Reduced Levels of NAD in Skeletal Muscle and Increased Physiologic Frailty Are Associated With Viral Coinfection in Asymptomatic Middle-Aged Adults

Thanh Tran, PhD,[a,b] Karol M. Pencina, PhD,[a,b,c] Michael B. Schultz, PhD,[d] Zhuoying Li, PhD,[b] Catherine Ghattas, PhD,[b] Jackson Lau, PhD,[b] David A. Sinclair, PhD, AO,[d] and Monty Montano, PhD[a,b,c]

Background: People living with HIV (PLWH) are disproportionately burdened with multimorbidity and decline in physiologic function compared with their uninfected counterparts, but biological mechanisms that differentially contribute to the decline in muscle function in PLWH compared with uninfected people remain understudied.

Setting: The study site was Brigham and Women's Hospital, Harvard Medical School, Boston, MA.

Methods: We evaluated skeletal muscle tissue for levels of total nicotinamide adenine dinucleotide (NAD), NAD^+, and nicotinamide adenine dinucleotide (NADH) in middle-aged asymptomatic PLWH, coinfected with hepatitis C virus and/or cytomegalovirus and compared them with uninfected control participants.

Results: Of the 54 persons with muscle biopsy data, the mean age was 57 years with 33% women. Total NAD levels declined in skeletal muscle in association with HIV infection and was exacerbated by hepatitis C virus and cytomegalovirus coinfection, with lowest levels of total NAD, NAD^+, and NADH among persons who were coinfected with all 3 viruses ($P = 0.015$, $P = 0.014$, and $P = 0.076$, respectively). Levels of total NAD, NAD^+, and NADH in skeletal muscle were inversely associated with inflammation ($P = 0.014$, $P = 0.013$, and $P = 0.055$, respectively). Coinfections were also associated with measures of inflammation (CD4/CD8 ratio: $P < 0.001$ and sCD163: $P < 0.001$) and immune activation (CD38 and human leukocyte antigen-DR expression on CD8 T cells: $P < 0.001$). In addition, coinfection was associated with increased physiologic frailty based on the Veteran Aging Cohort Study 1.0 index assessment ($P = 0.001$).

Conclusions: Further research is warranted to determine the clinical relevance of preclinical deficits in NAD metabolites in skeletal muscle in association with viral coinfection and inflammation, as well as the observed association between viral coinfection and physiologic frailty.

Key Words: HIV, NAD, muscle, inflammation, physiologic frailty

(*J Acquir Immune Defic Syndr* 2022;89:S15–S22)

INTRODUCTION

The success of effective combination antiretroviral therapy has dramatically increased life expectancy in people living with HIV (PLWH).[1] However, as PLWH age, they are prematurely burdened by multiple comorbid conditions, including a higher prevalence of functional limitations compared with their uninfected counterparts[2] and chronically elevated biomarkers for inflammation [eg, C-reactive protein (CRP), interleukin-6 (IL-6), CD163, and CD14] and immune activation [eg, CD38 and human leukocyte antigen-DR [HLA-DR]).[3] PLWH also have a higher prevalence of viral coinfections, notably with hepatitis C virus (HCV) and cytomegalovirus (CMV) infection. HCV coinfection is common among PLWH in the United States with a prevalence of 20%–25%.[4] Despite the recent success in sustained virologic repression with direct-acting agents, HCV continues to accelerate liver disease, adversely affecting mortality and quality of life and increasing the risk of physiologic frailty.[5] CMV coinfection is nearly universal in PLWH and is associated with elevated inflammation, accelerated immune senescence, and also an increased risk of frailty.[6]

Received for publication September 23, 2021; accepted September 27, 2021.

From the [a]Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA; [b]Men's Health: Aging and Metabolism, Brigham and Women's Hospital, Harvard Medical School, Boston, MA; [c]Boston Claude D. Pepper Older Americans Independence Center, Boston, MA; and [d]Department of Genetics, Blavatnik Institute, Paul F. Glenn Labs for the Biology of Aging, Harvard Medical School, Boston, MA.

Supported by the National Institute on Aging (R21 AG055415), the National Institute of Allergy and Infectious Diseases (R01 AI08541), the Boston Older Americans Independence Center (P30 AG031679), and the Harvard University Center for AIDS Research (P30 AI060354).

D.A.S is a consultant to, inventor of patents licensed to, board member, and investor in EdenRoc Sciences and affiliates, Life Biosciences and affiliates, MetroBiotech, Animal Biosciences, and Jumpstart Fertility, who are developing NAD-related medicines. He is an inventor on a patent application licensed by Harvard Medical School to Elysium Health. For additional unrelated affiliations, see https://genetics.med.harvard.edu/. The other authors have no conflicts of interest to disclose.

T.T.: conducting experiments, acquiring data, analyzing data, and preparing the manuscript. M.B.S.: conducting experiments, analyzing data, providing reagents, and editing the manuscript. K.M.P.: analyzing data, providing statistical insights, and reviewing the manuscript. Z.L.: analyzing data, providing statistical insights, and reviewing the manuscript. C.G.: conducting experiments, acquiring data, and editing the manuscript. J.L.: conducting experiments, acquiring data, and editing the manuscript. D.A.S.: designing research studies and reviewing the manuscript. M.M.: designing research studies, analyzing data, and writing the manuscript.

Correspondence to: Monty Montano, PhD, Brigham and Women's Hospital, Harvard Medical School, Boston, MA (e-mail: mmontano@bwh.harvard.edu).

We recently reported that the skeletal muscle phenotype of a cohort of asymptomatic middle-aged PLWH exhibits elevated internalized nuclei, reduced nuclear PGC-1α, a master regulator of mitochondrial biogenesis, and subclinical deficits in physical function[7]—features that become increasingly common with advanced age. However, in the same cohort, skeletal muscle cross-sectional area, fiber-type distribution, and fiber size did not differ from uninfected participants of similar age and sex. Collectively, this is consistent with an asynchronous aging phenotype, with some but not all features of age occurring prematurely.[8] In genetic studies using nonhuman model systems, mice lacking nicotinamide adenine dinucleotide (NAD^+) in skeletal muscle because of ablation of the rate limiting NAD biosynthetic enzyme [nicotinamide phosphoribosyltransferase (NAMPT)] displayed similar features to that observed in the skeletal muscle of our cohort of PLWH (eg, internalized nuclei and reduced PGC-1α) with a dramatic decline in physical function as the mice aged.[9]

