Title

Cerebrospinal fluid CXCL10 is associated with the presence of low level CSF HIV during suppressive antiretroviral therapy

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Study sponsorship:

National Institutes of Health R21 MH085610 (Principal Investigator: S. Letendre), R01 MH058076 (Principal Investigator: R. Ellis), R01 MH107345 (Co-Principal Investigators: R. Heaton, S. Letendre), K23 MH095679, R21 MH118092, R01 AG062387 (Principal Investigator: A. Anderson), and K24 MH097673 (Principal Investigator: S. Letendre). H. Zetterberg is a Wallenberg Scholar.

Abstract

Surrogate markers of HIV central nervous system (CNS) persistence are needed because direct HIV measurements from the CNS require specialized protocols and are not always detectable or quantifiable. We analyzed paired plasma and CSF samples from people with HIV (PWH) on suppressive therapy (ART) with a validated HIV single copy RNA assay. Two potential markers of CNS persistence were measured (CXCL10 and sCD30). We then examined associations with CSF HIV RNA positivity in univariable and multivariable analyses. Among 66 individuals, 18.2% had detectable CSF HIV. Individuals who had detectable HIV in CSF had higher CSF CXCL10 concentrations (median 514 pg/ml versus median 317 pg/ml, p = 0.019), but did not have significantly different CSF sCD30 concentrations (median 7.5 ng/ml versus median 7.6 ng/ml, p = 0.78). In the multiple logistic analysis, both higher CSF CXCL10 (p = 0.038) and plasma HIV detectability (p = 0.035) were significantly associated with detectable CSF HIV. Both sCD30 and CXCL10 correlated positively with NfL and NSE, two neuronal markers. This study demonstrates that CSF CXCL10 concentrations reflect low level HIV CNS persistence despite virologic suppression on ART. Given that it is readily detectable and quantifiable, this chemokine may be a promising biomarker to evaluate HIV eradication therapies that target the CNS.

Introduction

Despite the dramatic successes of combination antiretroviral therapy (ART), HIV cure has proved extremely elusive.(Hutter *et al*, 2009) Mounting evidence supports the hypothesis that the central nervous system (CNS) is a reservoir of HIV persistence and thus a barrier to viral eradication. HIV DNA is commonly found in brain tissue from HIV-infected individuals on suppressive ART,(Lamers *et al*, 2016) which suggests that some CNS cells allow for reverse transcription and could be latently infected. However, given the lack of brain tissue availability from living people with HIV (PWH), more research is needed to identify biomarkers of CNS persistence that could be potentially used in studies of HIV cure efforts.

Given its direct communication with brain interstitial fluid,(Ratner *et al*, 2017) the cerebrospinal fluid (CSF) is part of the CNS and a potential window into HIV brain persistence. Several CSF studies have provided important evidence that support the HIV CNS persistence hypothesis. This includes multiple independent reports of CNS virologic escape, a condition in which CSF HIV RNA concentrations are elevated despite relative virologic suppression in the blood during ART.(Canestri *et al*, 2010; Peluso *et al*, 2012) Additionally, viral genetic compartmentalization occurs in the CSF and may be associated with adverse clinical consequences.(Joseph *et al*, 2015; Oliveira *et al*, 2017) Multiple studies have also demonstrated that HIV RNA can be detected at very low levels in CSF during suppressive ART, which provides more evidence of HIV CNS persistence.(Anderson *et al*, 2017; Dahl *et al*, 2014) This low level presence of HIV in the CSF is also associated with increased markers of inflammation,(Dahl *et al*, 2014) and thus is potentially detrimental to the CNS. HIV DNA can also be found in CSF cells, and this finding was recently shown to be associated with neurocognitive impairment in individuals with HIV.(Spudich *et al*, 2019)

While it is possible to detect HIV RNA and DNA from the CSF during ART, these markers are undetectable even with the use of highly sensitive techniques in 50% or more of PWH.(Anderson *et al*, 2017; Spudich *et al*, 2019) For the purposes of HIV cure studies, other

markers that reflect HIV CNS persistence may be needed that are more readily quantifiable and can be measured without the need for advanced molecular techniques. CXCL10 is a chemokine induced by gamma interferon and is produced by macrophages, astrocytes, and other cells.(Majumder *et al*, 1996) In the absence of virologic suppression, CSF CXCL10 concentrations correlate with CSF HIV RNA concentrations but not plasma HIV RNA concentrations, making this protein a potential indicator of CNS HIV replication.(Cinque *et al*, 2005) Another potential marker of CNS HIV is soluble CD30 (sCD30). This is a cell surface marker that is shed from activated (but not resting) immune cells.(Fernandez-Ruiz *et al*, 2017) In CSF, sCD30 is elevated in HIV-infected individuals despite suppressive ART(Peluso *et al*, 2020) and thus may also be a marker of HIV CNS persistence.

