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# Higher CSF Ferritin Heavy-Chain (Fth1) and Transferrin Predict Better Neurocognitive Performance in People with HIV

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## Abstract

HIV-associated neurocognitive disorder (HAND) remains prevalent despite antiretroviral therapy and involves white matter damage in the brain. Although iron is essential for myelination and myelin maintenance/repair, its role in HAND is largely unexplored. We tested the hypotheses that cerebrospinal fluid (CSF) heavy-chain ferritin (Fth1) and transferrin, proteins integral to iron delivery and myelination, are associated with neurocognitive performance in people with HIV (PWH). Fth1, transferrin, and the pro-inflammatory cytokines TNF-α and IL-6 were quantified in CSF at baseline (entry) in 403 PWH from a prospective observational study who underwent serial, comprehensive neurocognitive assessments. Associations of Fth1 and transferrin with Global Deficit Score (GDS)-defined neurocognitive performance at baseline and 30–42 months of follow-up were evaluated by multivariable regression. While not associated with neurocognitive performance at baseline, higher baseline CSF Fth1 predicted significantly better neurocognitive performance over 30 months in all PWH ($p<0.05$), in PWH aged <50 at 30, 36, and 42 months (all $p<0.05$), and in virally suppressed PWH at all three visit time-points (all $p<0.01$). Higher CSF transferrin was associated with superior neurocognitive performance at all visits, primarily in viremic individuals (all $p<0.05$). All associations persisted after adjustment for neuro-inflammation. In summary, higher CSF Fth1 is neuroprotective over prolonged follow-up in all and virally suppressed PWH, while higher CSF transferrin may be most neuroprotective during viremia. We speculate that higher CSF levels of these critical iron-delivery proteins support improved myelination and consequently, neurocognitive performance in PWH, providing a rationale for investigating their role in interventions to prevent and/or treat HAND.

**Keywords**

HIV; prospective study; neurocognitive performance; CSF; myelination; iron metabolism

## INTRODUCTION

HIV-associated neurocognitive disorder (HAND) remains extremely common, despite effective combination antiretroviral therapy (cART) [1]. HIV crosses the blood-brain barrier via infected monocytes/macrophages early in infection, leading to infection of glia within the brain. While neurons are uninfected, sustained glial activation and release of neurotoxic viral proteins promote damage to white matter (myelinated nerve fiber tracts) in the brain, as well as neuroinflammation, and synaptodendritic loss [1–3]. Risk factors for neurocognitive impairment (NCI) in people with HIV (PWH) include older age, a lower nadir $CD4^+$ T-lymphocyte count, detectable HIV RNA in plasma, substance abuse, anemia and other comorbidities [4]. Chronic neuroimmune activation due to HIV latency, which is not impacted by cART [1], and potential cumulative effects of antiretroviral drugs on mitochondrial function, protein processing, and organellar stress in the central nervous system, have also been implicated [5–9]. Increased risk of HAND associated with the Alzheimer's disease-related *APOE-ε4* allele has been observed mainly in older PWH and in individuals with milder forms of HAND on cART [1,10,11]. Despite intensive research, however, the key pathophysiologic mechanism(s) driving white matter damage and development of NCI, particularly when HIV replication is suppressed on cART, are not well understood.

Myelin synthesis and maintenance, as well as neuronal oxidative metabolism, require bioavailable iron, but reactive (non-protein-bound) forms of iron can also mediate oxidative injury. Iron homeostasis and compartmentalization are therefore critical for brain health [12–15]. Among brain cells, oligodendrocytes have the highest metabolic requirement for iron, which is needed both for normal differentiation of oligodendrocyte precursors and for support of the myelinating function of mature oligodendrocytes [14]. Dysregulated myelin production, maintenance, and repair are linked to cognitive as well as motor deficits via slowed transmission of neural action potentials [16], and in PWH, smaller white matter volumes on brain imaging have been associated with HAND/NCI [17,18]. While transferrin is the principal iron-delivery protein for most cells in the body, including differentiating oligodendrocytes and neurons, mature myelinating oligodendrocytes lack transferrin receptors entirely and cannot obtain iron from transferrin [16]. Recent studies have demonstrated that ferritin heavy-chain-1 (Fth1), a functionally distinct subunit and component of the much larger iron-carrier and storage protein ferritin, is the principal source of iron for mature oligodendrocytes. The routinely measured, standard form of ferritin is a large, globular protein comprising 24 heavy- and light-chain subunits in proportions that depend upon the tissue of origin, and circulating ferritin is made up almost entirely of the light-chain form [19,20]. Fth1 is capable of far more efficient iron delivery than transferrin and binds to the T-cell immunoglobulin and mucin domain (Tim)-1 receptor present on oligodendrocytes in humans, though it may also replace transferrin in binding (with less avidity than transferrin) to the transferrin receptor present on other brain cells

[21]. Fth1 has therefore been suggested to deliver iron to cells other than oligodendrocytes under conditions of extreme iron demand, such as rapid growth early in life, and brain development [22]. Inflammation also results in reduced circulating levels of transferrin, which is required for myelin biosynthesis and may stimulate oligodendrocyte maturation independent of its iron-binding/delivery properties [23–25].

HIV infection and some antiretroviral drugs dysregulate cellular iron homeostasis: release of the iron-regulatory hormone hepcidin due to inflammation leads to sequestration of iron within monocyte-macrophages and microglia, substantially reducing its bioavailability to other cells, while benefiting viral replication within monocyte-macrophages and glia [4,26]. Functional iron deficiency (reduced bioavailable iron) in the brain during HIV-mediated inflammation, with damage to and/or loss of myelinated nerve fibers and neurons, may therefore contribute to HIV-associated NCI [27,28]. Although we and others have previously quantified iron-binding proteins in cerebrospinal fluid (CSF) from PWH, the ability of specific iron-delivery proteins important for the synthesis and preservation of myelin to predict neurocognitive function over time in PWH has not been investigated in epidemiologic studies [12,29–32]. This study, in meticulously characterized participants from a large, prospective HIV cohort study, tested the hypotheses that higher levels of CSF iron-delivery proteins of established importance to oligodendrocytes and myelination (Fth1 and transferrin) are associated with better neurocognitive performance over time, independent of CSF biomarkers of inflammation and *APOE-ε4*, and that such effects differ by age, comorbidity, and/or viral suppression on cART. Our results indicate that both of these proteins exert significant, independent, neuroprotective effects in PWH under differing clinical scenarios (*e.g.,* viral suppression *versus* ongoing viremia).

