CONCISE COMMUNICATION

Plasma galectin-9 relates to cognitive performance and inflammation among adolescents with vertically acquired HIV

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Objective: Adolescents with perinatally acquired HIV (AWH) are at an increased risk of poor cognitive development yet the underlying mechanisms remain unclear. Circulating galectin-9 (Gal-9) has been associated with increased inflammation and multimorbidity in adults with HIV despite antiretroviral therapy (ART); however, the relationship between Gal-9 in AWH and cognition remain unexplored.

Design: A cross-sectional study of two independent age-matched cohorts from India [AWH on ART ($n = 15$), ART-naive ($n = 15$), and adolescents without HIV (AWOH; $n = 10$)] and Myanmar [AWH on ART ($n = 54$) and AWOH ($n = 22$)] were studied. Adolescents from Myanmar underwent standardized cognitive tests.

Methods: Plasma Gal-9 and soluble mediators were measured by immunoassays and cellular immune markers by flow cytometry. We used Mann–Whitney *U* tests to determine group-wise differences, Spearman's correlation for associations and machine learning to identify a classifier of cognitive status (impaired vs. unimpaired) built from clinical (age, sex, HIV status) and immunological markers.

Results: Gal-9 levels were elevated in ART-treated AWH compared with AWOH in both cohorts (all $P < 0.05$). Higher Gal-9 in AWH correlated with increased levels of inflammatory mediators (sCD14, TNFα, MCP-1, IP-10, IL-10) and activated $CD8^+$ T cells (all $P < 0.05$). Irrespective of HIV status, higher Gal-9 levels correlated with lower

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cognitive test scores in multiple domains [verbal learning, visuospatial learning, memory, motor skills (all $P < 0.05$)]. ML classification identified Gal-9, CTLA-4, HVEM, and TIM-3 as significant predictors of cognitive deficits in adolescents [mean area under the curve (AUC) = 0.837].

Conclusion: Our results highlight a potential role of Gal-9 as a biomarker of inflammation and cognitive health among adolescents with perinatally acquired HIV.

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Keywords: antiretroviral therapy, galectin-9, inflammation, neurocognition, perinatal HIV

Introduction

Early diagnosis and initiation of antiretroviral therapy (ART) are essential to suppress viral replication and reduce HIV-related mortality but often fails to resolve systemic inflammation and immune activation [1–5]. While persistent inflammation and comorbidities, such as cognitive deficits, are prevalent among adults with HIV on ART [6–8], the risk of chronic multisystem comorbidities is magnified in adolescents living with HIV (AWH), particularly in resource-limited settings where early diagnosis and treatment are not always available [9]. Moreover, AWH have an increased risk of variable degrees of developmental delay and cognitive deficits [10,11] compared with adolescents without HIV (AWOH) [12–15], as they are exposed to HIV during a period of rapid brain development. However, the underlying cause of central nervous system (CNS) injury and neurodevelopmental deficits among AWH is proposed to be multifactorial [16]. Soluble immune mediators, such as IL-6 and sCD14, are shown to associate with poor cognitive performance in AWH [17–26]. Further understanding the involvement of immunoproteins in the context of CNS injury and neurodevelopmental deficits could provide key insights into the intricate molecular processes influencing cognitive outcomes.

Galectin-9 (Gal-9), a β-galactosidase binding mammalian lectin, is involved in several immunological processes, including cytokine production and leukocyte activation, and a component of the first wave of the cytokine storm in acute HIV [27]. Gal-9 plasma levels remain elevated in adults with HIV despite ART and associates with the extent of viremia, multimorbidity, and transcriptionally active HIV reservoirs [27–31]. Interestingly, recent findings demonstrating elevated levels of Gal-9 in cerebrospinal fluid (CSF) and brain tissues suggest that Gal-9 and its related pathways may be involved in HIV-associated CNS pathogenesis in adults [32]. We previously reported higher expression of Tim-3, a receptor of Gal-9, correlated directly with viral burden in adolescents with vertically acquired HIV [33]. Based on these results, Gal-9 may play a vital role in HIV-associated comorbid outcomes at an earlier age in children or adolescents living with HIV. Utilizing cohorts of AWH on suppressive ART and age-matched AWOH from India and Myanmar, we investigated the relationship between Gal-9, HIV status, and cognition in this population.

