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NEUROCOGNITION, PLASMA LEVEL OF TUMOUR NECROSIS FACTOR-RELATED APOPTOSIS INDUCING LIGAND (TRAIL), AND PHAGOCYTIC ACTIVITY IN HIV PATIENTS ON LONG-TERM ANTIRETROVIRAL THERAPY

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ABSTRACT

Background: **The neuropathological process responsible for neurocognitive disorders in people living with HIV (PLHIV) on long-term antiretroviral therapy (ART) is not well elucidated. Presently, there is a dearth of information on the roles of altered immune response in the pathogenesis of HIV-associated neurocognitive disorders. To investigate the interplay between immune response alteration and neuropathological mechanisms underlying neurocognitive disorders in PLHIV on long-term ART, neurocognition, phagocytic activity and plasma levels of tumor necrosis factor-related apoptosis inducing ligand (TRAIL) and nitric oxide (NO) were determined in PLHIV on long-term ART.**

Methods: **Eighty eight adults comprising 48 PLHIV on long-term ART and 40 controls, were enrolled into this case-control study. Neurocognition was assessed using the Mini-Mental State Examination (MMSE) while the plasma levels of TRAIL and nitric oxide were determined using ELISA and spectrophotometric method respectively. Phagocytic activity was determined using the neutrophil Nitroblue Tetrazolium (NBT) Reduction Test.**

Results: **The plasma TRAIL level and phagocytic activity were significantly lower while the plasma level of NO was significantly higher in PLHIV compared with the controls. However, the mean MMSE score was similar in PLHIV and controls. There were no significant differences in the mean TRAIL levels, phagocytic activity, NO and MMSE score in PLHIV who have been on ART for less than 10 years compared with patients who have been on ART for 10 years or more.**

Conclusion: **Phagocytic activity and plasma levels of TRAIL and NO are altered in PLHIV on long-term ART. However, these alterations appear not to be forerunners to neurocognitive impairment in Nigerians living with HIV on long-term ART.**

INTRODUCTION

Human immunodeficiency virus (HIV) infection causes progressive degeneration of the immune system, leading to the development of acquired immune deficiency syndrome (AIDS).[1] HIV infects vital cells of the human immune system such as the $CD4^+$ T cells, macrophages, and dendritic cells.[2]

The global increasing coverage of antiretroviral therapy (ART) continues to change the landscape of HIV infection worldwide and it is greatly reducing the incidence of AIDS-related morbidity and mortality.[3] However, reports abound that people living with HIV infection, even after suppressive ART, still experience high incidence of non-AIDS associated comorbidities including cardiovascular disease, liver and kidney disease, osteoporosis and neurocognitive disorders among others.[4]

The risk factors underlying the non-AIDS associated comorbidities are multifactorial and poorly understood. However, chronic immune activation and inûammation have been identiûed as the most common risk factors.[5-8]

Despite effective ART with virological suppression and minimal or no neuropsychiatric confounds, reports continue to show that HIV associated neurocognitive disorders (HAND) remain major neurological complications associated with HIV infection.[3, 6, 9]

The neuropathological process responsible for neurocognitive disorders in patients on long-term ART use is not well elucidated but it is clear that several mechanisms are involved. One of the mechanisms is continued immune activation resulting from HIV

persistence despite long-term ART use. It is known that HIV persistence causes low-level immune activation driven by HIV DNA reservoirs and persistent low-level viremia.[6] The persistence could cause brain damage via two mechanisms; first, residual HIV viremia and DNA reservoirs in the systemic compartment activate monocytes/macrophages lineage which are then trafficked into the central nervous system (CNS) to cause activation-induced damage; and second, HIV reservoirs within brain cells (macrophages, glial cells and astrocytes) trigger chronic immune activation within the CNS separately from the systemic compartment.[6] It has been shown that monocytes and macrophages are susceptible targets for HIV-1 infection, and have a pivotal role in latency and diffusion of the infection.[10]

A number of mechanisms have been identified in viral elimination by the immune system. One of such mechanisms is extrinsic induction of cell death via the TNF-related apoptosis-inducing ligand (TRAIL/Apo2L); a member of the tumor necrosis factor (TNF) family. TRAIL is expressed by various cells of the immune system, including the natural killer (NK) cells,[11] activated T cells,[12, 13] natural killer T cells (NKT cells), dendritic cells and monocytes/macrophages.[13] TRAIL has the ability to induce apoptosis preferentially in transformed cells such as tumor cells, while in non-transformed cells the actions of TRAIL are less characterized.[13, 14]