## METHODS

### Study design and participants

The U.S. CNS HIV Anti-Retroviral Therapy Effects Research (CHARTER) Study is a prospective, multi-center observational study of neuro-HIV outcomes [33]. The CHARTER Study was approved by the Human Subjects Protection Committees of each participating institution, and all participants provided written informed consent. Participants were selected from the CHARTER/National NeuroAIDS Tissue Consortium (NNTC) biospecimen repository based on availability of longitudinal assessment data and stored CSF, collected by lumbar puncture, from the baseline (entry) visit. All CSF was collected within a 4–5-hour time window to avoid significant diurnal differences. CHARTER Study protocols adhere to the ethical principles set forth in the Helsinki Declaration.

### Assessment of HAND/NCI

CHARTER Study participants without severe comorbidity (such as a learning disability, epilepsy, or traumatic head injury with prolonged loss of consciousness) that could confound a diagnosis of HAND, as previously described [34], underwent comprehensive neurocognitive testing at baseline and follow-up visits every 6 months. The battery included 15 tests, which assess 7 neurocognitive ability domains, allowing for calculation of a Global Deficit Score (GDS). The GDS incorporates up-to-date norms for age and other

demographics, and corrects for learning/practice effects [34]. Neurocognitive outcomes evaluated in this study included the GDS as either a continuous or a dichotomous variable, using an established cut-off of GDS ≥0.50 to define NCI [35]. Frascati criteria for HAND were used to classify individuals as having *no impairment*, asymptomatic neurocognitive impairment (ANI), mild neurocognitive disorder (MND), or HIV-associated dementia (HAD) [35].

### Quantification of CSF iron-delivery proteins and inflammation

CSF Fth1 and transferrin levels were determined using commercially available, colorimetric enzyme-linked immuno-absorbent assay (ELISA) kits from Abnova (Human H-Ferritin ELISA kit, Taipei, Taiwan) and Abcam (Human Transferrin ELISA kit, ab108902, Cambridge, MA), respectively, according to the manufacturer's protocol [12]. For Fth1, 20 μl of standards, subject CSF samples (in duplicate), and controls were combined with 100 μl of Enzyme Conjugate Reagent. The solution was mixed for 30 seconds and incubated at room temperature for 45 min. The incubation mixture was removed and the plate was rinsed 5 times with distilled water. The TMB Reagent (100 μl) was added to each well, the solution mixed for 10 seconds and incubated at room temperature in the dark for 20 min. Stop Solution (100 μl) was added to each well, and the solution was mixed for 30 seconds. A series of standards provided by the vendor between 0 ng/mL and 150 ng/mL of Fth1 were used to generate a standard curve for this assay, which was linear in the range of values obtained in patient samples; the intra-assay coefficient of variation (CV) of the Fth1 assay (precision) was 4.27%, and the spike recovery (accuracy) was 98%. The CSF Fth1 concentration in the subject samples was determined by comparison of the optical density at 450 nm to the standard curve within 15 minutes. For transferrin, the intra-assay CV was 4.8% and spike recovery, 97%. CSF samples (in duplicate) were first diluted 1:1000 with mix diluent. Briefly, transferrin standard or diluted CSF sample (50 μl) was added to each well, and the solution was incubated for 2 hours. Following incubation, the plate was washed five times with 200 μL of 1X Wash Buffer. Following the wash step, 1X Biotinylated Transferrin Antibody (50 μl) was added to each well and the solution was incubated for 1 hour. The plate was washed again, as described above. The 1X Streptavidin-Peroxidase Conjugate (50 μl) was added to each well, and the solution was incubated for 30 minutes. The microplate was then washed again, as described above. The Chromogen substrate (50 μl) was added to each well, and the solution was incubated for 12 minutes. Stop solution (50 μl) was added to each well, and the absorbance was read immediately using a microplate reader at 450 nm. Similar to Fth1, transferrin concentration was determined by comparison to the standard curve, which was generated using vendor-supplied standards.

Concentrations of inflammatory cytokines and chemokines were measured in CSF by commercial, high-sensitivity multiplex assay [interleukin-6 (IL-6), tumor-necrosis-α (TNF-α)], according to the supplier's protocol (Luminex FlexMap 3D platform, Millipore, Billerica, MA). The stability and solubility of iron-carrier proteins in stored human biospecimens is well established [19,36–39]. Laboratory personnel who performed the assays were unaware of participant clinical outcomes.

## Statistical Methods

The GDS as a continuous variable was compared across baseline demographic and HIV disease factors using either analysis of variance (factors or variables with two or more categories), linear regression (normally distributed continuous variables), or the Wilcoxon Rank Sum test (non-normally distributed variables). Pearson's *r*-values were used to assess iron-delivery-protein correlations with biomarkers of inflammation. The multivariable analyses performed and participant subsets included in each analysis are summarized in Fig. 1.

Primary analyses were performed by repeated-measures analysis of variance (ANOVA) regression to evaluate the influence of CSF Fth1 and transferrin at baseline on differences in GDS-defined neurocognitive function over 30, 36, and 42 months of follow-up, normalized to baseline GDS values. In these longitudinal analyses, iron-related biomarkers (Fth1, transferrin, and iron) were categorized into tertiles to facilitate interpretation. The *p*-values at specific follow-up visits reflect differences in the GDS between the highest and lowest biomarker tertiles, summarized over all visits up to and including that visit; however, all three tertiles were included in regression models. Effect sizes for associations were estimated using the *eta*-squared ($\eta^2$) statistic, interpreted as the proportion of total variance in the dependent variable (GDS) that is accounted for by membership in a biomarker tertile.

All models were adjusted for comorbidity and plasma HIV RNA; analyses involving transferrin were also adjusted for zidovudine use, as use of this antiretroviral drug was previously associated with CSF transferrin [12]. Models additionally adjusted for *APOE-ε4* carrier status, and analyses stratified by age (<50 *vs.* ≥50 years), comorbidity severity (none/minimal *vs.* mild-to-moderate), or plasma HIV RNA (viral suppression (yes/no), <200 copies/mL *vs.* ≥200/mL) were also evaluated. This age cut-off was chosen due to low numbers of study participants 60 years of age and older (the age group in which *APOE-ε4* associations with NCI were previously reported) [10]; the higher (well-accepted) plasma HIV RNA cut-off was used in order to maximize power for detection of associations within strata. To avoid confounding by neuro-inflammation, which is associated with NCI and can affect the levels of iron-binding proteins, CSF TNF-α and/or IL-6 at baseline were included as covariate(s) in multivariable regression models; results including these biomarkers were essentially unchanged. (In some final models presented, these levels were therefore not included, in order to optimize power.) Due to previously identified relationships of CSF iron biomarkers with comorbidity, and the potential of residual confounding by inflammation or altered iron transport in the setting of clinically significant comorbidity, we also stratified Fth1 and transferrin analyses by this variable and evaluated multiplicative interactions between iron biomarkers and comorbidity, based on our prior findings [12]. Adjustment for self-reported race/ethnicity did not alter results, and the GDS incorporates corrections for demographic norms, so race/ethnicity was not included in final models. Neither current substance abuse nor a lifetime history of substance abuse was associated with the GDS at baseline, 30, 36, or 42 months for either biomarker, so these variables were not included in final models. Finally, since Mauchly's test of the sphericity assumption for repeated-measures ANOVA was statistically significant, ANOVA results are presented with

*p*-values incorporating the Huynh-Feldt *epsilon* correction [40]. The Huynh-Feldt correction minimizes the Type I error rate but is not overly stringent or biased for smaller samples.

