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Heme oxygenase-1 promoter (GT)_n polymorphism associates with HIV neurocognitive impairment

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Abstract

Objective

To determine whether regulatory variations in the heme oxygenase-1 (HO-1) promoter (GT)_n dinucleotide repeat length could identify unique population genetic risks for neurocognitive impairment (NCI) in persons living with HIV (PLWH), we genotyped 528 neurocognitively assessed PLWH of European American and African American descent and linked genotypes to cognitive status.

Methods

In this cross-sectional study of PLWH (the CNS HIV Antiretroviral Therapy Effect Research cohort), we determined HO-1 (GT)_n repeat lengths in 276 African Americans and 252 European Americans. Using validated criteria for HIV-associated NCI (HIV NCI), we found associations between allele length genotypes and HIV NCI and between genotypes and plasma markers of monocyte activation and inflammation. For comparison of HO-1 (GT)_n allele frequencies with another population of African ancestry, we determined HO-1 (GT)_n allele lengths in African PLWH from Botswana (n = 428).

Results

PLWH with short HO-1 (GT)_n alleles had a lower risk for HIV NCI (OR = 0.63, 95% CI: 0.42–0.94). People of African ancestry had a lower prevalence of short alleles and higher prevalence of long alleles compared with European Americans, and in subgroup analyses, the protective effect of the short allele was observed in African Americans and not in European Americans.

Conclusions

Our study identified the short HO-1 (GT)_n allele as partially protective against developing HIV NCI. It further suggests that this clinical protective effect is particularly relevant in persons of African ancestry, where the lower prevalence of short HO-1 (GT)_n alleles may limit induction of HO-1 expression in response to inflammation and oxidative stress. Therapeutic strategies that enhance HO-1 expression may decrease HIV-associated neuroinflammation and limit HIV NCI.

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Glossary

ADL = activities of daily living; **ANI** = asymptomatic neurocognitive impairment; **ART** = antiretroviral therapy; **GT** = promoter; **HAD** = HIV-associated dementia; **HAND** = HIV-associated neurocognitive disorders; **HIV NCI** = HIV-associated NCI; **HO-1** = heme oxygenase-1; **MND** = mild neurocognitive disorder; **NCI** = neurocognitive impairment; **NCN** = neurocognitive normal; **NNTC** = National NeuroAIDS Tissue Consortium; **NP** = neuropsychological; **PLWH** = persons living with HIV; **sHO-1** = soluble HO-1; **SNP** = single nucleotide polymorphism.

The syndrome of HIV-associated neurocognitive disorders (HAND), like many CNS disease states, is linked to neuroinflammation and oxidative stress. Induction of the antioxidant isoenzyme, heme oxygenase-1 (HO-1), can reduce oxidative stress and neuroinflammation within the CNS.^{1,2} HO-1 expression is rapidly induced in response to injury, and induced HO-1 expression is neuroprotective in vitro and in vivo in different injury models.^{3,4}

Transcriptional regulation of the HO-1 gene (*HMOX1*) depends partly on HO-1 promoter region genetic variations, including a (GT)_n dinucleotide repeat and single nucleotide polymorphisms (SNPs). Promoters with short HO-1 (GT)_n repeats express higher basal activity and inducibility,^{5–8} and cells with these repeats are more resistant to oxidative injury.⁶ An A(-413)T SNP can affect HO-1 promoter transcriptional activity and disease outcomes,⁹ albeit less so than the HO-1 (GT)_n repeat length.^{10–12} Short HO-1 (GT)_n repeat alleles associate with better outcomes in inflammatory and oxidative stress-associated diseases,^{7,8,13–15} and the prevalence of these short alleles differs significantly among different populations. We recently determined HO-1 (GT)_n alleles in individuals in an HIV autopsy cohort (n = 554) and showed that the presence of 1 or more short HO-1(GT)_n alleles associated with a lower risk of HIV encephalitis and less brain inflammation (type I interferon responses and T-cell activation).¹⁶ No effect of the A(-413)T SNP was observed.

We hypothesized that the HO-1 (GT)_n allele genotype also associates with risk for neurocognitive impairment (NCI) in persons living with HIV (PLWH) and that genotype effects vary among different populations. We genotyped a large, clinically characterized cohort of PLWH, the CNS HIV Antiretroviral Therapy Effect Research (CHARTER) cohort,^{17,18} and associated genotype with neurocognitive diagnosis. We report significant associations indicating a protective effect of short HO-1(GT)_n alleles against HIV-associated NCI (HIV NCI), particularly in African Americans.

Methods

Cohorts

For analysis of HO-1 (GT)_n allele genotypes, neurocognitive diagnosis, and plasma biomarkers, 606 PLWH were selected from the CHARTER Genetics Cohort,¹⁹ based on reported availability of neurocognitive diagnosis data and DNA samples, and without other inclusionary or exclusionary criteria,

to avoid potential bias. Of the 606 DNA samples, 3 were of insufficient quality to obtain reliable (GT)_n sequence data; therefore, HO-1 (GT)_n allele data from 603 individuals were presented (table 1 and figure 1A). CHARTER is an ongoing, observational study of PLWH enrolled between 2003 and 2007 (1,561 individuals) from 6 HIV treatment centers: Johns Hopkins University (Baltimore, MD), Mt. Sinai School of Medicine (New York, NY), University of California at San Diego (San Diego, CA), University of Texas Medical Branch (Galveston, TX), University of Washington (Seattle, WA), and Washington University (St. Louis, MO). Our required sample size was estimated at ~400, based on our analysis of the National NeuroAIDS Tissue Consortium (NNTC) autopsy cohort, which revealed the presence of short HO-1 (GT)_n allele associated with an increased risk of HIV encephalitis.¹⁶

Among these 603 individuals, 595 had sufficient neuropsychological (NP) test data for diagnostic HIV NCI assignment. Therefore, NCI data from those 595 individuals were analyzed for HIV NCI associations with genotype and subgroup analyses. Among these 595 individuals, data from 528 total self-identified African Americans and European Americans were analyzed to distinguish differences between these groups.

HO-1 (GT)_n allele genotyping was also performed on whole blood-derived DNA from a Botswana cohort of PLWH on antiretroviral therapy (ART), randomly selected irrespective of neurocognitive function,^{20,21} and under separate IRB approval.

Data availability

Anonymized data are available from the corresponding author (D.L.K.) on reasonable request.

Neurocognitive and functional assessments

Individuals from CHARTER completed baseline assessments, including NP testing (7 cognitive domains, corrected for age, education, sex, and ethnicity), substance abuse history, psychiatric diagnosis, self-reported cognition assessment, vocational function assessment, and assessment of independence with instrumental activities of daily living (ADLs).¹⁷ Specimens and data were obtained with permission of the CHARTER steering committee. Neurocognitive diagnosis was first determined by the Frascati criteria for HIV-associated neurocognitive disorders.²² Under these criteria, the diagnoses of asymptomatic neurocognitive impairment (ANI) and mild neurocognitive disorder (MND) require the presence of mild impairment in ≥2 NP domains that is not attributed to

Table 1 Cohort demographics and HIV disease parameters

Characteristic	Cohort group		HO-1 (GT) _n allele genotype		p Value
	All	Presence of Short Allele (SS, SM, SL)	Absence of Short Allele (MM, ML, LL)		
No. of individuals	606	294	309		—
Age (y, mean ± SD)	43.8 ± 8.3	43.4 ± 8.4	44.2 ± 8.1		0.227 ^a
Duration of infection	9.2 ± 6.4	9.0 ± 6.6	9.4 ± 6.3		0.493 ^a
Sex (%)					
Male	80.0%	80.6%	79.3%		0.685 ^b
Race (%)					
Non-Hispanic European American	41.6%	46.6%	36.6%		0.013 ^b
African American	45.5%	38.8%	52.1%		0.001 ^b
Other/unknown	12.9%	14.6%	11.3%		0.228 ^b
Education (y)	12.7 ± 2.5	12.8 ± 2.4	12.7 ± 2.5		0.401 ^a
Antiretroviral treated (%)	72.6%	74.7%	70.2%		0.215 ^b
HIV RNA <50 copies/mL	41.7%	40.5%	41.5%		0.798 ^b
Disease parameters (mean ± SD)					
Log plasma HIV copies/mL	2.8 ± 1.3	2.8 ± 1.3	2.8 ± 1.3		0.833 ^a
Log CSF HIV copies/mL	2.1 ± 0.8	2.1 ± 0.8	2.2 ± 0.8		0.474 ^a
CD4⁺ T lymphocytes (cells/μL)	458 ± 267	464 ± 245	455 ± 288		0.694 ^a
CD8⁺ T lymphocytes (cells/μL)	962 ± 503	975 ± 502	954 ± 505		0.602 ^a
CD3⁺ T lymphocytes (cells/μL)	1,476 ± 641	1,494 ± 623	1,464 ± 659		0.593 ^a
CD4/CD8 T lymphocyte ratio	0.6 ± 0.8	0.6 ± 1.2	0.6 ± 0.4		0.447 ^a
CD4⁺ T lymphocyte nadir (cells/μL)	207 ± 190	212 ± 181	204 ± 198		0.578 ^a

Abbreviations: L = long allele; M = medium allele; S = short allele.