NAD^+ is a key cofactor, both in cellular energy metabolism[10] and in modulation of inflammatory signaling.[11] In multiple species, NAD decline with age has been linked to deficits in mitochondrial function and metabolic capacity and decline in the activity of sirtuins, a class of NAD^+-dependent enzymes that control inflammation, mitochondrial metabolism, and aging.[12] Age-related decline of NAD is due in part to hydrolysis by an intrinsic NADase activity of the activation marker CD38.[13] Notably, NAD deficits in skeletal muscle have been linked to reduced capillary density and physical endurance.[14] In mice, restoring NAD levels in skeletal muscle[15] reduces centrally located myonuclei, decreases inflammation, and increases mitochondrial biogenesis and physical activity.[16] Thus, repletion of NAD is an attractive therapeutic modality for potentially reducing age-related inflammation (ie, inflammaging) and improving physical function.[12]

Given the increased risk of functional decline and elevated inflammation in PLWH and skeletal muscle phenotypes that resemble nonhuman models for reduced levels of NAD in skeletal muscle, this study sought to investigate whether NAD levels in skeletal muscle of asymptomatic PLWH, in the context of prevalent HCV and CMV coinfection, differ from their uninfected counterparts. We also sought to determine whether potential differences could be related to other variables present in this asymptomatic cohort of preclinical middle-aged PLWH.

MATERIALS AND METHODS

Study Population

The Muscle and Aging in Treated Chronic HIV infection (MATCH) study has been described elsewhere.[7,17,18] In brief, it is a prospective observational study conducted at Brigham and Women's Hospital in Boston, MA (clinical trials registration no. NCT03011957). The protocol was approved by the Partners Human Research Committee. Written informed consent was obtained from all participants. Men and women were recruited from the Boston metropolitan area, with 170 participants enrolled from April 2015 through October 2016. To be eligible for study entry, participants had to meet the following criteria: 50–65 years of age, sufficient lower extremity mobility to participate in functional assessment, HIV-negative (nonreactive to the HIV-1/2 antigen/antibody fourth generation test, Quest Diagnostics, MA) or HIV-positive on effective ART with no detectable virus (≤200 copies/mL, confirmed by HIV-1 RNA quantitative real-time PCR, Quest Diagnostics, MA), and CD4 levels ≥350 copies/mL (Quest Diagnostics, MA). Participants with acute illness in the past 60 days or use of anabolic therapy or corticosteroids within the past 6 months were excluded.

NAD^+/NADH Measurements

NAD^+ and nicotinamide adenine dinucleotide (NADH) were measured with a commercially available kit (Promega). In brief, banked muscle biopsy-derived tissue[7] was homogenized and lysed in a 1:1 solution of PBS and 0.2-N NaOH containing 1% dodecyltrimethylammonium bromide (wt/vol) (Sigma-Aldrich) and centrifuged (maximum speed for 5 minutes) to remove insoluble materials. For NAD^+ measurements, the pH was adjusted with addition of 0.4-N HCL in a 1:2 ratio with the sample; for NADH measurements, the pH was left basic. Samples were heated at 60°C for 15 minutes, allowed to cool, and neutralized with Tris base (Sigma-Aldrich). Finally, samples were incubated with a mixture containing lactate, lactate dehydrogenase, proluciferin, and an NADH-dependent proluciferin reductase.[19] NAD^+ or NADH levels were calculated by comparing luminescence with a standard curve and were normalized to protein concentration, measured with the bicinchoninic acid assay (Thermo Fisher Scientific). The Veteran Aging Cohort Study (VACS) index was determined for all participants based on a blood chemistry panel (Quest Diagnostics) and lymphocyte subset panel (Quest Diagnostics), including participant age, CD4 cell count, viral load, hemoglobin level, and renal and hepatic biomarkers, as previously described.[7,20]

Biomarker Values

Methods for assessing inflammatory biomarkers (high-sensitivity CRP, soluble CD14 (sCD14), soluble CD163 (sCD163), and IL-6) through ELISA, T-cell activation biomarkers ($CD8^+CD38^+$, $CD8^+$HLA-DR^+, and $CD8^+$HLA-DR^+CD38^+) through flow cytometry, and blood profile (VACS 1.0 index) through Quest Diagnostics. A composite score for inflammatory score was calculated based on measured serum levels of CRP, sCD14, sCD163, and IL-6 as previously described.[7] HCV status was reported by participants in the self-report medical history sheet and questionnaire and confirmed by a rapid antibody test (OraQuick). CMV status was determined by ELISA. Participants were assigned as CMV− or CMV+ based on CMV value <0.99 or ≥0.99, respectively. The composite viral score (0–3) reflects number of infections (ie, 0 = no infection; 1 = monoinfection with HIV, CMV, or HCV; 2 = coinfection with any combination of HIV, HCV, or CMV; and 3 = coinfection with all: HIV, HCV, and CMV).

Statistical Analysis

Descriptive statistics were presented as mean and SD or median and quartile range for normally and non-normally distributed variables, respectively. To combat skewness, data were log-transformed and compared between infected and noninfected individuals with respect to HIV, HCV, and CMV status using the Student *t* test. Univariate regression analyses were performed for each variable. All hypotheses were tested using the 2-sided alpha level of 0.05, except the hypothesis-driven prediction of reduced NAD metabolites with HIV infection which used a 1-sided *t* test. Analyses were performed using SAS v.9.4 (SAS Institute, Cary, NC) and STATA v.15.

RESULTS

Study Participant Characteristics

The participants in this study have been previously described.[7,17,18] In brief, the cohort consists of asymptomatic adults living with HIV infection on effective antiretroviral therapy and individuals without infection, both men and women, all aged between 50 and 65 years. This substudy consists of participants with skeletal muscle biopsy specimens (n = 54). Among the 54 participants, viral infection status was as follows: HIV+ (n= 29, 54%), HCV+ (n = 9, 17%), and CMV+ (n = 31, 57%). Among HIV+, coinfection was as follows: HIV+HCV+ (n = 9, 31%) and HIV+CMV+ (n = 24, 83%). Among HIV−, coinfection was as follows: HIV−HCV+ (0%, 0%) and HIV−CMV+ (n = 7, 28%) (Table 1). Among HCV−, n = 20 were HIV+ and n = 25 were HIV−. HCV participants reported previous treatment for infection. Study characteristics based on HIV, HCV, and CMV infection status are listed in Table 1.