Robustness of all observed associations was further evaluated by excluding iron-biomarker outlier values ≥2 standard deviations above or below the mean and performing a quartile rather than tertile analysis. Results of the tertile analysis are primarily reported, however, due to loss of 16% of study participants and hence, significant loss of power, in analyses by quartile. To evaluate potential selection bias, HIV disease characteristics and CSF iron-biomarker levels at baseline were also compared between study participants who had follow-up at 30–42 months and individuals who did not have longitudinal follow-up at ≥30 months. Results of these analyses are presented in Supplemental Material.

Statistical significance for all analyses was set at a two-tailed $p$-value<0.05. Statistical analyses were conducted using STATA Statistical Software, Release 13 (College Station, Texas: StataCorp LP).

## RESULTS

### Study Participants

Of 403 evaluated PWH, 120 (29.8%) had Global Deficit Score (GDS)-defined NCI at baseline, while 283 were not impaired. Among PWH who were longitudinally followed, older age (≥50 years), non-African ancestry, and longer durations of HIV infection were associated with higher (worse) GDS values (all $p$-values<0.05). Characteristics of these CHARTER study participants at baseline are shown in Table 1.

Participants with minimal or no comorbidity had significantly lower (better) GDS values (median 0.21 *vs.* 0.42, $p$<0.001). In the entire sample, 294 (72.9%) were on cART at baseline, and 184 (62.5%) of these individuals were virally suppressed. Of 398 genotyped participants, just under one-third were *APOE-ε4* risk allele carriers, but *APOE* genotype was not associated with the GDS at baseline.

### CSF Fth1, transferrin, and biomarkers of inflammation at baseline

Among all 403 study participants, median CSF Fth1 was 2.7 ng/mL (IQR: 1.4–4.2), and median CSF transferrin was 17.2 μg/mL (IQR: 10.3–27.6). Levels of Fth1 and transferrin did not differ significantly by comorbidity severity, cART use, or *APOE-ε4* genotype, and their correlations with contemporaneously measured CSF TNF-α and IL-6 at baseline and with plasma hemoglobin were weak (all $r$-values <0.20). Levels of both IL-6 and TNF-α were significantly higher in individuals with detectable plasma virus (ongoing viremia) than in virally suppressed individuals, using either an HIV RNA cut-off of <200 copies/mL, or a more stringent cut-off of <50 copies/mL to define viral suppression (all $p$-values <0.01).

Cellular iron transport and metabolism are influenced by inflammation via the hepcidin pathway, and some iron-binding proteins are positive or negative acute-phase reactants (levels of which increase or decrease during inflammation, respectively) [41,4]. Since higher CSF inflammation is also linked to worse neurocognitive performance [2,29], we measured levels of the pro-inflammatory cytokines TNF-α and IL-6, in CSF at baseline to facilitate

adjustment for inflammation in analyses of the iron-delivery proteins. Median TNF-α and IL-6 in CSF from study participants were 0.44 pg/mL (IQR: 0.33–0.63) and 3.4 pg/mL (IQR: 2.5–4.7), respectively.

## Multivariable-adjusted associations of CSF iron-delivery proteins with GDS differences over time

Neurocognitive function in PWH is a highly complex and often fluctuating phenotype, and comparison of each individual to his/her baseline level of performance is optimal for detection of biomarker associations. Stable changes in neurocognitive performance, particularly those involving altered myelination, may require extended periods of time to manifest. We therefore identified a subset 157 CHARTER Study participants for these analyses with the highest proportion of comprehensive neurocognitive assessments at later visit time-points. All 157 PWH were fully assessed at 30 months, 131 of the same individuals were assessed at 36 months, and 110 were assessed at 42 months. Changes in the GDS over 30–42 months of follow-up among PWH in the highest and lowest tertiles of CSF Fth1 at baseline are shown in Table 2 and plotted in Fig. 2, to better show the direction of effects. As shown in Table 2a and Fig. 2a, CSF Fth1 tertile had a statistically significant impact on the GDS in all longitudinally followed study participants: levels in the highest tertile (≥3.55 ng/mL: median 5.4, IQR 4.0–7.1) were associated with lower (better) GDS values over 30 and 42 months of follow-up than levels in the lowest tertile (≤2.15 ng/mL: median 1.0, IQR 0.5–1.5; *p*-values 0.037 at 30 months and 0.043 at 42 months for Fth1; Huynh-Feldt-corrected *p*-values 0.049 and 0.084, respectively). The Fth1 association was even stronger and/or easier to discern among individuals with minimal or no comorbidity, in whom CSF Fth1 in the highest tertile was associated with significantly better neurocognitive performance (lower GDS values) over 42 months, compared to participants in the lowest tertile (Fig. 2b; Huynh-Feldt-corrected *p*-values 0.024, 0.032 and 0.020 at 30, 36 and 42 months, respectively). CSF Fth1 levels were not significantly associated in PWH who had significant comorbidity, but a multiplicative interaction effect with comorbidity was not statistically significant. However, among virally suppressed study participants (plasma HIV RNA <200 copies/mL), CSF Fth1 levels were associated with highly significant differences in neurocognitive performance (Table 2b and Fig 2c). Virally suppressed PWH in the highest tertile had significantly better GDS values than individuals in the lowest tertile over 30, 36, and 42 months of follow-up (uncorrected and Huynh-Feldt-corrected *p*-values <0.01 at all three time-points). (Supplemental Fig. S1 and S2, for GDS-defined neurocognitive performance, plotted over time in all three tertiles of CSF Fth1.) Associations of CSF Fth1 with GDS differences over time remained statistically significant in PWH who had viremia ($p<0.05$ at 36 and 42 months; Supplemental Table S1).

