**Study Design and Settings**

A cross sectional study nested in a parent ongoing study HIV Drug resistance babies' study (DRIBS) was conducted between May 2019 and January 2022 to determine the association of CCR5 promoter genotype and virological failure among infants on cART. The study was conducted at the Centre for AIDS Research Laboratory at the Joint Clinical Research Centre (CFAR/JCRC) in Lubowa, Kampala, Uganda.

Study Population and Sample Size

Frozen buffy coat samples under DRIBS study were retrieved from the -80°C freezer and genomic DNA was extracted using QIAamp DNA mini kit (Qiagen) spin-column-based protocol according to the manufacturer's instructions. Genomic DNA was then PCR amplified using one-step PCR amplification Superscript III One-step PCR kit with Platinum High Fidelity Taq (Invitrogen,

ThermoFisher). Primers specific to the regions of interest were either designed using PerlPrimer software version 1.21 or primer sequence obtained from previous studies (11) then blasted in NCBI nucleotide blast to align to the human CCR5 promoter genes (**Supplementary table 1**). A conventional PCR was performed to amplify the region spanning nucleotides 58105 – 60274 on the short arm of chromosome 3 known to host the CCR5 promoter. A 12.5µl PCR total volume comprising of 0.25µl primers (25pmol/ul), 6.25ul 2X SuperScript III reaction buffer, 0.5ul MgSO₄, 0.25ul SuperScript III enzyme mix, and 2.5ul of DNA extracts was used. PCR cycling conditions were set as follows: 95°C for 3 minutes initial denaturation; 30 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 68°C for 2 minutes and 40 seconds; then a final extension at 68°C for 7 min and a 4°C hold. Finally, PCR amplicons were evaluated qualitatively by visualizing through agarose gel electrophoresis system using cyber safe nucleic acid staining, as per the laboratory Standard Operating Procedures (**Supplementary figure 1**). A 9 µl cycle sequencing master mix was prepared with a total of 4 primers using BigDye Terminator v3.1 kit. A 2.18kb target region was sequenced. The four primer sequence fragments (Supplementary table 1) per sample were then trimmed, base called and contigs aligned to make a one consensus sequence that was analysed for Single Nucleotide Polymorphisms (SNPs) by mapping it to the reference sequence (GenBank accession No: U95626.1) using SEQUENCHER software version 4.5.6 (Gene Codes Corporation, Ann Arbor, MI, USA).

Laboratory Procedures

Frozen buffy coat samples under DRIBS study were retrieved from the -80°C freezer and genomic DNA was extracted using QIAamp DNA mini kit (Qiagen) spin-column-based protocol according to the manufacturer's instructions. Genomic DNA was then PCR amplified using one-step PCR amplification Superscript III One-step PCR kit with Platinum High Fidelity Taq (Invitrogen, ThermoFisher). Primers specific to the regions of interest were either designed using PerlPrimer software version 1.21 or primer sequence obtained from previous studies (11) then blasted in NCBI nucleotide blast to align to the human CCR5 promoter genes (**Supplementary table 1**). A conventional PCR was performed to amplify the region spanning nucleotides 58105 – 60274 on the short arm of chromosome 3 known to host the CCR5 promoter. A 12.5µl PCR total volume comprising of 0.25µl primers (25pmol/ul), 6.25ul 2X SuperScript III reaction buffer, 0.5ul MgSO₄, 0.25ul SuperScript III enzyme mix, and 2.5ul of DNA extracts was used. PCR cycling conditions were set as follows: 95°C for 3 minutes initial denaturation; 30 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 68°C for 2 minutes and 40 seconds; then a final extension at 68°C for 7 min and a 4°C hold. Finally, PCR amplicons were evaluated qualitatively by visualizing through agarose gel electrophoresis system using cyber safe nucleic acid staining, as per the laboratory Standard Operating Procedures (**Supplementary figure 1**). A 9 µl cycle sequencing master mix was prepared with a total of 4 primers using BigDye Terminator v3.1 kit. A 2.18kb target region was sequenced. The four primer sequence fragments (Supplementary table 1) per sample were then trimmed, base called and contigs aligned to make a one consensus sequence that was analysed for Single Nucleotide Polymorphisms (SNPs) by mapping it to the reference sequence (GenBank accession No: U95626.1) using SEQUENCHER software version 4.5.6 (Gene Codes Corporation, Ann Arbor, MI, USA).