Biomarkers for Inflammation and Immune Activation

Among biomarkers tested (ie, IL-6, hsCRP, sCD14, and sCD163), the level of sCD163 (a biomarker for monocyte

TABLE 1. Characteristics of Study Population Based on HIV, HCV, or CMV Infection

	HIV− (n = 25)	HIV+ (n = 29)	*P*	HCV− (n = 45)	HCV+ (n = 9)	*P*	CMV− (n = 23)	CMV+ (n = 31)	*P*
Demographic									
Male	14 (56.0%)	22 (75.9%)	0.12	30 (66.7%)	6 (66.7%)	0.99	17 (73.9%)	19 (61.3%)	0.33
Age, yr	57.1 ± 3.9	56.9 ± 4.4	0.87	57.0 ± 4.0	57.0 ± 4.8	0.99	56.4 ± 4.1	57.4 ± 4.2	0.40
BMI, kg/m^2	27.4 ± 4.3	26.9 ± 5.0	0.66	27.6 ± 4.6	24.6 ± 4.4	0.07	27.4 ± 3.8	26.9 ± 5.2	0.72
VACS 1.0	22.0 (12.0–28.0)	23.0 (22.0–33.0)	**0.044**	22.0 (12.0–28.0)	29.0 (27.0–39.0)	**0.002**	22.0 (12.0–27.0)	24.0 (22.0–34.0)	**0.016**
Viral									
HIV	0 (0%)	29 (100%)	NA	20 (44.4%)	9 (100%)	**0.002**	5 (21.7%)	24 (77.4%)	**<0.001**
HCV	0 (0%)	9 (31.0%)	**0.003**	0 (0%)	9 (100%)	NA	3 (13.0%)	6 (19.4%)	0.72
CMV	7 (28%)	24 (83%)	**<0.001**	25 (56%)	6 (67%)	0.88	0 (0%)	31 (100%)	NA
Immune									
Inflammatory									
IL-6	0.99 (0.56–1.50)	0.97 (0.68–1.36)	0.59	0.98 (0.65–1.54)	1.02 (0.75–1.15)	0.68	0.98 (0.63–1.47)	1.02 (0.68–1.54)	0.15
hsCRP	1.17 (0.91–1.65)	1.08 (0.61–3.67)	0.72	1.08 (0.70–1.73)	1.51 (0.11–3.67)	0.56	1.17 (0.61–1.65)	1.08 (0.68–3.72)	0.25
sCD14	2.39 (2.13–2.79)	2.34 (2.04–3.23)	0.55	2.36 (2.07–2.79)	3.01 (2.29–3.40)	0.19	2.36 (2.04–2.70)	2.52 (2.14–3.23)	0.12
sCD163	0.37 (0.28–0.47)	0.47 (0.35–0.64)	**0.026**	0.40 (0.28–0.51)	0.62 (0.44–0.75)	**0.018**	0.37 (0.26–0.51)	0.44 (0.35–0.63)	0.11
Activation									
CD8$^+$ and CD38$^+$	2.86 (1.61–3.64)	3.38 (2.76–4.31)	**0.013**	3.28 (1.79–4.24)	3.59 (2.76–4.02)	0.22	3.28 (1.61–3.98)	3.35 (2.44–4.31)	0.19
HLA-DR$^+$	4.00 (2.42–5.96)	8.04 (6.12–14.3)	**<0.001**	5.10 (3.18–8.04)	8.21 (6.34–9.35)	0.22	3.83 (2.42–6.82)	6.87 (4.95–13.0)	**<0.001**
HLA-DR$^+$CD38$^+$	0.76 (0.57–1.18)	1.57 (1.00–2.07)	**<0.001**	0.97 (0.62–1.67)	1.49 (1.23–2.49)	**0.036**	0.79 (0.57–1.31)	1.50 (0.82–2.07)	**0.007**
Liver biomarker									
ALB	4.30 (4.20–4.50)	4.30 (4.20–4.60)	0.20	4.30 (4.20–4.50)	4.20 (4.20–4.40)	0.52	4.30 (4.10–4.50)	4.30 (4.20–4.60)	0.29
FIB4	1.26 (1.08–1.42)	1.28 (1.10–1.80)	0.42	1.24 (1.07–1.56)	1.80 (1.15–1.81)	0.23	1.22 (1.08–1.38)	1.48 (1.07–1.83)	0.18
BUN	16.0 (12.0–20.0)	13.0 (12.0–16.0)	0.35	14.0 (12.0–19.0)	16.0 (14.0–16.0)	0.61	16.0 (12.0–20.0)	13.0 (11.0–16.0)	**0.040**

Values in mean ± STD or median (IQR) for continuous variables, n (%) for categorical variables. Participants were assigned as CMV− or CMV+ based on CMV value <0.99 or ≥0.99, respectively. Values for BMI, IL-6, hsCRP, sCD14, sCD163, CD8$^+$CD38$^+$, HLA-DR$^+$, HLA-DR$^+$CD38$^+$, ALB, FIB4, and BUN were log-transformed before statistical analysis (Student *t* test).

activation and liver function[21]) was significantly increased for HIV and HCV, but not for CMV, when compared with uninfected control groups ($P = 0.026$, $P = 0.018$, and $P = 0.110$, respectively). Participants infected with HIV exhibited a significant elevated immune activation profile [$CD8^+CD38^+$ ($P = 0.013$), HLA-DR^+ ($P < 0.001$), and HLA-DR^+CD38^+ ($P < 0.001$)]. Those infected with HCV (100% were HIV-coinfected) displayed significant immune activation compared with HCV-uninfected. Also, those infected with CMV (83% were HIV-coinfected) displayed significant immune activation compared with CMV-uninfected (Table 1).

Physiologic Frailty Using the VACS Index

The VACS index is a composite score reflecting the status of multiple organ systems and has been associated with mortality risk and physiologic frailty.[20,22] VACS indices were calculated as described in the Methods section. VACS index 1.0 scores for individuals infected with HIV, HCV, or CMV were significantly higher than those for uninfected individuals ($P = 0.044$, $P = 0.002$, and $P = 0.016$, respectively) (Table 1).

Biomarkers for Liver Function

With the exception of elevated sCD163, a monocyte activation biomarker associated with liver function,[20] other circulating biomarkers for liver function (ALB, FIB4, and BUN; all $P > 0.100$) (Table 1) did not differ significantly based on HIV and HCV infection, suggesting asymptomatic and potentially compensated liver function given previous HCV infection. However, BUN was significantly lower in CMV-infected ($P = 0.040$) (Table 1).

