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No reliable gene expression biomarkers of current or impending neurocognitive impairment in peripheral blood monocytes of persons living with HIV

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Abstract

Events leading to and propagating neurocognitive impairment (NCI) in HIV-1-infected (HIV+) persons are largely mediated by peripheral blood monocytes. We previously identified expression levels of individual genes and gene networks in peripheral blood monocytes that correlated with neurocognitive functioning in HIV+ adults. Here, we expand upon those findings by examining if gene expression data at baseline is predictive of change in neurocognitive functioning 2 years later. We also attempt to validate the original findings in a new sample of HIV+ patients and determine if the findings are HIV specific by including HIV-uninfected (HIV−) participants as a comparison group. At two time points, messenger RNA (mRNA) was isolated from the monocytes of 123 HIV+ and 60 HIV− adults enrolled in the Multicenter AIDS Cohort Study and analyzed with the Illumina HT-12 v4 Expression BeadChip. All participants received baseline and follow-up neurocognitive testing 2 years after mRNA analysis. Data were analyzed using standard gene expression analysis and weighted gene co-expression network analysis with correction for multiple testing. Gene sets were analyzed for GO term enrichment. Only weak reproducibility of associations of single genes with neurocognitive functioning was observed, indicating that such measures are unreliable as biomarkers for HIV-related NCI; however, gene networks were generally preserved between time points and largely reproducible, suggesting that these may be more reliable. Several gene networks associated with variables related to HIV infection were found (e.g., MHC I antigen processing, TNF signaling, interferon gamma signaling, and antiviral defense); however, no significant associations were found for neurocognitive function. Furthermore, neither individual gene probes nor gene networks predicted later neurocognitive change. This study did not validate our previous findings and does not support the use of monocyte gene expression profiles as a biomarker for current or future HIV-associated neurocognitive impairment.

Keywords HIV-associated neurocognitive disorders · neuroHIV · Monocyte · WGCNA · Gene expression · Biomarker

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Introduction

HIV-associated neurocognitive disorders (HAND) represent a significant public health issue as they affect as many as half of the estimated 1.2 million HIV-1-infected individuals within the USA alone (Heaton et al. 2011; Sacktor et al. 2016). A key aspect of the neuropathogenic process leading to HAND is the increased migration across the blood-brain barrier of monocytes (Pulliam et al. 1997; Ellery et al. 2007) driven both by chemokine gradients originating in the CNS and from a peripheral immune response (Peluso et al. 1985; Ancuta et al. 2004; Kraft-Terry et al. 2009). Once in the CNS compartment, monocytes typically differentiate into macrophages which can release pro-inflammatory cytokines and chemokines; if infected with HIV, they may also release viral proteins that are harmful to nearby neurons and other cells (Kedzierska and Crowe 2002; Glass et al. 1995; Adle-Biassette et al. 1999; Lindl et al. 2007; Kaul and Lipton 2006; Kraft-Terry et al. 2009). Macrophage density in brain is associated with severity of HAND (Boven 2000), further underscoring the important role of monocyte/macrophages in HAND.

Because the crosstalk between the CNS and circulating blood monocytes is a central mechanism underlying HAND neuropathogenesis, monocytes may hold useful biomarkers of impending or current HAND. For example, CD14+/CD69+ monocytes were a strong indicator of neurologic injury among patients with HIV-associated dementia in the pre-HAART era (Pulliam et al. 1997), although this relationship appears to be weaker in the current HAART era (Kusdra et al. 2002). Considering that the vast majority of HAND cases are mild (Heaton et al. 2011; Sacktor et al. 2016), our group previously examined global gene expression within peripheral blood monocytes to identify transcriptional changes associated with not only in HIV-associated dementia but also neurocognitive functioning in general (Levine et al. 2013). By focusing on peripheral molecular genetic mechanisms that may be prodromal to HAND or indicative of mild HAND, this approach was potentially useful because it might enable deeper understanding of early neuropathogenic processes, and open the possibility of preventative therapies. Findings from our cross-sectional study of 86 HIV+ cases implicated a variety of dysregulated genes, most notably Kelch-like ECH-associated protein-1 (KEAP1), hypoxia upregulated-1, and interleukin 6 receptor, implicating oxidative stress as an underlying pathogenic process. In addition, weighted gene co-expression network analysis (WGCNA) (Zhang and Horvath 2005; Langfelder and Horvath 2008), a system biologic approach devised to arrive at a biologically meaningful reduction of high dimensional transcriptomic data, implicated mitotic cell cycle and translational elongation as biological processes correlated with neurocognitive functioning. Those results led successful preclinical trials of compounds that elicit broad antioxidant and anti-inflammatory responses in monocytes, enhance neuroprotective factors, and decrease viral replication (unpublished data presented by Gruenewald et al., at the 14th meeting of the International Society on NeuroVirology, 2016). Here, we expanded upon the previous findings in three ways. First, we attempted to validate the original findings in an independent sample of HIV+ adults. Second, we determined if gene expression changes within monocytes at baseline predicted neurocognitive status 2 years later. Third, we included a HIV-uninfected comparison group, which allowed us to determine if any associations between the biological signals and clinical variables are HIV-specific. Our hypotheses were (1) the findings from the initial study would be validated; (2) baseline gene expression characteristics would be predictive of neurocognitive change measured 2 years later; and (3) these findings would be HIV-specific; that is, they would not be observed in the HIV− group.

Materials and methods

Participants

This study was conducted in accordance with the University of California, Los Angeles Medical Institutional Review Board rules and regulations (IRB#10–001099). All MACS participants who completed the full neuropsychological test battery within 3 weeks of blood draw were eligible. Between 2011 and 2015, 206 participants in the Multicenter AIDS Cohort Study (MACS) in Los Angeles, California, were recruited for this sub-study. The total sample was composed of middle-aged males from white, black, and Hispanic racial groups, all of whom were on ART at the time of the study. Of these, 146 were HIV+ and 60 HIV-seronegative. Monocytes were extracted from the blood of 121 HIV+ cases at baseline (herein referred to as time point 1), and then 67 HIV+ (39 new and 28 returning) and 60 HIV-uninfected cases approximately 2 years later (herein referred to as time point 2). Due to specific procedural issues (platelets or red blood cell contamination and/or messenger RNA (mRNA) degradation), several samples were omitted from further analysis. After additional data quality control steps (described below), gene expression data from time point 1 included 89 HIV+ cases and from time point 2 included 62 HIV+ cases (28 of whom were also seen at time point 1) and 60 HIV− cases. Group characteristics are shown in Table 1, and participant and sample flow from baseline and follow-up visits are detailed in Fig. 1. All participants completed comprehensive self-report questionnaires assessing drug use, medication use, and medical co-

