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CONCISE COMMUNICATION

Plasma galectin-9 as a predictor of adverse non-AIDS events in persons with chronic HIV during suppressive antiretroviral therapy

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Background: People with HIV (PWH) on antiretroviral therapy (ART) still experience an increased risk of morbidity and mortality, presumably driven by chronic inflammation, yet predictors of discrete or combinatorial outcomes remain unclear. Galectin-9 (Gal-9), a driver of both inflammatory and immunosuppressive responses, has been associated with HIV disease progression and multimorbidity.

Objective: To determine whether plasma Gal-9 levels are associated with the occurrence of specific non-AIDS events (NAEs) in PWH initiating ART.

Design: We performed a nested case–control study of PWH enrolled from 2001 to 2009 and evaluated pre-ART (66 cases, 97 controls), a year post-ART (112 cases, 211 controls), and immediately preceding an event (89 cases, 162 controls). Events included myocardial infarction/stroke, malignancy, serious bacterial infection, or death.

Methods: Plasma Gal-9 levels were assessed by ELISA. Conditional logistic regression assessed associations with NAEs and Spearman's correlations compared Gal-9 with other previously assessed biomarkers.

Results: NAEs occurred at a median of 2.8 years (1.7–4.6) after ART initiation. Higher Gal-9 levels were associated with increased risk of NAEs at year 1 and preevent [odds ratio (OR) per 1 interquartile range = 1.4–1.6; all $P < 0.05$], specifically myocardial infarction/stroke at year 1 (OR = 1.9; $P = 0.029$). Gal-9 also correlated with multiple inflammatory and immune activation predictors of NAEs (all timepoints).

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Conclusion: Elevated Gal-9 levels are predictive of deleterious NAEs, particularly cardiovascular complications. Whether the Gal-9 pathway, potentially binding to its putative ligands, is active in the pathogenesis of these outcomes warrants further investigation to determine if targeting Gal-9 may slow or reverse the risk of NAEs.

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Keywords: antiretroviral therapy, biomarkers, galectins, HIV, morbidity viral suppression

Introduction

People with HIV (PWH) experience non-AIDS events (NAEs), such as cardiovascular disease, non-AIDS-defining malignancies, and mortality, earlier and at a higher rate than people without HIV despite the use of antiretroviral therapy (ART) [1,2]. Persistent inflammation and immune activation are hallmarks of HIV infection and are thought to be significant contributors to these comorbid events [3,4], and the cause of this chronic inflammation is likely multifactorial and varies by individual and over time [3]. Although several markers of inflammation, coagulation, and immune activation are predictive of NAEs [5,6], mechanisms involved in the progression of morbidities in HIV is still not fully understood.

Glycoimmunology, glycan–lectin interactions governing immune responses, is an emerging field in host–pathogen research, with several glycan and lectin members historically being associated with HIV pathogenesis [7,8]. Galectin-9 (Gal-9), a glycan-binding immunomodulatory protein, is elevated in plasma of PWH on ART [9] and associates with pathogenesis, increased risk of mortality, and the extent of multimorbidity [10–12]. Gal-9 has been linked to several age-related complications in the general population, including cardiovascular, kidney, and liver dysfunction [13–16], and proposed as a promising biomarker for various cancers and autoimmune diseases [17–24]. Given the pleiotropic nature of Gal-9 in immunosuppression and inflammation [25–31], and in line with studies on HIV pathogenesis and morbidity, the significance of Gal-9 in the occurrence of NAEs warrants consideration.

To investigate the predictive efficacy of plasma Gal-9 for NAEs, we conducted a case–control study of participants enrolled in the AIDS Clinical Trials Group (ACTG) Longitudinal Linked Randomized Trials (ALLRT) [32], evaluated prior to ART, 1 year after ART initiation, and a timepoint prior to an NAE. We also explored potential Gal-9 relationships with pre-ART factors and biomarkers previously linked to NAEs. Further elucidating novel biomarkers, such as Gal-9, that track immune perturbations and associate with NAEs will help identify and/or monitor interventions aimed to reduce morbidity and mortality in PWH on effective ART.

Methods

Cohort descriptions

NWCS 411 is an ALLRT-nested case–control study to examine potential predictive biomarkers and their relationships with NAEs in PWH enrolled from 2001 to 2009 [32]. This study builds off a previous case–control study, NWCS 329, which found associations between several biomarkers of immune activation and NAEs [6]. NAEs in participants (cases) include myocardial infarction/stroke, malignancy, serious bacterial infections, or mortality. For each case, 1–3 participants (controls) with an event-free follow-up equal or greater than the relevant case, were matched for age, sex, pre-ART $CD4^+$ T-cell count, and ART regimen. All participants were ART-naive when enrolled and had plasma HIV RNA less than 400 copies/ml a year post-ART initiation.