CSF transferrin at baseline also had a significant impact on neurocognitive performance in PWH over 30–42 months of follow-up, as presented in Table 3; results are also plotted in Fig. 3 to show directionality. Higher transferrin levels [tertile 3 (≥24.0 μg/mL: median 33.1, IQR 26.9–39.9) *vs.* tertile 1 (≤12.5 μg/mL: median 6.7, IQR 3.0–9.5)] were associated with better neurocognitive function among all study participants over 30, 36, and 42 months (uncorrected and corrected *p*-values all <0.01; Table 3a and Fig. 3). A statistically significant, multiplicative transferrin-by-comorbidity interaction was also

observed at all visit time-points (all *p*-values ≤0.05). (See Supplemental Fig. S1, for neurocognitive performance plotted over time in all three tertiles of CSF transferrin.) Higher CSF transferrin was most significantly associated with better GDS-defined neurocognitive performance in PWH who had detectable HIV RNA in plasma (≥200 copies/mL), as shown in Table 3b (all uncorrected and corrected *p*-values <0.001 for transferrin over 30, 36, and 42 months). Transferrin associations with neurocognitive performance in individuals with mild-to-moderate (*i.e.,* clinically significant) comorbidity were very similar to those in viremic PWH, and highly significant transferrin-by-comorbidity interaction effects were also observed at all three time-points in viremic individuals (all *p*-values for multiplicative interaction terms <0.001 at all visit time-points). Interestingly, CSF transferrin was not significantly associated with GDS-defined neurocognitive differences over time in the virally suppressed subset (Supplemental Fig. S3).

Due to possible influences of substance abuse on both neurocognitive performance and iron biomarkers in PWH, we also determined that including substance use disorder (either current or active) as a covariate in longitudinal analyses of CSF Fth1 and transferrin did not alter the observed associations.

We repeated longitudinal analyses at 42 months in all and younger study participants, after excluding biomarker outlier values (Supplemental Tables S2a-c). The observed associations were robust, as results of all of these analyses were essentially unchanged. Finally, demographic and HIV disease characteristics were compared between all CHARTER Study participants who were and were not followed longitudinally after the baseline assessment, as shown in Supplemental Table S3. Losses to follow-up included significantly more aviremic PWH ($p$=0.039). Finally, results of quartile analyses, which compared differences in normalized GDS values over time between participants in quartile 4 to GDS values of participants in quartile 1 of each CSF biomarker, were similar to the results of tertile-based analyses for Fth1 and transferrin, despite significant loss of statistical power. For example, higher CSF Fth1 at baseline remained associated with GDS-defined neurocognitive performance at 30 months ($p$=0.004), 36 months ($p$=0.053) and 42 months ($p$=0.025) in the entire sample and in younger PWH (aged <50; *p*-values 0.011, 0.016, and 0.025 at 30, 36, and 42 months, respectively). Higher CSF Fth1 levels also remained associated with better neurocognitive function at 30 months in virally suppressed PWH ($p$=0.037).

### Age-related CSF Fth1 and transferrin associations with neurocognitive performance

Since body iron stores and inflammation generally increase with age, and blood-brain-barrier integrity is believed to decline with age [12], we determined whether the impact of CSF iron-delivery proteins on neurocognitive performance in this study population differed between PWH aged 50 and over *vs.* PWH aged <50 years. The impact of CSF Fth1 at baseline on GDS differences over time was pronounced in PWH under 50 years of age, adjusting for comorbidity, a Fth1*comorbidity interaction term, and plasma HIV RNA (Huynh-Feldt-corrected *p*-values 0.034, 0.013, 0.006 at 30, 36 and 42 months, respectively; Supplemental Table S4a). A borderline statistical significance was observed for Fth1 interaction with comorbidity at 42 months. By contrast, CSF transferrin at baseline predicted differences in the GDS over time mainly in individuals aged 50 and older (Huynh-

Feldt-corrected *p*-values 0.038, 0.087, 0.078 at 30, 36, and 42 months, respectively); see Supplemental Table S4b). Corrected *p*-values for a multiplicative transferrin-by-comorbidity interaction term remained statistically significant at 30 and 36 months. Associations of CSF Fth1 with neurocognitive performance over time in PWH aged 50 or over, and of transferrin in PWH aged <50 years, were not statistically significant. Results of these analyses were unchanged by additional adjustment of analyses for either TNF-α or IL-6.

## DISCUSSION

People with HIV remain at high risk of neurocognitive decline, despite cART, and the prevalence of HAND has increased [33,42,43]. White matter alterations, which figure prominently in HAND regardless of the age at HIV infection, are characterized by thinning of the corpus callosum, white matter volume loss, myelin pallor, and reduced structural integrity of myelin. These changes develop and often progress, despite viral suppression on cART [44,45]. It has been debated whether these changes, which are strongly linked to NCI, are the legacy of primary, acute (untreated) HIV infection, or result from persistent immune activation in the setting of latent HIV reservoirs in the brain, antiretroviral drug toxicity, or vascular and metabolic comorbidities [2,46,47,18]. Their timing, however, suggests that direct effects on oligodendrocytes and myelin are likely to be responsible [48]. Iron metabolism is intimately linked to myelin synthesis/repair, glial function, and inflammatory responses and is dysregulated by HIV [49–51,4]. A role for iron dysregulation in HAND/NCI, which might be addressed by interventions that modulate iron transport, has not been sufficiently explored. Due to the challenges of extrapolating from animal models of neuro-HIV to humans, prospective epidemiologic studies can be highly complementary in addressing this important question. This study in a comprehensively characterized HIV cohort, predominantly on cART, showed that higher CSF levels of two proteins critical for iron delivery and myelin homeostasis in the brain – Fth1 and transferrin – predict better neurocognitive outcomes over time in virally suppressed and/or unsuppressed individuals. CSF Fth1 was particularly protective in virally suppressed PWH, individuals under the age of 50 or who had minimal comorbidity, whereas CSF transferrin was most protective in individuals with detectable plasma virus, in whom levels of inflammation were also higher. Importantly, none of these associations was diminished by adjustment of multivariable models for viral load and neuro-inflammation (CSF TNF-α and/or IL-6), measured at the same baseline visit. The fact that Fth1 and transferrin effects on neurocognitive performance were both in the same (neuroprotective) direction, despite the known opposite effect of inflammation on these proteins, is further evidence that their levels in the CNS are not merely reflecting inflammation. Rather, our observations are consistent with trophic roles for Fth1 and transferrin in the CNS, favoring the concept that these proteins are performing important iron-delivery and myelin-maintenance functions, which in turn can influence the trajectory of neurocognitive performance in PWH over time. This potential mechanism for our findings is speculative, however, and requires confirmation in larger epidemiologic studies and in experimental models.