Sample size within demographic and disease parameters may be less than the overall sample size due to missing data (%) in categories (duration HIV, 26.5%; antiretroviral treated, 0.2%; plasma HIV, 0.6%; CSF HIV, 5.8%; CD4⁺, 0.6%; CD8⁺, 2.7%; CD3⁺, 8.6%; CD4/CD8, 2.5%).

^a p Values represent comparisons between (SS, SM, SL) and (MM, ML, LL) HO-1 (GT)_n genotype groups by the Student *t* test.

^b p Values represent comparisons between (SS, SM, SL) and (MM, ML, LL) HO-1 (GT)_n genotype groups by the χ^2 test.

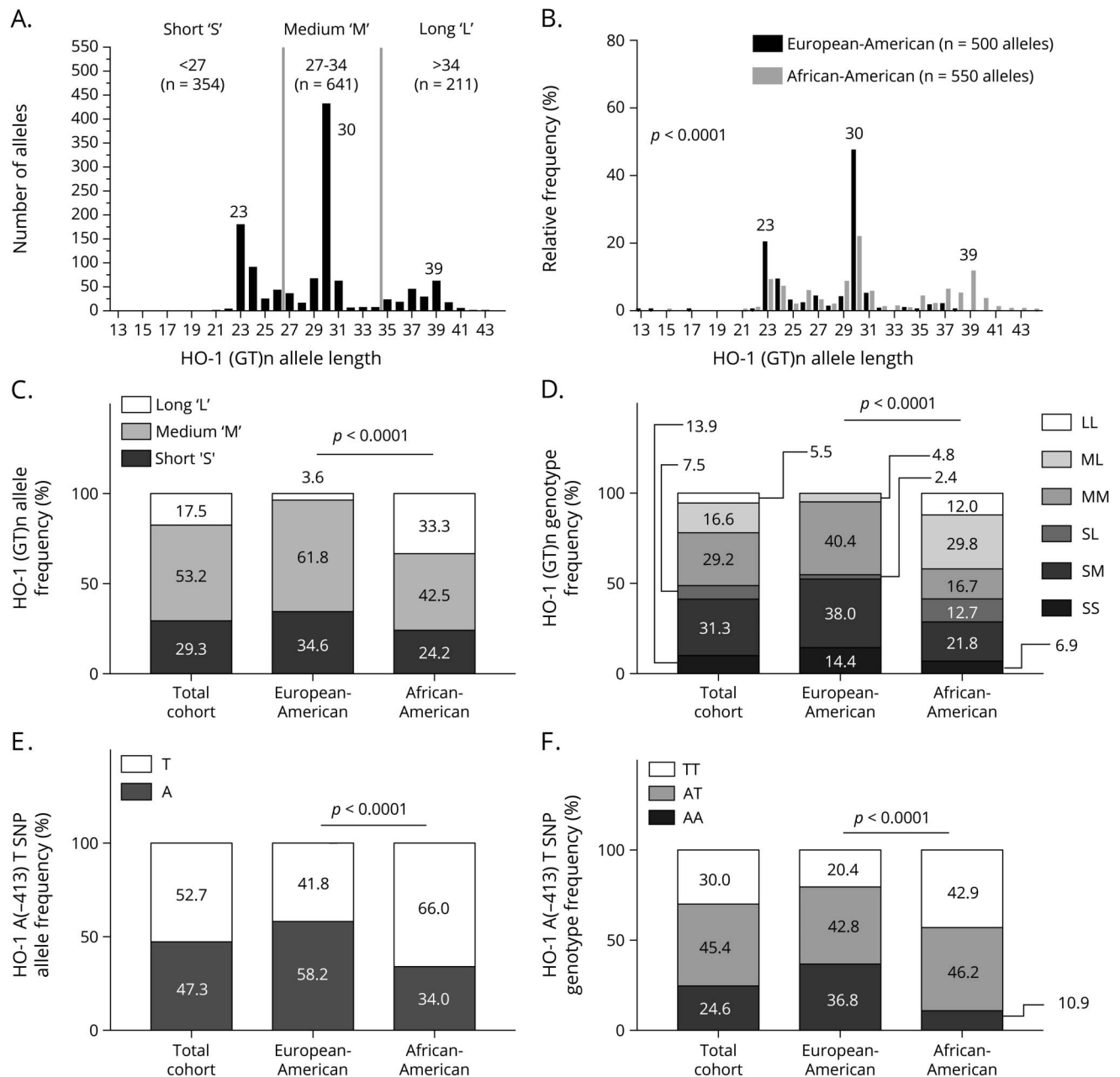
comorbid conditions. In addition, MND requires functional decline by 2 measures of functioning in ADLs. HIV-associated dementia (HAD) requires functional decline with greater severity than that of MND. Persons having no or only 1 abnormal NP domain test performance are classified as neurocognitive normal (NCN). We grouped MND and HAD individuals as functional HIV NCI individuals, whereby functional impairment of ADL was required for this grouping.

We further classified HIV NCI by distinguishing the presence or absence of comorbidity factors that were deemed severe enough to contribute to NCI in conjunction with HIV infection, according to CHARTER guidelines.^{17,22} Accordingly, PLWH were stratified by the severity by which these factors (e.g., depression, traumatic brain injury, developmental disability, substance abuse, opportunistic CNS infections, non-HIV-related neurologic conditions, systemic disease, and hepatitis C virus coinfection) are thought to affect NCI, as adjudicated by an experienced neuropsychologist. NCI without

such contributing factors is considered incidental HIV NCI (i.e., incidental to HIV infection alone). HIV NCI with comorbidities contributing a minor level of impairment is defined as HIV NCI with contributing factors.^{17,22,23} Individuals with comorbidities contributing a major level of impairment (confounding factors) were excluded. Thus, HIV NCI within the incidental group is considered to be a consequence of HIV infection itself, whereas HIV NCI within the contributing group is considered to be a consequence of HIV infection with a minor additional component due to contributing factors.²³

To include an appropriate subgroup analysis of HIV NCI vs no NCI, we grouped individuals without NCI in each of 2 ways: (1) NCN and (2) no functional NCI (NCN and ANI), because impairment of ADL is not a criterion for the diagnosis of ANI. All procedures were approved by the Human Subjects Protection Committees of the respective academic centers where patients were enrolled. Informed consent was obtained from all individuals.

Figure 1 HO-1 (GT)_n promoter repeat allele length and A(-413)T SNP allele frequencies differ significantly between African Americans and European Americans



HO-1 (GT)_n promoter repeats and A(-413)T SNPs were assessed in 603 and 601 PLWH, respectively, in the CHARTER cohort. (A) Distribution of HO-1 (GT)_n promoter allele lengths among all cohort individuals; alleles were defined as short "S" (<27 (GT)_n repeats), medium "M" (27–34 repeats), and long "L" (>34 repeats). (B) HO-1 (GT)_n allele length frequencies in European American and African American subgroups compared by the Kolmogorov-Smirnov test. (C) Allele frequencies and (D) genotype frequencies of the HO-1 promoter (GT)_n alleles and (E) allele frequencies and (F) genotype frequencies of the HO-1 promoter A(-413)T SNP in the total cohort and in African American and European American subpopulations. Allelic and genotypic frequency distributions were compared by the χ^2 test. CHARTER = CNS HIV Antiretroviral Therapy Effect Research; HO-1 = heme oxygenase-1; PLWH = persons living with HIV; SNP = single nucleotide polymorphism.