Results

Infant Baseline Characteristics and CCR5 Promoter Frequencies

It is important to note that the parent study experienced slow participant accrual, occasional participant visit misses and loss to follow-up translating into missed study data which was largely attributed to the COVID-19 lockdown measures that made movement extremely difficult for both participants and healthcare workers between 2020 and 2021. A total of 100 babies (males $n=40$) were studied. To augment our hypothesis, we collected data on more outcome variables other than viral load including infant weight, Mid-upper arm circumference (MUAC) for nutritional assessment and CD4+ cell count. Briefly, median baseline infant age was 4 months (IQR 1, 16), weight 6.1 kg (IQR 3.9, 9.5), CD4+ count 2127.5 (IQR 447, 5011), viral load 4.5 log RNA copies/mL (IQR 3.2, 7.2). 30% of the babies were the first born while 9% of the babies were of a birth order equal to or greater than 5; 55% of the mothers were urban dwellers while 60% of the mothers had at most primary education. 79% of infants had viral load above 1000 RNA copies/mL and only 12.2% were malnourished (**table 1**). At baseline, 21% of the babies had a baseline

viral load below 1000 copies/ml. This is because in an effort to reduce mortality in HIV positive babies, the Ministry of Health recommends that all babies with a high index of suspicion to be HIV positive are initiated on CART at the time a sample for DNA PCR is taken off.

87 infants (87%) had their samples amplified and genotyped for CCR5 promoter single nucleotide polymorphisms. 10 SNPs were identified with frequencies as follows; 58227G/A (21.8%), 58636A/G (28.7%), 58934T/G (74.7%), 59029G/A (48.3%), 59353T/C (55.2%), 59356C/T (23.0%), 59402G/A (94.2%) 59653C/T (66.7%), 59802A/C (34.5%), and 60024A/G (34.5%) (**table 1**). SNP 59402G/A was observed most frequently whereas 58227G/A was least frequently typed in this population. Furthermore, in this study, we identified four novel CCR5 promoter SNPs: CCR5 (58227G/A-21.8%, 58636A/G-28.7%, 59802A/C-34.5% and 60024A/G-34.5%).

Table 1: Frequency of mutations among different CCR5 promoter polymorphisms

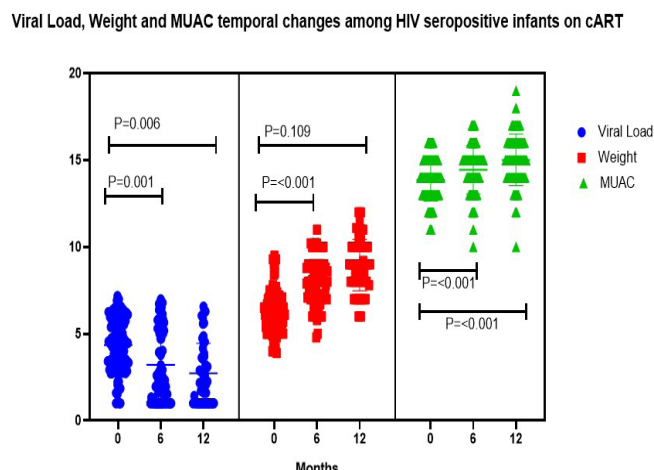
Variable	Median	IQR
Age in months	4	(1,16)
Weight in kgs	6.1	(3.9, 9.5)
CD4 count	2127.5	(447, 5011)
Mother's age	26	(19,35)
Variable	Frequency	Percent
Sex of the child		
Male	40	40
Female	60	60
Mother's education level		
No formal/primary education	60	60
Secondary/tertiary education	40	40
Mother's residence		
Urban	55	55
Rural	45	45
Nutrition assessment		
Red	3	3.3
Yellow	8	8.9
Green	79	87.8
Child birth order		
1	30	30
2 to 4	61	61
≥5	9	9
Chronic conditions in mother		
Yes	84	84
No	16	16
Viral load levels		
Suppressed	79	79
Non suppressed	21	21
59356C/T		

CC	67	77
TT	20	23
59402G/A		
GG	5	5.8
AA	82	94.2
59653C/T		
CC	58	66.7
TT	29	33.3
59802A/C		
AA	57	65.5
CC	30	34.5
60024A/G		
AA	57	65.5
GG	30	34.5
59356C/T		
CC	67	77
TT	20	23
59402G/A		
GG	5	5.8
AA	82	94.2
59653C/T		
CC	58	66.7
TT	29	33.3
59802A/C		
AA	57	65.5
CC	30	34.5
60024A/G		
AA	57	65.5
GG	30	34.5

Viral Load, Weight and MUAC Temporal Changes Among HIV Seropositive Infants on Cart

