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A CONCISE PANEL OF BIOMARKERS IDENTIFIES NEUROCOGNITIVE FUNCTIONING CHANGES IN HIV-INFECTED INDIVIDUALS

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Abstract

Background—Neurocognitive (NC) impairment (NCI) occurs commonly in people living with HIV. Despite substantial effort, no biomarkers have been sufficiently validated for diagnosis and prognosis of NCI in the clinic. The goal of this project was to identify diagnostic or prognostic biomarkers for NCI in a comprehensively characterized HIV cohort.

Methods—Multidisciplinary case review selected 98 HIV-infected individuals and categorized them into four NC groups using normative data: stably normal (SN), stably impaired (SI), worsening (Wo), or improving (Im). All subjects underwent comprehensive NC testing, phlebotomy, and lumbar puncture at two timepoints separated by a median of 6.2 months. Eight biomarkers were measured in CSF and blood by immunoassay. Results were analyzed using mixed model linear regression and staged recursive partitioning.

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An earlier version of the analyses was presented at the 19th Conference on Retroviruses and Opportunistic Infections, March 2012, Seattle, WA.

Results—At the first visit, subjects were mostly middle-aged (median 45) white (58%) men (84%) who had AIDS (70%). Of the 73% who took antiretroviral therapy (ART), 54% had HIV RNA levels below 50 c/mL in plasma. Mixed model linear regression identified that only MCP-1 in CSF was associated with neurocognitive change group. Recursive partitioning models aimed at diagnosis (i.e., correctly classifying neurocognitive status at the first visit) were complex and required most biomarkers to achieve misclassification limits. In contrast, prognostic models were more efficient. A combination of three biomarkers (sCD14, MCP-1, SDF-1 α) correctly classified 82% of Wo and SN subjects, including 88% of SN subjects. A combination of two biomarkers (MCP-1, TNF- α) correctly classified 81% of Im and SI subjects, including 100% of SI subjects.

Conclusions—This analysis of well-characterized individuals identified concise panels of biomarkers associated with NC change. Across all analyses, the two most frequently identified biomarkers were sCD14 and MCP-1, indicators of monocyte/macrophage activation. While the panels differed depending on the outcome and on the degree of misclassification, nearly all stable patients were correctly classified.

Keywords

HIV; Neurocognitive Disorders; Cerebrospinal Fluid; Biomarkers

Background

Antiretroviral therapy (ART) has marked increased life expectancy and reduced the incidence of HIV-associated dementia (HAD). (McPhail ME 2011; Rackstraw 2011) Despite these successes, up to 60% of people living with HIV disease develop at least mild neurocognitive impairment (NCI). (Heaton et al. 2011; Letendre 2011; McArthur J 2013; Rodriguez-Penney AT 2013; Antinori A 2007; Simioni et al. 2010) Such impairments can be associated with impaired daily functioning, including reduced ART adherence and worse survival, compared with unimpaired people. (Marcotte TD 2004; Poquette AJ 2013)

The pathogenesis of HIV-associated NCI likely involves interactions of host, viral, comorbidity, and treatment factors, but the specific mechanisms and pathways are not fully understood. (Burdo TH 2013; Ellis RJ and Morgello S 2011) One critical gap in neuroAIDS research, similar to many other neuropsychiatric and neurodegenerative conditions, is the identification of reliable molecular biomarkers. Discovery of disease-specific biomarkers has been a significant advance in biomedical research as well as clinical medicine. However, while biomarkers can have great utility in diagnostic, prognostic, and therapeutic decisions, the unique cellular and phenotypic complexity of the brain has hindered biomarker identification in neurological and psychiatric disorders. Reliable biomarkers would not only provide valuable insights into the pathogenesis of HIV-associated NCI but could lead to much needed clinical diagnostic and prognostic tests. Such tests might help bridge the “therapeutic gap” in neuroAIDS, i.e., they might help explain why ART does not consistently reverse neurocognitive deficits.

Many past biomarker analyses were hampered by issues, such as cross-sectional design, suboptimal patient characterization, use of blood-derived fluids only, relatively narrow assay methodology, and limited statistical analysis. (Yuan L 2013) To identify clinically relevant biomarkers and address these shortcomings, we assayed biomarkers in plasma and cerebrospinal fluid (CSF) from a well-characterized cohort of individuals with longitudinal neurobehavioral and neuromedical data using multiple assay methods (immunoassays, mass spectrometry for lipids and peptides, multiple reaction monitoring, nucleic acid amplification) and analyzed the results using multiple statistical methods. This manuscript summarizes the results of the immunoassays, which measured a panel of eight protein biomarkers that have been previously linked to HAND: monocyte chemotactic protein

(MCP)-1, interleukin (IL)-6, stromal-derived factor (SDF)-1 α , tumor necrosis factor (TNF)-1 α , interferon-inducible protein (IP)-10, fractalkine, soluble TNF receptor-II (sTNFR-II, p75), and soluble CD14 (sCD14).