Quantification of viral loads and lymphocyte counts

HIV infection determined by ELISA was confirmed by Western blot. Clinical chemistry panels, complete blood counts, and CD4⁺ T cells (flow cytometry) were performed at the respective enrollment site's clinical, or equivalent, medical center laboratory. Plasma and CSF HIV RNA was quantified by reverse transcriptase PCR (Roche Amplicor,

v. 1.5, lower limit of quantitation 50 copies/mL) in a central laboratory.¹⁷

HO-1 (GT)_n repeat and HO-1 A(-413)T SNP sequencing

For the CHARTER and Botswana cohorts, genomic DNA isolated from whole blood (DNA Extraction Kit, Agilent Technologies). Detailed protocols used for amplification and

determination of genotype were previously described by our group.¹⁶ Briefly, HO-1 (GT)_n repeat lengths were determined by PCR of the (GT)_n repeat region with a 6-fluorescein amidites labeled forward primer, followed by fragment size determination on a capillary sequencer. In a subset of samples, PCR products were run on a 2% agarose gel to assess amplification of the target sequence predicted sizes (homozygotes and heterozygotes). All samples were run at least twice from independent PCR reactions to ensure accurate and reproducible sizing and were determined to be accurate within ± 1 GT repeats. In rare (<4%) cases in which identical GT repeat lengths were not obtained in duplicate, samples were run additional times to confirm accurate repeat lengths. A(-413)T SNP (rs2071746) genotyping was performed using TaqMan SNP Genotyping Assay (ThermoFisher, Assay ID: C__15869717_10) per the manufacturer's instructions.

Determination of plasma sCD163 and sHO-1

We selected sCD163 as a macrophage-specific associative biomarker for HIV NCI/HAND groups inclusive of MND and HAD.^{24,25} Also, CD163 receptor engagement (scavenging hemoglobin/haptoglobin complexes) is a stimulus for production of HO-1.²⁶ Plasma soluble HO-1 (sHO-1) serves as a marker of inflammation and oxidative stress.^{27,28} Each was quantified in duplicate by commercial ELISA: (1) sCD163 (R & D Systems, #DY1607; LOD 156 pg/mL) and (2) sHO-1 (ENZO Life Sciences, #ADI-960-800; LOD 49 pg/mL) in stored, frozen (-80°C) plasma samples.

Statistical analyses

For comparisons between groups, differences are expressed as median ± 95% CI. Comparisons of distributions of categorical variables were determined by χ^2 and method for OR analysis. Comparisons of continuous variables between groups were performed using the Student *t* test or 2-way analysis of variance with the multiple comparisons test. Confounding and effect modification was assessed by including individual variables associated with the outcome with a *p* value < 0.10 using the Mantel-Haenszel procedure. Analyses of linear trends were performed by Spearman rank correlation. Comparisons of allele distributions were analyzed by the Kolmogorov-Smirnov test.

Results

HO-1 promoter (GT)_n allele repeat lengths and the A(-413)T SNP genotype have modal distributions that suggest higher-risk genotypes in African Americans compared with European Americans

To define relationships between HIV NCI, immune activation, and HO-1 (GT)_n allele length in PLWH, we studied individuals (*n* = 606) in the CHARTER Genetics Cohort (table 1).^{19,29} Of the 606 DNA samples, 3 were of insufficient quality to obtain reliable sequence data; therefore, HO-1 (GT)_n allele data from 603 individuals were presented (table

1 and figure 1A). The distribution of HO-1 (GT)_n allele lengths was trimodal (peaks at 23, 30, and 39), with a range of 13–44, and we assigned HO-1 (GT)_n alleles as short “S” (<27), medium “M” (27–34), or long “L” (>34) (GT)_n repeats (figure 1, A and B).^{8,16} HO-1 (GT)_n allele and genotype distributions (figure 1, C and D) differed significantly between self-identified African Americans and European Americans. African Americans expressed more HO-1 (GT)_n “L” alleles (33.3% vs 3.6%, *p* < 0.001), fewer HO-1 (GT)_n “M” alleles (42.5% vs 61.8%, *p* < 0.001), and fewer “S” alleles (24.2% vs 34.6%, *p* < 0.001) than European Americans.

We also genotyped the HO-1 promoter region A(-413)T SNP (rs2071746) in 601 (99.2%) individuals. The A(-413)T SNP can affect HO-1 promoter transcriptional activity and disease outcomes,⁹ albeit less so than the HO-1 (GT)_n repeat length.^{10–12} The frequency of the “T” A(-413)T SNP was significantly higher in African Americans than in European Americans (66.0% vs 41.8%), whereas the frequency of the “A” SNP was lower (34% vs 58.2%) (figure 1E), and, as expected for Hardy-Weinberg equilibrium, the TT and AT genotypes were more prevalent in African Americans (figure 1F). Also, the A(-413)T SNP and the HO-1 (GT)_n allele genotypes were not independently expressed; the “A” SNP associated with medium “M” HO-1 (GT)_n alleles, whereas the “T” SNP associated with both short “S” and long “L” HO-1 (GT)_n alleles (not shown). These findings are consistent with previous studies of HO-1 (GT)_n repeats and the A(-413)T SNP.^{8,16}

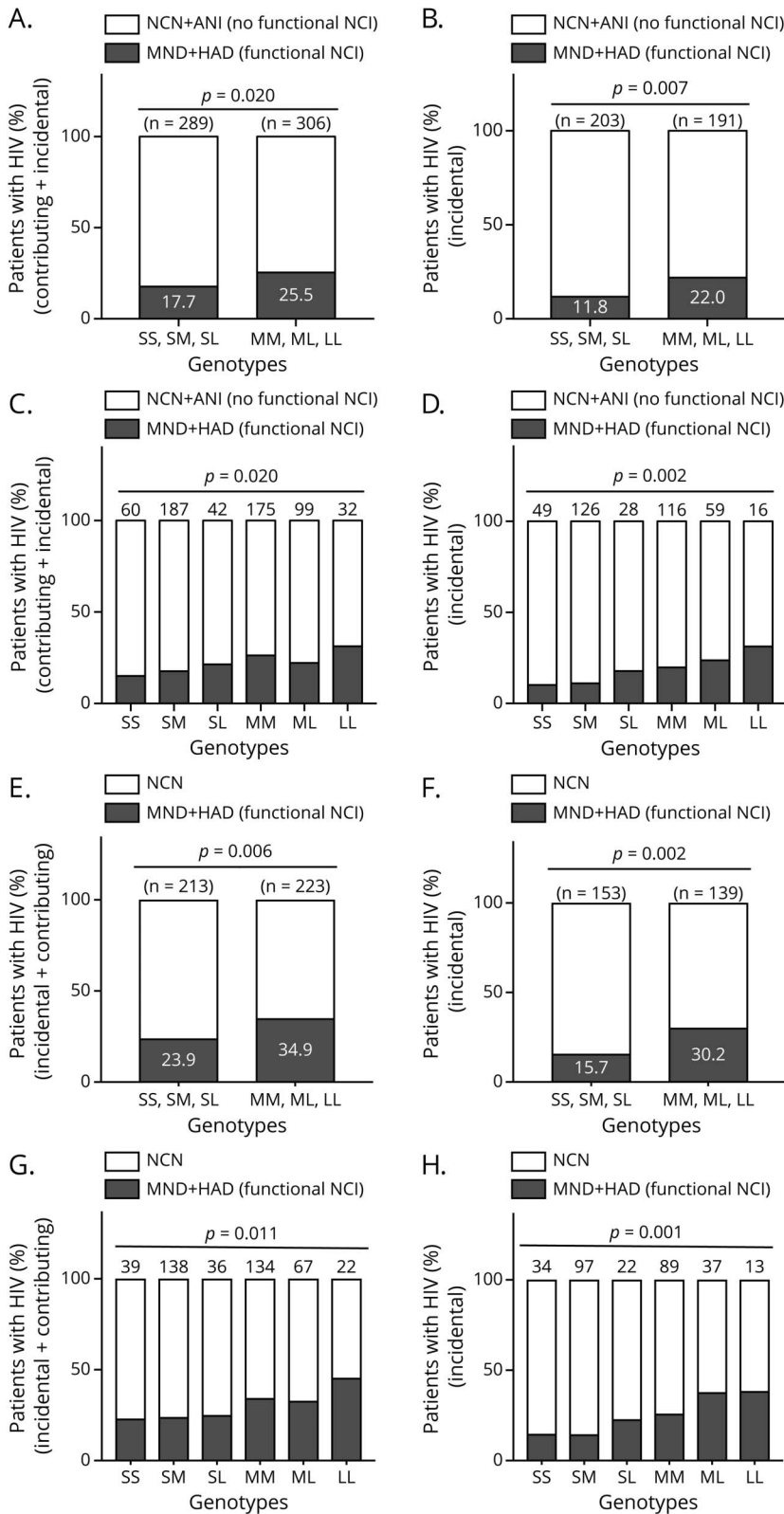
Neither the HO-1 (GT)_n allele genotype nor the A(-413)T SNP genotype correlates with plasma or CSF viral load, T-cell (CD4⁺ and CD8⁺) counts, or historical CD4⁺ nadir in PLWH