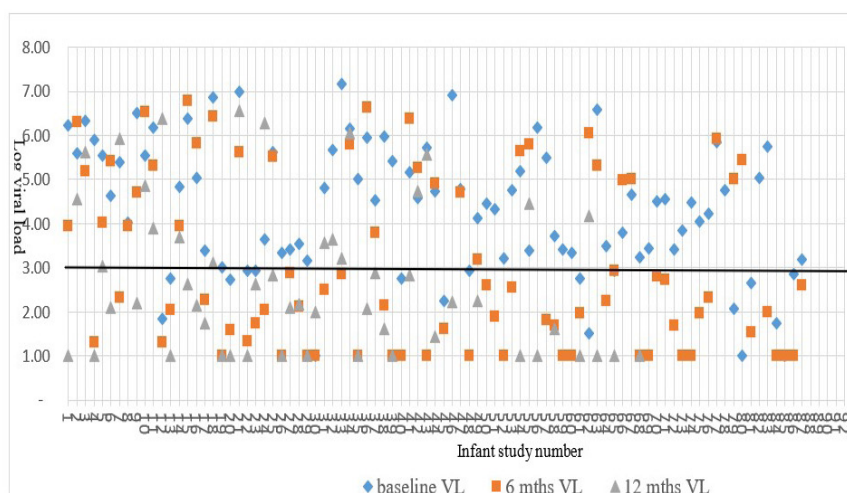
From cART initiation, infant HIV plasma load significantly reduced after 6 months (p-value=0.001) and 12 months (p-value=0.006) (**figure 1**). However, it is important to note that although viral suppression was significant from base line to six months and 12 months after initiation of cART, only 58.8% and 64.2% of the babies achieved viral suppression at month 6 and 12 after ART initiation respectively. Further analysis of the babies who attained viral suppression (less than 1000 copies/ml), revealed that about 50% of these babies harbored incompletely suppressed viruses between 50-1000 copies/ml (1.5-3 logs, **figure 2**). Also from figure 2, we see some babies who had viral load less than 1000 copies/ml at baseline (enrollment into study). This is because of the stringent Ministry of Health policy which recommends that any baby with a high index of suspicion of being HIV positive is initiated on therapy at a time a blood sample for DNA PCR is taken off for early infant diagnosis (EID). All these babies supposedly virally suppressed but harboring low level viremia lie below the bold line in **figure 2**. Interestingly, although infants gained significant weight from baseline to 6 months (p-value=<0.001), it was not the case at 12 months (p-value=0.109). Generally, infants portrayed an improved nutritional status throughout the study with significant increases in Mid-upper arm circumference from baseline to 6 months (p-value=<0.001) and 12 months (p-value=<0.001) (**Figure 1**). Weight gain as a measure of nutritional status was statistically significant at 6 months. At 12 months, although there was a gain in weight, it did not reach statistical significance (**Figure 1**).

Figure 1: Viral load, weight, and MUAC temporal changes among HIV seropositive infants on CART



Viral load measurements (blue circles), were done at baseline six months and twelve months as a way to monitor response to therapy. Indicated are log values. Weight was measured at each of the visits as a measure of nutritional status (red squares). Mid upper arm circumference was measured at each visit as an indicator of nutritional status (green triangles).

Figure 2: Infant HIV plasma load suppression rates following CART initiation



Viral load measurements were taken every six months. At baseline (blue diamonds), 6 months (orange squares) and 12 months (grey triangles). A log scale was used. According to the National Treatment guidelines, 1000 copies/ml (which translates into 3 on the log scale and is represented by a bold line that cuts across) has been set as cutoff. Between logs 1 and 3 are babies with low level viremia indicative of incomplete viral suppression.

Association of CCR5 Promoter Single Nucleotide Polymorphisms and Infant Virological Failure

None of the CCR5 promoter SNPs was significantly associated with infant virological failure at 6 months nor 12 months (Table 2). Nevertheless, presence of CCR5 58636A/G, 59029G/A, 59353T/C, 59802A/C, AND 60024A/G showed consistent higher odds of infant virological failure at both 6 and 12 months. Conversely, CCR5 58227G/A, and 59356C/T showed relatively decreased odds of infant virological failure at both time points. On the other hand, CCR5 58934T/G, 59402G/A and 59653C/T showed inconsistent odds of causing virological failure among infants at both 6 and 12 months. On analyzing other variables such as mother’s age, level of education, and residence, child’s birth order and self-reported adherence, they did not have a significant effect on viral non-suppression (Table 2). Furthermore, in this study, we identified four novel CCR5 promoter SNPs: CCR5 (58227G/A-21.8%, 58636A/G-28.7%, 59802A/C-34.5% and 60024A/G-34.5%). These are indicated in bold in table 1