Methods

Overall Design and Case Selection

The manuscript describes a longitudinal analysis of 98 HIV seropositive participants who were selected from participants enrolled in the longitudinal components of the CNS HIV Antiretroviral Effects Research (CHARTER) and HIV Neurobehavioral Research Center research programs. Cases were identified by screening nearly 3,500 visits from 430 participants who were assessed between August 1999 and December 2008. The Institutional Review Board approved the study protocol and all participants provided informed consent.

Case records were reviewed by a multidisciplinary team that included an infectious disease specialist (SLL), neuropsychologist (TDM), a senior study coordinator (DF), a senior lab scientist (DRC), and a research nurse. Cases were selected in this initial screening if they had at least three consecutive visits with: 1) complete neuromedical and neurobehavioral characterization (see below), and 2) availability of at least 6 mL of blood and CSF specimens stored at -80°C . Cases were excluded if they had severe neurocognitive co-morbidities (e.g., history of severe traumatic brain injury, mental retardation, as described by published criteria (Antinori A 2007)). In descending order of importance, the priorities for selecting participants were (a) those who best met the stringent neurocognitive change criteria, (b) those who had the least neurocognitive co-morbidities, (c) those who reported stable ART regimens for at least three months prior to the first observation and throughout the period of observation, and (d) those who were hepatitis C virus (HCV) seronegative. ART changes were allowed if only a single drug was changed within the same drug class (e.g., lamivudine to emtricitabine). All participants had a third visit, which was used to verify the stability of the neurocognitive grouping. For the work of this project, only the first two visits were used in analyses.

Neurobehavioral Methods

All participants completed a comprehensive neurocognitive test battery, covering seven cognitive domains known to be commonly affected in HIV-associated NC dysfunction. (Heaton et al. 2010) The best available normative standards were used to convert the scores to demographically-corrected standard scores (T-scores), which correct for effects of age, education, sex and ethnicity. The presence and severity of NCI was determined using the Global Deficit Score (GDS) approach, where a GDS ≥ 0.5 was impaired. (Carey et al. 2004) All follow-up visits were corrected for practice effects. (Cysique et al. 2011)

In order to determine neurocognitive change, we utilized a multivariable change score approach. (Cysique et al. 2011; Cysique et al. 2009; Temkin et al. 1999) Using regression formulas (Cysique et al. 2011), we generated a Z-score for each of 15 neuropsychological variables. These Z-scores reflect how well or poorly the participant performed at follow-up, relative to the expectation for someone with the same baseline neuropsychological and other relevant characteristics. The Z-scores were then summed to provide a summary regression change score. The central 80% of the summary regression change score distribution defined “stable” on the test battery. The top 10% of the summary regression change score distribution defined the ‘improved’ range and the cut-off for the bottom 10% defined the ‘declined’ range. (Cysique et al. 2011) A change status from visit to visit was generated for each participant. Participant were classified as “improved”, “declined”, or “stable” using groups of three consecutive visits in the following manner: 1) Declined: if a participant was

“stable” at the first visit used in this analysis compared with the prior visit, had a “decline” at the second visit, and was either “stable” or “decline” at the third visit; 2) Improved: if a participant was “stable” at the first visit compared with the prior visit, “improved” at the second visit, and was either “stable” or “improved” at the third visit; 3) Stable: if a participant was “stable” across all visits. This process identified four groups of participants: stably normal (SN), stably impaired (SI), reliably worsening (Wo), or reliably improving (Im).

Laboratory Procedures

Blood was collected by phlebotomy at all visits and processed for routine clinical labs, T-cell subsets, and HIV RNA (Roche Amplicor; lower limit of quantitation (LLQ) 50 copies/mL). Plasma for biomarker assays was collected using EDTA vacuum tubes. Specimens were centrifuged at 1,800 relative centrifugal force for 8 minutes at room temperature and aliquoted for storage at -80°C . CSF was collected at all visits by lumbar puncture using a non-traumatic spinal needle and aseptic technique. Specimens were also processed for routine clinical labs, including cell counts and HIV RNA (Roche Amplicor; LLQ 50 copies/mL). CSF was centrifuged at low speed to separate cells. Both supernatants and cells were aliquoted and stored at -80°C and were not thawed until the time of assay.