Galectin-9 quantification

Stored plasma aliquots were measured for Gal-9 in duplicate using the solid-phase Human Galectin-9 Quantikine ELISA kit (R&D Systems, Minneapolis, Minnesota, USA) according to manufacturer's instructions. Optical density was read with a microplate spectrophotometer (Bio-Rad, Hercules, California, USA) and data analysis, including four parameter logistic standard interpolation, was carried out using MyAssays Ltd. data analysis. Average intra-assay coefficient of variation (CV) was 4.11% and inter-assay CV was 9.95%.

Statistical analyses

Demographic and clinical characteristics are presented using median (IQR) for continuous variables and frequency for categorical variables. Baseline to year 1 Gal-9 distributions were compared using Wilcoxon matched-pairs signed rank test to calculate *P* values and Hodges–Lehmann estimate of the location shift (with 95% CI). Conditional logistic regression analysis assessed associations of Gal-9 and NAEs in models unadjusted and adjusted for pertinent covariates at every timepoint. As participants were matched by pre-ART $CD4^+$ count and suppressed by week 48, adjusted analyses considered HIV-RNA levels at baseline and $CD4^+$ count for postbaseline analyses. Relationships among biomarkers at each timepoint among controls were assessed by Spearman correlations. Soluble

markers were $\log_{10}$ transformed prior to analyses. All statistical tests used a two-sided 5% type-I error rate, without adjustment for multiple testing, and were performed in SAS version 9.4 (SAS Institute, Cary, North Carolina, USA).

Results

Cohort and plasma galectin-9 distributions

Three time points were considered in this analysis: baseline (pre-ART; 66 cases, 97 controls), a year post-ART initiation (112 cases, 211 controls), and immediately preceding an event (89 cases, 162 controls). Overall, 84% of participants were men, median (Q1–Q3) age was 45 (39–51) years, $CD4^+$ T-cell count was 213 (79–334) cells/μl, and plasma HIV RNA was 4.8 (4.4–5.4) $\log_{10}$ copies/ml (Table 1). $CD4^+$ T-cell count at year 1 for controls and cases were 404 (269–561) and 347 (229–479) cells/μl, respectively, reflecting an expected rebound of counts after ART initiation. NAEs occurred at a median of 2.8 years (1.7–4.6) after ART initiation and 10.5 (6, 19) weeks from the preevent timepoint. Among cases, 13.4% were nonaccidental deaths ($n = 18$), 28.4% were myocardial infarction (MI)/strokes ($n = 38$), 37.3% were malignancies ($n = 50$), and 26.9% were serious bacterial infections ($n = 36$). Distributions of plasma Gal-9 among cases and controls at each timepoint are shown in Fig. 1a. Baseline plasma Gal-9 levels were similar for cases and controls: median value 15.39 (IQR = 10.35–24.61) and 15.00 (10.05–24.03) μg/ml, respectively. As previously observed [33], plasma Gal-9 levels after ART initiation (year 1) differed significantly compared with baseline values, here in cases [95% CI = 0.20 (0.13–0.27), $P < 0.001$) and to a greater extent in controls [95% CI = 0.28 (0.22–0.33), $P < 0.001$].

Galectin-9 associations with pre-antiretroviral therapy factors and previously assessed biomarkers among controls

Higher Gal-9 levels at baseline were correlated with higher HIV RNA ($r = 0.49$, $P < 0.0001$) and with lower $CD4^+$ T-cell counts ($r = -0.58$, $P < 0.0001$), as previously reported (Fig. 1b, Supplementary Table 1, http://links.lww.com/QAD/C257) [34]. We also found several strong associations between plasma Gal-9 and previously analyzed biomarkers. Baseline levels of Gal-9 highly correlated with interleukin-6 (IL-6), soluble tumor necrosis factor receptor (sTNFR) I and II, soluble urokinase plasminogen activator receptor (suPAR), sCD14, interferon gamma-induced protein 10, and D-dimer (all $r \geq 0.45$, $P < 0.0001$), and were moderately associated with 1,3-β-D-glucan (BDG), liposaccharide-binding protein, intestinal fatty-acid binding protein, and sCD163 (all $r = 0.22–0.36$, $P \leq 0.008$). Strong associations among Gal-9 with sTNFR-I, sTNFR-II, and suPAR remained at year 1 post-ART initiation and preevent (all $r \geq 0.45$, $P < 0.0001$).