During HIV infection, increased expression of TRAIL and its receptors especially, DR5 has been observed in infected cells compared with uninfected cells. Miura *et al.*[15] showed that macrophages found in the brain tissue of patients with AIDS express high levels of TRAIL. This increased expression was however, reversed following antiretroviral therapy (ART).[16, 17] In contrast, TRAIL can provoke an immune suppression through induction of massive death of different immune cell populations. It has been shown in an experimental study that blocking TRAIL through systemic anti-TRAIL monoclonal antibody (mAb) administration, prevents the pathogen-induced immune suppression, keeping entire T cell-mediated immune function intact to clear the infection, and increase the survival. It was demonstrated that the inoculation of TRAIL-deficient mice with different pathogens or challenge with Toll like receptor (TLR) agonists improve their ability to clear the infection, due to the increased production of pro-inflammatory-cytokines such as interleukin (IL)-12 and interferons (IFNs).[13,18] However, a contradictory observation was reported in human. Schnepple *et al.*[17] showed that blockade of TRAIL-receptor interaction did not alter HIV-induced cell death but resulted in a novel splice variant; TRAIL-short (TRAIL-s), which antagonizes TRAIL-receptor. They reported that the plasma TRAIL-s concentration increases with increasing viral load and rendered cells resistant to TRAIL-induced death in HIV patients. This resistance was abrogated via knockdown of TRAIL-s.

HIV infection has been shown to impair the number and phagocytic functions of neutrophils thereby making HIV patients vulnerable to serious bacterial infections.[19] The reports of Ukibe *et al.*[20] and Yakubu and Kalgo[21] showed that there is lower phagocytic activity of neutrophils, assessed using the neutrophil ingestion rate of NBT, in HIV patients compared with the controls. They also showed that there is direct correlation between $CD4^+$ count and NBT in HIV patients.[20, 21]

Nitric oxide (NO) plays important roles in intracellular killings. It also combats viral infection by causing nitrosation of cysteine residues of proteins essential for viral replication and infection. These essential proteins, including integrases and nucleocapsid proteins, undergo nitrosation and become unable to bind DNA hence, cannot function as topoisomerases thereby preventing the integration of viral DNA to the host cell DNA.[22] The report of Olaniyi and Arinola[23] showed that the plasma level of NO was significantly higher in patients with HIV compared with the controls. Similarly, Soccal *et al.*[24] reported higher level of NO in HIV patients and that the level declined with the use of ART. Pietraforte *et al.*[25] showed that HIV-1 stimulates NO production by human macrophages. *In vitro*, they showed that recombinant gp 120 HIV envelope glycoprotein increase production of NO by human monocyte-derived macrophages. Surprisingly, increase in NO production has been shown to contribute directly to the pathogenesis of HIV-associated dementia.[22]

Presently, there is a dearth of information on the roles of altered immune response, including TRAIL levels and phagocytic activities, in the pathogenesis of HIV-associated neurocognitive disorders in patients on long-term ART. Therefore, the objective of this study was to investigate the interplay between immune response alteration and neuropathological mechanisms underlying neurocognitive disorders in PLHIV on long-term ART. Specifically, we aim to assess cognitive function, evaluate phagocytic activity, and measure plasma levels of TRAIL and NO, with a focus on understanding the potential roles of altered immune responses in the pathogenesis of HIV-associated neurocognitive disorders.

MATERIALS AND METHODS

Ethical Consideration

Ethical approval (UI/EC/21/0698) was obtained from the University of Ibadan/University College Hospital Joint Ethics Committee (UI) before the commencement of the study. Also, written informed consent was obtained from each study participant before enrollment into the study.

Sample size calculation

A significance level (α) of 0.05 and a statistical power ($1-\beta$) of 80% were used to calculate the required sample size. Estimates were derived from the neurocognitive scores reported by Nyongesa *et al.* [26] The following formula was employed for the calculation:

$$n = \frac{(Z_{\alpha/2} + Z_{1-\beta})^2 \times (\sigma_1^2 + \sigma_2^2)}{(\mu_1 - \mu_2)^2}$$

Where:

n = Sample size

$Z_{1-\beta}$ = Percentage point of the normal distribution corresponding to 90% power = 1.28

$Z_{\alpha/2}$ = Percentage point of the normal distribution corresponding to the (two-sided) significance level = 1.96 (95% confidence level)