Protein biomarkers for this analysis were measured using commercially available immunoassays according to the instructions of the manufacturer. MCP-1, IL-6, TNF- α , IP-10, and fractalkine were measured by a multiplex bead array and SDF-1 α and sTNFR-II were assayed using individual single-plex kits (EMD Millipore, Billerica, MA). Soluble CD14 was measured using a quantitative sandwich enzyme immunoassay (Quantikine; R&D Systems, Minneapolis, MN USA). Biomarker precision was ensured by assaying specimens in duplicate and repeating measurements with coefficients of variation greater than 20% or outliers that were more than 3 standard deviations from the mean. In addition, 10% of all assays were repeated to assess operator and batch consistency. Aberrant results were excluded from analysis. Of note, one CSF IL-6 assay plate demonstrated a substantial batch effect and repeating the assay did not remedy this to an acceptable extent. Since these specimens were then depleted, no additional assays were performed and CSF IL-6 values were not included in multivariable analyses.

Statistical Analysis

Demographics, disease characteristics, and antiretroviral therapy use at the first visit (baseline) for each of the four subject groups (SN, Wo, SI, Im) were summarized descriptively. Distributions were tested using Shapiro-Wilk tests, homogeneity of variance was tested using Levene tests, and transformations or nonparametric tests were applied to compare groups when appropriate. Group comparisons were made using chi-square tests, Fisher exact tests, one-way analysis of variance, or Kruskal-Wallis tests. Tukey's honestly significant difference (HSD) tests identified the direction of significant group effects. When ANOVA showed significantly different groups but Tukey's HSD did not identify which groups were different, pairwise Student t-tests based on pooled standard deviation were used to indicate group differences. Effects of cognitive change group, time between visits, and their interaction on levels of the eight biomarkers under study in each of the two body fluids were tested at the 5% significance level using mixed model linear regression with random intercepts to control for the within-subject correlation of the repeated measures. Suitable transformations of biomarkers were made when needed. No further adjustments were made for multiple testing.

Recursive partitioning decision trees (Breiman et al. 1984; Zhang et al. 2010) were developed to determine which combination of biomarkers, or biomarkers plus clinical

variables, would correctly classify individual subjects to their corresponding classification of cognitive stability or change. Five-fold cross-validation was used to guard against overfitting the sample data that could lead to degraded classification potential. Separate models were developed for each of three input settings based on measurement values at the first visit: (1) All biomarkers under study in both CSF and plasma, except IL-6 in CSF as noted above, (2) biomarkers only in plasma, and (3) biomarkers in plasma plus clinical variables (current and nadir CD4+ T-cell count, HIV RNA in plasma above or below the LLQ, using or not using ART, age, and HCV serostatus). Within each of these three input settings, cognitive change groups were categorized into either (a) diagnostic (impaired vs. unimpaired at the first visit), (b) prognostic for worsened (SN vs. Wo), or (c) prognostic for improved (SI vs. Im).

To build models for each of these combinations, first a full model was generated to achieve the best fit, sometimes approaching or achieving 100% accuracy. This “decision tree” often contained many “branches” resulting from “splits,” or decision points, and was typically complex and poorly validated. This tree was then “pruned,” or reduced, until maximum classification accuracy was reached without exceeding a pre-specified maximum misclassification limit (10% or 20%) to produce a smaller, less complex and better validated model. Selected classification trees and decisions based on the decision rules are reported, and the corresponding confusion matrices are presented for some models.

Results

Participant characteristics

As summarized in Table 1, the 98 participants in the four neurocognitive change groups did not differ in demographic, disease, treatment, or HCV characteristics, except that both groups who remained neurocognitively stable had higher current CD4+ T-cell counts at the first visit ($p < 0.01$). Nadir CD4+ T-cell counts also differed across all groups ($p < 0.05$) but, after adjustment for multiple comparisons, specific pairwise differences were not found with the Tukey HSD test. The less conservative pairwise Student t-tests based on a pooled standard deviation identified that the changing groups (Im and Wo) had lower nadir CD4+ T-cell counts at the first visit than the SN group. First visits occurred a median of 6.2 months (interquartile range 5.5–6.9 months) prior to second visits.

Mixed model linear regression analysis

Results are summarized in Table 2. Worsening was associated with higher MCP-1 in CSF ($p = 0.015$). Biomarker levels increased between visits for sTNFR-II ($p = 0.03$) and TNF- α ($p = 0.017$) in plasma and decreased for fractalkine in CSF ($p < 0.0001$). Significant group or time effects were not present for the other biomarker/fluid combinations (p -values ranged from 0.06 to 0.93), and no significant interactions between group and time were detected (all $p > 0.11$).