Galectin-9 as a predictor of non-AIDS events

Higher plasma levels of Gal-9 were associated with having a NAEs at year 1 (unadjusted odds ratio (OR) per 1 IQR = 1.4; 95% CI, 1.0–1.9; $P = 0.036$) and preevent (OR = 1.6, 1.0–2.3; $P = 0.029$) (Fig. 1c). Although not statistically significant, a similar effect size was observed at

Table 1. Cohort demographic and clinical characteristics.

Characteristic	Case ($N = 134$)	Control ($N = 292$)	Total ($N = 426$)
Age at parent study entry	47 (40–53)	44 (39–50)	45 (39–51)
Regimens evaluated, by parent study			
ACTG 384: (AZT + 3TC vs. d4T + ddI) + (EFV vs. NFV vs. NFV + EFV) ACTG 388: (AZT + 3TC vs. d4T + 3TC) + (IDV vs. NFV vs. IDV + NFV) A5014: NVP + [LPV/r vs. (ABC + 3TC + d4T)] A5095: AZT/3TC + (ABC vs. EFV vs. ABC + EFV) A5142: (EFV + AZT/d4T + 3TC) vs. (LPV/r + AZT/d4T + 3TC) vs. (EFV + LPV/r) A5202: (ABC/3TC vs. TFV/FTC) + (ATV/r vs. EFV)	32 (24%)	63 (22%)	95 (22%)
Sex			
Male	112 (84%)	247 (85%)	359 (84%)
Female	22 (16%)	45 (15%)	67 (16%)
Race/ethnicity			
White non-Hispanic	70 (52%	138 (47%)	208 (49%)
Black non-Hispanic	48 (36%)	82 (28%)	130 (31%)
Hispanic (regardless of race)	15 (11%)	61 (21%)	76 (18%)
Asian, Pacific Islander	0 (0%)	6 (2%)	6 (1%)
Native American, Alaskan native	1 (1%)	2 (1%)	3 (1%)
Participant does not know	0 (0%)	1 (0%)	1 (0%)
More than one race	0 (0%)	2 (1%)	2 (0%)
Baseline $CD4^+$ T-cell count (cells/μl)	207 (87–334)	220 (76–332)	213 (79–334)
Baseline $\log_{10}$ HIV-1 RNA (copies/ml)	4.8 (4.4–5.3)	4.8 (4.4–5.4)	4.8 (4.4–5.4)

Categorical variables are represented as frequency and continuous variables as median (Q1–Q3). 3TC, lamivudine; ABC, abacavir; ATZ/r, ritonavir-boosted atazanavir; AZT, zidovdine; d4T, stavudine; ddI, didanosine; EFV, efavirenz; FTC, emtricitabine; IDV, indinavir; LPV/r, ritonavir-boosted lopinavir; NFV, nelfinavir; NVP, nevirapine; TFV, tenofovir.