To our knowledge, this represents the first prospective study to evaluate the role of iron-delivery proteins in NCI among PWH, and it is also the first study of CSF Fth1 levels in relation to longitudinal neurocognitive performance in humans. The identification of

CSF biomarkers of neurocognitive *improvement* in PWH was previously highlighted as a research priority in the neuro-HIV field, since milder forms of HAND are often non-progressive [1,52,53]. Most prior CSF iron-related studies in PWH have been anecdotal, cross-sectional reports in very small numbers of PWH in the pre-cART era who had advanced HIV disease, and none evaluated the risk of NCI or neurocognitive decline in relation to iron-delivery protein levels [31,32]. Only serum or CSF *total* ferritin, primarily made up of light-chain (L) ferritin subunits, was measured in these studies. Deisenhammer *et al* reported elevated CSF *total* ferritin in PWH who had diverse neurological conditions, including opportunistic infections and HAD; no associations of *total* CSF ferritin levels with severity of NCI or abnormalities on brain imaging were observed [31]. In a previous descriptive analysis, we reported the distributions of CSF iron, Fth1, and transferrin in PWH and determined their cross-sectional relationships to demographic and HIV disease factors [12]. By contrast, the present study specifically assessed independent associations of these iron-delivery proteins with cross-sectional and longitudinal neurocognitive performance in PWH who underwent detailed medical, neurocognitive, and neuropsychiatric follow-up for up to 42 months.

Oligodendrocytes, which are the most iron-laden cells within the CNS, have a particular need for iron, due to the essential role of this micronutrient in cholesterol synthesis (a key component of myelin) and as a cofactor for numerous enzymes involved in myelination and re-myelination [54,55]. Immature oligodendrocytes, neurons, and astrocytes, like the vast majority of cells in the body, express transferrin receptors and derive iron mainly via receptor-mediated endocytosis of transferrin-bound iron [24,56]. Even in the absence of bound iron, (apo)transferrin is important for the viability, differentiation, and proliferation of oligodendrocyte precursors [21,24]. On the other hand, transferrin receptors are absent in mature myelinating oligodendrocytes (and adult human brain white matter); these cells must obtain iron from another source, which was recently discovered to be Fth1, via its binding to the Tim-1 receptor in humans (Tim-2 in mice) [54,57,55]. Iron-replete microglia in the brain are believed to be the primary source of this trophic Fth1, and activated microglia, which play a central role in the pathogenesis of HAND, secrete significantly less Fth1, potentially interrupting the supply of iron to oligodendrocytes [1,58]. Even immature, differentiating oligodendrocytes may utilize Fth1 as an iron source in times of extreme iron demand, or as their expression of transferrin receptors wanes. Unlike the L-subunit of ferritin, Fth1 is also able to bind the transferrin receptor, and each Fth1 molecule can deliver up to 4500 iron atoms, compared to only two iron atoms per molecule of transferrin [59,57,60,55]. Deletion of the *FTH1* gene specific to cells of the oligodendrocyte lineage in mice has been reported to result in significantly impaired remyelination in the adult mouse brain [61].

Despite their iron avidity, the limited antioxidant defenses of oligodendrocytes also make them highly vulnerable to iron-mediated oxidative stress and direct injury due to inflammation [55]. High basal expression of cellular iron-efflux proteins such as ferroportin, and internalization and/or expression of Fth1, may enable these cells to balance their need for iron with the risk of iron-mediated cytotoxicity, since Fth1 acts as a highly effective antioxidant *in vivo.* Fth1 contains the entire ferroxidase activity of ferritin, which is essential to its antioxidant properties [22,19,62]. In addition, oligodendrocytes provide substantial metabolic and structural support to neurons and have been reported to prevent axonal

damage in *Drosophila* and in mice via the release of Fth1 [63]. Fth1 may also prevent toxicity to oligodendrocytes by other mechanisms. We previously demonstrated that the levels of semaphorin 4A, a direct oligodendrocyte toxin which causes demyelination and is produced by activated T-cells, are significantly increased in both serum and CSF in PWH, compared to HIV-seronegative individuals without known demyelinating disease [64,65]. Recent studies by Chiou *et al* demonstrated that semaphorin 4A also utilizes the Tim-1 receptor on human oligodendrocytes, and furthermore, that Fth1 can block semaphorin 4A-mediated cytotoxicity [16]. Higher CSF Fth1 may therefore promote oligodendrocyte survival in PWH by competing with semaphorin 4A for Tim-1 binding.

Taken together, these lines of evidence suggest that HIV-mediated inflammation may induce a *functional iron deficiency* state in oligodendrocytes via several potential mechanisms: 1) disruption of the transferrin-mediated route of iron-delivery to differentiating oligodendrocyte precursors, since transferrin levels decline during inflammation; 2) reduction in release of trophic Fth1 by activated microglia, leading to loss of mature oligodendrocytes and impaired remyelination; 3) reduced Fth1-mediated antioxidant defense for both oligodendrocytes and the neurons they support; and 4) increased binding of semaphorin 4A to Tim-1, reducing iron delivery to oligodendrocytes via Fth1 and culminating in oligodendrocyte loss and white matter damage [16,22,64,66,67,27]. Transferrin also confers neuroprotection by supporting differentiation and lineage commitment of oligodendrocytes, reducing mitochondria-mediated cell death and upregulating cell-survival pathways [68,23]. We therefore speculate that Fth1 and transferrin act to preserve iron bioavailability to and survival of oligodendrocytes and the neurons they support, resulting in improved neurocognitive performance. Since the effect of transferrin was most apparent in viremic PWH, it is possible that this protein's non-iron-dependent trophic effects on oligodendrocytes are compromised in these individuals due to inflammation-depressed transferrin levels [67]. These concepts require significantly further study, however, to clearly determine the biological mechanisms underlying the novel associations we observed between neurocognitive performance in PWH and the levels of these iron-delivery proteins in CSF.