Recursive partitioning analysis

For all biomarker input settings, the diagnostic decision trees that were produced to classify subjects as unimpaired or impaired at the first visit for both specified misclassification limits (10% or 20%) included at least five of the eight biomarkers under study with between up to 12 splits, or decision points, and proved to be complex to interpret. Of note, diagnostic models were unable to attain the 10% misclassification rate, instead achieving at best 12% misclassification. Examples of three diagnostic trees, and their confusion matrices, are shown in Figures 1, 4, and 7. Figure 1 is the simplest decision tree that was produced using all 98 subjects with no more than 20% misclassified using the diagnostic model containing biomarkers from both CSF and plasma. This particular tree required measurement of 6

different biomarker-body fluid combinations. The figure demonstrates that interpretation can be complicated depending on the path a subject takes through the tree.

Prognostic models (i.e., those limited to two neurocognitive change groups) were simpler and provided more easily interpretable information. Between 1 and 5 assays were required and resulted in between 3 and 9 splits. Examples of decision trees corresponding to the 20% misclassification rate limit produced by models for worsening or improvement when biomarkers from CSF and plasma were used as input variables are displayed in Figures 2 and 3. Confusion matrices are also shown.

Tables 3, 4, and 5 summarize the results from the best classification models that emerged from recursive partitioning. Prognostic R^2 values for the 10% threshold in misclassification are moderately large (between 0.60 and 0.72). For the 20% misclassification limit, R^2 values were lower, ranging from 0.35 to 0.46. When CSF biomarkers were included (Table 3, Figures 1, 2, and 3), both specificity and positive predictive values were 100% for the 10% cutoff, as well as for the model with improved subjects at the 20% misclassification limit. For models that included only plasma biomarkers (Table 4, Figures 4, 5, and 6), specificity and positive predictive value were also 100% for the “Prognostic for Improved” models at the 20% misclassification limit. When clinical variables were combined with the plasma biomarkers in the prognostic models (Table 5, Figures 7, 8, and 9), the analyses that are the most clinically relevant, classification accuracy on all measures was high and only required between 1 and 3 biomarker assays. Diagnostic efficiency remained suboptimal, requiring that up to 7 biomarkers be measured and yielding relatively complex models with a larger number of splits.

Discussion

This report is the first from an NIMH-funded project that aims to address common limitations of prior biomarker analyses of neurocognitive complications of HIV disease, namely the project was longitudinal instead of cross-sectional; participants were assessed using comprehensive neurocognitive testing; a multidisciplinary team carefully selected cases who were either stably on or off ART and had neurocognitive functioning that was either clearly stable or clearly changing over time; biomarkers were measured in both CSF and blood; multiple assay methods were used to measure biomarkers; and multiple statistical methods were used to analyze the results. This report focuses on proteins measured by immunoassay and future reports will focus on other assay methods, such as mass spectrometry and multiple reaction monitoring. When using the more common statistical method, mixed model linear regression, only one biomarker, MCP-1 in CSF, was associated with neurocognitive change group, confirming the findings of many prior publications. (Gonzalez et al. 2002; Kamat et al. 2012; Kelder W 1998; Letendre et al. 2011; Yuan L 2013) The particularly informative findings resulted from recursive partitioning.

Recursive partitioning modeling uncovered complex relationships between our concise panel of biomarkers and the diagnosis of neurocognitive impairment. The panel of eight biomarkers was relatively inefficient in diagnosing neurocognitive impairment at the first visit, highlighting the challenges of cross-sectional analyses. The reasons for this are uncertain but likely include the heterogeneity of the preceding clinical course (despite our attempts to account for this) and the static nature of the impairment in half of the impaired subjects as well as the between-group differences in current and nadir CD4+ T-cell counts and between-subject differences in HIV RNA suppression and HCV seropositivity. In addition, our panel focused on biomarkers of immune activation. While persistent immune activation during effective therapy has been strongly implicated in neurocognitive

impairment in people living with HIV disease, the current panel did not include biomarkers that reflect activation or injury of other, likely influential cells, particularly neurons.

