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ORIGINAL ARTICLE

Effect of Antiretroviral Therapy on Neutrophil Oxidative Burst in Children

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Abstract

Objective To ascertain the effect of human immunodeficiency virus (HIV) infection, as well as, antiretroviral therapy (ART) on neutrophil oxidative burst in children.

Methods Fifty-five children living with HIV infection (30 receiving ART for ≥ 2 y, 25 treatment-naïve) and 30 healthy controls, aged 18 mo–18 y, were assessed for hemogram and neutrophil oxidative burst. The treatment-naïve children were followed up and the above tests were repeated after 6 mo of ART.

Results Mean (SD) serum MPO activity at 6 mo after ART [32.1 (± 19.9) U/L] was comparable to that at disease onset [17.2 (± 23.0) U/L], although it was significantly higher compared to that in children on ART ≥ 2 y [13.3 (± 15.8) U/L] and controls [12.1 (± 11.9) U/L]. Median fluorescence intensity (MFI) of unstimulated DHR was highest at 6 mo after ART and in the treatment-naïve group, which was significantly higher than in the controls, as well as, children receiving ART ≥ 2 y. Stimulation index was highest in the control group [442.4 (341.9–562.9)], which was comparable to that in children on ART ≥ 2 y [304.2 (153.2–664.8)], but was significantly higher than the treatment-naïve cohort [266.1 (148.2–339.4)] and children on ART for 6 mo [318.8 (154.9–395.6)].

Conclusion A hyperinflammatory state caused by an increased serum myeloperoxidase enzyme activity and increased basal neutrophil oxidative burst was seen in untreated HIV infection and during initial 6 mo of ART. ART given for ≥ 2 y normalized the impaired neutrophilic phagocytic functions.

Keywords Dihydrorhodamine · Innate · Immunity · Myeloperoxidase

Introduction

Neutrophils are the most abundant immune cells and are also the first responder to the infection, particularly against bacterial and fungal pathogens [1]. Neutrophils can contribute to anti-HIV response in several ways like the release of human neutrophil peptides (HNP) or α-defensins, which directly inactivate the virus or by blocking viral replication [2]. Additionally, antimicrobial effects of neutrophils are mediated by several processes including phagocytosis, release of proteolytic enzymes, and production of reactive oxygen species (ROS) [3]. The release of myeloperoxidase enzyme and reactive oxygen species by neutrophils to form hypochlorous acid has been demonstrated to be virucidal to HIV-1 in vitro [4].

A subset of peripheral neutrophils has been shown to express clusters of differentiation 4 (CD4) on their surface in a subset of individuals, and hence these may be a target for HIV infection, in addition to the lymphocytes, especially T-helper lymphocytes [5]. Advanced HIV infection has shown an association with neutropenia [6]. Additionally, antiretroviral therapy (ART) with zidovudine has also been shown to be associated with lower neutrophil counts [6, 7]. HIV infection has been shown to have a dual effect on neutrophil functions as well. On the one hand, HIV infection leads to functional impairment of neutrophils in the form of reduced chemotaxis, cytokine production, decreased degranulation, impaired production of ROS, and impaired antibody-mediated cytotoxicity [8, 9]; on the other hand, HIV infection has been shown to

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delay apoptosis of neutrophils [10], as well as, cause hyperactivation of neutrophils associated with an increased production of proinflammatory cytokines [11] and increased ROS production leading to chronic systemic inflammation [12, 13]. ROS also contributes to HIV pathogenesis, disease progression, and transmission [14]. Upregulation of neutrophil inflammasome in HIV-infected individuals has been shown to contribute to persistent inflammation and immunological non-response [15] as well as increased morbidity [16]. The effect of HIV infection on neutrophil oxidative burst and phagocytic functions have also been inconsistent [8, 9, 12, 13]; moreover, these have been evaluated only sparingly in children living with HIV (CLHIV) [9, 17–20]. Further, the effect and duration of ART on reversing the neutrophil dysfunction has been inconsistent [11, 21–23]. Hence, this study was conducted to ascertain the effect of HIV infection as well as ART on neutrophil oxidative burst in children.