NAD Levels in Skeletal Muscle

Because multiple studies indicate declines in NAD with disease conditions, we hypothesized a directional effect (lower level) outcome in NAD metabolites (ie, total NAD, NAD^+, and NADH) in skeletal muscle biopsies from PLWH compared with uninfected. As shown in Figure 1, levels of total NAD ($P = 0.0492$) (but not NAD^+ or NADH) differed marginally in 1-tailed tests in skeletal muscle between PLWH and uninfected participants (Figs. 1A–C). Notably, when HIV+ participants were evaluated based on HCV coinfection status (no participants were monoinfected with HCV), in 2-tailed tests, we observed that total NAD ($P = 0.0211$), NAD^+ ($P = 0.0314$), and NADH ($P = 0.0483$) all differed significantly in HIV+HCV+ compared with HIV+HCV− (Figs. 1D–F). When HIV+ participants were evaluated based on CMV coinfection, NAD metabolites did not differ significantly between HIV+ with CMV compared with HIV+ without CMV coinfection (data not shown). A composite score for viral infections (eg, HIV, HCV, and CMV) was significantly associated with reduced total NAD, NAD^+, and a trend reduction in NADH (Figs. 2A–C). Interestingly, a composite score for increased inflammation was significantly associated with reduced total NAD and NAD^+ ($P = 0.013$ and $P = 0.014$, respectively) and a trend decline in NADH ($P = 0.055$) (Figs. 2D–F).

Univariate Regression Analysis for Viral Associations With NAD Metabolites and Immune Biomarkers for Inflammation and Activation

To identify variables associated with NAD metabolite levels in skeletal muscle, univariate regression analysis was performed with total NAD, NAD^+, and NADH as dependent variables with variables related to demographics [sex, age, and body mass index (BMI)], viral infection (HIV, HCV, and CMV), and a composite viral score (0–3) for number of infections (ie, 0 = no infection; 1 = monoinfection with HIV, CMV, or HCV; 2 = coinfection with any combination of HIV, HCV, or CMV; and 3 = coinfection with all: HIV, HCV, and CMV). As shown in Figure 2, a composite score for viral burden was associated with reduced total NAD and NAD^+ and a trend decline in NADH. A composite score for increased inflammation was also significantly associated with reduced total NAD and NAD^+ and a trend decline in NADH (Table 2).

To identify variables associated with viral coinfection, we measured biomarkers for inflammatory (CD4/CD8 ration and sCD163) and immune activation (CD8 T-cell expression of CD38 and HLA-DR) (Fig. 3). Among the tested demographic variables, age and biological sex did not differ significantly; however, BMI differed significantly in total NAD and NAD^+ ($P = 0.013$ and $P = 0.005$, respectively) (Table 2). Among viral infection variables, HCV and a composite viral score for infections were strongly associated with lower total NAD, NAD^+, and NADH levels with HCV: $P = 0.008$, $P = 0.012$, and $P = 0.026$, respectively (Table 2). The composite viral score was also associated with immune variables, including the CD4/CD8 ratio (a generic biomarker for inflammation, Fig. 3A), sCD163 (specific marker for inflammation, Fig. 3B), HLA-DR^+CD38^+ (biomarker for immune activation on CD8 T cells, Fig. 3C), and the VACS 1.0 index (a measure of physiologic frailty) (Fig. 3D).

DISCUSSION

A cornerstone of healthy aging is the maintenance of mobility and functional independence, and yet although PLWH have an increased life expectancy, they nevertheless experience premature loss in mobility and increased frailty risk.[23] Physiologic mechanisms that underlay this functional decline in PLWH are poorly understood, but accumulating evidence points toward dysregulated inflammation and bioenergetics.[24–27] Identifying targetable pathways contributing to these potential drivers of functional decline, especially before the onset of clinical symptoms, would provide an opportunity to improve health outcomes as PLWH age.

In our previous studies of asymptomatic middle-aged PLWH,[7,17,18] we reported modest deficits in gait speed and stair climb power. Interestingly, we also observed increased internalized nuclei more typical of skeletal muscle in older persons[28] and reduced levels of nuclear PGC-1α (a master regulator of mitochondrial biogenesis), suggesting compromised bioenergetics.[7] Notably, in a follow-up study, physical activity measured with accelerometry revealed a significantly

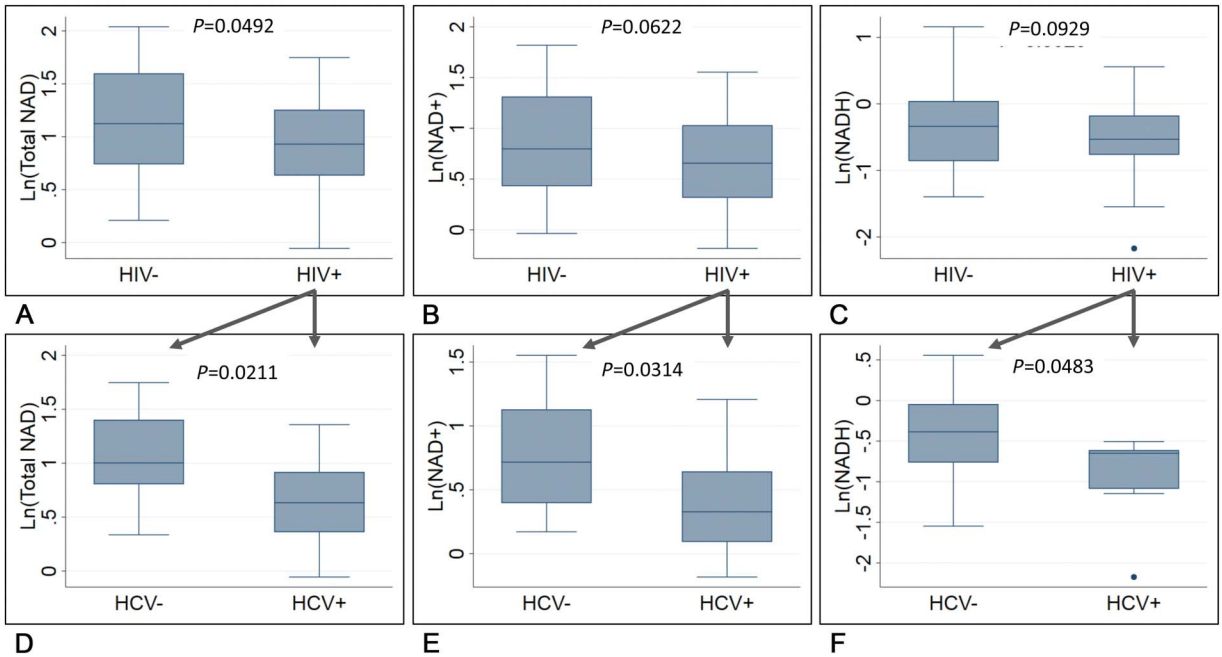

FIGURE 1. Levels of NAD metabolites (total NAD, NAD+, and NADH) in skeletal muscle. Shown are levels of total NAD (A, D), NAD+ (B, E), and NADH (C, F) in skeletal muscle for HIV+ vs HIV− (A–C) and HCV coinfection (D–F). *P* = *P* value of the Student *t* test; n = 25 HIV−, n = 29 HIV+, n = 9 HIV+HCV+, and n = 20 HIV+HCV−. CMV coinfection data are not shown.