Table 1 Descriptive statistics of sample sets

		Time point 1 HIV+			Time point 2 HIV+			Time point 2 HIV-		
		N	Mean	Standard deviation	*N*	Mean	Standard deviation	*N*	Mean	Standard deviation
Age (years)		89	52.7	9.1	62	51.7	10.5	60	57.2	10.3
GNF T-score		89	50.1	7.1	62	49.12	6.67	60	52.00	6.26
Log10 viral load		89	1.41	0.99	55	1.44	1.01			
CD4 count		88	601	189	54	644	265	60	965	271
Nadir CD4 count		89	258	164	62	290	164	60	616	192
Duration of infection (years)		89	19.9	8.7	62	17.1	10.2			
CNS penetration effectiveness		66	1.36	0.80	31	1.13	0.88			
		N	Percent		*N*	Percent		*N*	Percent	
Viral load	Detectable	69	78%		42	76				
	Undetectable	20	22%		13	24				
HAND	0	65	73%		44	71%		51	85%	
	1	10	11%		13	21%		6	10%	
	2	12	13%		3	5%		3	5%	
	3	2	2%		2	3%		0	0%	
Education (years)	< 8	4	4%		1	2%		0	0%	
	< 12	5	6%		3	5%		3	5%	
	12	10	11%		6	10%		5	8%	
	< 16	25	28%		28	45%		13	22%	
	16	20	22%		13	21%		17	28%	
	> 16	25	28%		11	18%		22	37%	
Ethnic group	White non-Hispanic	54	61%		27	44%		42	70%	
	White Hispanic	11	12%		9	15%		4	7%	
	Black non-Hispanic	12	13%		12	19%		5	8%	
	Black Hispanic	0	0%		1	2%		0	0%	
	Other non-Hispanic	0	0%		2	3%		1	2%	
	Other Hispanic	12	13%		11	18%		8	13%	
Smoke tobacco	Never	18	21%		12	20%		17	29%	
	Former	49	56%		33	56%		34	59%	
	Current	20	23%		14	24%		7	12%	
Alcohol	< Monthly	47	54%		30	51%		29	52%	
	Monthly	14	16%		9	15%		7	13%	
	Weekly	12	14%		13	22%		11	20%	
	Daily	14	16%		7	12%		9	16%	
Cannabis	< Monthly	64	74%		45	76%		47	84%	
	Monthly	4	5%		4	7%		3	5%	
	Weekly	7	8%		4	7%		4	7%	
	Daily	12	14%		6	10%		2	4%	
Cocaine	< Monthly	85	98%		57	97%		54	96%	
	Monthly	0	0%		1	2%		0	0%	
	Weekly	2	2%		1	2%		0	0%	
	Daily	0	0%		0	0%		2	4%	

morbidities, as well as comprehensive neuropsychological testing and assessment of activities of daily living from which their HAND status was determined. All participants returned after 2 years for follow-up questionnaires and procedures. Procedures and assays were identical to those described in the previous study (Levine et al. 2013).

Blood processing, monocyte isolation, mRNA extraction, and gene expression profiling

Of the fresh blood, 24 mL was collected from participants. Blood was drawn into three 8-mL cell preparation tubes (CPTs) containing sodium citrate. Peripheral blood mononuclear cells (PBMCs) were then isolated through centrifugation within 6 h of collection (Salazar-Gonzalez et al. 1997). PBMCs were washed with phosphate-buffered saline, and then, monocytes were isolated through Rosette separation (RosetteSep®; Stem Cell Technologies, British Columbia, Canada) according to the manufacturer instructions and purity estimates. This led to an approximately 80% purity of isolate monocyte fractions, per the manufacturer's data. Monocytes were then pelleted, lysed, and RNA extracted using the Qiagen RNeasy Kit including a DNase treatment to eliminate any potentially confounding genomic DNA contamination (Shay et al. 2003). RNA purity was assessed via the 260/280 ratio, with values of greater than 1.5 accepted for further analysis. RNA was stored at − 80 °C and sent in batches to the Southern California Genotyping Consortium (SCGC) for microarray analysis, which was performed with the Illumina Human HT-12 v4 gene expression BeadChip. The expression data and sample characteristics, including all information required by the MIAME standard, are available from the NCBI Gene Expression Omnibus (GEO accession #GSE104640).

Variables included in the gene expression analysis

Neurocognitive functioning Participants completed a comprehensive battery of neuropsychological tests as part of the standard MACS protocol, as previously described (Levine et al. 2014b). This includes measures of working memory, learning, memory, executive functioning, motor functioning, and information processing speed. T-scores were calculated using normative data derived from the HIV-seronegative MACS cohort, with demographic corrections for age, education, ethnicity, and number of times they had undergone neurocognitive testing. For this study, we calculated a global neurocognitive functioning (GNF) score based on the average of all available domain T-scores. GNF was our primary phenotype.

HAND severity HAND status was determined via an algorithm developed by MACS investigators. The algorithm is based on neurocognitive test performance and self-reported deficits in activities of daily living (Lawton and Brody 1969) in accordance with current research criteria (Antinori et al. 2007). Participants were rated as neurocognitively normal, mildly impaired, moderately impaired, or severely impaired. The latter three correspond to established research criteria, respectively, asymptomatic neurocognitive impairment, minor neurocognitive disorder, and HIV-associated dementia. Because of the poor reliability and specificity of the HAND from a diagnostic standpoint (Woods et al. 2004), we limited this variable to secondary analyses.

CNS penetration effectiveness CNS penetration effectiveness (CPE) scores for the regimen reported at the time of neurocognitive testing were calculated (Letendre 2011). Higher scores indicate a regimen with increased penetration of the blood-brain barrier.

Substance use We considered the effects of alcohol, marijuana, and cocaine use on gene expression. MACS participants completed a substance use questionnaire that assesses frequency of use during the 6 months prior to the visit. Participants were considered *active* users of alcohol, stimulants, or marijuana if they report daily or weekly use and *non-users* if they report monthly or less use in the 6 months preceding the visit. Tobacco use was also considered.

Depression Depression was determined with the Center for Epidemiologic Studies Depression Scale (CES-D) (Radloff 1977). Scores on the CES-D were entered as a continuous variable, with higher scores indicating greater degree of depression.