Table 2: Effect of CCR5 mutations and baseline characteristics on virologic failure among infants < 2years after six and twelve months of ART

CCR5 promoter polymorphisms	RNA level>1000 copies/mL	RNA level>1000 copies/mL ²
	6 months	12 months
58227G/A	0.92(0.31-2.73)	0.50(0.12-2.14)
58636A/G	1.33(0.50-3.56)	1.45(0.45-4.66)
58934T/G	0.89(0.32-2.48)	3.70(0.72-19.10)
59029G/A	1.06(0.43-2.62)	1.51(0.48-4.69)
59353T/C	1.46(0.58-3.68)	1.43(0.45-4.54)
59356C/T	0.81(0.27-2.37)	0.43(0.10-1.82)
59402G/A	1.52(0.13-17.55)	0.56(0.03-9.54)
59653C/T	0.84(0.32-2.22)	1.06(0.31-3.59)
59802A/C	2.21(0.84-5.84)	1.45(0.45-4.66)
60024A/G	2.21(0.84-5.84)	1.45(0.45-4.66)
Mother's age		
≤26	1	1
≥27	0.55(0.23-1.32)	0.90(0.29-2.77)
Mother's education level		
No formal/primary education	1	1
Secondary/tertiary education	0.96(0.40-2.35)	0.94(0.30-3.01)
Mother's residence		
Urban	1	1
Rural	1.59(0.66-3.80)	0.74(0.23-2.34)
Child birth order		
1	1	1
2 to 4	1.29(0.49-3.39)	2.14(0.56-8.17)
≥5	0.21(0.02-2.02)	0.55(0.05-6.27)
Chronic conditions in mother		
Yes	1	1
No	2.54(0.81-7.95)	0.87(0.22-3.37)
Adherence		
Good (≥ 95%)	1	1
Poor(< 95%)	0.60(0.16-2.26)	2.40(0.25-23.18)

Discussion

In this study, we found no significant association between CCR5 SNP genotype and infant virological failure. This is similar to two early Kenyan studies by Katz et al 2008, and John et al 2001 who found no significant differences in plasma HIV loads by CCR5 promoter genotype among pregnant and breast feeding mothers. The relationship between CCR5 promoter polymorphisms and HIV disease progression has been extensively studied and sound associations between certain CCR5 SNPs and HIV disease progression have been reported (12). But, most pediatric genetic studies of this kind have been conducted among the Caucasian

and Asian origins and less remains explored in the African race, in particular Uganda. Moreover, the few studies conducted in sub-Saharan Africa have used allele specific-PCR or amplification refractory mutation system (ARMS-PCR) for SNP typing (9, 10), a genotypic method unable to detect novel SNPs as its biased to capture only known ones (13). Our study used capillary electrophoresis (Sanger sequencing) to characterize and determine CCR5 promoter polymorphisms including novel mutations that could be associated with persistent viremia or viral non-suppression among HIV-1 infected infants on combination antiretroviral therapy. Indeed, we report for the first time, four novel CCR5 promoter SNPs observed in our study population including CCR5 (58227G/A, 58636A/G, 59802A/C and 60024A/G). Closer analysis of these SNPs reveal interesting features, for instance, the first pair (58227G/A, 58636A/G) and last pair (59802A/C, 60024A/G) sit up-stream and down-stream of the previously reported SNPs respectively. The last pair seems to be in a linkage disequilibrium as it occurred in same frequencies and in the same infant IDs. In addition, the same SNP pair showed relatively higher odds of infant virological failure at both six and 12 months. A study by Lu, Sheng, et al. 2012, found a complete linkage disequilibrium between CCR2-V641 with SNPs CCR5 58755G/A, 59029G/A, 59353C/T and 59402G/A in a Northern Han Chinese population, suggesting that the two SNPs may jointly influence the process of HIV-1 infection.

6 of the 10 CCR5 SNPs including 58934T/G, 59029G/A, 59353T/C, 59356C/T, 59402G/A and 59653C/T have been reported before. SNP 59402G/A (94.2%) was most frequent in our population. This was in agreement with a similar study in neighboring Kenya by Katz and colleagues (2010) who identified the same SNP at 80% (14) among HIV-1 seropositive pregnant women. On the contrary, two other studies by Kaur *et al.* (2007) and Lu *et al.* (2012) reported the SNP at relatively lower frequencies of 47.8% and 66.4% among HIV-1 infected patients in a Northern Han Chinese population and North Indians (11, 15). These findings emphasize the impact of ethnicity and geographic location on human population genetics as we observe data similarities in regions in close proximities.