We examined associations between HO-1 (GT)_n allele genotype, A(-413)T SNP genotype, plasma and CSF viral load, T-cell (CD4⁺, CD8⁺) counts, and CD4⁺ nadir and found no significant correlations (table 1). We conclude that neither the HO-1 (GT)_n allele genotype nor the A(-413)T SNP genotype affects HIV replication or severity of HIV-driven immune suppression.

PLWH who have short HO-1 (GT)_n alleles have a lower prevalence of HIV NCI

We determined associations between the HO-1 (GT)_n allele genotype and functional HIV NCI (grouped as total MND and HAD individuals) in comparison to those without functional HIV NCI (grouped either as total ANI and NCN individuals or only those categorized as NCN, figure 2). Comparing the no functional HIV NCI groups with the functional HIV NCI groups, the presence of at least 1 short HO-1 (GT)_n allele associated with a lower functional HIV NCI prevalence (OR = 0.63, 95% CI: 0.42–0.94, figure 2A). The protective effect of the short allele was greater in individuals with functional HIV NCI without other contributing factors (OR = 0.48, 95% CI: 0.28–0.84, figure 2B). Furthermore, the group with 2 short alleles, “SS,” had the lowest

Figure 2 PLWH with short HO-1 (GT)_n alleles have significantly decreased risk of functional HIV NCI



Individuals underwent a comprehensive neurocognitive test battery (7 domains) and were assigned functional neurocognitive diagnoses by Frascati criteria for HIV-associated neurocognitive disorders. (A) Within the total cohort (contributing and incidental risk(s)), the presence of at least 1 short “S” HO-1 (GT)_n allele associated with lower functional HIV NCI (groups MND and HAD) vs no functional HIV NCI (NCN and ANI groups) by an OR of 0.63 (95% CI: 0.42–0.94) (χ^2 test, $p = 0.020$). (B) Within the HIV NCI incidental group (without other contributing comorbidities), the presence of at least 1 short “S” HO-1 (GT)_n allele associated with lower functional HIV NCI by an OR of 0.48 (95% CI: 0.28–0.84, χ^2 test, $p = 0.007$). The prevalence of functional HIV NCI increased linearly with stratification of genotypes from homozygous short “SS” to homozygous long “LL” within (C) the total cohort and within (D) the functional HIV NCI group without contributing comorbidities (χ^2 tests for linear trend, $p = 0.020$, $p = 0.002$, respectively). Subgroup analyses were similarly performed by comparing function HIV NCI groups MND and HAD with NCN alone (E–H), with similar results. ANI = asymptomatic neurocognitive impairment; HAD = HIV-associated dementia; HO-1 = heme oxygenase-1; MND = mild neurocognitive disorder; NCI = neurocognitive impairment; NCN = neurocognitive normal; PLWH = persons living with HIV.

functional HIV NCI prevalence (15.0% and 10.2%, respectively, figure 2, C and D) among all HO-1 (GT)_n allele genotypes, whereas those with 2 long alleles, “LL,” had the

highest functional HIV NCI prevalence (31.3% for both). No effect of the A(-413)T SNP genotype was observed in any group analysis (not shown).

We performed a subgroup analysis by comparing the NCN group alone (removing the ANI subgroup) with the MND and HAD group. Again, the presence of at least 1 short allele associated significantly with a lower prevalence of functional HIV NCI (OR = 0.59, 95% CI: 0.38–0.89, figure 2E) with a lower OR for functional HIV NCI without contributing factors (OR = 0.43, 95% CI: 0.24–0.75, figure 2F). Similarly, individuals with 2 short alleles, “SS,” had the lowest functional HIV NCI prevalence (23.9%, 14.7%, respectively), whereas those with 2 long alleles, “LL,” had the highest prevalence of functional NCI (45.4, 38.5%, respectively) (figure 2, G and H). Again, no effect of the A(-413)T SNP genotype was observed (not shown).

The significant risk reduction for HIV NCI by short HO-1 (GT)_n alleles is observed in African Americans and not in European Americans

Within the CHARTER cohort, non-Hispanic, self-identified European Americans represented 41.6%, and self-identified African Americans represented 45.5%; the remaining 12.9% represented other/unknown racial groups (table 1). African Americans had lower mean CD4⁺ T-cell counts, CD4⁺/CD8⁺ T-cell ratio, and CD4⁺ T-cell nadirs compared with European

Americans, with no difference in the absolute mean CD8⁺ T-cell count (table 2).

Because African Americans have a different HO-1 (GT)_n allele distribution with fewer short and more long alleles than European Americans, we hypothesized that the protective effect of a short HO-1 (GT)_n allele may be more easily detected in African Americans. Comparing the no functional HIV NCI group with the functional HIV NCI group (figure 3), and further analyzing those with HIV NCI without other contributing factors, we observed a different effect between African Americans and European Americans. The presence of at least 1 short allele associated with a strong protective effect in African Americans (OR = 0.38, 95% CI: 0.16–0.84, figure 3A) but not in European Americans (figure 3B). However, the effect difference between the races was not significant (Mantel-Haenszel test, *p* = 0.31). Also, although the prevalence of functional HIV NCI was similar in both racial subgroups with at least 1 short allele (11.7% and 12.2%, respectively), there was no significant difference in functional HIV NCI prevalence in African Americans without a short allele (26.0%) compared with European Americans (16.5%) (*p* = 0.11). Within the African American