We acknowledge some limitations of this study, as well as the need for further confirmatory studies in this area. First, although this is the largest study to date involving measurement of CSF iron-delivery proteins in PWH, our study sample was still limited by its modest size and relatively small number of older participants, preventing investigation of age-related interactions with iron-delivery protein levels on neurocognitive performance. Small numbers of women in the sample also precluded assessment of differences in the relative importance of Fth1 and transferrin to neurocognitive performance over time by sex; this question begs further investigation, as it has recently been suggested that postnatal brain acquisition of iron may differ between men and women, with men relying more on Fth1 and women more on transferrin, for brain iron uptake [13]. Secondly, the source of Fth1 and transferrin in CSF cannot be determined with certainty in a study of this type. However, published studies have consistently indicated that these proteins are generated largely within the CNS, since CSF and serum levels correlate poorly or not at all [69,12]. Although our analyses relied on a single measurement at baseline rather than serial CSF measurements, normalization of GDS values at follow-up to baseline values within individuals, and the lengthy follow-up

duration, mitigate concerns about sampling error or misclassification of NCI. The consistent direction of associations of CSF Fth1 and transferrin with GDS-defined neurocognitive performance over 30, 36 and 42 months of follow-up, despite declining sample sizes at later visits, are all-the-more compelling given the single baseline measurement. Furthermore, associations were unchanged following a sensitivity analysis that included a re-analysis of the longitudinal data after removing outlier values of CSF Fth1 and transferrin, and by performing quartile- rather than tertile-based, biomarker analyses, suggesting robust effects. Finally, while inclusion of serum biomarker or CSF:serum albumin ratio data as covariates in our analyses would have been ideal, these data were not available in most CHARTER Study participants, and adjustment of models for age and plasma HIV RNA – two major factors that influence blood-brain-barrier integrity – did not alter the observed associations [12]. Overall, these findings support the validity of the neuroprotective effects we observed with higher CSF Fth1 and transferrin levels in PWH.

In conclusion, results from this prospective study suggest that higher CSF Fth1 and transferrin predict significantly better neurocognitive performance over time in PWH, possibly due to trophic and other salutary effects on oligodendrocytes. Importantly, the benefits of higher Fth1 extend to virally suppressed PWH on cART, while higher transferrin levels are primarily protective in older PWH and in viremic individuals, who have increased levels of inflammation. Future large-scale studies that include greater numbers of women and older PWH, as well as neuroimaging modalities which can assess structural integrity of myelin, will clarify the relative contributions of HIV, sex, and age to iron dysregulation in the brain and neurocognitive function over time. Further studies in PWH are also needed to test the hypotheses generated by this study regarding the impact of HIV-mediated changes in iron transport and CSF iron-transport protein levels on myelination. Novel findings from this study, however, suggest that iron-modulating interventions such as transferrin (or apotransferrin), and Fth1, might have a role to play in preventing NCI, or halting its progression, in PWH.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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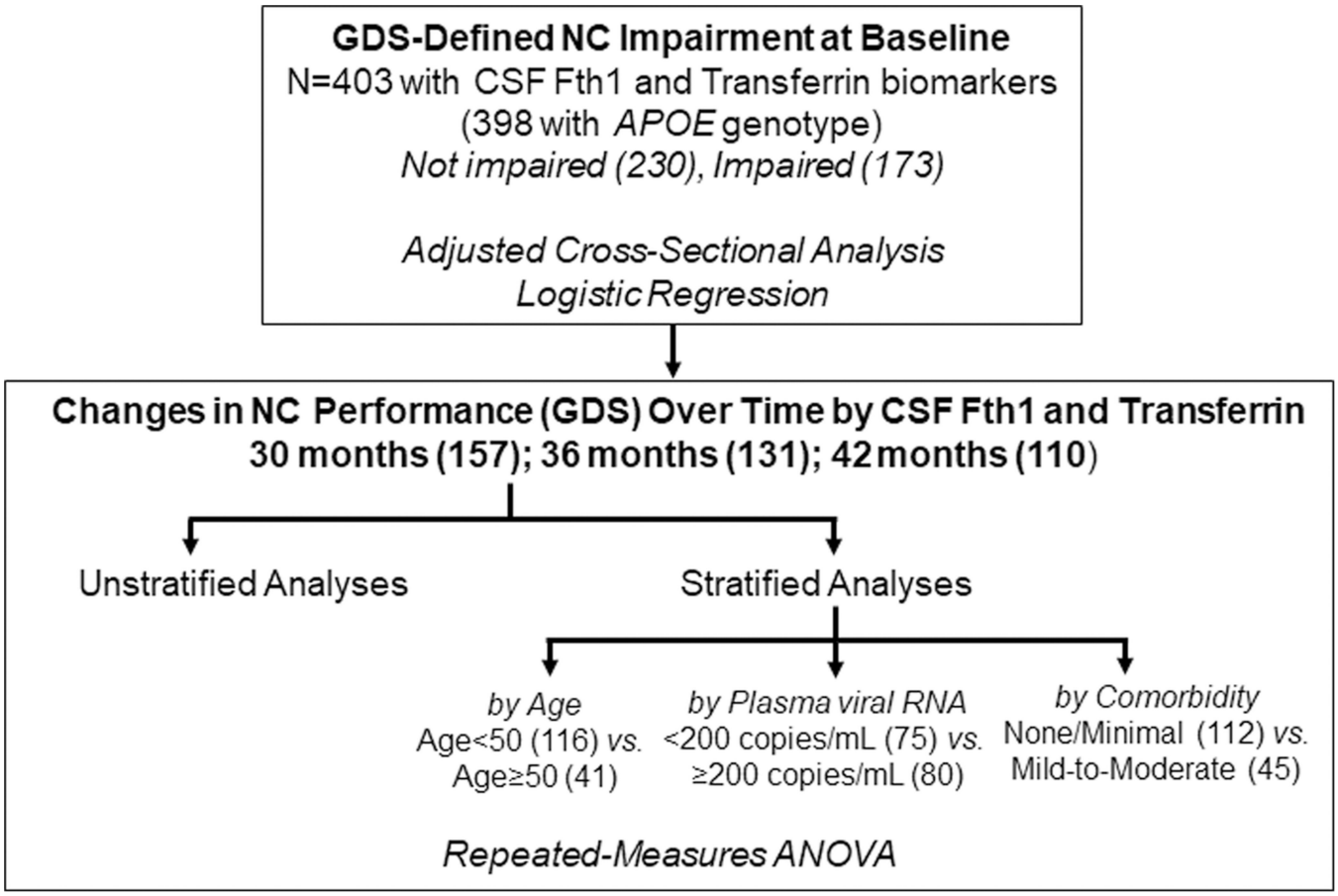


**Fig 1:**

Study schema showing numbers of participants (in parentheses) who were included in individual analyses.

*Abbreviations: CSF*, Cerebrospinal fluid; *GDS,* Global Deficit Score; *NC*, Neurocognitive; *ANI*, Asymptomatic Neucocognitive Impairment; *MND*, Mild Neurocognitive Disorder; *HAD*, HIV-Associated Dementia; *ANOVA*, Analysis of variance.