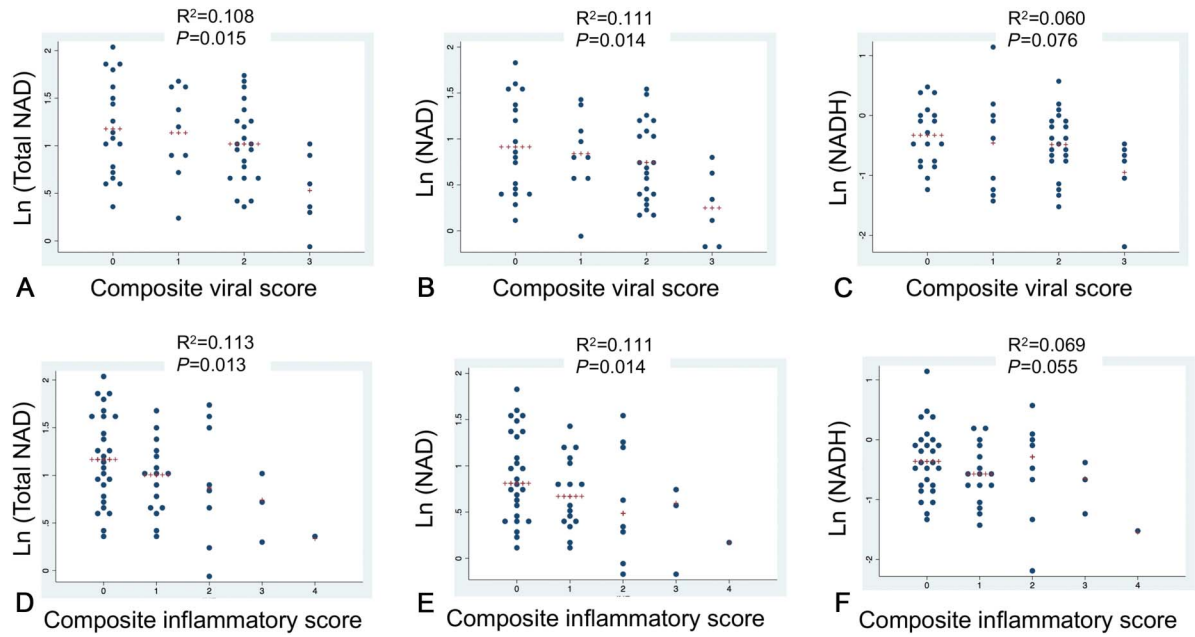

FIGURE 2. Total NAD, NAD+, and NADH in skeletal muscle vs a composite viral score or inflammatory score. A–C, Images show the correlation between the composite viral score and total NAD (A), NAD+ (B), or NADH (C). The composite viral score (0–3) reflects number of infections (ie, 0 = no infection; 1 = monoinfection with HIV, CMV, or HCV; 2 = coinfection with any combination of HIV, HCV, or CMV; and 3 = coinfection with all: HIV, HCV, and CMV). D–F, Images show the correlation between INF and total NAD (D), NAD+ (E), or NADH (F). The composite inflammatory scores were calculated based on quartiles of expression for each biomarker (ie, CD163, CD14, CRP, and IL-6), with the top quartile and the bottom 3 quartiles dichotomized and summed to generate unique scores. R^2 and *P* values shown for univariate regression analysis are listed in Table 2.

TABLE 2. Univariate Linear Regression Analysis for NAD Metabolite Levels

	Ln (Total NAD)			Ln (NAD^+)			Ln (NADH)		
	Estimate (95% CL)	*P*	R^2	Estimate (95% CL)	*P*	R^2	Estimate (95% CL)	*P*	R^2
Demographic									
Male	−0.15 (−0.43 to 0.14)	0.308	0.020	−0.07 (−0.36 to 0.22)	0.611	0.005	−0.26 (−0.61 to 0.10)	0.152	0.039
Age	0.01 (−0.03 to 0.04)	0.657	0.004	0.01 (−0.03 to 0.04)	0.664	0.004	0.01 (−0.03 to 0.05)	0.650	0.004
BMI	0.03 (0.01 to 0.06)	**0.013**	0.106	0.04 (0.01 to 0.07)	**0.005**	0.142	0.03 (−0.01 to 0.07)	0.120	0.046
Viral									
HIV	−0.22 (−0.48 to 0.04)	0.098	0.052	−0.21 (−0.48 to 0.06)	0.124	0.045	−0.23 (−0.56 to 0.11)	0.186	0.033
HCV	−0.47 (−0.81 to −0.13)	**0.008**	0.129	−0.45 (−0.80 to −0.10)	**0.012**	0.116	−0.50 (−0.94 to −0.06)	**0.026**	0.092
CMV	−0.19 (−0.46 to 0.08)	0.161	0.038	−0.23 (−0.50 to 0.04)	0.092	0.054	−0.12 (−0.47 to 0.22)	0.470	0.010
Composite viral score (0 to 3 infections)	−0.15 (−0.27 to −0.03)	**0.015**	0.108	−0.16 (−0.28 to −0.03)	**0.014**	0.111	−0.14 (−0.30 to 0.16)	0.076	0.060
Composite inflammatory score	−0.16 (−0.29 to −0.03)	**0.013**	0.113	−0.16 (−0.29 to −0.04)	**0.014**	0.111	−0.16 (−0.33 to 0.33)	0.055	0.069

Univariate regression analysis was performed with total NAD, NAD^+, and NADH as dependent variables with variables related to demographics (sex, age, and BMI), viral infection (HIV, HCV, and CMV), a composite viral score (0–3) for number of viral infections, and a composite score for inflammation. Values were log-transformed before statistical analysis. Values shown for regression are coefficient β [95% confidence level (CL); R^2 = R-squared; and *P* values].