The unique findings of these analyses derive from the prognostic models, i.e., those predicting future stability or change in neurocognitive functioning (as summarized in Tables 3 to 5 and in Figures 2, 3, 5, 6, 8, and 9). Compared with diagnostic models, prognostic models were generally more efficient, explaining similar degrees of variance in neurocognitive outcomes but with fewer biomarkers. For example, once clinical variables were taken into account, simply measuring two biomarkers, IL-6 and sTNFR-II, in blood enabled correct distinction of Stably Normal from Worsening subjects in 92% of subjects (see Table 5). A second conclusion deriving from these analyses is that the correlates of neurocognitive worsening and neurocognitive improvement differ. For instance, Table 4 shows that the biomarkers associated with neurocognitive worsening (sCD14, sTNFR-II) do not overlap (using the 20% misclassification limit) with those for neurocognitive improvement (TNF- α , IP-10, fractalkine). Historically, many have assumed that these processes were similar if not identical, a justification for grouping all subjects together. However, if the biomarkers of neurocognitive change – and by extension the pathogenesis – truly differ based on the trajectory, then clearly distinguishing people who are stable from those who are either worsening or improving will be critical in future analyses. A third conclusion centers on the value of CSF. While most of our analyses focused on biomarkers measured in blood because of the logistical difficulty of obtaining CSF in the typical clinic, when CSF biomarkers were included as candidate covariates, modeling procedures often preferentially selected them (see Table 3). While models that included CSF biomarkers did not by design explain more variance in our outcome, they were more likely to achieve 100% specificity and positive predictive value.

From a pathogenesis standpoint, the two biomarkers that most consistently appeared in combined CSF and plasma models (Table 3) were sCD14 and MCP-1, emphasizing the continuing importance of monocyte/macrophage lineage cells in HAND pathogenesis. (Kennedy 1988) A subset of activated monocytes, which expresses CD14+low/CD16+high in common with tissue macrophages, expand with HIV infection;(Thiebaut N 1995); may be more likely to migrate into the CNS; and are found in higher percentages in neurocognitively impaired individuals. (Gartner S 2002; Pulliam L 1997) Serum levels of sCD14 are associated with HIV disease progression *in vivo* (Lien E 1998; Nockher WA 1994) and correlate with lower CD4+ T-cell counts, older age, (Thiebaut R 2012), and worse neurocognitive functioning. (Kamat et al. 2012; Lyons et al. 2011; Sun et al. 2010; Ryan et al. 2001) Soluble CD14 may also reflect ongoing immune responses in the CNS, which may be due to low-level HIV replication or to prior or continuing cellular injury. (Fields J and Adame A 2013) MCP-1 is a potent inducer of chemotaxis of monocytes across endothelial barriers (Weiss et al. 1999); is expressed by astrocytes in response to HIV *in vitro* (Conant et al. 1998); and has been identified on CNS macrophages (Sanders et al. 1998). MCP-1 levels in CSF have been previously reported to correlate with HAND disease severity (Kelder W 1998) and to decline along with HIV RNA levels in CSF in response to ART (Monteiro de Almeida et al. 2005). Moreover, polymorphisms in the gene encoding MCP-1(Gonzalez et al. 2002) and its receptor, CCR2(Singh et al. 2004), confer increased risk of HAD. Our findings are consistent with these prior reports and extend them to identify that combinations of biomarkers better predict outcomes than one biomarker alone.

After accounting for clinical variables, the simplest prognostic models identified that lower sTNFR-II concentrations were associated with neurocognitive worsening and higher IL-6 concentrations were associated with neurocognitive improvement. TNF- α stimulation of cells, such as macrophages and CD4+ T-cells, can result in solubilization of the p75 TNF receptor. (Zelova and Hosek 2013) TNF- α can also induce the synthesis of IL-6. (Gruol and

Nelson 1997) Together, these observations suggest that TNF- α may link neurocognitive progression and remission. TNF- α , sTNFR-II, and IL-6 have all been previously implicated in HIV disease progression (Nixon and Landay 2010; Crowe et al. 2010) and in HIV-associated neurocognitive complications. (Achim et al. 1993; Mastroianni et al. 1990; Perrella et al. 1992; Vullo et al. 1995; Wesselingh et al. 1994) Supporting the central importance of TNF- α , TNF- α concentrations in plasma correlated with both sTNFR-II ($\rho = 0.48$, $p < 0.0001$) and IL-6 ($\rho = 0.41$, $p < 0.0001$), but sTNFR-II and IL-6 did not correlate with each other ($\rho = 0.01$, $p = 0.92$). Further, among the 16 biomarker-fluid combinations measured, only concentrations of TNF- α and sTNFR-II in plasma increased over time (Table 2).

If TNF- α is the central mediator of these processes, the question follows, “Why was it not selected by the most concise partitioning models?” The answer may lie in the limitations of our cohort and analytical methods but an alternative answer is that its concentrations in body fluids may not fully reflect its *in vivo* biological activity since extracellular TNF- α is strictly regulated by multiple mechanisms, including soluble receptor binding and protease-mediated degradation. These processes can continue *ex vivo* after specimen collection and, as a result, surrogate proteins, such as sTNFRs, may better reflect *in vivo* TNF- α activity and thereby serve as better clinical biomarkers. A role for TNF- α in progressive neurocognitive disease might have therapeutic implications in light of the availability of FDA-approved, anti-TNF- α drugs.