Table 2 Demographics and parameters

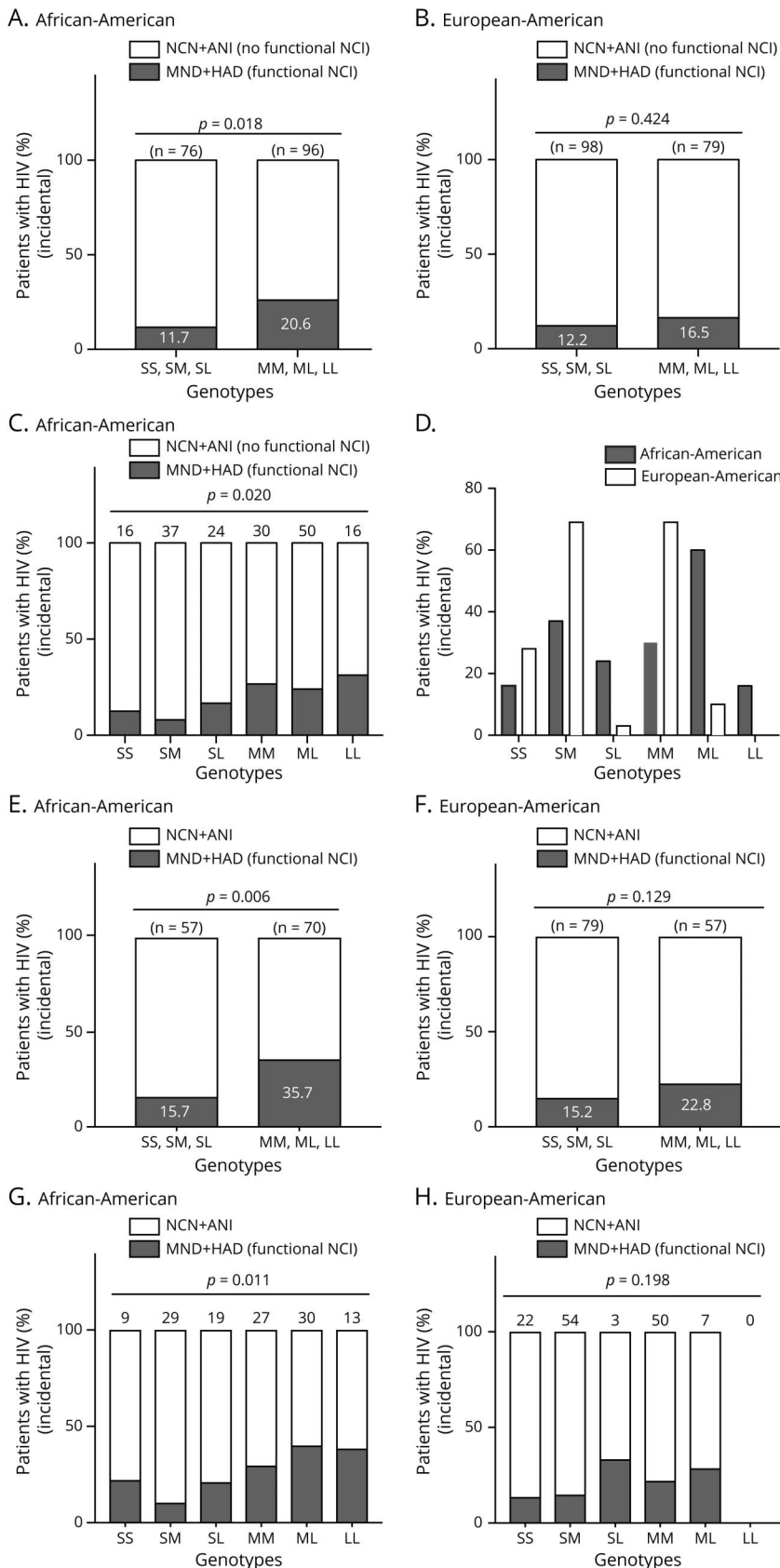
Parameter	Racial group		<i>p</i> Value
	European American	African American	
No. of individuals	252	276	—
Age (y, mean ± SD)	43.8 ± 9.1	44.3 ± 7.4	0.496 ^a
Duration of infection	8.8 ± 6.7	9.8 ± 6.1	0.120 ^a
Sex (%)			
Male	71.4%	88.9%	<0.0001 ^b
Education (y)	13.4 ± 2.5	12.1 ± 2.2	<0.0001 ^a
Antiretroviral treated (%)			
HIV RNA <50 copies/mL	74.3%	70.6%	0.380 ^b
HAND (%)	39.2%	43.1%	0.376 ^b
MND and HAD	22.1%	20.5%	0.670 ^b
Disease parameters (mean ± SD)			
Log plasma HIV copies/mL	2.8 ± 1.3	2.8 ± 1.3	0.690 ^a
Log CSF HIV copies/mL	2.1 ± 0.8	2.1 ± 0.8	0.881 ^a
CD4 ⁺ T lymphocytes (cells/μL)	504 ± 272	419 ± 261	0.000 ^a
CD8 ⁺ T lymphocytes (cells/μL)	944 ± 499	960 ± 470	0.707 ^a
CD3 ⁺ T lymphocytes (cells/μL)	1,499 ± 651	1,429 ± 602	0.217 ^a
CD4/CD8 T lymphocyte ratio	0.7 ± 1.2	0.5 ± 0.4	0.025 ^a
CD4 ⁺ T lymphocyte nadir (cells/μL)	237 ± 199	183 ± 172	0.001 ^a

Abbreviations: HAD = HIV-associated dementia; HAND = HIV-associated neurocognitive disorders; MND = mild neurocognitive disorder. Sample size within demographic and disease parameters may be less than the overall sample size due to missing data (%) in categories (duration HIV, 28.6%; HAND, 1.9%; plasma HIV, 0.6%; CSF HIV, 6.1%; CD4⁺, 0.6%; CD8⁺, 2.8%; CD3⁺, 8.9%; CD4/CD8, 2.7%).

^a *p* Values represent comparisons between the Caucasian and African American groups by the Student *t* test.

^b *p* Values represent comparisons between the Caucasian and African American groups by the χ^2 test.

Figure 3 African American PLWH with 1 or more short HO-1 (GT)_n alleles have significantly decreased risk of functional HIV NCI in the absence of contributing comorbidity factors



Within the group of African Americans (A), but not the European American group (B) with functional HIV NCI without contributing comorbidities, the presence of at least 1 short "S" HO-1 (GT)_n allele associated with lower functional HIV NCI (MND and HAD) vs no functional HIV NCI (NCN and ANI), by an OR of 0.38 (95% CI: 0.16–0.84) (χ^2 test, $p = 0.018$). (C) Functional NCI frequency increased linearly with stratification of genotypes from homozygous short "SS" to homozygous long "LL" within the African American group (χ^2 test for linear trend, $p = 0.020$), but not in the European American group (not shown). (D) African Americans have a lower prevalence of HO-1 (GT)_n genotypes containing a short "S" allele and a higher prevalence of genotypes containing a long "L" allele than European Americans (χ^2 test, $p < 0.0001$). Subgroup analyses were similarly performed by comparing function HIV NCI groups MND and HAD with NCN alone (E–H), with similar results. ANI = asymptomatic neurocognitive impairment; HAD = HIV-associated dementia; HO-1 = heme oxygenase-1; MND = mild neurocognitive disorder; NCI = neurocognitive impairment; NCN = neurocognitive normal; PLWH = persons living with HIV.

group, there was a significant linear trend of increased prevalence of functional HIV NCI with genotypes expressing alleles of increasing HO-1 (GT)_n repeats from “SS” to “LL” ($p = 0.020$; figure 3C), which was not observed in the European American cohort (not shown). We note that no European Americans in our cohort had the “LL” genotype (figure 3D).

In a subgroup analysis of functional HIV NCI without other contributing factors, we compared the NCN group with the functional NCI (MND and HAD) group and again observed similar effects. The presence of at least 1 short allele associated with a lower prevalence of NCI (OR = 0.34, 95% CI: 0.14–0.81) in African Americans but not in European Americans (figure 3, E and F). Again, within the African American group, there was a significant linear trend of increased prevalence of functional HIV NCI with genotypes expressing alleles of increasing HO-1 (GT)_n repeats from “SS” to “LL” ($p = 0.011$; figure 3G). No significant trend was observed in European Americans (figure 3H). No effect of the A(-413)T SNP genotype was observed in comparisons among any groups (not shown).