Material and Methods

This case–control study was conducted in the pediatric ART clinic of a tertiary hospital in Delhi, between November 2019 and October 2021. The study was approved by the Institutional Ethics Committee for Human Research (IECHR/2021/41/106).

The study included CLHIV, aged 18 mo to 18 y attending the ART clinic and healthy age- and sex-matched controls after obtaining appropriate consent and assent. The participants included CLHIV who had been receiving ART for ≥ 2-y duration, treatment-naïve CLHIV who were followed up for 6 mo after initiating ART, and healthy controls. Children with critical illnesses, opportunistic infections, acute infections, and chronic diseases were excluded. Control population included healthy siblings of CLHIV who volunteered to participate in the study.

Demographic details including age, sex, date of initiation of ART, ART regimen given and anthropometry (weight, height, weight for height, body mass index) were recorded. All CLHIV received combination ART as per the national guidelines [24].

Laboratory assessment included complete hemogram, absolute neutrophil count (ANC), and neutrophil to lymphocyte ratio (NLR) estimation. Neutropenia was defined as ANC below 1500 neutrophils per mm^3. Neutrophil oxidative burst was estimated by dihydrorhodamine (DHR) assay, as well as, serum myeloperoxidase (MPO) activity.

Oxidative burst was determined quantitatively using 2 mL of whole blood freshly collected in heparin-coated pyrogen-free tube for DHR assay. Stock solution of DHR was prepared by adding 200 µL of dimethyl sulfoxide (DMSO) to 2 mg DHR-123 and that of phorbol 12-myristate 13-acetate (PMA) was made by adding 1 mg PMA to 1 mL of DMSO, which were stored at −20ºC. The stock solutions were stored as 5 µL aliquots; each aliquot was diluted with 45 µL of normal saline prior to usage. For each sample, two tubes were prepared each containing 100 µL of blood to which 0.8 µL of DHR-123 was added and the tubes were incubated for 5 min at 37ºC. To one of the tubes (stimulated DHR tube), 2 µL of PMA was added followed by incubation for 15 min at 37ºC; followed by addition of 2 mL of red cell lysis fluid and vortexing, followed by keeping in the dark room for 10 min after which it was centrifuged for 5 min at 1500 rpm. The supernatant was decanted and 2 mL sheath fluid was added, vortexed and centrifuged for 5 min at 1500 rpm. The supernatant was decanted and 0.5 mL of sheath fluid was added. Flowcytometric analysis was carried out on 5 color 2 laser instrument Beckman Coulter FC500. The authors assessed the median fluorescence intensity (MFI) of the unstimulated tube, MFI of the stimulated tube (after stimulation with PMA), stimulation index (SI) (SI = MFI of stimulated tube/MFI of unstimulated tube) as well as percentage of neutrophils which underwent stimulation wherein DHR-123 was converted to fluorescent rhodamine-123.

One mL blood was collected in plain vial from each participant at enrollment, sera separated and stored at −20ºC; myeloperoxidase estimation was done using commercial ELISA-based kits (Human Myeloperoxidase ELISA kit, Shanghai Coon Koon Biotech Co. Ltd., Shanghai, China).

For the treatment-naïve cohort, laboratory evaluation was repeated at 6 mo after initiation of ART. CD4 counts were estimated by BD FACS Presto™ System and the viral load was estimated using Abbott RealTime HIV-1 assay, an in vitro reverse transcription–polymerase chain reaction (RT-PCR) assay for all participants at enrollment.

The sample size was calculated using G-power software version 3.1.9.2. Based on the study conducted by Ross et al. [25], wherein serum MPO activity was estimated in the persons living with HIV (PLHIV) receiving ART, ART-naïve PLHIV, and healthy controls, and was reported as median of 4665 (960–137,700), 5590 (828–20,845) and 1684 (1124–10,865) pg/mL, respectively. At 95% power and 5% alpha error, a sample size of 8 per group was needed to compare serum MPO levels between the treatment-naïve CLHIV with those receiving treatment. At 95% power and 5% alpha error, a sample size of 2 per group is needed to compare serum MPO levels between controls with the treatment-naïve CLHIV. For establishing meaningful statistical comparisons, at least 25 children were recruited per group.