$\mu 1$ = mean estimate of neurocognitive score in cases = 16.82

$\mu 2$= mean estimate of neurocognitive score in controls = 19.81

$\sigma 1$ = Standard deviation of neurocognitive score for cases = 5.39

$\sigma 2$= Standard deviation of neurocognitive score for controls = 7.28

Substituting the values into the equation;

$$n = \frac{(1.96 + 1.28)^2 \cdot (5.39^2 + 7.28^2)}{(16.82 - 19.81)^2}$$

$$n = \frac{10.4976 \cdot (29.05 + 53.02)}{(-2.99)^2}$$

$$n = \frac{10.4976 \cdot 82.07}{8.9401} \approx 29.74$$

The calculated sample size was rounded up to 30 participants per group but to account for potential attrition, the final sample size was increased to 48 cases and 40 controls.

Study participants

A total number of 88 adults comprising 48 HIV patients on long-term ART (ART initiation $\geq$ 2 years with no detectable viral load) and 40 apparently healthy adults, who served as controls, were enrolled into this case-control study. Patients with HIV were enrolled from the Infectious Diseases Institute, College of Medicine, University of Ibadan while the controls were enrolled from among the staff of University College Hospital and its environs. All the HIV patients had non-detectable viremia (<50 copies/ml) for at least 2 months before enrolment into the study.

Assessment of neurocognition

Neurocognition was assessed u1sing the Mini-Mental State Examination (MMSE); a neuropsychological test used to assess speed of information processing, executive function, learning, memory, motor, and verbal function.[27] The test yields a single composite score that reflects disease severity. Scores <10, 10 – 20, 21 – 24 and $\geq$25 indicate severe impairment, moderate impairment, mild impairment and no impairment, respectively.

Exclusion criteria

Patients with history of substance abuse and/or dependence, schizophrenia, severe depression, post-traumatic stress disorder, learning disability and central nervous system (CNS) opportunistic infections such as Toxoplasmosis and Meningitis were excluded from the study. Also, patients with unstable general medical conditions were excluded from the study.

Data collection

Demographic data and medical history were obtained using a short-structured questionnaire.

Blood sample collection

Five millilitres (5 ml) of venous blood was obtained from each participant and dispensed into heparin containing sample bottles. Twenty five microliter (25 µL) of the whole blood was taken for nitroblue tetrazolium (NBT) reduction test.. Thereafter, the blood samples were centrifuged and the plasma obtained were stored at -20^{0}C until analyzed.

Laboratory Analysis

Plasma level of TRAIL was determined using Sandwich ELISA following the Manufacturer's instructions (ElabScience Biotechnology Inc., USA). The plasma level of NO was determined using the Griess reagent as described by Green *et al.*,[28] while the neutrophil phagocytic activity was determined using the modified semi-quantitative NBT procedure as described by Feigin *et al.*[29] and Edem and Arinola.[30] Briefly, 25 µL of well-mixed heparinized blood was added to 50 µL of NBT solution in a vial. Thereafter, 25 µL of stimulant solution (lipopolysaccharide; LPS) was added and mixed gently and adequately. The mixture was incubated for 10 minutes at 37^{0}C and further incubated for 10 minutes at room temperature. The vial content was mixed again and a moderately thick smear was prepared. The smear was air-dried and treated with Wright stain by flooding with 1 ml

of stain for 30 seconds. Distilled water (1 ml) was added to the flooded smear and the mixture was allowed to stand for 30 seconds and then, air-dried. The stained smear was viewed with a microscope using oil immersion objective. Neutrophils showing formazan deposits were recorded as positive. Thereafter, %NBT was calculated as:

$$\frac{\text{Number of neutrophil showing formazan deposit}}{\text{Total number of neutrophils counted}} \times 100$$

Data Analysis

Data analysis was done using the Statistical Package for Social Sciences (SPSS), version 23.0. Before analysis, the data were assessed for Gaussian distribution and thereafter, appropriate statistical tool was applied. Student's t-test was used to determine differences in means of the variables with Gaussian distribution. Mann-Whitney *U* was used to determine differences in medians of the variables without Gaussian distribution. Correlation between the variables was determined using Spearman's Rho correlation. P-values less than 0.05 (2-tailed) were considered as statistically significant. Results are presented as median (interquartile range), mean ± standard deviation or number (percentage).

RESULTS

The characteristics of the study participants are shown in Table 1. None of the study participants had history of dementia, metabolic disorders, stroke, or any common immune disorders. Higher proportion of the study participants had no impaired neurocognition (Table 1).