Genetic ancestry analysis confirms self-identified racial assignments and ancestry-specific associations between the HO-1 (GT)_n genotype and functional HIV NCI

Previously, ancestry-informative nuclear DNA SNP variant analysis of the CHARTER PLWH cohort defined categories of ancestry assignment by genetic ancestry clusters using principal component ancestry-based stratifications (European, African, or admixed Hispanic).³⁰ In the CHARTER cohort, 98.1% of self-identified African Americans and European Americans were assigned to a genetic ancestry cluster, resulting in 97.7% concordance with African and European ancestry, respectively. No self-identifying African Americans were classified as European ancestry, and no self-identifying European Americans were classified as African ancestry. The 10 discordant self-identified European Americans and 5 discordant self-identified African Americans were classified as admixed Hispanic ancestry. In addition, 7 PLWH who did not self-identify as European American or African American were classified as European or African ancestry (4 and 3, respectively). When the racial subgroup associations between HO-1 (GT)_n allele genotype and functional HIV NCI were repeated using genetically assigned ancestry clusters, the results and statistical significance were nearly identical to the analyses using self-identification (figure e-1, [links.lww.com/NXI/A231](https://www.lww.com/NXI/A231)). Specifically, in African Americans, the presence of at least 1 short allele associated with a lower prevalence of functional HIV NCI in both NCN and ANI (OR = 0.31, 95% CI: 0.12–0.78) and NCN-only subgroup analyses (OR = 0.28, 95% CI: 0.11–0.69), whereas no statistical significance was observed in these associations in the European ancestry group (both $p > 0.5$). Although the ORs were lower in the genetically assigned African ancestry group compared with the self-identified African American group, no significant change in ORs was observed ($p > 0.5$, Mantel-Haenszel test).

Short HO-1 (GT)_n alleles associate with lower plasma sHO-1 in virally suppressed African American PLWH.

A previous report associated short HO-1 (GT)_n repeat lengths with lower plasma soluble CD14 (sCD14) levels in ART-treated African American PLWH, but not in European American PLWH, which suggests that short HO-1 (GT)_n alleles have a downmodulating effect against HIV-associated inflammation in African Americans.⁸ We determined relationships between HO-1 (GT)_n genotype, plasma sCD163 (monocyte activation),²⁶ and plasma sHO-1 (inflammation and oxidative stress)^{27,28} in ART-treated, virally suppressed individuals within the cohort. We did not observe significant differences in the total cohort (not shown). However, when we stratified groups according to current CD4⁺ T-cell count (above and below 350 cells/ μ L), as an indicator of relative level of immunosuppression,^{31,32} we observed significant differences. In African Americans (figure 4A), but not in European Americans (figure 4B), with current CD4 T-cell counts ≥ 350 cells/ μ L, the presence of 1 or more short alleles associated with lower plasma sHO-1. This effect was independent of HIV NCI diagnosis. An observed difference in plasma sCD163 in African Americans (figure 4C) was not statistically significant ($p = 0.076$), and no sCD163 difference was observed in European Americans (not shown). Furthermore, sCD163 associated positively with sHO-1 in African Americans (figure 4D), but not in European Americans (not shown).

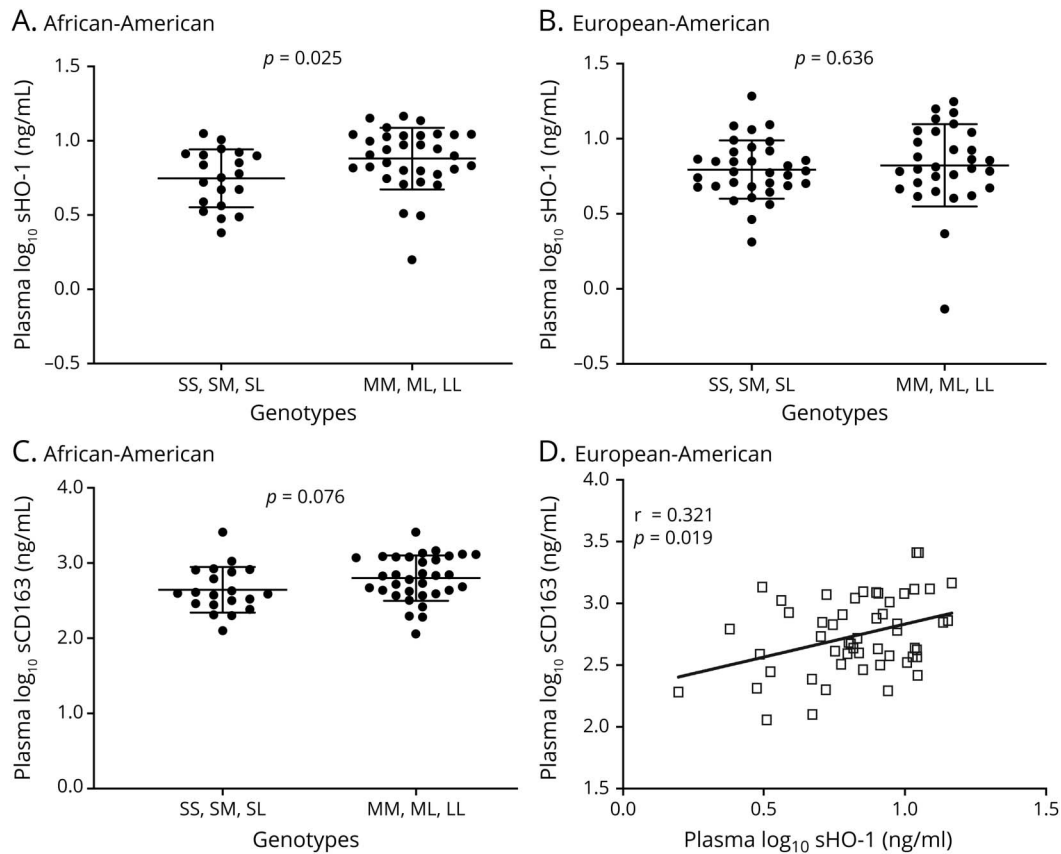
Africans from Botswana have a higher prevalence of long HO-1 (GT)_n alleles than African Americans and European Americans.

To confirm the apparent association between the trimodal HO-1 (GT)_n allele length distribution, genotype prevalence distribution, and African ancestry, we genotyped a cohort ($n = 428$) of PLWH from previous observational studies of Africans in Botswana.^{20,21} The allele distributions differed significantly from that observed in African Americans and European Americans genotyped in our CHARTER and NNTC cohorts (figure 5A).¹⁶ Individuals in the Botswana cohort had the highest prevalence of long “L” alleles (38.4%) compared with African Americans ($p < 0.01$) and European Americans (32.4%, 4.7%, $p < 0.01$, respectively; figure 5, B and C). Significantly different HO-1 (GT)_n allele genotype frequencies were also observed, with a higher prevalence of “LL” genotypes in the Botswana cohort (16.8%, 10.7%, and 0.7%, respectively), confirming a higher prevalence of long HO-1 (GT)_n alleles in individuals of African ancestry (compared with European Americans).

Discussion

The global burden of NCI from HIV infection remains high, despite the effectiveness of ART in suppressing HIV replication, increasing life expectancy, and reducing the severity of HIV NCI in PLWH.^{17,18,33} The spectrum of HAND has evolved with the use of ART. Despite the decreased prevalence

Figure 4 Short HO-1 (GT)_n alleles associate with lower oxidative stress (sHO-1) in virally suppressed African American PLWH



Virally suppressed individuals with current CD4⁺ T cell counts greater than 350 cells/ μ L were examined. African Americans (A), but not European Americans (B), with at least 1 short “S” HO-1 (GT)_n allele had lower mean plasma sHO-1. (A) statistically insignificant ($p = 0.076$) difference in favor of lower plasma sCD163 was observed in African Americans (C), but not in European Americans ($p = 0.676$, not shown). Plasma sCD163 associated with plasma sHO-1 in African Americans (D) but not in European Americans by Spearman rank correlation (not shown). HO-1 = heme oxygenase-1; sHO-1 = soluble HO-1; PLWH = persons living with HIV.