The project has important limitations. Firstly, even though the careful, multidisciplinary selection process increases confidence in the group assignments, any selection procedure can also reduce generalizability and introduce bias. For this reason, the findings should be validated in an independent cohort. Efforts to accomplish this are already underway in our group. Secondly, despite our efforts to limit confounds that might affect the relationship between biomarkers and cognition, approximately one-quarter of subjects were not taking ART and approximately one-fifth were HCV seropositive. While these characteristics improve generalizability to the heterogeneous clinical population, the findings may not generalize well to HCV seronegative subjects who are taking suppressive ART, an important patient population in the clinic. While ART use did not differ between the four neurocognitive change groups, they were not matched for CD4+ T-cell counts, an important shortcoming. Thirdly, while recursive partitioning was responsible for the most important insights in this report, it is also prone to overfitting. To reduce the possibility of overfitting, we performed five-fold cross-validation. Finally, the biomarker panel reported in this report is relatively sparse and does not reflect the substantial complexity of the immune response or other processes such as lipid oxidation or neuronal injury. Other biomarkers, such as hydroxynonenals or neurofilament-light, might perform even better than those reported here.

In summary, this project aimed to identify biomarkers associated with neurocognitive change and incorporated multiple methods to address the limitations of prior analyses, including those from our group. Analyses that focused on the diagnosis of neurocognitive impairment were complex and relatively inefficient. In contrast, models that focused on prognosis (i.e., identifying people at risk for neurocognitive worsening or improvement) were more efficient, requiring fewer biomarker measurements and simpler classification decision rules. Models were particularly accurate in correctly classifying neurocognitive stability. While most prior biomarker studies of HAND have focused on identifying biomarkers of worsening or improvement, correctly classifying all patients who will remain neurocognitively stable (either stably normal or stably impaired) has clinical value since assessment and intervention efforts can then be focused on those who are less likely to remain stable. While our findings are potentially important, they should be validated in independent cohorts before attempting to translate them to the clinical environment.

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Figure 1. Diagnostic model and confusion matrix with a 20% misclassification limit and biomarkers in CSF and plasma as input variables

Terminal nodes display classification decisions. Values adjacent to classification decisions are the number correctly classified with the number incorrectly classified in parentheses for that classification.

True classification ↓	Classification decision	
	Worsened	Stably normal
Worsened	19	6
Stably normal	3	22

Figure 2. Prognostic model for “worsened” and confusion matrix with a 20% misclassification limit and biomarkers in CSF and plasma as input variables

Terminal nodes display classification decisions. Values adjacent to classification decisions are the number correctly classified with the number incorrectly classified in parentheses for that classification. As seen in the confusion matrix, this tree correctly classified 22 of 25 (88%) of Stably Normal participants.

True classification ↓	Classification decision	
	Stably Impaired	Improved
Stably impaired	25	0
Improved	9	14

Figure 3. Prognostic model for “improved” and confusion matrix with a 20% misclassification limit and biomarkers in CSF and plasma as input variables

Terminal nodes display classification decisions. Values adjacent to classification decisions are N correctly classified (N incorrectly classified) for that classification. As seen in the confusion matrix, this tree correctly classified all 25 (100%) of Stably Impaired participants.

True classification ↓	Classification decision	
	Impaired	Normal
Impaired	51	5
Normal	14	28

Figure 4. Diagnostic model and confusion matrix with a 20% misclassification limit and biomarkers in plasma as input variables

Terminal nodes display classification decisions. Values adjacent to classification decisions are the number correctly classified with the number incorrectly classified in parentheses for that classification.

True classification ↓	Classification decision	
	Worsened	Stably normal
Worsened	24	1
Stably normal	8	17

Figure 5. Prognostic model for “worsened” and confusion matrix with a 20% misclassification limit and biomarkers in plasma as input variables

Terminal nodes display classification decisions. Values adjacent to classification decisions are the number correctly classified with the number incorrectly classified in parentheses for that classification. As seen in the confusion matrix, this tree correctly classified 24 of 25 (96%) of Worsened participants.

Figure 6. Prognostic model for “improved” and confusion matrix with a 20% misclassification limit and biomarkers in plasma as input variables
 Terminal nodes display classification decisions. Values adjacent to classification decisions are N correctly classified (N incorrectly classified) for that classification. As seen in the confusion matrix, this tree correctly classified all 25 (100%) of Stably Impaired participants.

True classification ↓	Classification decision	
	Impaired	Normal
Impaired	50	6
Normal	12	30

Figure 7. Diagnostic model and confusion matrix with a 20% misclassification limit and biomarkers in plasma and clinical variables as input variables

Terminal nodes display classification decisions. Values adjacent to classification decisions are the number correctly classified with the number incorrectly classified in parentheses for that classification.