Methods

Participants were recruited in two independent cohorts in Asia. Cohort I at St. John's Medical College & Hospital, Bengaluru, India included AWH on suppressive ART, ART-naive AWH, and AWOH (Table S1, http://links.lww.com/QAD/D182). Cohort II at Yangon Children's Hospital, Yangon, Myanmar included AWH on ART and AWOH (Table S1, http://links.lww.com/QAD/D182). Ethics permissions were obtained from the Institutional Review Board of Weill Cornell Medicine, New York, USA (WCM- 20-07022376 and 20-07022375), Yangon Children's Hospital, Yangon, Myanmar (Ethics/DMR/2019/027), St. Johns Medical College & Hospital at Bangalore, India (IEC Study Ref No. 254/2016) and Jawaharlal Nehru University at New Delhi, India (IERB Ref. No. 2015/Faculty/102), and all procedures were carried out in accordance with the approved guidelines. Informed written consent was obtained from parents/guardian of all participants including adolescents without HIV. Whole blood was obtained from the participants in both cohorts, plasma and peripheral blood mononuclear cells (PBMCs) were isolated and stored at −80 °C and liquid nitrogen, respectively, until analysis.

Soluble biomarkers were measured in the plasma using immunoassays, per manufacturer's instructions (Table S2, http://links.lww.com/QAD/D182). All samples were analyzed in duplicate. Additional immunophenotyping was performed on cryopreserved PBMCs from 13 ART-naive and 13 ART-suppressed AWH in Cohort I using a BD FACSAria Fusion flow cytometer. Flow cytometry data were analyzed using FlowJo v.10 software. Biomarker data was analyzed using GraphPad Prism v.10. Significant changes were determined using nonparametric one-way analysis of variance (ANOVA) (Kruskal–Wallis test) and two-tailed Mann–Whitney *U*-test. The strength and direction of association between paired variables were

assessed by Spearman's rank correlation coefficient (ρ) [34]. Asterisk signs (*) represent P values: $^{*}P$ less than 0.05; $^{**}P$ less than 0.01; $^{***}P$ less than 0.001; $^{****}P$ less than 0.0001.

Participants in Cohort II additionally underwent standardized neuropsychological assessments adapted for cultural relevance. Cognitive tests (15 total) examined multiple domains, including executive function, psychomotor/processing speed, memory, gross motor, visuospatial, and learning skills (Table S3, http://links.lww.com/QAD/D182). Z scores were calculated as [(individual raw score) − (AWOH mean)]/(AWOH standard deviation (SD)). Participants were considered 'impaired' if they performed at least 1SD below the norm on more than five assessments. Supervised machine learning decision tree (Sklearn v.1.2.0; DecisionTreeClassifier function) was implemented to identify biological and demographic factors that discriminated cognitive status (i.e. impaired vs. not impaired). Group imbalance was adjusted by setting class_weight parameter to 'balanced'. Soluble biomarkers were removed if 40% of the observations were missing or below the lower limit of detection. Spearmen correlations assessed multicollinearity between features and for each highly correlated pair (correlation coefficient $\geq$0.65), one feature (the first alphabetically) was removed. SelectKBest (Sklearn v.1.2.0) retains k number of features based upon scoring criteria (ANOVA F-value by default) and was employed to reduce model complexity. To determine optimum k, models were fitted, plotted the receiver-operating characteristic (ROC), and calculated the area under the curve (AUC) to assess performance for each k number of features. Given the small sample size, models were trained in triplicate with newly generated training and test sets used for each replicate, with the average AUC serving as the final measure of model performance.