reduced activity profile in PLWH compared with uninfected participants.[7,17] These data are consistent with independent studies, reporting that PLWH display reduced oxidative enzyme activity in skeletal muscle[29] that may reduce aerobic capacity and exercise tolerance. Additional studies have also reported that PLWH also experience a disproportionate decline in grip strength and gait speed.[26,27]

NAD^+ is a key cofactor in cellular energy metabolism.[10] In mice, loss of NAD in skeletal muscle results in centrally located nuclei associated with myopathy and aging, and reduced activity of PGC-1α, resulting in a progressive decline in physical function and activity.[9] NAD also influences inflammatory signaling, in part, through NAD-dependent SIRT1 deacetylation of the p65 subunit of NF-κB, a heterodimeric transcription factor regulating multiple inflammatory genes.[30] With aging, NAD levels gradually decline in multiple tissues,[31] in part, because of age-related increases in NAD^+-consuming enzymes, such as CD38.[13] Notably, CD38 NADase activity has been reported to increase with HIV infection in vitro, thereby reducing levels of NAD

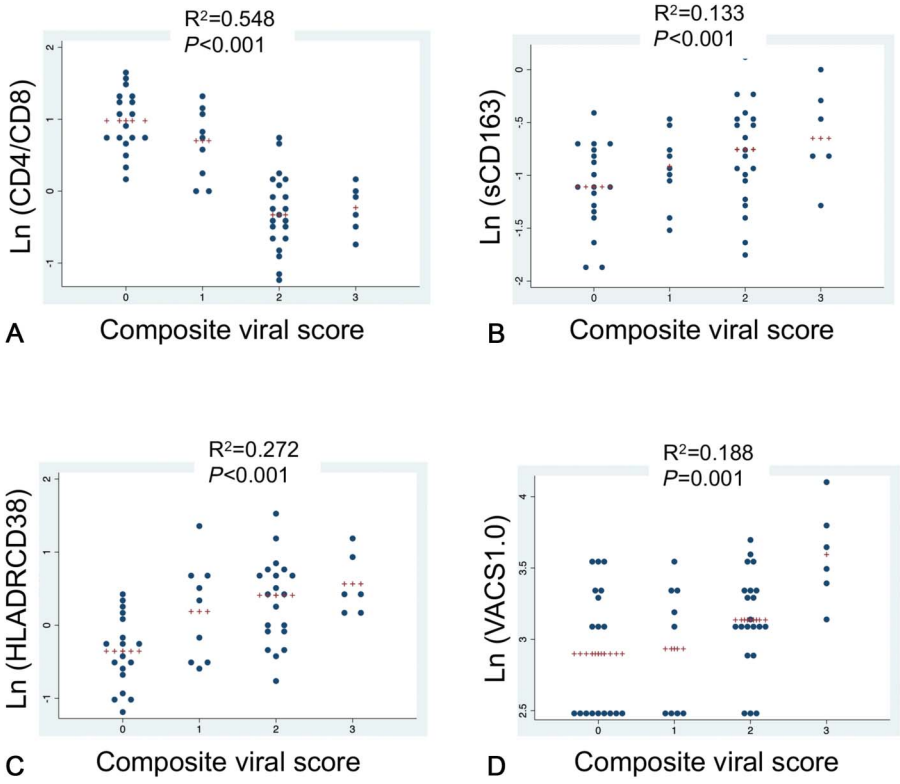

FIGURE 3. CD4/CD8 ratio, sCD163, and HLA-DR^+CD38^+ in peripheral blood and VACS index vs composite viral score. Shown are R^2 and *P* values for univariate regression analysis for CD4/CD8 ratio (A), sCD163 (B), HLA-DR^+CD38^+ (C), and the VACS 1.0 index (D).

in leukocytes.[32] Deficits in NAD may be amenable to therapeutic intervention. For example, restoring NAD levels (eg, with nicotinamide riboside or nicotinamide mononucleotide[15]) in nonhuman models was shown to reverse the level of central nuclei, reduce inflammation in skeletal muscle, and increase mitochondrial biogenesis and physical activity.[16]

In this report, levels of NAD in skeletal muscle differed based on infection status, with reductions in total NAD, NAD^+, and NADH strongly associated with viral coinfection (Fig. 2). In univariate analysis to identify predictors of NAD levels in skeletal muscle, the strongest predictors of total NAD, NAD^+, and NADH were HCV and CMV coinfection and BMI (Table 2).

Multiple previous studies have observed functional impairment and frailty in PLWH compared with uninfected controls of similar age.[23] Frailty has been defined as a loss in reserve capacity and increased vulnerability to stressors.[33] Although frailty has most often been characterized as either a clinical syndrome[34] or as an accumulation of deficits,[35] there are currently as many as 29 different measures for frailty.[36] Alternatively, the VACS score is based on standardized routinely collected clinical measures of multiorgan systems that as an index reflect physiological frailty.[37] Physiologic frailty is an important subclinical landmark that may precede overt evidence of frailty.[22,38] Indeed, the VACS score predicts frailty-related outcomes (eg, hospitalizations, fractures, and falls) and is associated with measures of functional performance,[39] inflammation,[40] and more recently HCV infection.[5] In this study, measurement of physiologic frailty using the VACS 1.0 index indicated that viral coinfection was associated with a higher score for physiologic frailty (Fig. 3).

Notably, inflammation and immune activation were associated with viral coinfection burden and inversely with NAD levels in skeletal muscle. A composite viral score reflecting burden of viral infections was associated with a reduced CD4/CD8 ratio (a general biomarker for inflammation), increased sCD163 (a monocyte biomarker of inflammation), increased $HLA\text{-}DR^+CD38^+$ (a biomarker for immune activation), and an increase in the VACS 1.0 index (a measure of physiologic frailty) (Fig. 3). Thus, the composite burden of asymptomatic infections affect drivers of aging (inflammation and immune activation) and warrant further study to determine a potential role for coinfection burden on biomarkers of aging, particularly in the context of asymptomatic or treated infections. In addition, levels of NAD in skeletal muscle were inversely associated with circulating inflammatory factors in blood, based on evaluation of a composite score for inflammation (IL-6, CRP, sCD163, and sCD14) (Table 2 and Fig. 2) and in sCD163 evaluated separately (data not shown). The mechanistic relationship between reduced NAD levels in skeletal muscle and elevated inflammation in blood remains unclear and requires further study.