Virologic measures The percentage of lymphocytes that were CD4+ T cells was determined by flow cytometry. HIV viral load was determined via either the COBAS TaqMan HIV-1 Test, version 2.0, or Roche Amplicor HIV-1 MONITOR Test, version 1.5. Both tests quantify HIV-1 RNA based on in vitro amplification of the highly conserved HIV-1 gag gene. Nadir CD4+ T cell count was obtained either by self-reports or, for those who seroconverted during the course of the study, their lowest CtaD4+ count according to study records. Duration of infection was calculated based on self-reported year of conversion or study records if they seroconverted while in the MACS.

Statistical analysis

Data preprocessing

Raw gene expression data was processed in Illumina BeadStudio software, and the *lumi* R package was used to log2-transform and quantile normalize the expression profiles to stabilize variance and to normalize inter-sample expression profile distributions, respectively. Probe reannotations provided by the illuminaHumanv3.db R package were used to filter out poor probe hybridization specificity. Probes with significant detection in less than 80% of samples were omitted from further analysis. The data was then batch-corrected for sample chip effects using the *ComBat* R function from the *R package sva* (freely available from http://www.bioconductor.org).

Outliers identified by hierarchical clustering of samples using standardized Euclidean distance and single linkage were

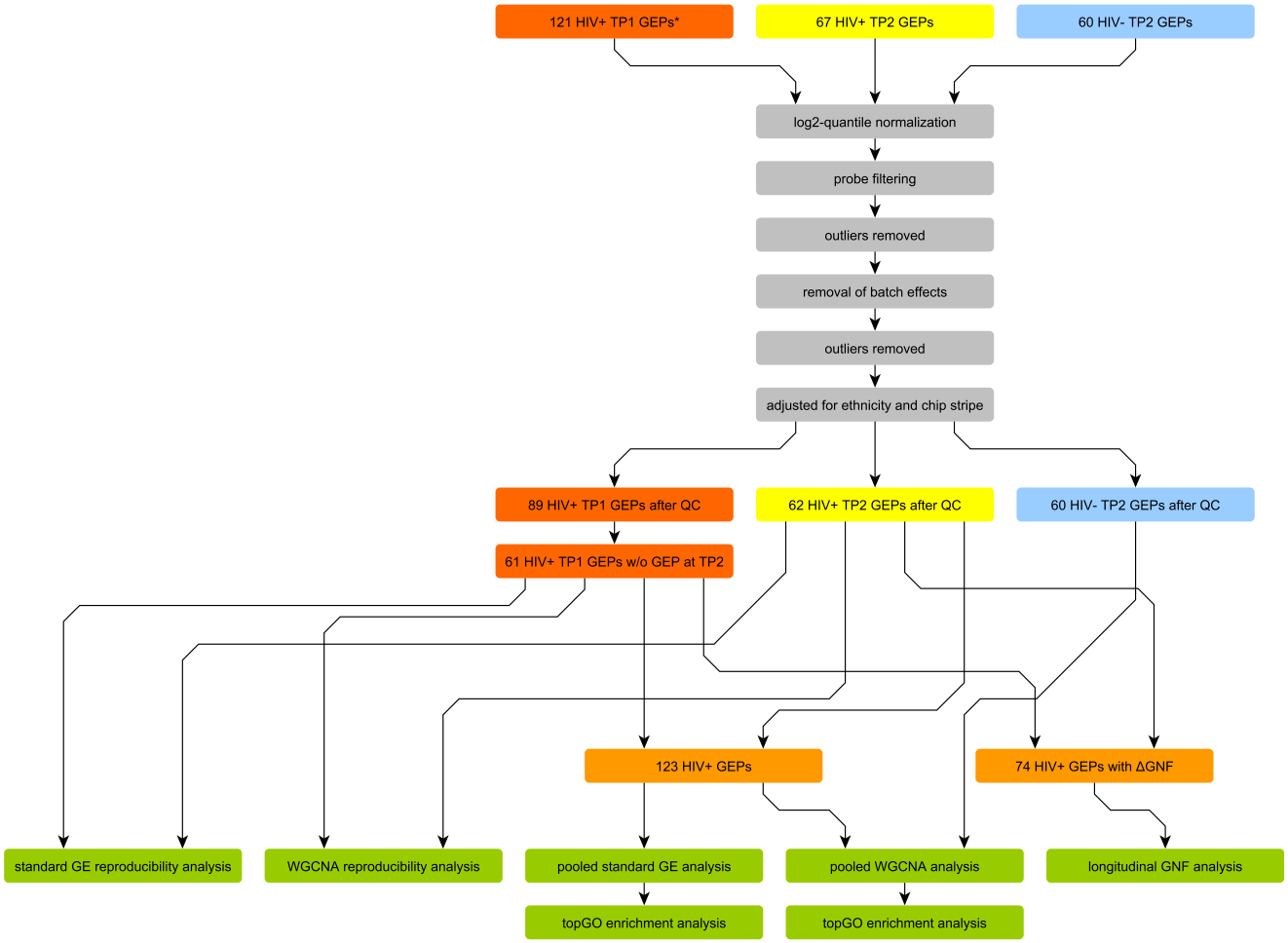

Fig. 1 Study workflow diagram. The workflow for the time point 1 (TP1) HIV+ (dark orange), time point 2 (TP2) HIV+ (yellow), and time point 2 HIV− (blue) sample set are illustrated in the workflow diagram. The gene expression profiles (GEPs) for the three sample sets all undergo processing steps (gray); some GEPs are omitted after quality control (QC) steps. More information on these steps can be found in the "Materials and methods" section. The input sample sets to the various analyses (green) are denoted by arrows. Asterisks indicate that the samples from our previous transcriptome study are included in this sample set

removed both before and after batch correction. The expression data was then adjusted for race and chip stripe by retaining the residuals from robust multivariable linear regression on these covariates.

As a final quality control measure, we determined the correlation between the gene expression profiles of all samples. We found strong consistency between the gene expression profiles within and between individuals (Supplemental Fig. 1). Inter-individual variation was greater than the variation between repeat measurements on the same individual between time points; however, even then, the lowest inter-sample correlation was strong ($r = 0.93$).

Differential expression analysis

In our previous study, we found significant correlations between several gene transcript and GNF in a HIV+ sample (Levine et al. 2013). Here, we assessed the consistency of these findings in an independent sample of HIV+ participants, and also in the HIV− participants in order to determine if the correlations were specific to HIV. Towards these ends, we first correlated gene expression with GNF in the time point 1 samples (excluding samples with repeat measurements at follow-up), and in the HIV+ and HIV− samples at time point 2. These probe-GNF correlations were then correlated among these subsets to determine the reproducibility of between different HIV+ samples and the agreement between HIV+ and HIV− samples.