As expected, infant HIV plasma load decreased significantly with cART initiation by six months ($p=0.001$) and twelve months ($p=0.006$). This is a scientific fact as past studies have shown cART to suppress HIV multiplication by blocking multiple steps in the virus replication cycle (16, 17). On the other hand, however, it is imperative to note that only 50 of 85 infants (58.8%) and 34 of 53 infants (64.2%) achieved viral suppression by six and twelve months respectively. These findings are in agreement with the earlier cited viral suppression rate in six months among infants of 59.3% versus 88.4% in the general population by the Uganda Viral load dashboard (Central Public Health Laboratory of Republic of Uganda, 2018). We have shown that mother's age, level of education, residence, child's birth order and self-reported adherence did not have a significant effect on the levels of viral non-suppression. The global community continues to set tougher targets to end the HIV pandemic, recently the Joint United Nations Program on HIV/AIDS (UNAIDS) shifted the 90-90-90 goals to 95-95-95, implying that 95% for all HIV infected people on ART should be virally suppressed by year 2030 (UNAIDS). Additionally, Chan M. K et al reported 89% viral suppression rate by 12 months of cART initiation among infants in Europe and Thailand (18). It is against this background that our findings emphasize the urgent need for enhanced and robust measures to improve cART coverage and adherence, vis-à-vis scientific research aimed at bridging knowledge gap on the likely factors associated with virological failure among the HIV infected paediatric population in Uganda. It is also important to note that while viral suppression in the adult Uganda population is approaching 90%, the pediatric suppression rates are still lagging behind (19). In addition to the low viral suppression rates we have seen in these babies compared to adults, there is a big proportion of babies who are harboring low level viremia (50-999 copies/ml). It is possible that such virus continues to replicate and also harbor drug resistant mutations that may prevent total viral suppression. More indepth analysis using Next Generation Sequencing (NGS) is being done on these patient samples to see if they harbor any drug resistant mutations even at levels less than 20% that could explain the incomplete viral suppression.

Although we did not find any significant associations between CCR5 promoter SNPs and virological failure, we picked interest in certain SNPs that showed consistency with earlier studies. CCR5 59029G/A and 59353T/C showed higher odds among non-suppressors whereas CCR5 59356C/T and 59402G/A showed to be protective with lower odds among the same group. This is in agreement with John et al, 2001 who reported similar findings among an HIV seropositive mother-infant cohort in Kenya (10). Additionally, several other studies (11, 20, 21) have linked CCR5 59029G/A and 59353T/C to high HIV plasma loads.

In summary, following sanger sequencing, we have re-characterised the CCR5 promoter single nucleotide polymorphism genotype including four novel SNPs. No significant association was found between CCR5 promoter SNPs and infant virological failure, however, in agreement with earlier studies SNPs CCR5 59029G/A, and 59353T/C correlated with high plasma HIV loads whereas CCR5 59356C/T and 59402G/A appear protective against viral non-suppression. Similar to Uganda national viral load data, we also report a 59% viral suppression rate among infants on cART at six months and showing the need for more enhanced measures and scientific work to be done in this population. Our study augments previous studies that CCR5 promoter SNPs play a role in modulating patient virological status however, strong conclusions could be drawn from either utilizing in-vitro studies or large epidemiological studies.

Limitations

The small study population was attributed to the slow participant accrual into the parent study which in turn could have been attributed to (1) the effectiveness of PMTCT programs in the urban areas of Uganda as compared to rural-Uganda which has greatly reduced numbers of newborns acquiring HIV infection from their mothers (22). (2) the COVID-19 pandemic that caused total lockdowns and almost put the parent study to halt as both study participants and healthcare workers could hardly move to meeting points.

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Authors' contributions

All authors read and approved the manuscript. DK, IN, and BSB conceptualized and made the study design; IN, DR, RCB, PA and CM collected data and managed the project; DK, EN, and PA performed the laboratory experiments. DK and IN analyzed data, DK wrote the manuscript. IN edited the manuscript.

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Availability of Data and Materials

All data generated or analyzed during this study is available

Declarations

Ethics Approval and Consent to Participate

Ethical approval was obtained from Makerere University School of Biomedical Sciences Higher Degrees Research and Ethics Committee (SBS-HDREC-607). Amendments were made to the parent study (DRIBS) protocol to incorporate genetic studies and ethical clearance obtained from the Joint Clinical Research Centre Institutional Review Board (JC3617). Study participants provided written informed consent for participation, genetic study and consent for sample storage and future use at enrolment in the DRIBS study.

Consent for Publication

Not applicable

Competing Interests

The authors declare that they have no competing interests.

Supplementary Information

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