HIV/HCV coinfection has been associated with a higher prevalence of clinically significant liver fibrosis.[41] Although direct-acting agents have improved liver function in HIV/HCV coinfection,[42] adverse patient-reported outcomes remain significant.[43] Interestingly, HCV proteins are reported to inhibit the SIRT1-AMPK signaling pathway,[44] and more recently, the HCV serine protease NS3/4A was shown to inhibit quinolinate phosphoribosyl transferase, a key enzyme in the de novo NAD synthesis pathway,[45] suggesting a direct link between HCV infection and NAD. Notably, NAD treatment inhibited HCV replication in vitro and in vivo.[45]

The observed reduction in NAD levels in skeletal muscle of persons with viral coinfection calls into question the mechanism by which a primarily hepatotropic virus (ie, HCV) influences skeletal muscle NAD levels but does pose testable possibilities: (1) The de novo synthesis of NAD occurs primarily in the liver, with other tissues relying almost exclusively on circulating nicotinamide made by the liver.[46] Therefore, subclinical liver function despite effective antiviral therapy (ie, direct-acting agents) may result in reduced bioavailable skeletal nicotinamide (and consequently reduced levels of skeletal NAD). Also, (2) the kynurenine–tryptophan pathway is disrupted by HIV infection and may be further exacerbated in HCV[47] and/or CMV coinfection,[48] compromising NAD levels in skeletal muscle tissue.

There are limitations to this substudy. First, the sample population is relatively small and will need to be validated in a larger cohort. Second, because there were no participants with HCV monoinfection, we cannot exclude potential differences in HCV monoinfection vs HIV/HCV coinfection. Third, our age range of 50–65 years in this study was not sufficient to identify potential age and NAD level associations in skeletal muscle. Finally, the sample size and asymptomatic health status for all infection in this population were insufficient to directly test NAD levels of traditional measures of geriatric syndrome such as frailty. These limitations restrict our ability to infer mechanism and underscore the need for a larger comprehensive mechanistic study but do point the way toward hypothesis-driven assessments.

CONCLUSIONS

In conclusion, a cohort of middle-aged, asymptomatic PLWH compared with uninfected participants displayed reduced levels of total NAD, NAD^+, and NADH in skeletal muscle that was in part explained by viral coinfection with HCV and/or CMV. A composite score for viral infection indicated associations with pathophysiologic frailty and circulating biomarkers for inflammation and immune activation. Collectively, the findings in this study support the presence of preclinical deficits that may help to explain previously observed inflammatory and bioenergetic derangements and support clinical follow-up studies to replete skeletal muscle NAD levels to improve physical function, quality of life, and overall healthspan in PLWH.

REFERENCES

1. Wada N, Jacobson LP, Cohen M, et al. Cause-specific life expectancies after 35 years of age for human immunodeficiency syndrome-infected and human immunodeficiency syndrome-negative individuals followed simultaneously in long-term cohort studies, 1984–2008. *Am J Epidemiol.* 2013;177:116–125.
2. Greene M, Covinsky K, Astemborski J, et al. The relationship of physical performance with HIV disease and mortality. *AIDS*. 2014;28:2711–2719.
3. Deeks SG, Tracy R, Douek DC. Systemic effects of inflammation on health during chronic HIV infection. *Immunity*. 2013;39:633–645.