Results

Circulating levels of galectin-9 remain elevated in adolescents with perinatally acquired HIV despite antiretroviral therapy and associate with inflammation and T-cell activation

We examined plasma Gal-9 levels in two independent cohorts from Asia. Cohort I from India included AWH on suppressive ART ($n=15$; median duration of ART = 5.25 years, range 0.83–8.25 years), ART-naive ($n=15$), and AWOH ($n=10$), with median age of 10 years (6–15 years). ART-suppressed AWH had undetectable plasma viral load (<50 copies/ml) but $CD4^+$ counts were variable (median = 1157 cells/μl, range 86–2406 cells/μl). Cohort II included AWH on ART (total $n=54$; ART duration available for $n=16$; median = 4.42 years, range 0.83–13 years), and AWOH ($n=22$), with median age 12 years (11–13 years). AWH on ART in this cohort had undetectable plasma viral load in 88.46% of the individuals and variable $CD4^+$ count (median = 814 cells/μl, range 290–1480 cells/μl) [Table S1, http://links.lww.com/QAD/D182].

ART-naive AWH in cohort I had significantly higher levels of Gal-9 ($n=15$; median 13.64 ng/ml), compared with AWOH ($n=10$; median 6.72 ng/ml) (Fig. 1a, $P<0.0001$). Although Gal-9 levels in ART-suppressed AWH ($n=15$; median 10.81 ng/ml) were lower than ART-naive AWH, levels still remained significantly higher compared with AWOH ($P=0.0143$). Similarly, in Cohort II, plasma levels of Gal-9 were elevated in AWH on ART ($n=54$; median 2.53 ng/ml) compared with AWOH ($n=22$; median 1.53 ng/ml) (Fig. 1c, $P=0.0027$).

We next evaluated for associations between Gal-9 and markers of inflammation and immune activation. Among the AWH from Cohort I (ART-naive and ART-treated, $n=30$), circulating Gal-9 positively correlated with plasma levels of sCD14 ($r=0.39$ $P=0.03$), TNFα ($r=0.58$ $P=0.005$), MCP-1 ($r=0.50$ $P=0.02$), IP-10 ($r=0.65$ $P=0.001$), and IL-10 ($r=0.47$ $P=0.03$; Fig. 1b). Further, immunophenotyping revealed that plasma Gal-9 levels correlated positively with the frequency of activated ($HLA\text{-}DR^+$ $CD38^+$) $CD8^+$ T cells ($n=26$; $r=0.52$ $P=0.005$). Among the virally suppressed adolescents in cohort II, Gal-9 positively correlated with sCD14 ($r=0.25$; $P=0.06$), TNFα ($r=0.37$; $P=0.005$), and MCP-1 ($r=0.36$; $P=0.008$; Fig. 1d). Correlations with other markers of inflammation and immune activation were not significant (data not shown).

Higher Galectin-9 levels were associated with poor cognitive performance in adolescents

Plasma Gal-9 levels exhibited modest inverse correlations with performances in the domains of learning (HVLT-R learning total, $r=-0.31$; $P=0.0163$; BVMT-R total, $r=-0.26$; $P=0.0439$), psychomotor speed (Digit Symbol, $r=-0.31$; $P=0.0176$) and visuospatial (Block Design, $r=-0.30$; $P=0.0225$) (Fig. 2a). These associations were not significant when Gal-9 levels and cognitive performances were examined among AWH (data not shown). Among the 59 individuals who underwent cognitive testing in cohort II (AWH $n=44$; AWOH $n=15$), 23 were defined as cognitively impaired. Among these, 21 (91.3%) were AWH on ART representing 47.7% (21/44) of AWH on ART. The participants defined as cognitively impaired had higher Gal-9 levels ($n=23$; median 2.47 ng/ml) compared with those who were nonimpaired ($n=36$; median 1.767 ng/ml; Fig. 2b, $P=0.0423$). None of the other soluble markers evaluated were significantly different based on cognitive status (data not shown).