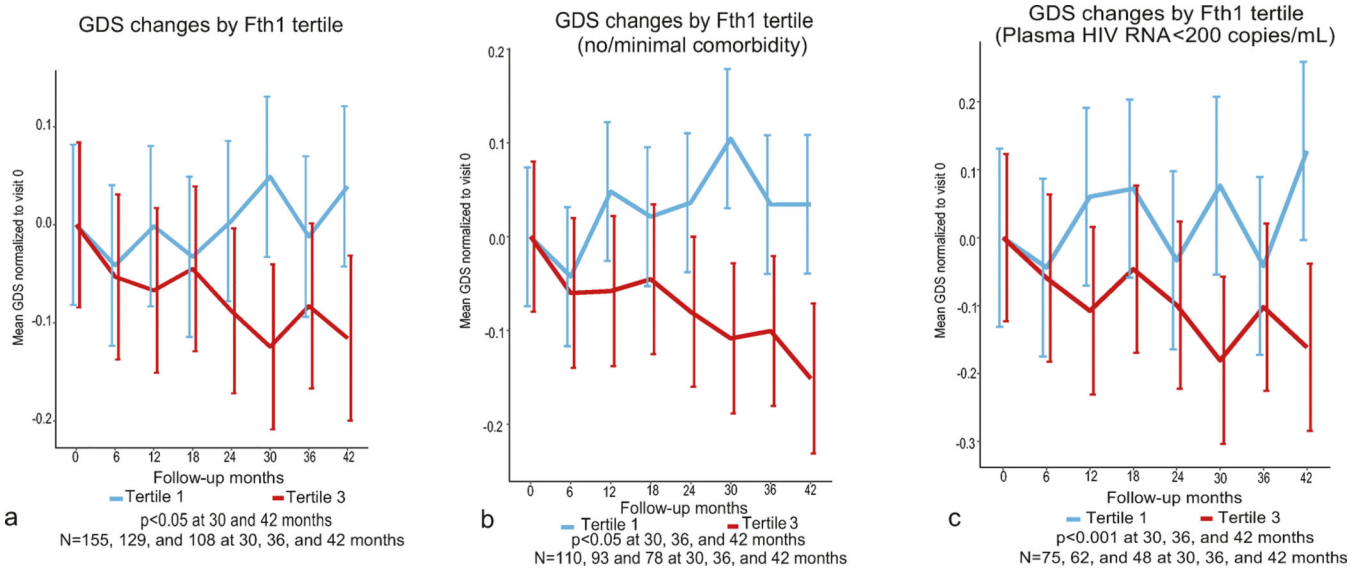


**Fig 2:**

Changes in the Global Deficit Score (GDS) over time in tertiles 3 *vs.* 1 of CSF Fth1, in all study participants (**panel a**, $p<0.05$ for Fth1 at 30 and 42 months, adjusting for comorbidity and plasma HIV RNA). **Panel b** shows changes in the GDS in participants with no comorbidity or minimal comorbidity ($p<0.05$ for Fth1 at 30, 36 and 42 months), adjusting for plasma HIV RNA. **Panel c** shows changes in the GDS by Fth1 tertile among virologically suppressed participants ($p<0.01$ at 30, 36 and 42 months adjusting for comorbidity).Tertile 2 is omitted for clarity from these plots but all tertiles were included in ANOVA analyses. The mean GDS at each visit was normalized to the baseline GDS for individuals in each tertile. (Results were unchanged even after repeating the analysis, excluding tertile 2, shown in Supplemental, Fig. S1.)

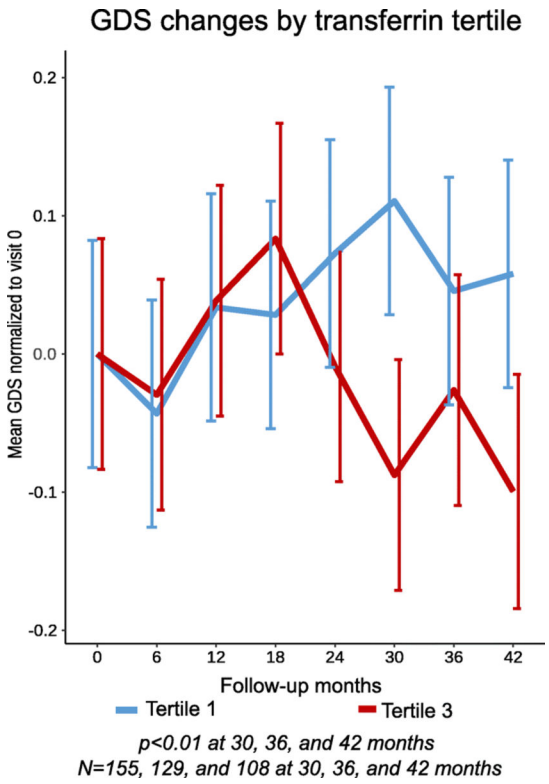


**Fig 3:**

Changes in the Global Deficit Score (GDS) over time in study participants with CSF transferrin in tertiles 3 *vs.* 1 ($p$<0.01 for transferrin at 30, 36 and 42 months, adjusting for comorbidity, plasma HIV RNA, and zidovudine use). (Associations remained unchanged even after repeating the analysis, excluding tertile 2, shown in Supplemental, Fig S1.)

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**Table 1:**

Baseline characteristics of all and longitudinally followed CHARTER Study participants.

| All Participant Characteristics at Baseline | | Subset with Longitudinal Follow-up | | |
|---|---|---|---|---|
| | | N | GDS at Baseline Median (IQR) | *p*-value[a] |
| Age (years) | <50 | 116 | 0.26 (0.11, 0.58) | 0.51 |
| | ≥50 | 41 | 0.32 (0.16, 0.47) | |
| Race/Ethnicity (self-report) | African-American | 69 | 0.21 (0.11, 0.42) | 0.15 |
| | Hispanic | 13 | 0.47 (0.05, 0.63) | |
| | White | 73 | 0.32 (0.16, 0.68) | |
| | Other | 2 | 0.29 (0.00. 0.58) | |
| Sex | Men | 138 | 0.26 (0.11, 0.58) | 0.70 |
| | Women | 19 | 0.32 (0.05, 0.42) | |
| Comorbidity[d] | None/Minimal | 112 | 0.21 (0.05, 0.42) | **<0.01** |
| | Mild/Moderate | 45 | 0.47 (0.26, 1.00) | |
| HIV RNA in plasma[1] | ≤50 copies/mL | 94 | 0.26 (0.11, 0.52) | 0.87 |
| | >50 copies/mL | 61 | 0.32 (0.11, 0.58) | |
| cART status | Off | 41 | 0.21 (0.05, 0.42) | 0.79 |
| | On | 116 | 0.29 (0.11, 0.58) | |
| Zidovudine use | Off | 127 | 0.26 (0.11, 0.58) | 0.28 |
| | On | 30 | 0.26 (0.05, 0.47) | |
| *APOE-ε4* allele status[1] | *ε*4 Non-carrier | 106 | 0.26 (0.11, 0.53) | 0.57 |
| | *ε*4 Carrier | 47 | 0.26 (0.11, 0.68) | |
| Estimated duration of HIV infection (months)[1c] | < 118 | 78 | 0.26 (0.06, 0.52) | 0.17[b] |
| | ≥ 118 | 77 | 0.32 (0.16, 0.58) | |
| Current substance abuse | No | 152 | 0.27 (0.07, 0.53) | 0.34 |
| | Yes | 5 | 0.07 (0.00, 0.33) | |
| Lifetime history of substance abuse | No | 53 | 0.36 (0.07,0.53) | 0.83 |
| | Yes | 104 | 0.20 (0.07, 0.53) | |