In order to maximize power, we then proceeded to test for differential gene expression across all HIV+ samples (excluding repeat measurements) using correlation tests with the variables of interest including GNF, HAND rating, CPE, CES-D, substance use (separately: alcohol, tobacco, marijuana, and cocaine), nadir CD4, and log10 viral load. To address our multiple testing across gene probes, we use a Bonferroni corrected significance threshold.

To examine whether or not individual gene probes measured at time point 1 (for the original sample of 89 HIV+ individuals) or time point 2 (for the second sample of 62 HIV+ individuals and the HIV− comparison group) predicted change in neurocognitive functioning at follow-up visits, we calculated the change in GNF by regressing follow-up GNF on current GNF, retaining the residuals to adjust for the potential confounding effects of regression to the mean. Change in GNF was then subject to correlation with individual gene probes, and module eigengenes in the WGCNA analyses (below).

WGCNA

WGCNA was employed in our previous study to reduce the data into smaller groups of co-expressing genes (modules) which generally represent biologically meaningful pathways (Horvath and Dong 2008; Langfelder et al. 2008). In WGCNA, highly correlated module genes are represented and summarized by the module eigengene, or ME (Langfelder and Horvath 2007), which can then be used in standard statistical analyses. In this study, we first attempted to reproduce the WGCNA results from our previous study by assessing the reproducibility of the gene co-expression network results. This was accomplished by computing the preservation of modules found in the first HIV+ sample (from time point 1), in the second, independent HIV+ sample (from time point 2), as described elsewhere (Langfelder et al. 2011). Briefly, we use the modulePreservation function from the WGCNA package, which computes a module preservation statistic for modules in a reference dataset within a new set of data along with an accompanying significance level (permutation test p value).

In order to examine associations between modules and variables of interest, we then used the entire sample of HIV+ and HIV− expression profiles (excluding repeat measurements) to construct a gene network using the WGCNA parameter settings power = 4 and deepCut = 4, which were chosen based on their qualitative optimality for scale-free topology and resolution of finer modules, respectively. We then correlated the identified modules with the variables of interest.

Gene-annotation enrichment analysis

Understanding the biological meaning of gene and module associations with GNF and other variables requires gene annotation enrichment analysis. For this, we used the topGO R package. For the differential expression analyses (which consider correlations between individual gene probes and variables of interest), we conducted enrichment analysis on the top 5% genes associated with GNF (and change in GNF) in the HIV+ samples and in the HIV− samples, regardless of statistical significance. We conducted an analogous enrichment analysis on the gene co-expression modules identified by the WGCNA analyses. TopGO was run using the Fisher's exact and Kolmogorov-Smirnov significance tests and the weight01 algorithm which takes into account the dependencies present in the GO topology and thus can be considered corrected for multiple testing.

Results

Cross-sectional and longitudinal associations between GNF and gene expression

Agreement between time points

We first sought to assess the reproducibility of the findings of our previous study by comparing gene expression probe-GNF associations between the previous and new study samples. After excluding the repeated measurements on the same individuals to avoid statistical dependency, the sample sizes for time point 1 HIV+ and time point 2 HIV+ groups were 61 and 62, respectively. Of the 89 HIV+ participants from time point 1, 28 also provided blood samples for gene expression analysis at time point 2; we did not include duplicate cases in this analysis. Thus, our sample size for time point 1 HIV+ is 89–28 = 61. None of the top genes identified in our previous study were validated in the independent HIV+ group. Furthermore, the correlation between all probe-GNF correlations for the two different groups was weak ($r = 0.07$), indicating that the reproducibility of the differential expression at the single probe level was unreliable (Supplemental Fig. 1, Panel A). In comparison, the probe-GNF correlations between the HIV+ groups and HIV− group indicated an inverse association of slightly greater magnitude, either when the HIV+ samples from time point 1 and time point 2 were combined ($r = -0.16$) or analyzed separately ($r = -0.09$ and $r = -0.15$, respectively) (Supplemental Fig. 1, Panel B). None of these correlations are statistically significant, as the listed p value for the correlation of correlations is massively inflated since it treats each GNF-probe correlation as independent ($n > 10$ k probes) when in reality there is only a sample size of 2 (HIV+ correlations versus HIV− correlations). As such, we find poor validation for gene expression between the HIV+ groups, whereas this correlation was somewhat stronger, yet inverse, between HIV+ and HIV− groups.

Correlations between GNF and gene probe levels among combined sample

In order to maximize statistical power, we combined the HIV+ samples from time points 1 and 2 (excluding repeat measurements) and correlated expression levels with GNF. No significant associations with GNF were found ($p > 1.9 \times 10^{-4}$) at the

Bonferroni adjusted significance threshold ($\alpha < 5 \times 10^{-6}$). Similarly, no significant associations between probes and GNF were found for the HIV− samples ($p > 2.5 \times 10^{-5}$) at the Bonferroni adjusted significance threshold.

To further leverage our data, we then focused on the top 5% genes with the strongest positive and negative correlations with GNF and change in GNF (regardless of statistical significance) and performed gene annotation enrichment analysis using the topGO package. Using this method, genes positively correlated with GNF in HIV+ subjects were found to be enriched for annotations related to c*omplement activation* and consistent with monocyte activation and proliferation (see Table 2 below and Supplemental Table 1 for full details). *Mitochondrial outer membrane permeability* was also a notable finding. Significant GO term enrichment observed for genes negatively correlated with GNF largely involved *regulation of transcription* and *negative regulation of production miRNA involved in gene silencing*, as well as other seemingly innocuous biological processes. GNF in HIV− cases was positively correlated genes related to *mitochondrial activation*, whereas negatively correlated genes were enriched for *morphogenic activities* (Table 2).

The top individual gene probes correlated with GNF, as well as HIV status and viral load, are displayed in Fig. 2. The more comprehensive list can be found in Supplemental Table 2.