Machine learning reveals Galectin-9 is important to estimate cognitive dysfunction

We next implemented a decision tree machine learning algorithm to classify individuals with cognitive

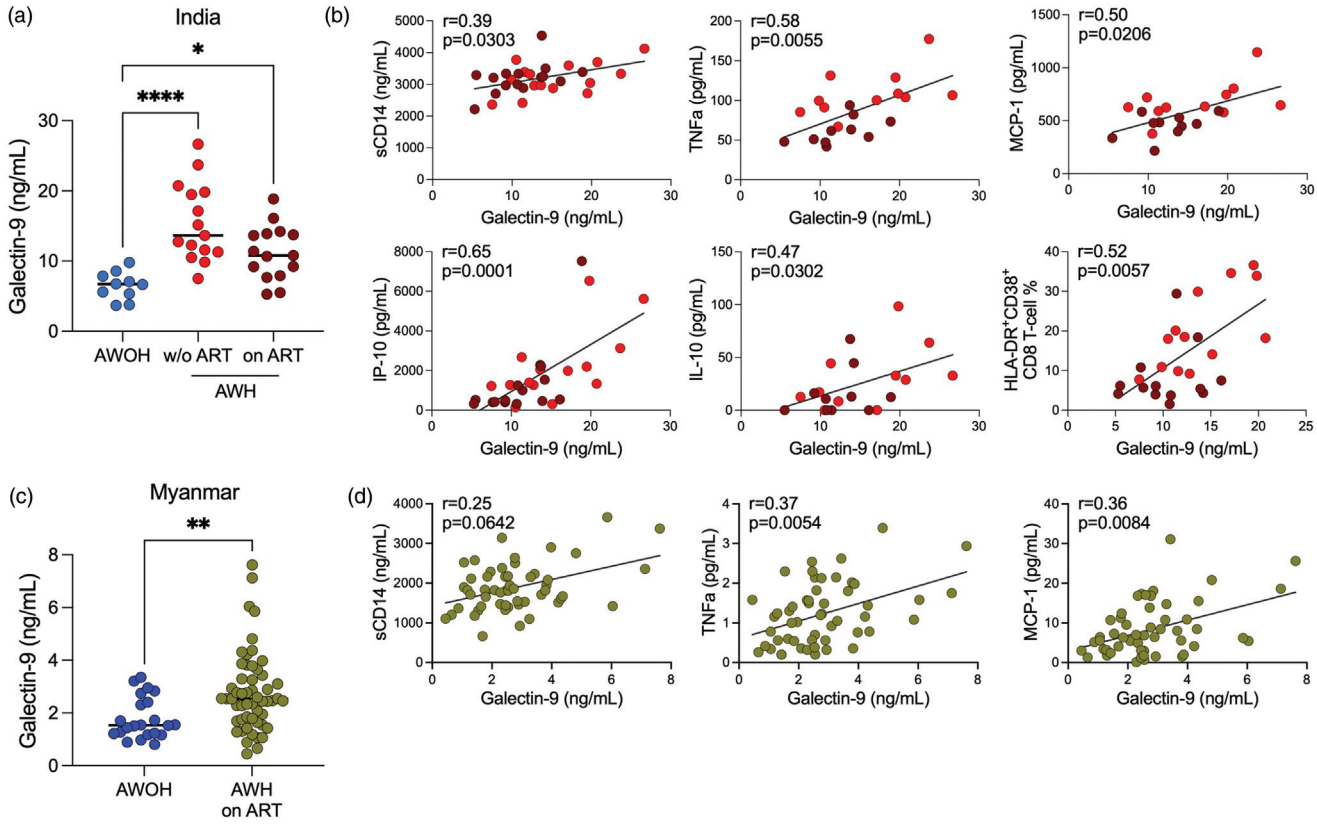

Fig. 1. Circulating galectin-9 levels in adolescents with perinatally acquired HIV and markers of inflammation and immune activation. (a) Comparison of galectin-9 (Gal-9) levels among AWOH ($n=10$), ART-naive AWH ($n=15$), and AWH on ART ($n=15$) in India. (b) Correlations of Gal-9 levels in AWH with sCD14, TNFα, MCP-1, IP-10, IL-10, and HLA-DR$^+$ CD38$^+$ CD8$^+$ T cell% in the India cohort. (c) Comparison of Gal-9 levels among AWOH ($n=22$) and AWH on ART ($n=54$) in Myanmar. (d) Correlations of Gal-9 levels in AWH with sCD14, TNFα, and MCP-1 in the Myanmar cohort. Statistical significance was determined using nonparametric Kruskal–Wallis and Mann–Whitney *U* tests. Asterisk signs (*) represent *P* values in the following manner: $^*P$ less than 0.05; $^{**}P$ less than 0.01; $^{***}P$ less than 0.001; $^{****}P$ less than 0.0001. Correlations were determined by Spearman's *r* value.

impairment using a total of 36 features, including 33 available biomarker measures, age, sex, and HIV status. To determine the optimum number of features to include, models were repeatedly trained to include increasingly more features until all features were included. When models containing *k* features were plotted against mean AUC, models containing the most significant eight or four features stood out as the most accurate (Fig. 2c). The 8 feature model consisting of Gal-9, the immune