The analysis was carried out using SPSS package version 26. Normality of data was tested using Shapiro–Wilk test. For continuous data, mean (standard deviation, SD) and median (interquartile range, IQR) were computed and comparisons between groups were done using unpaired t test and Mann–Whitney *U* test, respectively. The change in mean (SD) MFI of neutrophils (stimulated and unstimulated) detected by DHR assay, SI, and serum myeloperoxidase in the ART-naïve

group after 6 mo of ART using the Wilcoxon signed-rank test was also compared. For comparison of proportions between the groups, like male sex distribution, the chi-square test was used. Spearman correlation between neutrophil functions and viral load, CD4 count, ANC, NLR and duration of ART, was calculated. A p value < 0.05 was considered significant.

Results

Between November 2019 and October 2021, 55 CLHIV (30 children on ART $\geq$ 2 y and 25 treatment-naïve) and 30 controls were enrolled. Figure 1 depicts the flow diagram of the study participants.

Out of the 85 children analyzed, 48 (56.5%) were males. The mean age of the study participants was 100.9 ($\pm$ 57.4) mo, range 18–210 mo. The mean (SD) age of study participants in the study groups was comparable as shown in Table 1. Out of 55 CLHIV, 30 had been receiving ART for $\geq$ 2-y duration [median (interquartile range), duration of treatment being 37.5 (28–63.3) mo]. Twenty-five ART-naïve CLHIV were initiated on triple drug combination ART regimens and followed up for 6 mo. Overall, 20 (36.4%) children were receiving ZLE, 11 (20%) received ALE, 4 (7.3%) received ZLLpv/r, 1 (1.8%) received TLLpv/r, 4 (7.3%) received ALN, 6 (10.9%) received ALLpv/r, 2 (3.6%) received TLE, 5 (9.1%) received TLD, and 2 (3.6%) received ALD (A - abacavir, D - dolutegravir, E - efavirenz,