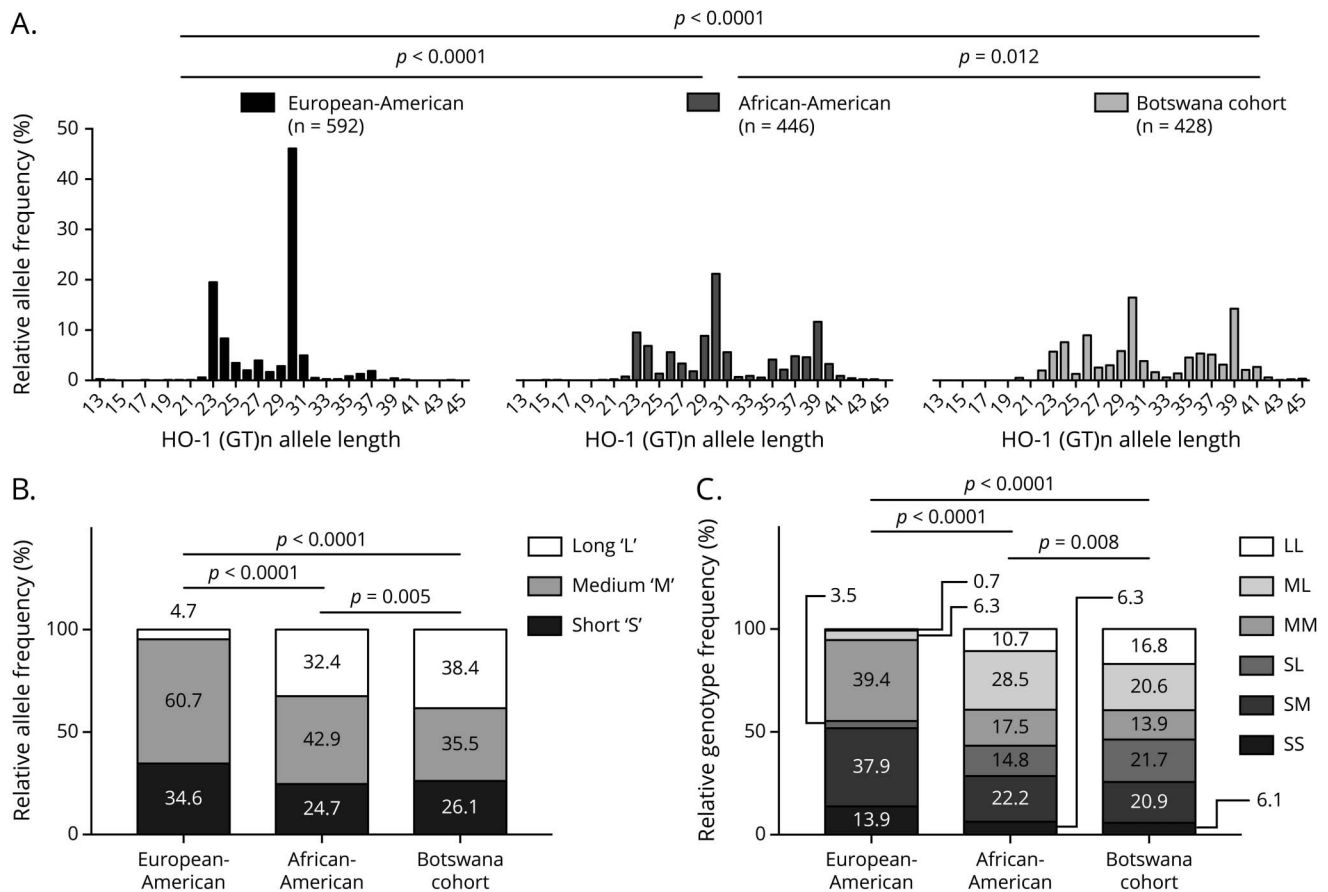
of its most severe form, HAD, from $\sim 20\%$ to $\sim 2\%$, its less severe, but nonetheless disabling form, MND affects $\sim 15\%$ of ART-suppressed PLWH. Thus, ART provides incomplete protection from developing functionally disabling HIV NCI, and this challenging therapeutic gap may widen as the population of PLWH ages.^{34–37} Closing this gap depends on identifying and managing those pathologic processes that drive HIV NCI in ART-suppressed PLWH and identifying individuals who are highly vulnerable to those processes.

Persistent oxidative stress and inflammation are risk factors for the development of HIV NCI in ART-suppressed PLWH,^{38–41} and we have identified the cytoprotective enzyme HO-1 as a potential therapeutic target.^{16,42–44} Our study identifies the HO-1 promoter (GT)_n repeat as a potential risk modulator for the development of HIV NCI in such individuals. We emphasize this effect in individuals with functional HIV NCI (i.e., impaired ADLs; subgroups MND and HAD) in comparison to PLWH without functional HIV NCI; our conclusions were confirmed by comparisons with either NCN or combined NCN and ANI group subgroup analyses. We specifically demonstrate that the presence of short HO-1

(GT)_n alleles, which are known to have higher HO-1 transcriptional activity, associates with a decreased risk for HIV NCI. Moreover, our subgroup analyses demonstrate that this HO-1 (GT)_n allele risk reduction is relatively specific for NCI due to the presence of HIV infection alone and not common comorbid cognitive risk factors. These findings suggest a therapeutic opportunity for further HIV NCI risk reduction through targeting HO-1 expression, function, and associated factors in PLWH who are receiving ART.

Although we observed that a protective effect of the short allele associated with NCI due to the presence of HIV infection alone, and not contributing comorbidity factors, we cannot rule out protective effects against other causes of NCI. The definition of contributing factors, as defined by CHARTER criteria, is that these factors are felt to affect NCI to a minor degree, as assigned by the CHARTER group (adjudicating Neurologists). Within these individuals, we speculate that the contribution of neuroinflammation and CNS oxidative stress, which are the downstream targets of HO-1 enzymatic products, to NCI is relatively low compared with that which is attributable to HIV infection itself.

Figure 5 Africans from Botswana have a higher prevalence of long HO-1 (GT)_n alleles than African Americans and European Americans



HO-1 (GT)_n allele lengths were determined in African PLWH in Botswana (n = 428) who were previously enrolled in cohort studies^{20,21} and allele distributions were compared with those determined in individuals in our CHARTER and NNTC cohorts (592 European Americans and 446 African Americans).¹⁶ (A) Distributions of HO-1 (GT)_n allele lengths within European American, African American, and Botswana cohort groups compared by the Kolmogorov-Smirnov test. Short "S," medium "M," and long "L" HO-1 (GT)_n allele and (B) allele and (C) genotype frequencies within European American, African American, and Botswana cohort groups compared by the χ^2 test. CHARTER = CNS HIV Antiretroviral Therapy Effect Research; HO-1 = heme oxygenase-1; NNTC = National NeuroAIDS Tissue Consortium; PLWH = persons living with HIV.

Previous studies suggested that HO-1 may inhibit HIV infection and/or replication,⁴⁵ suggesting a mechanism by which differential expression of HO-1 could affect HIV pathogenesis, both in the periphery and in the CNS. However, our previous studies did not confirm an effect of HO-1 induction or suppression on HIV-1 replication in human monocyte-derived macrophages, the primary HIV reservoir within the CNS.⁴³ These in vitro findings are consistent with the lack of significant associations between either the HO-1 (GT)_n allele genotype or the A(-413)T SNP and plasma HIV load or CSF HIV load.¹⁶ We also did not observe associations between either the HO-1 (GT)_n allele genotype or the A(-413)T SNP and blood CD4 T-cell counts. Thus, our studies indicate that HO-1 does not directly modulate HIV replication and associated immune suppression.

We therefore propose an indirect role for HO-1 in modifying HIV neuropathogenesis mechanisms that could modify the risk for HIV NCI. Through our previous analysis of autopsied brain specimens from PLWH with advanced HIV

disease, along with HIV-negative controls (n = 554), we showed that the presence of 1 or more short HO-1 (GT)_n alleles was associated with lower risk of HIV encephalitis (OR = 0.62) and lower brain expression of type I interferon responses and T-cell activation markers.¹⁶ In a subset of these autopsy cases (n = 156), we further demonstrated that brain HO-1 expression is reduced in PLWH with HIV NCI and that this HO-1 deficiency associates with brain HIV load, markers of type I interferon responses, and expression of immunoproteasome subunits.^{43,46} Together, these autopsy studies suggest that higher HO-1 expression may limit neuroinflammation, providing an indirect mechanism by which HO-1 modifies the risk for HIV NCI. In in vitro studies, we also demonstrated that HIV infection of macrophages, the primary CNS target of HIV infection, results in loss of HO-1 expression and that this is associated with the release of neurotoxic levels of glutamate.^{43,44} Correction of this HO-1 deficiency prevents this neurotoxic effect in vitro. Thus, a state of reduced HO-1 expression within the HIV-infected brain could promote both neuroinflammation and

neuronal injury through excitotoxic mechanisms, and therapeutic induction of HO-1 expression might therefore prevent or suppress these processes.