checkpoint proteins CTLA-4, Tim-3, LAG-3, and HVEM, lymphocyte activation receptor CD40$^+$, and myeloid-associated biomarkers CD163 and MCP-1 resulted in a mean AUC = 0.874 (Fig. 2d). Further reducing the complexity of model to the top four features (Gal-9, CTLA-4, HVEM, and TIM-3) resulted in the mean model performance remaining high (AUC = 0.837) when classifying adolescents by cognitive status (Fig. 2d). Besides Gal-9, among markers included in the eight-feature and four-feature models, only CD163 was significantly different between AWOH and AWH on ART [Figure S1, http://links.lww.com/QAD/D182].

Discussion

According to UNAIDS, globally in 2021, 1.7 million adolescents are currently living with HIV as a result of perinatal transmission. Studies conducted in the global north and south reveal persistent cognitive symptoms among AWH receiving ART [35–39]. Abnormal levels of several soluble immune mediators persist in AWH despite suppressive ART [40–42]. Given the emerging literature on the immunomodulatory effects of Gal-9 particularly in the CNS, the degree to which galectins are perturbed in this population remain unexplored. Consistent with our previous reports in adults with HIV on ART [27,29], in two independent cohorts from Asia, we found that plasma Gal-9 levels were elevated in AWH despite long-term viral suppression by ART, suggesting mechanisms similar to that in adults may be driving increased Gal-9 in AWH. We have also shown previously that Gal-9 is associated with worse cognition in adults with HIV [29,32], and we expand here demonstrating similar findings with cognitive status among AWH.