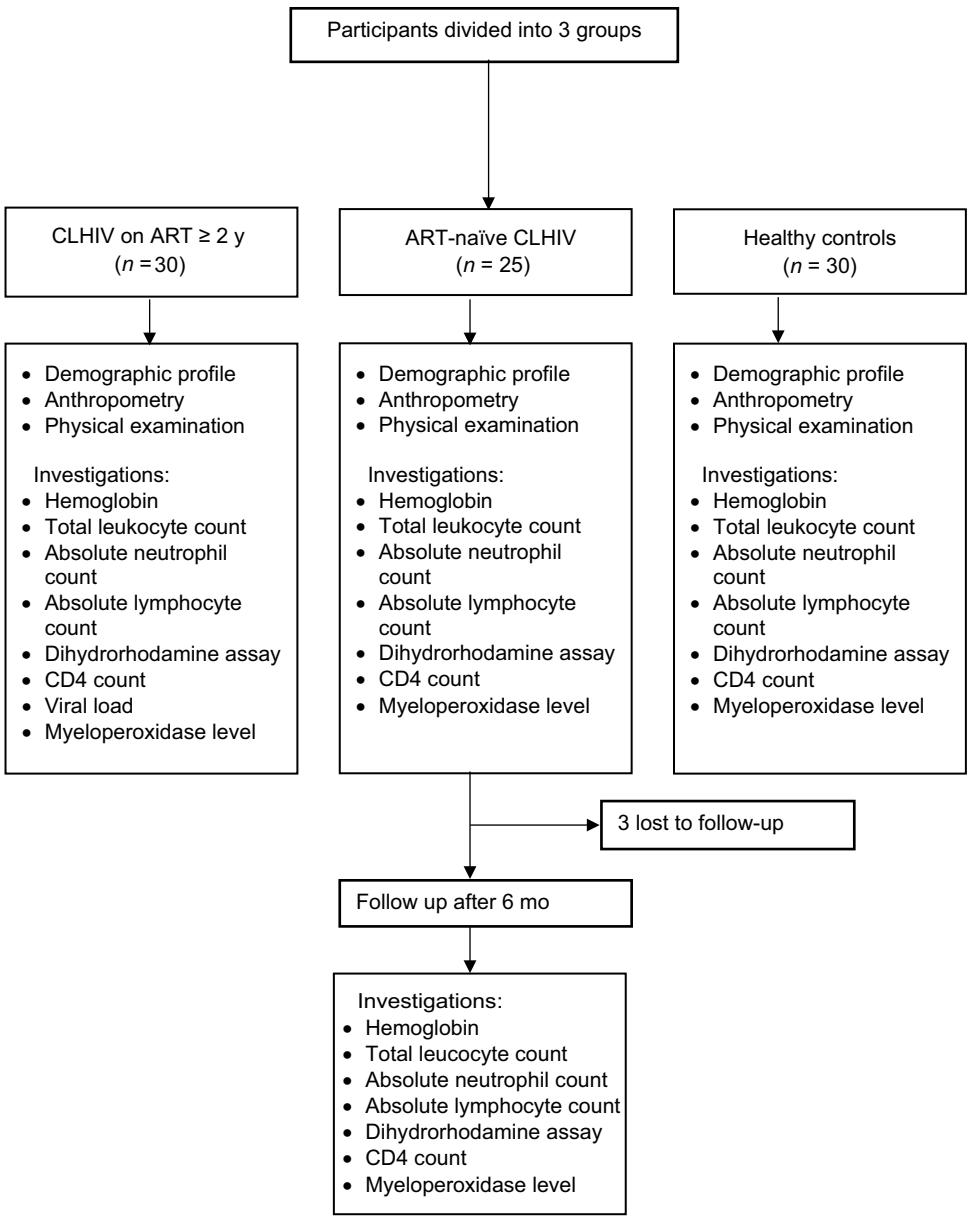

Fig. 1 Flow of participants in the study. *ART* Antiretroviral therapy, *CD4* Clusters of differentiation 4, *CLHIV* Children living with human immunodeficiency virus

Table 1 Baseline demographic characteristics of the study participants

	CLHIV receiving ART ≥ 2 y ($n=30$)	ART-naïve CLHIV ($n=25$)	Controls ($n=30$)
Age (months)	98.8 (40.2)	102.3 (75.2)	100.2 (38.3)
Male sex, *n* (%)	20 (66.7%)	12 (48%)	16 (53.3%)
Weight* (kg)	20.2 (7.3)	25.4 (16.2)	25.9 (11.1)
Height (cm)	120.4 (16.5)	123.0 (30.9)	130.4 (28.4)
Weight for age *z* score (WAZ)[#]	−1.8 (0.9)	−1.4 (1.4)	−0.8 (0.2)
Height for age *z* score (HAZ)[$]	−1.3 (1.4)	−0.7 (1.8)	−0.4 (2.2)
Weight for height *z* score (WHZ)	−2.5 (0.9)	−1.3 (1.7)	−2.6 (3.8)
Body mass index *z* score (BMIZ)	−1.5 (1.2)	−1.4 (1.8)	−0.8 (1.6)

Values expressed as mean (SD), except male sex expressed as *n* (%)

The *p* value for comparison between CLHIV on ART ≥ 2 y and controls is *0.02, [#]0.001, and.[$]0.02; For all other comparisons, $p > 0.05$

ART Antiretroviral therapy, *CLHIV* Children living with human immunodeficiency virus

L - lamivudine, Lpv/r - lopinavir–ritonavir, N - nevirapine, Z - zidovudine).