Table 1: Characteristics of the study participants

Variables	HIV Patients	Controls
Gender		
Male	8 (16.7)	20 (50.0)
Female	40 (83.3)	20 (50.0)
Educational Status		
No formal	1 (2.1)	0 (0.0)
Primary	16 (33.3)	4 (10.0)
Secondary	24 (50.0)	17 (42.5)
Tertiary	7 (14.6)	19 (47.5)
Cigarette		
Never Smoked	45 (93.8)	40(100.0)
Before	2 (4.2)	0(0.0)
Presently Smoking	1(2.1)	0(0.0)
Alcohol		
Never	44(91.7)	40(100.0)
Before	2(4.2)	0(0.0)
Present	2(4.2)	0(0.0)
History of dementia		
No	48(100.0)	40(100.0)
Yes	0(0.0)	0(0.0)
History of endocrine/metabolic disorders		
No	48(100.0)	40(100.0)
Yes	0(0.0)	0(0.0)
History of cardiovascular diseases		
No	47(97.9)	40(100.0)
Yes	1(2.1)	0(0.0)
History of immune disorders		
No	48(100.0)	40(100.0)
Yes	0(0.0)	0(0.0)
History of stroke		
No	48 (100.0)	40(100.0)
Yes	0 (0.0)	0(0.0)
MMSE Score		
No impairment	46 (95.83)	39 (97.5)
Mild impairment	2 (4.17)	1 (2.5)
Moderate impairment	0 (0.0)	0 (0.0)
Severe impairment	0 (0.0)	0 (0.0)

Values are in number (percentage)

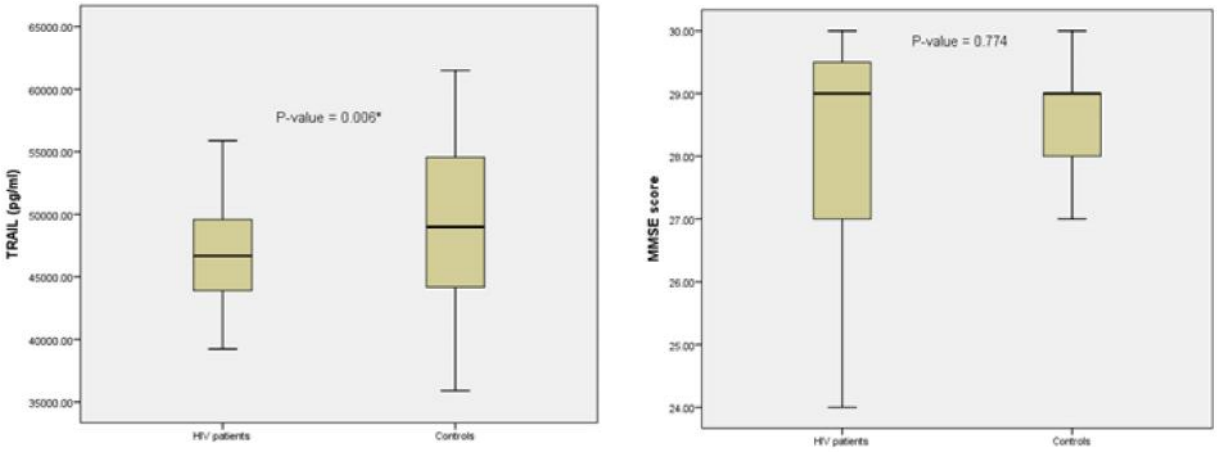

Figure 1: Plasma TNF-related apoptosis-inducing ligand levels and neurocognitive index in patients with HIV and controls

As shown in Figure 1 and Table 2, the mean TRAIL level and NBT were significantly lower in patients with HIV compared with the controls. In contrast, the mean plasma level of NO was significantly higher in HIV patients compared with the controls. The mean MMSE score was similar in HIV patients and controls (Figure 1).