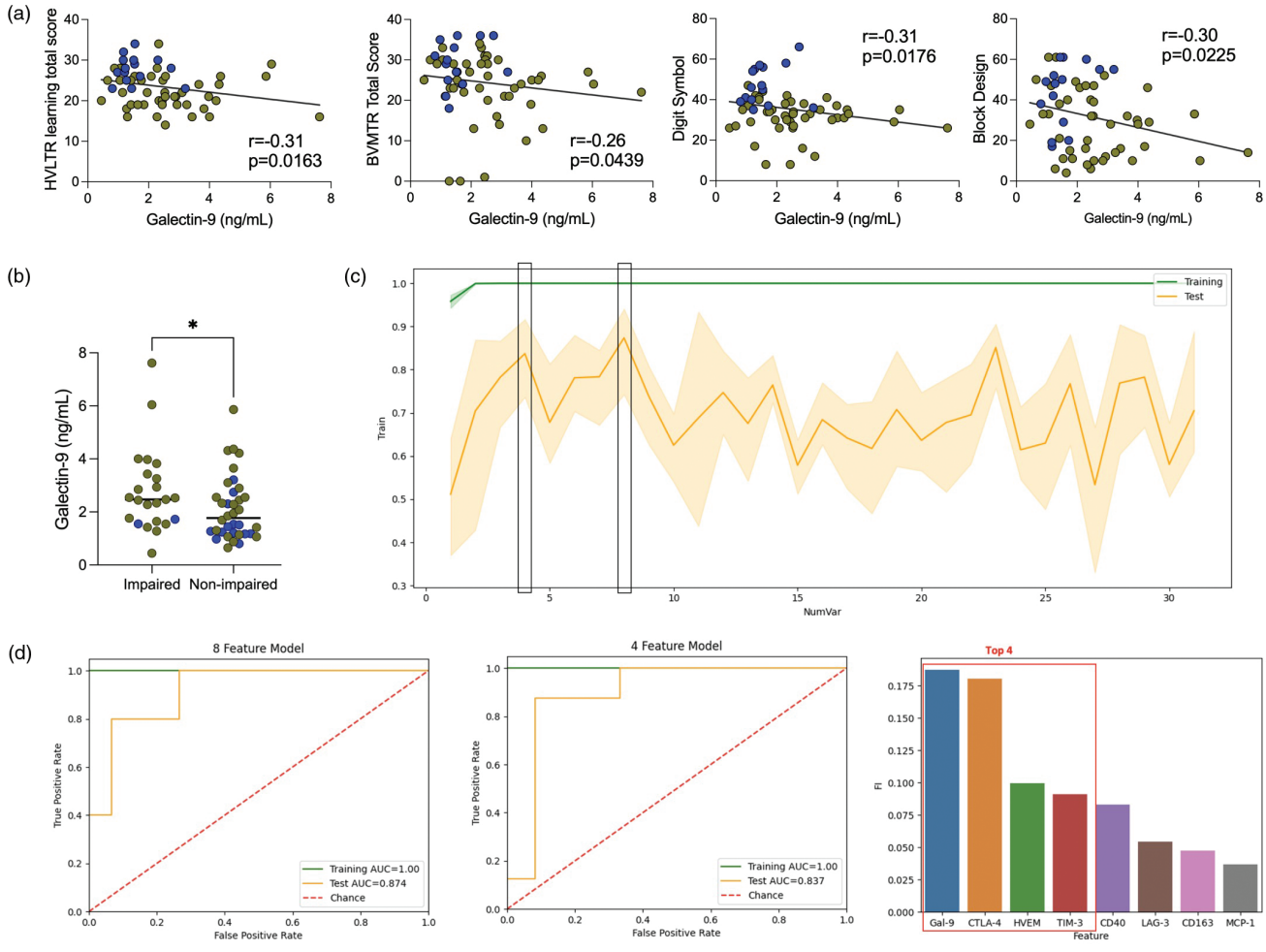

Fig. 2. Plasma levels of galectin-9 correlate with neurocognitive impairment and classifies cognitive status in adolescents, independent of HIV status. (a) Correlations of Gal-9 levels with HVLTR learning, BVMTR, Digital Symbol and Block Design test scores determined by Spearman's *r* value ($n = 59$). Raw scores from each cognitive test were used for correlation analysis. (b) Comparison of Gal-9 levels among individuals defined as neurocognitively impaired ($n = 23$) vs. nonimpaired ($n = 36$). (c) Decision tree models comprised of immune proteins were repeatedly trained to include increasingly more features to classify adolescents by cognitive status. Models including eight and four features were chosen for best performance with the least components. (d) ROC curves to evaluate the performance of eight-feature and four-feature decision tree models. AUC measuring the model performance for training and test sets are detailed in legend. Bar graph represents feature importance (FI) of biomarkers included in models.