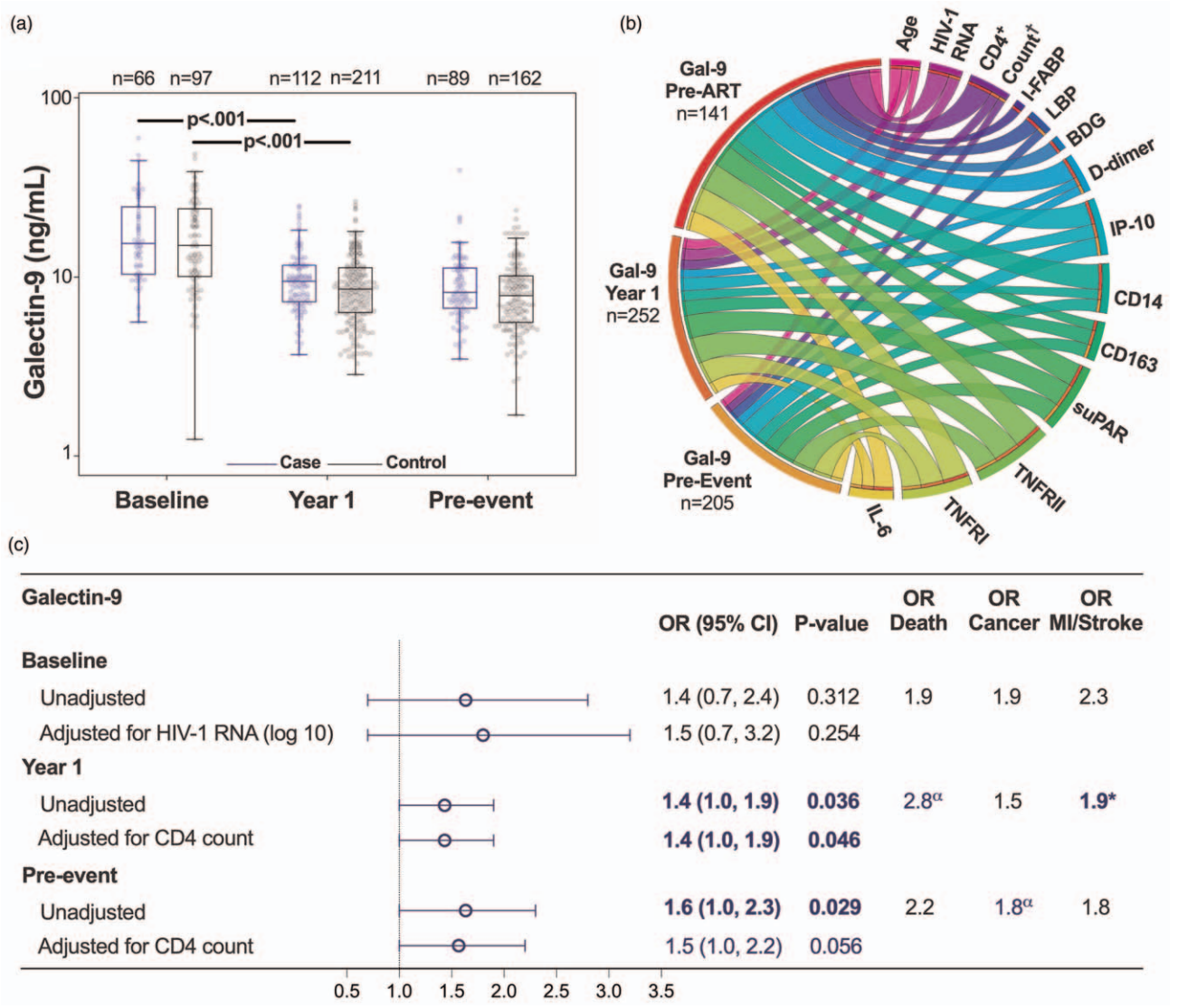

Fig. 1. Plasma galectin-9 predicts non-AIDS events post-antiretroviral therapy initiation. (a) Distribution of plasma galectin-0 (Gal-9) among cases (blue) and controls (black) at each time point. Jitter plots including median and interquartile range (box), and minimum and maximum (vertical lines) are displayed. (b) Spearman correlations for galectin-9 with pre-ART factors and previously assessed biomarkers among controls; all correlations *P* less than 0.05; ribbon width is proportional to respective *r* value (range: 0.17–0.64); band color represents associated parameter; $^{+}$Indicates inverse correlations. (c) Associations between galectin-9 levels and odds ratios (ORs) the occurrence of a non-AIDS event; adjusted analyses controlled for concurrent HIV viral load at baseline and CD4^{+} T-cell count at year 1 and preevent; $^{*}P$ less than 0.05, $^{\alpha}P = 0.10$ to less than 0.05. BDG, 1,3-β-D-glucan; I-FABP, intestinal fatty-acid binding protein; IL-6, Interleukin 6; IP-10, interferon gamma-induced protein 10; LBP, liposaccharide-binding protein; MI, myocardial infarction; suPAR, soluble urokinase plasminogen activator receptor; TNFR, tumor necrosis factor receptor.

baseline (OR = 1.4, 0.7–2.8; $P = 0.312$). Furthermore, the association at year 1 remained significant with adjustment for CD4^{+} count (OR = 1.4, 1.0–1.9; $P = 0.046$). However, adjustment in other biomarkers previously linked to NAEs, particularly IL-6, TNFRI/II, and suPAR, attenuated Gal-9 associations (Supplementary Tables 2–4, http://links.lww.com/QAD/C258, http://links.lww.com/QAD/C259, http://links.lww.com/QAD/C260). When examining component-specific analyses by type of NAEs (Fig. 1c, Supplementary Table 5, http://links.lww.com/QAD/C261), higher year 1 Gal-9 levels were associated with increased risk of MI/stroke ($n = 32$ events; OR = 1.9, 1.1–3.4; $P = 0.029$); while not statistically significant, higher levels of Gal-9 was associated with increased risk of death ($n = 14$ events; OR = 2.8, 0.9–8.2; $P = 0.069$) at year 1 and increased risk of malignancy at preevent ($n = 35$ events; OR = 1.8, 1.0–3.3; $P = 0.050$).