Predicting change in GNF

We were largely interested in identifying gene expression signals that might predict later neurocognitive change. Seventy-four HIV+ participants with baseline gene expression profiling at either time point 1 or time point 2 were assessed for neurocognitive function again approximately 2 years later (mean interval = 1.9 years). Correlations between gene expression at time point 1 and change in GNF across this period

Table 2 GO term enrichment of top genes correlated with GNF

		GO ID	Term	Annotated	Significant	Expected	Fold enrichment	Fisher's p-value	
HIV+ subjects	GNF	GO:0006958	complement activation, classical pathway	12	6	0.6	10.0	1E-5	GO terms enriched in negatively correlated genes
		GO:0006957	complement activation, alternative pathway	6	4	0.3	13.3	9E-5	
		GO:0097345	mitochondrial outer membrane permeabilization	47	5	2.34	2.1	2E-4	
		GO:0014066	regulation of phosphatidylinositol 3-kinase signaling	75	9	3.74	2.4	7E-4	
		GO:1901299	negative regulation of hydrogen peroxide-mediated programmed cell death	5	3	0.25	12.0	1E-3	
		GO:0038203	TORC2 signaling	5	3	0.25	12.0	1E-3	
		GO:0045916	negative regulation of complement activation	5	3	0.25	12.0	1E-3	
	Change in GNF	GO:2001223	negative regulation of neuron migration	6	5	0.29	17.2	2E-6	
		GO:0060441	epithelial tube branching involved in lung morphogenesis	14	6	0.68	8.8	3E-5	
		GO:0060259	regulation of feeding behavior	7	4	0.34	11.8	2E-4	
		GO:0006953	acute-phase response	21	6	1.01	5.9	4E-4	
		GO:0001656	metanephros development	27	7	1.3	5.4	7E-4	
		GO:0043303	mast cell degranulation	38	5	1.84	2.7	9E-4	
		GO:0007098	centrosome cycle	47	9	2.27	4.0	1E-3	
HIV- subjects	GNF	GO:0045930	negative regulation of mitotic cell cycle	156	8	7.65	1.0	1E-5	
		GO:0060571	morphogenesis of an epithelial fold	8	5	0.39	12.8	1E-5	
		GO:0019896	axonal transport of mitochondrion	5	4	0.25	16.0	3E-5	
		GO:0001922	B-1 B cell homeostasis	6	4	0.29	13.8	8E-5	
		GO:0032909	regulation of transforming growth factor beta2 production	6	4	0.29	13.8	8E-5	
		GO:0002052	positive regulation of neuroblast proliferation	11	5	0.54	9.3	1E-4	
		GO:0009855	determination of bilateral symmetry	34	8	1.67	4.8	1E-4	
HIV+ subjects	GNF	GO:0060065	uterus development	6	4	0.31	12.9	1E-4	GO terms enriched in positively correlated genes
		GO:0000122	negative regulation of transcription from RNA polymerase II promoter	410	38	21.04	1.8	3E-4	
		GO:0045944	positive regulation of transcription from RNA polymerase II promoter	569	52	29.2	1.8	3E-4	
		GO:0006355	regulation of transcription, DNA-templated	1886	143	96.8	1.5	6E-4	
		GO:0007064	mitotic sister chromatid cohesion	19	6	0.98	6.1	1E-3	
		GO:0051056	regulation of small GTPase mediated signal transduction	165	19	8.47	2.2	1E-3	
		GO:1903799	negative regulation of production of miRNAs involved in gene silencing by miRNA	5	3	0.26	11.5	1E-3	
	Change in GNF	GO:0048841	regulation of axon extension involved in axon guidance	9	4	0.45	8.9	6E-4	
		GO:0032007	negative regulation of TOR signaling	28	7	1.39	5.0	7E-4	
		GO:0046323	glucose import	43	4	2.13	1.9	1E-3	
		GO:0007602	phototransduction	24	5	1.19	4.2	1E-3	
		GO:0006417	regulation of translation	305	26	15.14	1.7	3E-3	
		GO:0071380	cellular response to prostaglandin E stimulus	13	4	0.65	6.2	3E-3	
		GO:0032094	response to food	16	5	0.79	6.3	4E-3	
HIV- subjects	GNF	GO:0070125	mitochondrial translational elongation	90	18	4.52	4.0	4E-7	
		GO:0006418	tRNA aminoacylation for protein translation	34	11	1.71	6.4	5E-7	
		GO:0070126	mitochondrial translational termination	89	17	4.47	3.8	2E-6	
		GO:0042776	mitochondrial ATP synthesis coupled proton transport	29	9	1.46	6.2	8E-6	
		GO:0030099	myeloid cell differentiation	232	12	11.66	1.0	3E-4	
		GO:0043985	histone H4-R3 methylation	8	4	0.4	10.0	4E-4	
		GO:0009584	detection of visible light	17	6	0.85	7.1	5E-4	

Fig. 2 Top correlation gene probes between gene probes and HIV status, viral load, and GNF. Traits of interest are listed in the leftmost column with each grouped set of gene probes described in the middle columns. These probes have the top 10 most significant correlations with their respective traits. Correlation coefficients are colored in blue and red for negative and positive correlations, respectively. *P* values are denoted in green with *p* values surpassing transcriptome-wide significance denoted in bold

	Illumina ID	Gene name	Symbol	cor	p
HIV+ status	ILMN_1763207	basic leucine zipper ATF-like transcription factor 3	BATF3	-0.38	**1E-7**
	ILMN_1655163	serine/threonine kinase 24	STK24	-0.36	**5E-7**
	ILMN_2103841	aryl hydrocarbon receptor interacting protein	AIP	-0.34	**3E-6**
	ILMN_1746704	tripartite motif containing 8	TRIM8	-0.34	**3E-6**
	ILMN_2373010	transmembrane protein 70	TMEM70	0.33	**4E-6**
	ILMN_1706273	MOB kinase activator 2	MOB2	-0.32	9E-6
	ILMN_1738938	translocase of inner mitochondrial membrane 8 homolog B	TIMM8B	0.32	9E-6
	ILMN_1739032	transmembrane protein 70	TMEM70	0.32	1E-5
	ILMN_2411897	Kruppel like factor 10	KLF10	0.32	1E-5
	ILMN_1793950	POTE ankyrin domain family member M	POTEM	0.31	2E-5
Viral laod	ILMN_1711030	5-oxoprolinase (ATP-hydrolysing)	OPLAH	0.42	**3E-6**
	ILMN_2132599	ankyrin repeat domain 22	ANKRD22	0.36	6E-5
	ILMN_1708672	acetyl-CoA acetyltransferase 2	ACAT2	0.35	9E-5
	ILMN_1762725	eukaryotic translation initiation factor 3 subunit L	EIF3L	-0.35	1E-4
	ILMN_1670305	serpin family G member 1	SERPING1	0.35	1E-4
	ILMN_2388547	epithelial stromal interaction 1	EPSTI1	0.35	1E-4
	ILMN_1713285	NSF attachment protein alpha	NAPA	0.35	1E-4
	ILMN_1748650	mitochondrial ribosomal protein L45	MRPL45	-0.35	1E-4
	ILMN_1655497	eukaryotic translation initiation factor 4B	EIF4B	-0.35	1E-4
	ILMN_1749629	cullin 1	CUL1	0.34	2E-4
GNF in HIV+	ILMN_1723020	mitogen-activated protein kinase kinase kinase 1	MAP3K1	0.33	2E-4
	ILMN_2137066	zinc finger protein 7	ZNF7	0.33	2E-4
	ILMN_1740716	RNA binding motif protein 26	RBM26	0.33	2E-4
	ILMN_1763663	HEAT repeat containing 3	AF086132	-0.32	2E-4
	ILMN_1807633	reactive intermediate imine deaminase A homolog	HRSP12	-0.32	3E-4
	ILMN_1801766	mitochondrial calcium uniporter dominant negative beta subunit	CCDC109B	0.32	3E-4
	ILMN_2151048	stromal antigen 1	STAG1	0.32	4E-4
	ILMN_1683313	ST3 beta-galactoside alpha-2,3-sialyltransferase 1	ST3GAL1	0.31	4E-4
	ILMN_1805646	SS18, nBAF chromatin remodeling complex subunit	SS18	0.31	4E-4
	ILMN_1679881	Werner syndrome RecQ like helicase	WRN	0.31	5E-4