Circulating immune markers have been associated with cognitive function in AWH [17–26]; however, associations between Gal-9 and cognition remain poorly defined. Gal-9 elicits multifactorial immune responses through interactions with various cognate ligands, like Tim-3, PDI, and CD44 [43–46]. Gal-9 levels are perturbed in many disease conditions, including viral infections [47–49], which may lead to increased immune activation and exhaustion, cell death, monocyte turnover rates, perturbation of lymphocyte effector functions, and altered cytokine production [50–58]. Our results demonstrating Gal-9 associations with multiple soluble mediators, indicate that Gal-9 may play a central role in immune mechanisms driving HIV-associated comorbidities in adolescents. Notably, the association of Gal-9 levels with sCD14 is interesting. $CD14^+$ in the plasma represents ongoing microbial translocation and compromised gut integrity, which is a hallmark of HIV infection in adults; however, studies in children with vertically acquired HIV are limited. It was recently reported that immune activation and inflammation were not linked to alterations in the gut in the setting of perinatal HIV [59]. In our study, the association of Gal-9 with sCD14 was only statistically significant in the Indian cohort (Fig. 1b and d). Further, we did not find any association of Gal-9 levels with I-FABP (Figure S2, http://links.lww.com/QAD/D182), which is also a marker of intestinal barrier dysfunction [60]. Decision tree ML algorithm revealed Gal-9 and its receptor, Tim-3, as

contributors in the classification of AWH with cognitive impairment, suggesting Gal-9 and related pathways may be potential drivers of cognitive dysfunction.

Study limitations include a small sample size, which restricted adjustments for correlation analysis. Gal-9 values differed between India and Myanmar cohorts because of assay variability; however, the values were comparable within assay. Such inter-assay variation is common [61] and does not affect our study findings. As cognitive test scores were not available for adolescents in India, it is unclear if findings in Myanmar AWH will generalize to those residing in India given potential differences in viral clade, treatment histories, and social determinants of health. Moreover, as our cohorts only included adolescents with vertically acquired HIV, it would be interesting to see if these associations persist in individuals with nonvertically acquired HIV from similar demographic background. As Gal-9, directly or via pro-inflammatory cytokines, can modulate CNS function [29,62], further studies to evaluate potential mechanisms of how peripheral Gal-9 may affect the brain are warranted. Monitoring changes of circulating Gal-9 levels may be beneficial in clinical practice to inform treatment decisions. Future research may explore the underlying mechanisms linking Gal-9, inflammation, and cognitive performance, paving the way for targeted interventions and therapeutic strategies to improve cognitive outcomes in adolescents.

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L.C.N., R.T., and P.M. conceived and designed the experiments; U.K.S. and B.P.G. were involved in recruitment of the Indian cohort and clinical data collection; K.L., E.E.S., T.S., H.H., D.C., C.S.H., E. H.K., C.T., Y.Y.M, N.N., J.M., J.B., S.M., and A.M.M. A. were involved in recruitment of the Myanmar cohort and clinical data collection; P.M., T.A.P., and U.K.S. performed experiments; L.C.N. and P.M. analyzed the data; L.C.N., and S.B. contributed reagents, materials, and analysis tools; L.C.N. and P.M. wrote the article. All authors reviewed and approved the article.

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Conflicts of interest

L.C.N. reports grants from the NIH and has received consulting fees from work as a scientific advisor for AbbVie, ViiV Healthcare, and Cytodyn where he also serves on the Board of Directors for work outside of the submitted work. L.C.N. interests were reviewed and are managed by Weill Cornell Medicine in accordance with their conflict-of-interest policies. All other authors declare that they have no conflicts of interest.

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