In Table 3, the phagocytic activity, TRAIL level and neurocognitive index in HIV patients of different duration of antiviral therapy are shown. There were no significant differences in the mean TRAIL levels, NBT, NO and MMSE score in HIV patients who have been an antiretroviral for less than 10 years

Table 2: Phagocytic activity and plasma level of nitric oxide in patients with HIV and controls

Parameters	HIV patients (n = 48)	Controls (n = 40)	*P*-value
NBT (%)	47.85 ± 14.52	66.68 ± 5.88	0.000*
NO (μM)	0.51 ± 0.10	0.46 ± 0.02	0.001*

**Significant at P<0.05, TRAIL = TNF-Related Apoptosis-Inducing Ligand, NBT = Nitroblue Tetrazolium, NO = Nitric oxide, MMSE = Mini-mental state examination, results are presented as mean ± standard deviation.*

Table 3: Phagocytic activity, plasma TRAIL levels and neurocognitive index in patients with HIV patients of different duration of antiviral therapy

Parameters	<10 years (n = 18)	≥10 years (n = 30)	*P*-value
TRAIL (pg/ml)	48808.42 ± 6826.00	46647.83 ± 3886.92	0.202
NBT (%)	49.50 ± 12.81	47.13 ± 15.36	0.640
NO (μM)	0.49 ± 0.04	0.53 ± 0.12	0.332
MMSE Score	28.33 ± 1.97	28.43 ± 1.25	0.873

TRAIL = TNF-related apoptosis-inducing ligand, NBT = Nitroblue tetrazolium, NO = Nitric oxide, MMSE = Mini-mental state examination, results are presented as mean ± standard deviation or median (interquartile range).

Table 4: Correlation between the neurocognitive index and selected immunological parameters in patients with HIV and controls

	HIV patients (n = 48)		Controls (n = 40)	
Parameters	r-value	*P*-value	r-value	*P*-value
MMSE vs TRAIL (pg/ml)	0.210	0.152	-0.062	0.705
MMSE vs NBT	-0.095	0.522	0.027	0.870
MMSE vs NO	0.144	0.328	-0.075	0.645

TRAIL = TNF-Related Apoptosis-Inducing Ligand, NBT = Nitroblue Tetrazolium, NO = Nitric oxide, MMSE = Mini-mental state examination

compared with patients who have been on antiretroviral for 10 years or more (Table 3).

Correlations between neurocognitive index, plasma TRAIL level, NBT and NO in the HIV patients and controls are shown in Table 4. No significant correlations were observed between the neurocognition index and the immunological parameters in the HIV group and in the controls (Table 4)

DISCUSSION

The incidence of severe HAND has been reduced by ART. However, nearly half the population of patients on ART continues to present with some form of cognitive impairment which can range from mild and asymptomatic to the severe form.[31, 32] The absence of overt neuropathologies that were common before the cART era suggests that the HIV-associated neurocognitive impairments result from functional alteration of neuronal connectivity and not as a result of gross neuronal loss or encephalitis.[33, 34] In this study, the mean MMSE score was similar in HIV patients and controls. This observation further buttresses the neuroprotective effects of ART as all the study participants with HIV infection had undetectable viral load. Elendu *et al.*[35] reported that reduction in viral load following ART prevents neurocognitive decline, and improves survival. Similarly, the report of Saylor *et al.*[34] showed that optimization of ART regimens, including the use of combination therapies and drugs with better CNS penetration as well as psychosocial support improved neurocognition.

TRAIL, a cytokine and a member of the TNF superfamily, is expressed by a variety of immune cells. It initiates apoptosis of transformed cells or tumor cells by binding to death receptors.[36, 37] Reports have shown that TRAIL triggers necroptosis, a programmed cell necrosis characterized by its caspase-independent activation and release of damage-associated molecular patterns (DAMPs) which result in proinflammation.[38-40]

In this study, the mean TRAIL level was significantly lower in patients with HIV compared with the controls. This observation supports the report of Herbeuval *et al.*[16] which showed that there was reduction in plasma TRAIL levels in HIV patients on ART and that the reduction correlated with reduction in viral load. This reduction in TRAIL level could be due to the long-term ART use which resulted in suppression of HIV replication. Shepard *et al.*[41] reported that TRAIL expression is downregulated and it is associated with reduction in TRAIL-sensitive cells following a successful antiretroviral therapy. Upregulation of TRAIL receptor has been shown to be induced by gp120.[41] Aside the possible reduction in TRAIL level as a result of viral suppression, the observed reduction in TRAIL level in this study could also be drug-associated. It is well known that comorbidities associated with HIV are not driven HIV infection itself only but also by the adverse effects of long-term ART.[42, 43] The report of Korencak *et al.*[43] showed slow proliferation and decreased cellular function in $CD4^+$ T cells in HIV patients on dolutegravir (DLG) or elvitegravir (EVG) antiretrovirals. This decreased cellular function in immune cells could result in decreased expression of TRAIL in patients on long-term ART as observed in this study.