Table 2 depicts the baseline hematological parameters in the study groups; all four groups were comparable with respect to TLC, ANC, ALC, and NLR. Two ART-naïve children had neutropenia which normalized after 6 mo of ART. CD4 counts were significantly higher in the healthy controls and those receiving ART ≥ 2 y, when compared to ART-naïve and children receiving ART for 6 mo. Six children in ART-naïve group had CD4 count < 350/mm^3 and they persisted to have CD4 count below 350/mm^3 even after 6 mo of ART. Two children amongst those receiving ART ≥ 2 y had CD4 counts < 350/mm^3 and another 4 were having HIV viral load exceeding 1000 copies/mL.

The neutrophil oxidative burst was quantitatively measured by DHR flow cytometric assay. The median (IQR) MFI of the unstimulated tubes in the treatment-naïve children was high and remained high even after 6 mo of ART. The children on ART for ≥ 2 y and controls had significantly lower median (IQR) MFI of unstimulated tubes (Fig. S1). Neutrophil oxidative burst in response to a stimulus was highest in controls as reflected by the median (IQR) MFI of stimulated tube. SI was highest in the controls, followed by the children receiving ART ≥ 2 y, ART for 6 mo, and the treatment-naïve cohort (Fig. S2). Myeloperoxidase activity was highest at 6 mo after starting ART and significantly lower in the controls and those receiving ART for ≥ 2-y duration (Fig. S3). Table 3 depicts the neutrophil phagocytic functions.

Six months of ART led to a significant improvement in the median (IQR) MFI of neutrophils on stimulated DHR ($p = 0.03$), as well as, increase in percentage of positive neutrophils on stimulated DHR ($p = 0.02$). The change in SI ($p = 0.10$), MPO ($p = 0.35$), NLR ($p = 0.96$), ANC ($p = 0.73$), CD4 ($p = 0.13$), or ALC ($p = 0.25$) was statistically insignificant.

The SI, median (IQR) MFI of stimulated DHR assay and ΔMFI on DHR assay were found to be significantly correlated with CD4 counts. Serum myeloperoxidase levels did

Table 2 Comparison of baseline hematological parameters in the study groups

	Group 1 (CLHIV on ART > 2 y) ($n=30$)	Group 2 (CLHIV on ART × 6 mo) ($n=22$)	Group 3 (ART-naïve CLHIV) ($n=25$)	Group 4 (Controls) ($n=30$)
Hemoglobin (g/dL)	10.9 (10–12)	11 (9.7–12.3)	10.3 (8.2–11.5)	11.9 (11.1–12.3)
Total leucocyte count (cells/mm^3)	7700 (6100–9925)	8250 (6525–10010)	8600 (5950–12000)	8450 (8675–10075)
Absolute neutrophil count (cells/mm^3)	3552 (2538–4715)	4058 (2856–5232)	4015 (3033–4986)	4008 (3271–5467)
Absolute lymphocyte count (cells/mm^3)	3634 (2152–5301)	3418 (2087–4492)	3480 (2195–5697)	3129 (2536–2568)
Neutrophil-to-lymphocyte ratio	1.1 (0.6–1.7)	1.1 (0.9–1.9)	1.2 (0.7–1.8)	1.5 (1–1.8)
CD4 count (cells/mm^3)	1133 (865–1530)*	727 (465–963)*	511 (318–767)*	1141 (871–1148)*

Values expressed as median (interquartile range)

*The *p* values for comparison of group 1 vs. 2, group 1 vs. 3, group 2 vs. 3, and group 3 vs. 4 are statistically significant, i.e., < 0.05; All other parameters are statistically comparable between the groups

ART Antiretroviral therapy, *CD4* Clusters of differentiation 4, *CLHIV* Children living with human immunodeficiency virus

Table 3 Quantitative dihydrorhodamine (DHR) flow cytometric assay (unstimulated DHR, stimulated DHR, and stimulation index), and serum myeloperoxidase in the study groups