were determined (Table 2). After adjusting for multiple comparisons, no significant associations were detected between probe levels and change in GNF ($p > 2.5 \times 10^{-5}$). The top GO terms for the top 5% of genes correlated with change in GNF in HIV+ subjects were *negative regulation of neuron migration* and *regulation of axon extension involved in axon guidance* for negatively and positively correlated genes, respectively (Table 2 and Supplemental Table 1).

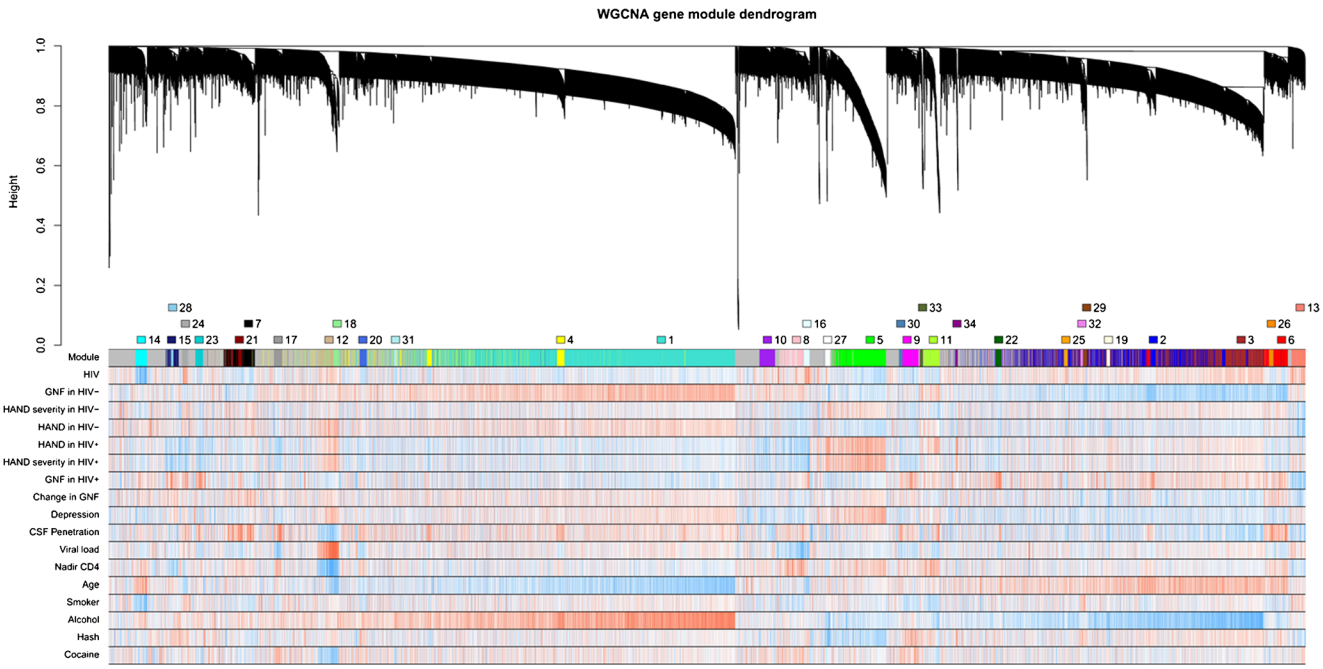

Fig. 3 Dendrogram of WGCNA gene modules constructed from pooled HIV+ and HIV− samples. The clustering of genes based on co-expression is represented in the dendrogram with individual gene probes represented as the vertical leafs (black lines) and descending branches indicating co-expression gene clusters. Module labels are shown in the first row by color along with numeric labels displayed above. Subsequent rows show correlations between traits and individual gene probe levels with blue and red denoting negative and positive correlations according to their magnitude

WGCNA

Preservation of gene modules between two separate HIV+ samples

We first conducted a WGCNA module preservation analysis between time points 1 and 2 for the nonoverlapping HIV+ participants. The majority of modules from the original sample exhibit significant preservation as indicated by their significant permutation p values (Supplemental Fig. 2). These results indicate that at the network level, expression data is reproducible between these two small HIV+ samples.

Cross-sectional WGCNA analysis

We conducted a WGCNA analysis of the gene expression data from all samples (HIV+ and HIV−, excluding repeat measurements). The dendrogram of the gene expression WGCNA analysis is shown in Fig. 3. There are several variables showing qualitative relationships with gene clusters. For example, module 1 is negatively correlated with age and positively correlated with reported alcohol intake and GNF in HIV− subjects, whereas modules 2 and 3 appear to have the reverse relationship; they are positively associated with age and negatively associated with alcohol and GNF in HIV− participants. Globally, the gene expression profiles of the HIV+ and HIV− cases show qualitatively different associations with GNF and HAND (as indicated by opposing red and blue bands on the heatmap).

The resulting eigengenes, each a quantitative value representing the level of a gene module, were then analyzed for correlations with virologic, immunologic, neurocognitive, and drug use variables (Fig. 4). With the Bonferonni corrected significance threshold of $p < 0.001$, significant associations were found between modules 12 and 18 and viral load (and nadir CD4 for module 12), and between modules 14, 16, and 24 and HIV status. Gene ontology analyses for these modules are shown in Table 3. More detailed results are provided in Supplemental Table 3.

Regarding GNF, several additional modules indicated trends towards significance ($p < 0.01$). For example, GNF in HIV+ individuals is positively correlated with modules 22 ($p = 0.008$) and 28 ($p = 0.006$), which appear to be enriched for genes involved in *protein ubiquitinylation process*, whereas module 13 has a negative correlation with GNF ($p = 0.002$) and is enriched for *gluconeogenic activity*. For HIV− individuals, only module 6 has a negative correlation ($p = 0.007$) with GNF and is enriched for adaptive immune response.