*Abbreviations: GDS*, the Global Deficit Score as a continuous variable; *IQR*, interquartile range; *CSF*, cerebrospinal fluid; *cART*, combination antiretroviral therapy (at the time of the baseline neurocognitive assessment); *APOE-ε4*, high-risk variant of the apolipoprotein E gene; *mL*, milliliter.

[a] *p*-value calculated by analysis of variance, unless otherwise noted.

[b] *p*-value calculated by linear regression

[c] GDS values for groups above or below the median are presented for consistency across variables.

[d] Comorbidity relevant to neurocognitive performance was ascertained, as previously described [Heaton 2010]

[1] *Individuals with unavailable data:* 2 (plasma HIV RNA); 4 (*APOE-ε4*-carrier status); 2 (estimated duration of HIV infection)

**Table 2:**

Associations of CSF Fth1 levels with GDS differences in all study participants who had comprehensive follow-up for at least 30 months (**panel a**). Additional adjustment for *APOE-ε4* carrier status at 30, 36, and 42 months, respectively, did not alter results (*p*-values for Fth1 = 0.02, 0.04, 0.03), TNF-α (*p*-values for Fth1 = 0.04, 0.12, 0.04) or IL-6 levels (*p*-values for Fth1 = 0.04, 0.12, 0.04). Shown in **panel b** are associations confined to virally suppressed study participants (plasma HIV RNA <200 copies/mL). Again, additional adjustment for either baseline CSF TNF-α did not significantly alter results (*p*-values for Fth1 all <0.01 at 30, 36, and 42 months) or IL-6 (*p*-values for Fth1 <0.05 at all visit time-points).

**Panel a**. All Study Participants

| Variable | 30 months (N=155) | | 36 months (N=129) | | 42 months (N=108) | |
|---|---|---|---|---|---|---|
| | *#1p* | *$1p* | *#1p* | *$1p* | *#1p* | *$1p* |
| CSF Fth1 | **0.04** | **0.05** | 0.11 | 0.16 | **0.04** | 0.08 |
| Comorbidity | 0.43 | 0.42 | 0.56 | 0.51 | 0.49 | 0.45 |
| Fth1*Comorbidity Interaction | 0.26 | 0.28 | 0.29 | 0.31 | 0.13 | 0.17 |
| Plasma HIV RNA | 0.94 | --- | 0.91 | --- | 0.84 | --- |

**Panel b**. Virally Suppressed Participants

| Variable | 30 months (N=75) | | 36 months (N=62) | | 42 months (N=48) | |
|---|---|---|---|---|---|---|
| | *#2p* | *$2p* | *#2p* | *$2p* | *#2p* | *$2p* |
| CSF Fth1 | **<0.001** | **<0.01** | **<0.001** | **<0.001** | **<0.001** | **<0.01** |
| Comorbidity | 0.37 | 0.36 | 0.36 | 0.35 | 0.16 | 0.20 |
| Fth1*Comorbidity Interaction | 0.33 | 0.33 | 0.40 | 0.39 | 0.44 | 0.42 |

*p*-values <0.05 (**bolded**) are statistically significant.

*#1p*-values were adjusted for comorbidity severity, plasma HIV RNA, and a Fth1*comorbidity interaction term.

*#2p*-values were adjusted for comorbidity and an Fth1*comorbidity interaction term.

*$p*-values incorporate the Huynh-Feldt correction.

**Table 3:**

Associations of CSF transferrin with GDS differences in all study participants (**panel a**) and in the subset with ongoing viremia (plasma HIV RNA ≥200 copies/mL, **panel b**) across all three evaluated visits (up to 30, 36, or 42 months).

**Panel a**. All Study Participants

| Variable | 30 months (N=155) | | 36 months (N=129) | | 42 months (N=108) | |
|---|---|---|---|---|---|---|
| | *#1p* | *$1p* | *#1p* | *$1p* | *#1p* | *$1p* |
| CSF Transferrin | **<0.01** | **<0.01** | **<0.01** | **<0.01** | **<0.01** | **<0.01** |
| Comorbidity | 0.13 | 0.16 | 0.21 | 0.23 | 0.26 | 0.28 |
| TF*Comorbidity Interaction | **0.03** | 0.05 | **<0.01** | **0.02** | **<0.01** | **0.02** |
| Plasma HIV RNA | 0.98 | --- | 0.34 | --- | 0.87 | --- |
| Zidovudine use | 0.26 | | **0.03** | | 0.71 | --- |

**Panel b**. Viremic Individuals

| Variable | 30 months (N=80) | | 36 months (N=67) | | 42 months (N=60) | |
|---|---|---|---|---|---|---|
| | *#2p* | *$2p* | *#2p* | *$2p* | *#2p* | *$2p* |
| CSF Transferrin | **<0.0001** | **<0.001** | **<0.0001** | **<0.001** | **<0.0001** | **<0.001** |
| Comorbidity | **<0.001** | **<0.001** | **<0.0001** | **<0.0001** | **<0.001** | **<0.0001** |
| TF*Comorbidity Interaction | **<0.001** | **<0.001** | **<0.0001** | **<0.001** | **<0.001** | **<0.001** |
| Zidovudine use | 0.94 | --- | 0.95 | --- | 0.97 | --- |

*p*-values <0.05 (**bolded**) are statistically significant.

*#1p*-values were adjusted for comorbidity severity, plasma HIV RNA, zidovudine use, and a transferrin (TF)*comorbidity interaction term. Additional adjustment for baseline CSF TNF-α (all *p*-values for transferrin <0.01) and/or IL-6 levels (all *p*-values for transferrin <0.01) did not alter observed associations at 30, 36, and 42 months.

*#2p*-values were adjusted for comorbidity severity, zidovudine use, and a transferrin (TF)*comorbidity interaction term. Additional adjustment for baseline CSF TNF-α did not alter transferrin associations (*p*-values for transferrin <0.01 at at 30, 36, and 42 months, respectively) or IL-6 levels (*p*-values for transferrin <0.01 at all time-points).