	Group 1 (CLHIV on ART ≥ 2 y) $n=30$	Group 2 (CLHIV on ART < 6 mo) $n=22$	Group 3 (ART-naïve CLHIV) $n=25$	Group 4 (Controls) $n=30$
MFI of unstimulated DHR tube (UDHR)	0.5 (0.5–0.7)[*]	0.9 (0.8–1.0)[*#]	0.8 (0.5–0.9)	0.6 (0.5–0.7)[#]
MFI of stimulated DHR tube (SDHR)	180.5 (88.3–338.2)	277 (159.5–310.5)	220 (122–279)	259.5 (216.2–309.7)
ΔMFI (MFI of SDHR–MFI of UDHR)	169.9 (87.2–337.5)	276.2 (158.5–309.6)	219.0 (121.6–278.1)	259.0 (215.3–309.1)
Stimulation index	304.2 (153.2–664.8)	318.8 (154.9–395.6)[#]	266.1 (148.2–339.4)[$]	442.4 (341.9–562.9)[#$]
Percent (%) positive neutrophils on SDHR	93 (91–95)	93.5 (92.7–95)	92 (88.5–94)[$]	95 (93–96)[$]
Myeloperoxidase (U/L)^	13.3 (± 15.8)[*]	32.1 (± 19.9)[*#]	17.2 (± 23.0)[$]	12.1 (± 11.9)[#$]

Values expressed as median (interquartile range), Values expressed as mean (standard deviation)^

[*] p value < 0.05 for comparison between group 1 and 2

[#] p value < 0.05 for comparison between group 2 and 4

[$] p value < 0.05 for comparison between group 3 and 4

ART Antiretroviral therapy, *CLHIV* Children living with human immunodeficiency virus, *DHR* Dihydrorhodamine, *IQR* Interquartile range, *MFI* Median fluorescence intensity, *SDHR* Stimulated dihydrorhodamine, *UDHR* Unstimulated dihydrorhodamine

not have significant correlation with CD4 count, viral load, ANC, TLC, or NLR as shown in Table 4.

Discussion

It was found that untreated HIV infection was associated with a state of hyperactivation of neutrophils with an increased myeloperoxidase activity and production of ROS (increased chemiluminescence in unstimulated neutrophils). Serum myeloperoxidase activity was higher in treatment-naïve children, peaked at 6 mo after starting ART and normalized after ≥ 2 y of ART. Hayani et al. [20] also reported that serum myeloperoxidase levels were higher in CLHIV compared to controls, although they did not include treatment-naïve children. Ross et al. [25] demonstrated that myeloperoxidase activity was higher in treatment-naïve PLHIV compared to those on ART and controls. Emokpae and Mrakpor [26] showed that myeloperoxidase activity in PLHIV on ART was significantly higher than controls and it correlated with CD4 counts. No correlation was found between CD4 counts and myeloperoxidase activity.

An increased basal ROS production as observed in the present study, has also been demonstrated previously in untreated HIV infection [9, 21, 22]. Lowe et al. [21] reported that treatment-naïve children had hyperstimulated neutrophils which eliminated *Mycobacterial tuberculosis* less effectively compared to controls. Bandres et al. [13] found that neutrophils from asymptomatic HIV-1–infected patients had a significantly increased capacity to phagocytose

Table 4 Correlation of neutrophil phagocytic functions (assessed by DHR and MPO assay) with CD4 counts, viral load, absolute neutrophil count, and neutrophil-to-lymphocyte ratio

Parameter	Stimulation index		MFI on SDHR		ΔMFI (MFI on SDHR–MFI on UDHR)		Percent (%) positive neutrophils on SDHR		Serum myeloperoxidase activity	
	r value	p value	r value	p value	r value	p value	r value	p value	r value	p value
CD4 count	0.43	< 0.001	0.21	0.04	0.27	0.005	0.17	0.12	0.07	0.51
Viral load	−0.03	0.81	0.11	0.43	0.11	0.43	−0.03	0.81	0.13	0.36
Absolute neutrophil count	−0.06	0.60	−0.04	0.70	−0.006	0.95	0.11	0.32	−0.07	0.53
Neutrophil-to-lymphocyte ratio	−0.05	0.63	−0.09	0.37	−0.17	0.08	0.01	0.87	0.08	0.45
Duration of ART	0.20	0.16	0.04	0.76	−0.08	0.38	0.23	0.09	0.15	0.26
Serum myeloperoxidase activity	0.23	0.01	0.28	0.11	0.20	0.03	0.06	0.57	-	-