For the current study, we used CSF samples from a cross-sectional subset of individuals with HIV RNA previously measured by single copy assay.(Anderson *et al*, 2017) We hypothesized that both CXCL10 and sCD30 would be associated with the presence of low level CSF HIV RNA in the setting of suppressive ART. We also hypothesized that these markers would be associated with markers of neuronal damage.

Methods

As in the parent study,(Anderson *et al*, 2017) inclusion criteria were: a) adults older than 18 years who had CSF and blood collected within one hour, b) On stable ART with HIV RNA ≤50 copies/ml in CSF and plasma, and c) SCA results available. Participants with sufficiently severe neuropsychiatric comorbid conditions to confound attribution of neurocognitive impairment (NCI) to HIV as per Frascati criteria(Antinori *et al*, 2007) were excluded. For the current analysis, participants were also only included if sufficient volume was available for the proposed new biomarkers. All participants provided written consent.

Neuropsychological (NP) performance was assessed using a comprehensive and standardized battery of tests, which has been described in detail elsewhere.(Carey *et al*, 2004;

Heaton *et al*, 2010) Briefly, the battery covers seven neurocognitive domains commonly affected by HIV: verbal fluency, executive functioning, processing speed, learning, delayed recall, attention/working memory, and motor skills.(Heaton *et al*, 2010) Demographically uncorrected scaled scores were converted to T scores (Mean= 50; Standard Deviation = 10) that corrected for the effects of age, education, sex, and race/ethnicity on neurocognition.(Heaton *et al*; Norman *et al*, 2011) In cases where participants had previous study visits with neuropsychological testing, adjustment for practice effects was made by using median practice effect data from previous work.(Cysique *et al*, 2011) A global T score was computed by average of the individual T scores for each test.

Laboratory investigation: For plasma/CSF HIV RNA concentrations by conventional testing, the Roche Amplicor platform was used (lowest level of quantification 50 copies/ml). Low level HIV-1 RNA was measured in CSF and plasma with the single copy assay (SCA, bioMONTR Labs Research Triangle Park, NC, USA) that has been validated and used in other CSF studies.(Santos *et al*, 2013) This method is based on a proprietary protocol which is used in conjunction with a commercial HIV-1 RNA easyQ reagent kit (bioMerieux Inc, Lyon, France). Briefly, a specimen of up to 2 ml of human plasma or CSF is added to lysis buffer containing guanidine thiocyanate. HIV-1 RNA is extracted in combination with the easyMAG platform (bioMerieux, Inc). Eluates containing HIV-1 RNA are aliquoted into 0.5 mL reaction tubes and amplified using 3 enzymes: T7 RNA polymerase, avian myeloblastosis virus reverse transcriptase, and RNase H. Molecular beacons targeting the pol/gag region of HIV-1 RNA are used for amplification and detection by isothermal reactions at 41^{o}C. HIV-1 RNA level is quantified in conjunction with the NucliSENS easyQ HIV-1 v2.0 Director software and a proprietary algorithm developed by bioMONTR Labs. The dynamic range of this HIV-1 assay is 1–5,000,000 copies/mL. sCD30 was measured by enzyme-linked immunosorbent assay (ELISA, Thermo Fisher). CXCL10 was measured using single molecule digital ELISA (Quanterix corporation). Two neuronal markers were selected. The first, neurofilament light chain (NFL), is

a major structural component of axons and thus a marker of neuronal injury. CSF concentrations of NFL are elevated in the setting of HIV-associated dementia.(Abdulle *et al*, 2007) Neuron-specific enolase (NSE) is the dominant enolase isoenzyme found in neuronal and neuroendocrine tissue. CSF concentrations of NSE increase in the setting of disease states such as seizures.(Correale *et al*, 1998) These two neuronal markers were also measured with single molecule digital ELISA (Quanterix corporation).