True classification ↓	Classification decision	
	Worsened	Stably normal
Worsened	16	9
Stably normal	1	24

Figure 8. Prognostic model for “worsened” and confusion matrix with a 20% misclassification limit and biomarkers in plasma and clinical variables as input variables

Terminal nodes display classification decisions. Values adjacent to classification decisions are the number correctly classified with the number incorrectly classified in parentheses for that classification. As seen in the confusion matrix, this tree correctly classified 24 of 25 (96%) of Stably Normal participants.

Figure 9. Prognostic model for “improved” and confusion matrix with a 20% misclassification limit and biomarkers in plasma and clinical variables as input variables

Terminal nodes display classification decisions. Values adjacent to classification decisions are N correctly classified (N incorrectly classified) for that classification. As seen in the confusion matrix, this tree correctly classified 19 of 25 (76%) Stably Impaired participants and 20 of 23 (87%) Improved participants.

Subject characteristics at first visit

Table 1

Values are mean $\pm$ standard deviation, median [interquartile range], or percent. SN = Stably Normal, Wo = Worsened, SI = Stably Impaired, Im = Improved, ART = antiretroviral therapy

Characteristic	SN N=25	Wo N=25	SI N=25	Im N=23	p- value	Group Comparison
Age, years	44.0 $\pm$ 10.5	44.6 $\pm$ 7.0	47.0 $\pm$ 9.5	43.0 $\pm$ 6.3	0.43 ^a	
Education, years	13.3 $\pm$ 2.0	12.7 $\pm$ 2.2	13.4 $\pm$ 2.5	13.3 $\pm$ 3.1	0.82 ^b	
Sex (men)	92%	80%	84%	78%	0.58 ^c	
Ethnicity (Caucasian)	56%	60%	48%	70%	0.49 ^d	
AIDS	56%	68%	80%	78%	0.23 ^d	
HCV seropositive	20%	18%	24%	27%	0.92 ^c	
Duration of current ART regimen, months *	9.2 [4.6, 31.2]	9.6 [4.3, 23.0]	10.7 [5.7, 28.6]	13.0 [2.4, 29.2]	0.96 ^a	
Current CD4 count, cells/mm ³ *	462 [312, 667]	335 [194, 481]	463 [304, 642]	260 [102, 540]	0.005 ^a	SN=SI > Im **
Nadir CD4 count, cells/mm ³ *	190 [95, 343]	85 [11, 231]	128 [46, 243]	79 [7, 217]	0.047 ^a	SN > Wo=Im ***
ART Use	76%	64%	84%	70%	0.40 ^d	
Among those taking ART						
-HIV RNA, Plasma, 50 copies/mL	61%	56%	62%	31%	0.23 ^d	
-HIV RNA, CSF, 50 copies/mL	95%	75%	81%	88%	0.36 ^d	

* Square-root transformed,

** Tukey HSD test for pairwise comparisons,

*** Pairwise Student's t-tests using a pooled standard deviation from all four groups

^a ANOVA,

^b Kruskal-Wallis test,

^c Fisher Exact test,

^d Chi-square test

Mixed model linear regression analysis

Table 2

Only MCP-1 was associated with neurocognitive change group. No Group by Time interactions were present. Wo = worsened, SN = Stably Normal, SI = Stably Impaired

Biomarker	CSF			Plasma		
	p-value	Effect	Direction of Effect	p-value	Effect	Direction of Effect
sCD14 ^{B,a}	-	-	-	-	-	-
sTNFR-II ^{C,b}	-	-	-	0.03	Time	Increased over time
TNF-α ^{C,b}	-	-	-	0.017	Time	Increased over time
IP-10 ^{B,b}	-	-	-	-	-	-
MCP-1 ^{A,c}	0.015	Group	Wo > SN = SI	-	-	-
SDF-1α ^{A,a}	-	-	-	-	-	-
IL-6 ^{*B,b}	-	-	-	-	-	-
Fractalkine ^{A,c}	<0.0001	Time	Decreased over time	-	-	-

* CSF IL-6 analysis included 59 subjects (see Methods for explanation)

^A Raw data in CSF,

^B Natural log transformation in CSF,

^C Square root transformation in CSF

^a Raw data in plasma,

^b Natural log transformation in plasma,

^c Square root transformation in plasma

Table 3
Models using biomarkers in both CSF and plasma as input variables

Diagnostic models classified neurocognitive status (unimpaired vs. impaired) at the first visit. Prognostic models included two change groups each (Prognostic for Worsened: SN vs. Wo; Prognostic for Improved: SI vs. Im). Contents of the cells to the right of the biomarker name indicate whether the indicated biomarker was included in the model, either measured in CSF (“CSF”) or in plasma (“PL”) or both. Dashes indicate that the biomarker was not included in the model.