ART Antiretroviral therapy, *CD4* Clusters of differentiation 4, *MFI* Median fluorescence intensity, *SDHR* Stimulated dihydrorhodamine, *UDHR* Unstimulated dihydrorhodamine

Staphylococcus aureus and *Escherichia coli* and produce ROS compared to healthy individuals. Further, it was also found that MFI of unstimulated DHR peaked at 6 mo following ART initiation which may reflect immune reconstitution syndrome (IRIS) as has been shown in a few other studies [8, 14, 21].

It was also found that compared to controls, CLHIV had impaired neutrophil oxidative capacity as evidenced by a lower SI; these changes were more pronounced in treatment-naïve CLHIV. Chen et al. [9] found that neutrophils from HIV-1–infected patients (18 children and 13 adults; of these 5 children and 6 adults were receiving ART) had a significantly diminished ability to produce ROS following stimulation as compared to controls.

Following ART for ≥ 2-y duration, the SI improved in CLHIV and was comparable to that of controls which suggests that HAART can normalize neutrophil oxidative burst in children. The present study evidenced that the duration of treatment has an impact on the neutrophil functions; SI in CLHIV on ≥ 2 y of ART was significantly higher than CLHIV on 6 mo of ART. Surprisingly, a study by Monari et al. [22] showed that 3 mo of ART led to a normalization of anticryptococcal activity, reduction in ROS formation by unstimulated cells, and restoration of oxidative burst after appropriate stimulation with reduction of IL-12 hypersecretion. Further, this restoration in oxidative burst was observed in only 14 out of 18 patients who had a high viral load. The differences observed with the present results may have been due to the differences in age profile of study groups, and CD4 counts and viral load of participants. Unfortunately, viral load in participants was not estimated at baseline although it is possible that viral load in the untreated adults might have been much higher than that in untreated children. It is also possible that nonoxidative mechanisms are involved in the killing of cryptococcus. A study from Greece showed that neutrophil phagocytic activity was lower in ART-naïve patients compared to treated patients; further a continued declining trend of neutrophil phagocytic activity was recorded despite ART, within 48 wk of observation [23].

A correlation was also found between SI, MFI of stimulated DHR, and ΔMFI with CD4 counts, as has been reported previously [8, 20]. Hayani et al. [20] found that unprimed chemiluminescence response of neutrophils to opsonized zymogen was decreased in severe immunosuppression while the primed chemiluminescence response of neutrophils to opsonized zymogen was decreased in both moderate and severe immunosuppression.

A significantly lower hemoglobin in the ART-naïve patients was noted, as compared to the controls ($p = 0.001$) highlighting the depressant effect of HIV on the hematopoiesis as seen before [27]. Significant neutropenia was not noted in CLHIV, which is consistent with another study [28] unlike certain other studies [29]. The NLR was also found to be comparable in the CLHIV and controls, although previous studies show that NLR in HIV-positive patients may be higher as compared to controls [30]; this might have been due to the fact that no child with overt opportunistic infections or illnesses was included.

The strength of the present study is the prospective component of this study, wherein the treatment-naïve cohort was followed up longitudinally. The present study population was homogenous as the controls were siblings of CLHIV participants having a similar sociodemographic profile. This also precluded undue genetic variability in the participants. The study was limited by the COVID-19 pandemic due to which the sample size was restricted, and only 22 out of 25 treatment-naïve children could be followed up at 6 mo after starting ART.

Conclusions

HIV infection impacts the phagocytic functions of neutrophils making them vulnerable to serious bacterial infections, although the neutrophil counts remain preserved. Low CD4 count is a predictor of impaired phagocytic function. A hyperinflammatory state caused by increased serum myeloperoxidase enzyme activity and increased basal neutrophil oxidative burst is seen in untreated HIV infection as well as during initial phase of ART. Prolonged ART normalizes the impaired neutrophilic oxidative burst.

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Declarations

Conflict of Interest None.

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