Discussion

Given that PWH on effective ART remain at an increased risk in developing age-related comorbidities or death [1,2], reliable screening to mitigate risk and monitor interventions is required. Uncovering effective biomarkers that predict adverse HIV outcomes at any time during the disease course prior to or during ART are key in this regard. We show here that plasma Gal-9 associates with an increased risk of overall NAEs post-ART initiation despite significant decreases in levels after treatment, in line with previous studies of Gal-9 levels reflecting severe outcomes in infectious diseases and associating with multimorbidity and mortality risk in treated HIV [11,35]. Furthermore, we found that Gal-9 levels predicted MI/stroke 1 year after ART, of importance as PWH have a higher risk of developing coronary heart disease and Gal-9 is associated with cardiovascular outcomes in the general population [15,16]. Overall, our data suggests that Gal-9 may serve as a novel, predictive, and easily measurable marker to evaluate alone or incorporated in a biomarker composite panel to strengthen the monitoring of PWH at high risk and interventional studies.

Elevated levels of Gal-9 levels have been shown to correlate with multiple biomarkers of inflammation and immune activation previously linked to NAEs (i.e. sTNFRI/II, suPAR) [5,6]. Gal-9 itself is known to drive multiple immune pathways, including both the immunosuppression and proinflammatory cytokine induction of myeloid cells [36,37] and the impairment of T, B, and natural killer (NK) lymphocyte function [25,29,30]. Gal-9 can elicit pathways involved in apoptosis [26] and immune negative checkpoint activation [38–40], which are relevant to outcomes in HIV infection [41–44]. Investigating the cellular expression of Gal-9 ligands, including TIM-3 and Dectin-1, will contribute to understanding the pathogenic effect of elevated Gal-9. Interestingly, both Gal-9 and BDG bind Dectin-1 on myeloid cells [45,46]. As BDG is the main ligand of Dectin-1 on myeloid cells, and also predictive of NAEs at year 1, unraveling their intertwined pathways would be particularly interesting. Ultimately, unraveling the role Gal-9 may play in HIV-associated adverse outcomes would reveal whether modulation through targeting Gal-9 and/or Gal-9-induced pathways would prove valuable in altering the trajectory of disease. However, given proinflammatory cytokines can induce the cellular release of Gal-9 [47], it remains unclear whether elevations observed could merely be a consequence of residual inflammation that persists during infection.

Limitations to this study include that our analysis was restricted to PWH during chronic infection with low baseline $CD4^+$ counts, limiting inference for patients today. Given comorbidity-free life expectancy is increased with early ART administration and rapid viral decline with current regimens that include integrase inhibitors [48,49], investigating whether Gal-9 still predicts NAEs following early modern ART intervention is warranted. Although Gal-9 was not significantly associated with NAEs at baseline, the effect size was notably consistent with our other findings and may be because of its strong link with viremia and immune suppression [9,12,34], as well as insufficient plasma for many participants at baseline. However, no major differences for baseline characteristics or Gal-9 levels were observed at year 1 among participants with a year 1 result who did or did not have one at baseline. We were also not able to evaluate Gal-9 changes from year 1 to preevent as the preevent time point varied across participants and sometimes occurred before year 1. Finally, the relative strength of the association between Gal-9 and disease outcomes were attenuated for and lower as compared with several previously assessed biomarkers, such as IL-6 and suPAR; although, calculated risks were relatively similar [5,6].

In conclusion, further dissecting Gal-9 pathways that interlink with viral persistence, immune activation, and inflammation leading to NAEs will determine the potential of Gal-9 as a target for intervention in the setting of suppressive ART. As Gal-9 is only one component of the emerging field of glycoimmunology, elucidating the importance of glycosylation and glycan–lectin interactions in HIV infection, particularly in the context of NAEs, should be pursued.

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Ethics approval and consent to participate: participants (or, for minors, their parent or legal guardian) provided written informed consent, and institutional review board approval for ALLRT was obtained by each ACTG site.

Availability of data and materials: Individual participant data and a data dictionary defining each field in the set will be made available to investigators on a case-by-case basis via request to the AIDS Clinical Trials Group (ACTG) via the link: https://submit.actgnetwork.org/. Completion of an ACTG Data Use Agreement may be required.

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Author contributions: T.A.P. and L.C.N. wrote the manuscript; T.A.P. conducted soluble marker acquisition and data interpretation; C.B.M. and A.M. performed data analysis and interpretation; M.H., E.I.L., M.M.L., and A.L.L. provided critical review of the manuscript. L.C.N., C.B.M., and S.G. contributed to study design and concept. All authors reviewed the manuscript.

Conflicts of interest

L.C.N. has served on an advisory board for Abbvie, ViiV, and Cytodyn for work unrelated to this project. M.H. has received project funding from Gilead, Pfizer, and Astellas. All of the other authors have no conflicts of interest.

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