4. Breskin A, Westreich D, Hurt CB, et al. The effects of hepatitis C treatment eligibility criteria on all-cause mortality among people with human immunodeficiency virus. *Clin Infect Dis*. 2019;69:1613–1620.
5. Zhang X, Hu Y, Justice AC, et al. DNA methylation signatures of illicit drug injection and hepatitis C are associated with HIV frailty. *Nat Commun.* 2017;8:2243.
6. Wang GC, Kao WH, Murakami P, et al. Cytomegalovirus infection and the risk of mortality and frailty in older women: a prospective observational cohort study. *Am J Epidemiol.* 2010;171:1144–1152.
7. Tran T, Guardigni V, Pencina KM, et al. Atypical skeletal muscle profiles in human immunodeficiency virus-infected asymptomatic middle-aged adults. *Clin Infect Dis*. 2018;66:1918–1927.
8. Montano M, Bhasin S, D'Aquila RT, et al. Harvard HIV and aging workshop: perspectives and priorities from claude D. Pepper centers and centers for AIDS research. *AIDS Res Hum Retroviruses*. 2019;35:999–1012.
9. Frederick DW, Loro E, Liu L, et al. Loss of NAD homeostasis leads to progressive and reversible degeneration of skeletal muscle. *Cel Metab.* 2016;24:269–282.
10. Canto C, Menzies KJ, Auwerx J. NAD(+) metabolism and the control of energy homeostasis: a balancing act between mitochondria and the nucleus. *Cel Metab.* 2015;22:31–53.
11. Elhassan YS, Kluckova K, Fletcher RS, et al. Nicotinamide riboside augments the aged human skeletal muscle NAD(+) metabolome and induces transcriptomic and anti-inflammatory signatures. *Cell Rep.* 2019; 28:1717–1728.e1716.
12. Rajman L, Chwalek K, Sinclair DA. Therapeutic potential of NAD-boosting molecules: the in vivo evidence. *Cell Metab*. 2018;27:529–547.
13. Camacho-Pereira J, Tarrago MG, Chini CCS, et al. CD38 dictates age-related NAD decline and mitochondrial dysfunction through an SIRT3-dependent mechanism. *Cell Metab.* 2016;23:1127–1139.
14. Donato AJ, Magerko KA, Lawson BR, et al. SIRT-1 and vascular endothelial dysfunction with ageing in mice and humans. *J Physiol.* 2011;589:4545–4554.
15. Trammell SA, Schmidt MS, Weidemann BJ, et al. Nicotinamide riboside is uniquely and orally bioavailable in mice and humans. *Nat Commun.* 2016;7:12948.
16. Ryu D, Zhang H, Ropelle ER, et al. NAD+ repletion improves muscle function in muscular dystrophy and counters global PARylation. *Sci Transl Med.* 2016;8:361ra139.
17. Hale TM, Guardigni V, Roitmann E, et al. Middle-aged men with HIV have diminished accelerometry-based activity profiles despite similar lab-measured gait speed: pilot study. *JMIR Mhealth Uhealth*. 2019;7:e11190.
18. Pencina K, Li Z, Montano M. Objectively measured physical activity in asymptomatic middle-aged men is associated with routine blood-based biomarkers. *J Gerontol A Biol Sci Med Sci*. 2019;74:S32–S37.
19. Vidugiriene J, Leippe D, Sobol M, et al. Bioluminescent cell-based NAD(P)/NAD(P)H assays for rapid dinucleotide measurement and inhibitor screening. *Assay Drug Dev Tech.* 2014;12:514–526.
20. Justice AC, Tate JP. Strengths and limitations of the veterans aging cohort study index as a measure of physiologic frailty. *AIDS Res Hum Retroviruses*. 2019;35:1023–1033.
21. Lidofsky A, Holmes JA, Feeney ER, et al. Macrophage activation marker soluble CD163 is a dynamic marker of liver fibrogenesis in human immunodeficiency virus/hepatitis C virus coinfection. *J Infect Dis.* 2018; 218:1394–1403.
22. Justice AC. HIV and aging: time for a new paradigm. *Curr HIV/AIDS Rep.* 2010;7:69–76.
23. Althoff KN, Jacobson LP, Cranston RD, et al. Age, comorbidities, and AIDS predict a frailty phenotype in men who have sex with men. *J Gerontol Ser A Biol Sci Med Sci.* 2014;69A:189–198.
24. Erlandson KM, Allshouse AA, Jankowski CM, et al. Comparison of functional status instruments in HIV-infected adults on effective antiretroviral therapy. *HIV Clin Trials*. 2012;13:324–334.
25. Erlandson KM, Schrack JA, Jankowski CM, et al. Functional impairment, disability, and frailty in adults aging with HIV-infection. *Curr HIV/AIDS Rep.* 2014;11:279–290.
26. Schrack JA, Althoff KN, Jacobson LP, et al. Accelerated longitudinal gait speed decline in HIV-infected older men. *J Acquir Immune Defic Syndr.* 2015;70:370–376.
27. Schrack JA, Jacobson LP, Althoff KN, et al. Effect of HIV-infection and cumulative viral load on age-related decline in grip strength. *AIDS*. 2016; 30:2645–2652.
28. Cristea A, Qaisar R, Edlund PK, et al. Effects of aging and gender on the spatial organization of nuclei in single human skeletal muscle cells. *Aging Cell*. 2010;9:685–697.
29. Ortmeyer HK, Ryan AS, Hafer-Macko C, et al. Skeletal muscle cellular metabolism in older HIV-infected men. *Physiol Rep.* 2016;4: e12794.
30. Yeung F, Hoberg JE, Ramsey CS, et al. Modulation of NF-kappaB-dependent transcription and cell survival by the SIRT1 deacetylase. *EMBO J.* 2004;23:2369–2380.
31. Massudi H, Grant R, Braidy N, et al. Age-associated changes in oxidative stress and NAD+ metabolism in human tissue. *PLoS One*. 2012;7: e42357.
32. Murray MF, Nghiem M, Srinivasan A. HIV infection decreases intracellular nicotinamide adenine dinucleotide [NAD]. *Biochem biophysical Res Commun.* 1995;212:126–131.
33. Walston J, Hadley EC, Ferrucci L, et al. Research agenda for frailty in older adults: toward a better understanding of physiology and etiology: summary from the American Geriatrics Society/National Institute on aging research conference on frailty in older adults. *J Am Geriatr Soc.* 2006;54:991–1001.
34. Fried LP, Ferrucci L, Darer J, et al. Untangling the concepts of disability, frailty, and comorbidity: implications for improved targeting and care. *J Gerontol A Biol Sci Med Sci.* 2004;59:255–263.
35. Rockwood K, Bergman H. FRAILTY: a report from the 3(rd) Joint Workshop of IAGG/WHO/SFGG, Athens, January 2012. *Can Geriatr J.* 2012;15:31–36.
36. Dent E, Kowal P, Hoogendijk EO. Frailty measurement in research and clinical practice: a review. *Eur J Intern Med.* 2016;31:3–10.
37. Justice AC, Modur SP, Tate JP, et al. Predictive accuracy of the Veterans Aging Cohort Study index for mortality with HIV infection: a North American Cross Cohort Analysis. *J Acquir Immune Defic Syndr.* 2013; 62:149–163.
38. Escota GV, Patel P, Brooks JT, et al. Short communication: the Veterans Aging Cohort Study Index is an effective tool to assess baseline frailty status in a contemporary cohort of HIV-infected persons. *AIDS Res Hum Retroviruses*. 2015;31:313–317.
39. John MD, Greene M, Hessol NA, et al. Geriatric assessments and association with VACS index among HIV-infected older adults in san francisco. *J Acquir Immune Defic Syndr.* 2016;72:534–541.
40. Mooney S, Tracy R, Osler T, et al. Elevated biomarkers of inflammation and coagulation in patients with HIV are associated with higher framingham and VACS risk index scores. *PLoS One*. 2015;10:e0144312.
41. Kirk GD, Mehta SH, Astemborski J, et al. HIV, age, and the severity of hepatitis C virus-related liver disease: a cohort study. *Ann Intern Med.* 2013;158:658–666.
42. Cheung MC, Walker AJ, Hudson BE, et al. Outcomes after successful direct-acting antiviral therapy for patients with chronic hepatitis C and decompensated cirrhosis. *J Hepatol.* 2016;65:741–747.
43. Marcellin F, Roux P, Protopopescu C, et al. Patient-reported outcomes with direct-acting antivirals for the treatment of chronic hepatitis C: current knowledge and outstanding issues. *Expert Rev Gastroenterol Hepatol.* 2017;11:259–268.
44. Yu JW, Sun LJ, Liu W, et al. Hepatitis C virus core protein induces hepatic metabolism disorders through down-regulation of the SIRT1-AMPK signaling pathway. *Int J Infect Dis.* 2013;17:e539–e545.
45. Wang Z, Gao Y, Zhang C, et al. Quinolinate phosphoribosyltransferase is an antiviral host factor Against hepatitis C virus infection. *Scientific Rep.* 2017;7:5876.
46. Liu L, Su X, Quinn WJ III, et al. Quantitative analysis of NAD synthesis-breakdown fluxes. *Cel Metab.* 2018;27:1067–1080.e1065.
47. Kardashian A, Ma Y, Yin MT, et al. High kynurenine:tryptophan ratio is associated with liver fibrosis in HIV-monoinfected and HIV/hepatitis C virus-coinfected women. *Open Forum Infect Dis.* 2019;6:ofz281.
48. Yap SH, Abdullah NK, McStea M, et al. HIV/Human herpesvirus co-infections: impact on tryptophan-kynurenine pathway and immune reconstitution. *PLoS One*. 2017;12:e0186000.