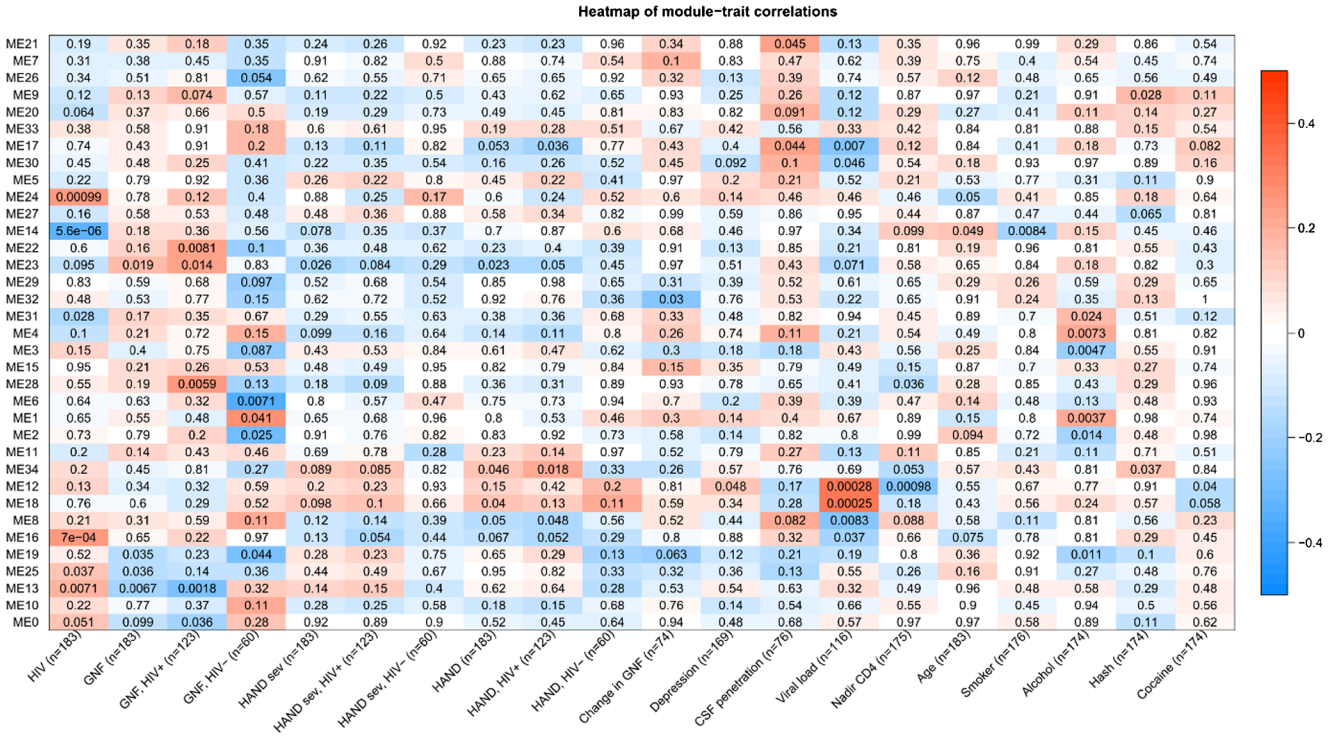

Fig. 4 Heatmap of correlations between modules and traits. Correlations between are illustrated in this grid with blue and red representing negative and positive correlations, respectively, according to magnitude as the color scale shows on the right. Module eigengenes are listed in the rows as labeled on the left, and traits are listed in the columns as labeled at the bottom with sample numbers described in parentheses (for example "HIV ($n = 183$)" designates the column of correlations between modules and HIV+ status in 183 samples). The correlation p values are printed within the grid; here, the Bonferroni significance threshold is $p < 0.0015$

Table 3 FO term enrichment of gene modules

Module	GO ID	Term	Annotated	Significant	Expected	Fold enrichment	Fisher's p-value
12	GO:0033209	tumor necrosis factor-mediated signaling pathway	138	17	1.75	9.7	3E-14
	GO:0002479	antigen processing and presentation of exogenous peptide antigen via MHC class I, TAP-dependent	80	15	1.02	14.7	4E-14
	GO:0060333	interferon-gamma-mediated signaling pathway	68	16	0.86	18.6	1E-12
	GO:0060337	type I interferon signaling pathway	69	13	0.88	14.8	2E-9
	GO:0006521	regulation of cellular amino acid metabolic process	65	10	0.83	12.0	7E-9
	GO:0051437	positive regulation of ubiquitin-protein ligase activity involved in regulation of mitotic cell cycl...	86	11	1.09	10.1	9E-9
	GO:0051436	negative regulation of ubiquitin-protein ligase activity involved in mitotic cell cycle	84	10	1.07	9.3	9E-8
14	GO:0043117	positive regulation of vascular permeability	5	3	0.05	60.0	1E-5
	GO:0038084	vascular endothelial growth factor signaling pathway	8	3	0.08	37.5	6E-5
	GO:0050672	negative regulation of lymphocyte proliferation	31	5	0.32	15.6	1E-4
	GO:0007219	Notch signaling pathway	87	6	0.91	6.6	2E-4
	GO:0050853	B cell receptor signaling pathway	44	5	0.46	10.9	3E-4
	GO:0002250	adaptive immune response	214	8	2.23	3.6	9E-4
	GO:0030035	microspike assembly	5	2	0.05	40.0	1E-3
16	GO:0006413	translational initiation	274	38	2.04	18.6	1E-30
	GO:0006614	SRP-dependent cotranslational protein targeting to membrane	181	36	1.35	26.7	1E-30
	GO:0000184	nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	212	36	1.58	22.8	1E-30
	GO:0019083	viral transcription	245	36	1.82	19.8	1E-30
	GO:0006364	rRNA processing	317	36	2.36	15.3	1E-30
	GO:0000027	ribosomal large subunit assembly	34	7	0.25	28.0	4E-9
	GO:0075713	establishment of integrated proviral latency	9	3	0.07	42.9	3E-5
18	GO:0051607	defense response to virus	186	29	1.7	17.1	9E-25
	GO:0045071	negative regulation of viral genome replication	42	16	0.38	42.1	6E-23
	GO:0035455	response to interferon-alpha	19	10	0.17	58.8	2E-12
	GO:0039530	MDA-5 signaling pathway	9	5	0.08	62.5	3E-6
	GO:0033159	negative regulation of protein import into nucleus, translocation	5	3	0.05	60.0	7E-6
	GO:0010847	regulation of chromatin assembly	5	3	0.05	60.0	7E-6
	GO:0034341	response to interferon-gamma	112	12	1.02	11.8	9E-6
24	GO:0032467	positive regulation of cytokinesis	12	2	0.07	28.6	2E-3
	GO:1902600	hydrogen ion transmembrane transport	88	5	0.52	9.6	3E-3
	GO:1900153	positive regulation of nuclear-transcribed mRNA catabolic process, deadenylation-dependent decay	14	2	0.08	25.0	1E-2
	GO:0042776	mitochondrial ATP synthesis coupled proton transport	29	2	0.17	11.8	1E-2
	GO:0006278	RNA-dependent DNA biosynthetic process	56	2	0.33	6.1	2E-2
	GO:0000398	mRNA splicing, via spliceosome	281	6	1.65	3.6	2E-2
	GO:0018279	protein N-linked glycosylation via asparagine	39	2	0.23	8.7	2E-2