Statistical analysis: Statistical analyses were performed with SAS JMP version 13 as well as Graphpad Prism 6. Normality of continuous variables was assessed with the Shapiro-Wilk test. Based on lack of normality in most instances, continuous variables were compared using Wilcoxon rank sum test (with the exception of NP T scores, which were compared with t tests). Also due to lack of normality, Spearman's rho was calculated for correlations. Fisher's exact test was used to evaluate categorical variables. For the outcome of detectable CSF HIV RNA by SCA, univariate logistic regression was first performed with each of the following variables: age, sex, nadir CD4+, current CD4+, months on current ART, CSF total protein, detectable plasma HIV RNA by SCA, CSF WBC, CSF sCD30, and CSF CXCL10. Multivariate logistic regression was then performed incorporating variables associated with detectable CSF HIV RNA in the univariate models with p value < 0.1. Linear regression was also performed for the Global T score variable with the same variables (except that age, sex, and race/ethnicity were not evaluated separately given that they are incorporated in T scores). P value of <0.05 was considered statistically significant for all results.

Results

Paired CSF/plasma samples from 66 participants who had sufficient sample volumes for the planned biomarkers were analyzed. The majority of these particular participant visits were between the years of 2005 and 2011. All participants were on three drug ART with the most common regimen being efavirenz/tenofovir/emtricitabine followed by

atazanavir/ritonavir/tenofovir/emtricitabine. As seen in Table 1, participants were mostly male with median current CD4+ of 483 cells/microliter and median nadir CD4+ of 98 cells/microliter. 33 participants out of 66 (50%) had positive plasma HIV RNA by SCA, while 12 out of 66 (18.2%) had detectable CSF HIV RNA (CSFHIV+) by SCA. Median CSF HIV RNA SCA value in this subset of 12 participants was 6.5 copies/ml (interquartile range 1-14).

There were no significant differences between CSFHIV+ and CSFHIVnegative in terms of age, race, sex, current CD4+, CD4+ nadir, estimated duration of HIV infection or months on current ART regimen. However, CSFHIV+ participants were more likely to be plasmaHIV+ than CSFHIVnegative participants (83% versus 43%, p= 0.023). Additionally, CSFHIV+ participants had higher CSF CXCL10 concentrations (median 514 picograms/milliliter versus median 317 picograms/milliliter, p= 0.019, see Figure 1a with Cohen's D result) and higher CSF WBC concentrations (median 4 cells/microliter versus median 2 cells/microliter, p= 0.035). There were no concentration differences for NFL, NSE, or sCD30 (see Figure 1b with Cohen's D result for sCD30) between CSFHIVnegative and CSFHIV+ groups. Between the four CSF biomarkers, there were significant positive correlations between sCD30 and NFL (rho=0.31, p=0.012), between NFL and NSE (rho=0.28, p=0.024), and between NFL and CXCL10 (rho= 0.25, p= 0.041). There was a correlation trend between CXCL10 and sCD30 (rho= 0.23, p= 0.064).

For NP performance, 65 participants (98.5%) had full testing results available. There was no difference in global T score between the groups (mean= 47.7, standard deviation= 5.9 for CSFHIV+ group and mean= 46.0, standard deviation= 6.6 for CSFHIVnegative group, p= 0.43). For the univariate linear regression analysis with Global T score in the cohort as a whole (n=66) as the outcome, none of the following variables were associated significantly: nadir CD4+, current CD4+, months on current ART regimen, Plasma HIV RNA detectable by SCA, CSFHIV detectable by SCA, sCD30, CXCL10, NFL, and NSE (all p>0.1).