Neutrophils mediate innate immune response via multiple processes including phagocytosis, release of proteolytic enzymes, production of reactive oxygen species, and neutrophil extracellular trap formation.[44] They are considered as the first line of defense against invading microorganisms, particularly bacterial and fungal pathogens. However, the importance of neutrophils in containing and eliminating viral infections is also being increasingly appreciated.[44] It is well known that HIV infection impairs the number and phagocytic functions of neutrophils thereby, making HIV patients vulnerable to serious bacterial infections.[19, 45] In this study, phagocytic activity, assessed using the neutrophil ingestion rate of NBT was significantly lower in patients with HIV compared with the controls. This observation corrborates previous reports. Ukibe *et al.*[20] and Yakubu and Kalgo[21] reported that there is lower phagocytic activity of neutrophils in HIV patients compared with the controls. Similarly, the report of Michailidis *et al.*[45] showed a significant decrease in monocytes and neutrophils phagocytic function in treatment naïve HIV patients and those on ART. Observation from our study suggests impaired phagocytosis in the HIV patients and that the vulnerability of HIV patients to opportunistic infections might not be totally reversed by ART. The reports of Kedzierska *et al.*[46] showed that HIV causes defects in phagocytosis by interfering with intracellular signalling of Fc-gamma receptors rather than interfering with the expression of phagocyte receptors or their binding to ligands. Similar observation suggesting defective formation of the phagosome in HIV patients has been reported.[47]

Nitric oxide (NO) combats viral infection by causing nitrosation of cysteine residues of proteins essential for viral replication and infection.[48] Overproduction of nitric oxide has been associated with HIV-1 infection and may contribute directly to the pathogenesis of HIV-1-associated dementia.[22, 49] In this study, there was significant elevation in NO level in

HIV patients compared with the controls. This observation is in line with the report of Olaniyi and Arinola[23] which showed that the plasma level of NO was significantly higher in patients with HIV compared with the controls. Similarly, Soccal *et al.*[24] reported higher level of NO in HIV patients and that the level declined with the use of ART. Observation from this study could indicate higher stimulation of NO producing cells or increased tissue damage and inflammation in patients with HIV, even after viral suppression, than in the controls. Pietraforte *et al.*[25] showed that HIV-1 stimulates NO production by human macrophages. They showed that recombinant gp120 HIV envelope glycoprotein increase production of NO by human monocyte-derived macrophages. The observed elevation of NO in the HIV patients in this study suggests that the risk of developing NO-mediated dementia may be persistent even, in HIV patients on long-term ART.

The observed insignificant difference in the mean TRAIL levels, NBT, NO and MMSE score in HIV patients who have been an antiretroviral for less than 10 years compared with patients who have been on antiretroviral for 10 years or more could suggest that the immunological and neurocognitive profiles of HIV patients are similar once viral suppression has been achieved. This observation also indicates that the duration of HIV infection is of less clinical importance as maximum health benefit is achieved via effective viral suppression.

Our observed insignificant correlations between the neurocognition index and the immunological parameters in patients with HIV could indicate that alterations in the immunological parameters as observed in the patients with HIV are not associated with neurocognitive impairment.

CONCLUSION

It could be concluded from this study that phagocytic activity and plasma levels of TRAIL and NO are altered in HIV patients on long-term ART. However, these alterations appear not to be forerunners to neurocognitive impairment in HIV patients on long-term ART

It is worthy of note that small sample size was a limitation in this study. The use of MMSE as a tool for assessing neurocognition in the HIV patients (due to the design of the study as a case-control study) as against tools such as FRASCATI criteria which can be used to categorize HAND into symptomatic Neurocognitive Impairment (ANI), Mild Neurocognitive Disorders (MND), and Severe Neurocognitive Disorders termed HAND was also a limitation. Therefore, a cross sectional study with large population of HIV patients on long-term ART using neurocognitive tools validated for assessing neurocognitive disorders in HIV patients is suggested with a view to understanding the interplay between alteration in immunological profile and neurocognitive disorders in HIV patients on long-term ART.

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AUTHORS' CONTRIBUTION

SKR and OGA conceived and designed the study; SKR, TBK, AAO, and SPO collected the samples; SKR, TBK and SPO did the laboratory analysis, SKR wrote the initial draft, SKR, TBK, AAO, SPO and OGA reviewed the final draft, SKR supervised the entire research.

Competing Interest

The authors have no competing interest to declare.

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