WGCNA at time point 1 as a predictor of later neurocognitive change

Change in GNF was not significantly associated with any time point 1 modules (Fig. 4).

Discussion

In this study, we attempted to replicate our previous findings that neurocognitive functioning in HIV+ persons was correlated with the expression of several oxidative-stress-related genes in peripheral blood monocytes. We also sought to expand those findings by determining if gene expression profiles in such cells could predict neurocognitive status 2 years later, and whether or not any associations or predictive markers were specific to HIV+ persons or were also observed in an HIV− comparison sample.

Contrary to our hypotheses, we were unable to replicate the findings from our earlier study (Levine et al. 2013), which had implicated several genes involved in anti-oxidant response. Despite some overlap between the current and previous study, there was a substantial number of samples that were different in the current study—only 61 out of the 123 samples were from the original analysis. The lack of reproducibility of our previous top associations is consistent with the weak agreement found between our two cross-sectional samples at the single gene level. Also contrary to our hypotheses, gene expression characteristics determined at baseline did not predict neurocognitive decline as measured 2 years later. This includes both individual gene transcripts, modules consisting of co-varying gene networks, and biological ontologies based on top correlations. These results, although unexpected, provide strong evidence that a useful concurrent or predictive biomarker of HIV-associated neurocognitive impairment is unlikely to be found in the gene expression profiles of monocytes, a finding also supported by past studies(Sun et al. 2010), as also reviewed in Kallianpur and Levine (2014) and Levine et al. (2014a).

An alternative reason for the null results may be that our primary phenotype (global neurocognitive functioning) is affected not only by HIV but also by other factors including

substance use, HCV co-infection, pre-existing cognitive deficits, and error due to psychometric characteristics of the tests and participant effort(Devlin et al. 2012; Antinori et al. 2007; Levine et al. 2017). This is especially true of mild neurocognitive deficits, which would generally be seen in the relatively healthy MACS participants (Sacktor et al. 2016). We chose GNF as our primary outcome variable because the diagnosis of HAND is unreliable, as demonstrated by Woods et al. (2004) and further indicated by the near equal number of HIV-seronegative control cases that meet criteria for this condition (Sacktor et al. 2016; Levine et al. 2017). Therefore, if one were to focus advanced HAND cases (e.g., HIV-associated dementia) in analyses such as ours, more consistent signals are more likely to be found. The problem with this approach, however, is that advanced cases are increasingly rare, thus being statistically underpowered for similarly sized studies. A power analysis indicates that in order to have 80% power to detect a weak correlation of $r = 0.3$ at a transcriptome-wide significance level of $p < 5 \times 10^{-6}$, we would need approximately 300 samples; analogously, a modular approach with a less stringent significance threshold of $p < 0.001$ would still require at least 170 samples. However, because we were searching for biomarkers of HAND, the value of weak associations would be insubstantial, considering that biomarkers require medium to large effect sizes.

Despite these null results, there are several indications that the findings from this are valid and meaningful. For example, we found that alcohol intake and GNF in HIV− participants appeared to have anti-aging gene expression signatures (i.e., increased mitochondrial function and decreased transcriptional activity), which is consistent with a growing body of literature establishing the healthful effects of moderate alcohol consumption (Quach et al. 2017; Reas et al. 2016). Additionally, the WGCNA results related to our other variables of interest as expected. The strong effects of HIV infection and viral load yielded clear correlations between HIV viral load and modules enriched for gene networks involved in immune response (e.g., MHC I antigen processing and presentation, TNF signaling, and interferon gamma signaling) and antiviral defense. Furthermore, HIV infection was associated with glycoprotein functioning and translation/transcription processes (e.g., SRP-dependent co-translational protein targeting to membrane, translation initiation, and viral transcription). Finally, the module preservation analysis showed that gene co-expression structure was preserved between our two samples, indicating that though the expression of individual genes is inconsistent, gene modules are reproducible.

It is worth noting that the non-significant trends between GNF and modules 6, 13, 22, and 28 broadly suggest a potential relationship with regulation of glucose metabolism and ubiquitin-proteasomal-based protein. It is unclear what relation this may have with previous studies of proteasomal regulation in brains of HIV+ cases with HIV-associated dementia (Nguyen et al. 2010), but our results suggest that upregulation of this process in monocytes is associated with better neurocognitive function. Additional biological functions associated with GNF that were implicated by the GO analysis, and that also have some support via previous studies, include activation of NFκβ-inducing kinase activity (Reddy et al. 2012), tumor necrosis factor-mediated signaling pathway (Reddy et al. 2012), and positive regulation of canonical Wnt signaling pathway and beta-catenin-TCF complex assembly (Al-Harthi 2012). However, while our findings may provide support for dysregulation of these processes in association with HAND, they strongly indicate that none are so crucial that they could serve as biomarkers, at least not based on transcript levels.

We acknowledge that there are limitations to this study beyond sample size and phenotype limitations. Here, we have focused specifically on monocytes given their previous implication in brain infiltration. However, it may be that gene expression levels in other blood cell types or tissues may be more predictive of HAND. Furthermore, additional steps could have been taken to ensure monocyte fraction purity (e.g., flow cytometry); that is, our monocyte samples may have also included other cells that obfuscated phenotype-related signals. We consider this unlikely, however, because samples from both time points were processed similarly.

In summary, the results from our study show that monocyte transcriptional profiles are not significantly predictive of future GNF or reliably associated with current GNF. While this may be due in part to an imperfect neurocognitive phenotype or underpowered sample, our results suggest that there are no strong relationships between gene expression in peripheral blood monocytes and GNF in HIV+ individuals.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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