Collectively, our studies strongly support a role for HO-1 dysregulation in the neuropathogenesis of HIV infection through regulation of both neuroimmune activation and excitotoxic injury, and they further identify HO-1 induction as a potential treatment approach for HIV NCI.^{16,43,44,46} This HO-1–targeting therapeutic opportunity is likely distinct from therapeutic opportunities that focus on risk reduction by targeting contributing comorbidity factors; this thus emphasizes the need for specific pharmacologic therapies that are adjuncts to suppressive ART.²² Our current and previous studies offer a rationale for targeting of HO-1 expression, particularly early after HIV infection, as one such adjunctive approach to address the gap in therapeutics for HIV NCI reduction by ART.

Our study highlights HO-1 induction as a therapeutic application to HIV NCI that might be particularly important in individuals of African ancestry. African Americans have a lower prevalence of protective short HO-1 (GT)_n alleles and a higher prevalence of higher-risk long alleles compared with European Americans. Because short HO-1 (GT)_n repeats produce higher basal HO-1 promoter activity and inducibility, the lower prevalence of short alleles and higher prevalence of long alleles in African Americans suggests greater vulnerability to oxidative stress and oxidative stress–induced injury in individuals of African ancestry. We note that racial assignment within the CHARTER cohort was defined by self-identification, and we confirmed the high concordance (97.7%) with previously genetically assigned racial ancestry clusters based on sequencing of ancestry-informative nuclear DNA SNP variants.³⁰ We found statistically significant associations between the presence of short alleles and lower OR for HIV NCI when individuals were classified by either genetic ancestry assignment or by self-identification. We thus report our observations primarily in self-identified groups, based on our original study design, the increased clinical and research utility of self-identification, and with confidence in the applicability to this patient cohort.

Our study suggests that individuals of African ancestry may be at a higher risk for developing HIV NCI, and indeed, some studies have found a higher incidence of HIV NCI in African American PLWH compared with European American PLWH, even after correcting for comorbidity factors.⁴⁷ The positive effect of a short HO-1 (GT)_n allele in our cohort was observed in African Americans and not in European Americans, perhaps because of the higher prevalence of long HO-1 (GT)_n alleles in African Americans, which may contribute to a higher risk for inflammation, oxidative stress, and HIV NCI. The protective effect of the short HO-1 (GT)_n allele appears to be driven by African ancestry, although the test for effect of race as a modifier was not statistically significant ($p = 0.11$), and our study might be underpowered to detect such an effect. The question of race itself as a contributing risk factor

warrants further investigation. Our data do support a previous report suggesting increased HIV disease progression risk in African American PLWH because of their higher prevalence of long HO-1 (GT)_n repeats.⁸ In that study, increased HIV disease risk was inferred from the detection of higher plasma soluble CD14 levels (suggested as an indicator of monocyte transition to an activated state⁴⁸) in PLWH on suppressive ART. These associations were observed in African American PLWH and not in European American PLWH.

Further supporting a role for the HO-1 (GT)_n allele genotype in HIV pathogenesis in individuals of African ancestry, we found associations between allele genotype, inflammatory and oxidative stress biomarkers, and HIV disease status. We assessed plasma soluble CD163 as a marker of inflammation and monocyte activation,²⁶ plasma sHO-1 as a marker of inflammation and oxidative stress,^{27,28} and current CD4⁺ T-cell count (cutoff of 350 cells/ μ L) as a marker of HIV immune status.^{31,32} We demonstrated that the presence of 1 or more short HO-1 (GT)_n alleles associates with lower plasma sHO-1 in virally suppressed African American PLWH, but not in European American PLWH, with CD4⁺ T-cell counts greater than 350 cells/ μ L. However, we did not observe differences in plasma sHO-1 in European Americans or in African Americans with CD4⁺ T-cell counts less than 350 cells/ μ L. These data suggest that the HO-1 (GT)_n allele genotype may modulate inflammation, oxidative stress, and risk for HIV NCI in African American PLWH and that these protective effects are particularly expressed in individuals without severe immunosuppression.

We believe that the association between lower plasma sHO-1 levels and the presence of ≥ 1 short HO-1 (GT)_n alleles in African American PLWH reflects an indirect effect of the short allele associating with lower levels of inflammation and/or oxidative stress, which, in turn, associate with less induction of HO-1 expression. Thus, there may be less HO-1 available for cleavage and release from its membrane-bound state within cells. We do not have direct proof of this hypothesis, but we do find some evidence supporting this in the published literature.^{27,28,49} Several reports of increased plasma sHO-1 levels in human disease states associated with systemic inflammation provide such evidence: acute HIV infection,⁴⁹ *Strongyloides stercoralis* infection,²⁸ and coronary artery disease.²⁷

A major implication of our study is that individuals of African ancestry might be more vulnerable to HIV NCI than individuals of European ancestry because of differences in HO-1 (GT)_n allele genotype prevalence. However, determining this will require additional prospective cohort studies. The protective effect of a short HO-1 (GT)_n allele may be detected in African American PLWH and not in European American PLWH because of the markedly lower prevalence of long alleles in European Americans, which may contribute to a lower risk for inflammation, oxidative stress, and HIV NCI. Furthermore, the observation that Africans from Botswana express an even higher prevalence of long HO-1 (GT)_n

alleles than African Americans suggests that the effects of allele genotype on HIV NCI risk may be even more pronounced in native African PLWH. Thus, our studies support the need for studies of sufficiently large populations of PLWH worldwide to confirm risk-modifying effects of the HO-1 (GT)_n allele genotype in different racial groups and to more effectively define HIV NCI risk worldwide.⁵⁰ We suggest that adjunctive HO-1-inducing therapeutics may reduce HIV NCI in virally suppressed PLWH and that such therapies may be particularly beneficial to individuals of African ancestry, where endogenous HO-1 expression may be limited.

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Disclosure

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Rolando Garza, BS	University of Pennsylvania, Philadelphia	Conducted genotyping experiments; analyzed and interpreted genotype data; and drafted the original manuscript.
Alexander J. Gill, MD, PhD	University of Pennsylvania, Philadelphia	Conceptualized the study and designed experiments; analyzed and interpreted genotype data; and drafted the original manuscript.

Appendix (continued)

Name	Location	Contribution
Brandon L. Bastien, BA	University of Pennsylvania, Philadelphia	Conducted genotyping experiments; analyzed and interpreted genotype data; and critically reviewed, edited, and approved the final version of the manuscript.
Yoelvis Garcia-Mesa, PhD	University of Pennsylvania, Philadelphia	Analyzed and interpreted genotype data and critically reviewed, edited, and approved the final version of the manuscript.
Analise L. Gruenewald, BS	University of Pennsylvania, Philadelphia	Analyzed and interpreted genotype data and critically reviewed, edited, and approved the final version of the manuscript.
Benjamin B. Gelman, MD, PhD	University of Texas Medical Branch, Galveston, TX	Curated NNTC cohort samples and contributed to analysis of data and critically reviewed, edited, and approved the final version of the manuscript.
Billy Tsima, MD, MSCE	University of Botswana, Gaborone, Botswana	Curated the Botswana samples and critically reviewed, edited, and approved the final version of the manuscript.
Robert Gross, MD, MSCE	University of Pennsylvania, Philadelphia	Curated the Botswana samples and critically reviewed, edited, and approved the final version of the manuscript.
Scott L. Letendre, MD	University of California, San Diego, San Diego	Curated CHARTER cohort samples and provided support for analysis of neurocognitive data and critically reviewed, edited, and approved the final version of the manuscript.
Dennis L. Kolson, MD, PhD	University of Pennsylvania, Philadelphia	Conceptualized the study and designed experiments; drafted the original manuscript; and critically reviewed, edited, and approved the final version of the manuscript.

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