Misclassification Limits→	Diagnostic		Prognostic for Worsened		Prognostic for Improved	
	10%*	20%	10%	20%	10%	20%
sCD14	CSF	CSF	CSF	CSF	CSF	---
sTNFR-II	PL	PL	PL	---	---	---
TNF-α	---	---	---	---	CSF, PL	CSF, PL
IP-10	PL	PL	---	---	---	---
MCP-1	PL	PL	PL	PL	CSF, PL	CSF
SDF-1α	CSF, PL	CSF, PL	CSF	CSF	---	---
IL-6	PL	---	---	---	---	---
Fractalkine	---	---	---	---	---	---
Total biomarker assays	7	6	4	3	5	3
Number of splits	10	6	6	3	6	3
Correct classification	88%	83%	92%	82%	92%	81%
Sensitivity	88%	82%	84%	76%	83%	61%
Specificity	88%	83%	100%	88%	100%	100%
Positive predictive value	91%	87%	100%	86%	100%	100%
Negative predictive value	84%	78%	86%	79%	86%	74%
Model R ²	0.55	0.42	0.62	0.35	0.72	0.46

* The full model attained only a 12% misclassification rate.

Table 4
Models using biomarkers in plasma only as input variables (i.e., no CSF or clinical variables)

Diagnostic models classified neurocognitive status (unimpaired vs. impaired) at the first visit. Prognostic models included two change groups each (Prognostic for Worsened: SN vs. Wo; Prognostic for Improved: SI vs. Im). A checkmark indicates that the indicated biomarker was included in the model. Dashes indicate that the biomarker was not included in the model.

Misclassification Limits→	<u>Diagnostic</u>		<u>Prognostic for Worsened</u>		<u>Prognostic for Improved</u>	
	10%*	20%*	10%	20%	10%	20%
sCD14						
sTNFR-II						
TNF-α						
IP-10						
MCP-1						
SDF-1α						
IL-6						
Fractalkine						
Total biomarker assays	7	6	4	2	4	3
Number of splits	10	6	8	5	9	5
Correct classification	85%	81%	92%	82%	94%	81%
Sensitivity	86%	91%	96%	96%	96%	61%
Specificity	83%	67%	88%	68%	92%	100%
Positive predictive value	87%	78%	89%	75%	92%	100%
Negative predictive value	81%	85%	96%	94%	96%	74%
Model R ²	0.45	0.31	0.63	0.41	0.69	0.41

* The full model attained only a 15% misclassification rate.

Table 5
Models using biomarkers in plasma plus clinical variables as inputs (i.e., no CSF)

These models are the most clinically relevant since they account for readily available clinical information and do not require lumbar puncture. Diagnostic models classified neurocognitive status (unimpaired vs. impaired) at the first visit. Prognostic models included two change groups each (Prognostic for Worsened: SN vs. W0; Prognostic for Improved: SI vs. Im). A checkmark indicates that the indicated biomarker was included in the model. Dashes indicate that the biomarker was not included in the model.

Misclassification Limits→	<u>Diagnostic</u>		<u>Prognostic for Worsened</u>		<u>Prognostic for Improved</u>	
	10%*	20%	10%	20%	10%	20%
sCD14	---	---	---	---	---	---
sTNFR-II	---	---	---	---	---	---
TNF-α	---	---	---	---	---	---
IP-10	---	---	---	---	---	---
MCP-1	---	---	---	---	---	---
SDF-1α	---	---	---	---	---	---
IL-6	---	---	---	---	---	---
Fractalkine	---	---	---	---	---	---
Current CD4+ count	---	---	---	---	---	---
Nadir CD4+ count	V	---	---	---	---	---
Plasma HIV RNA < 50	---	---	---	---	---	---
ART use	---	---	---	---	---	---
Age	---	---	---	---	---	---
Hepatitis C status	---	---	---	---	---	---
Total biomarker assays	7	5	2	1	3	1
Number of splits	12	7	8	5	6	3
Correct classification	86%	82%	92%	80%	92%	81%
Sensitivity	89%	89%	88%	64%	96%	87%
Specificity	81%	71%	96%	96%	87%	76%
Positive predictive value	86%	81%	96%	94%	95%	77%
Negative predictive value	85%	83%	89%	73%	89%	86%
Model R ²	0.51	0.35	0.63	0.38	0.60	0.37

* The full model attained only a 14% misclassification rate.