In the univariate logistic regression analyses with CSFHIVRNA+ as the outcome (top part of table 2), two variables were significantly associated with CSFHIVRNA+. Specifically, the

presence of plasma HIV RNA and higher CSF CXCL10 both had statistically significant positive odds ratios in relationship to CSFHIVRNA+. There was no significant difference in CSF CXCL10 concentrations based on plasma HIV RNA detectability (median 373 picograms/milliliter for plasma HIV+ versus median 313 picograms/milliliter for plasma HIV negative, p=0.135). In the multivariate model using these two variables, both remained significantly associated with CSFHIVRNA+ (see bottom part of table 2). The odds ratio associated with detectable HIV RNA by SCA was 5.91 (95% confidence interval [CI]= 1.13-30.98). The odds ratio per unit increase of CXCL10 was 1.003, which translates to an odds ratio of 1.4 (95% CI= 1.02-1.93) per CXCL10 unit increase of 100.

Discussion:

Despite suppressive ART, HIV persists and is associated with adverse clinical outcomes.(Coghill *et al*, 2017) There is great interest in developing a cure of HIV, which if achieved would obviate the need for lifetime ART with its associated costs and toxicities. There is significant evidence that the CNS is an HIV reservoir and a barrier to HIV cure. Such evidence includes the finding of HIV DNA in brain tissue despite ART(Lamers *et al*, 2016) as well as the presence of HIV DNA in CSF cells and low level CSF HIV RNA during virologic suppression.(Anderson *et al*, 2017; Spudich *et al*, 2019) However, HIV DNA and RNA from CSF is often not detectable even with very sensitive methods such as single copy assay.(Dahl *et al*, 2014) Therefore, more research is needed to identify more markers of HIV CNS persistence. Ideal CNS HIV reservoir markers would be consistently detectable and quantifiable. Such markers may reflect the size of the HIV CNS reservoir through quantification. Quantification of markers before and after an HIV cure intervention could potentially indicate how effective the intervention is in decreasing the HIV CNS reservoir.

In the current study, we examined two biomarkers that have shown promise as indicators of HIV in the CNS. CXCL10 is a chemokine induced by gamma interferon and is

produced by macrophages, astrocytes, and other cells.(Majumder *et al*, 1996) This chemokine is one of the first inflammatory markers to become elevated in the acute HIV infection period and thus its production is very sensitive to the presence of HIV.(Stacey *et al*, 2009) In the absence of virologic suppression, CSF CXCL10 concentrations correlate with CSF HIV RNA concentrations but not plasma HIV RNA concentrations, and therefore may be particularly valuable as an HIV CNS reservoir marker.(Cinque *et al*, 2005) Another potential marker of CNS HIV is soluble CD30 (sCD30). The CD30 molecule is part of the tumor necrosis factor family and in healthy individuals is only rarely expressed on lymphocytes.(Falini *et al*, 1995) In the case of PWH however, CD4+CD30+ cells are common and HIV is particularly enriched in these cells.(Hogan *et al*, 2018) In CSF, the soluble form of the receptor (sCD30) is elevated in HIV-infected individuals despite suppressive ART.(Peluso *et al*, 2020) Therefore, sCD30 is another candidate marker of HIV CNS persistence.

In the current study, we evaluated these two markers in relationship to the presence of low level CSF HIV by SCA. We found that while CSF sCD30 was not associated with CSF HIV detectability, higher CSF CXCL10 was associated with CSF HIV detectability in both univariate and multivariate analysis. The fact that there was no relationship between the presence of HIV RNA in plasma and CSF CXCL10 supports the hypothesis that our findings were most likely driven by CNS HIV as opposed to peripheral HIV. Further investigation is indicated on CSF CXCL10 as a CNS HIV marker. For example, it could be measured from PWH whose CSF cells were tested for the presence of HIV DNA or from pre-mortem CSF samples from PWH who then had post mortem brain tissue measurement of HIV DNA. The advantage to CSF CXCL10 compared to HIV nucleic acid tests is that it is readily detectable and quantifiable. Such a marker is potentially very valuable because concentrations could be measured before and after HIV cure interventions to better understand the effect of such interventions on the CNS HIV reservoir.

We acknowledge the limitations of the current study. The study population was majority men, and the inclusion of more women would have made the study more representative of PWH. The study was relatively small (n=66) and less than 20% of participants had detectable CSF HIV RNA by SCA. Therefore, it is possible that the absence of significant association between CSF sCD30 and the presence of CSF HIV was influenced by lack of statistical power. A larger study may have shown a significant association. Other biomarkers could also reflect low level HIV in the CNS and may merit further study as well. For example, another study of the presence of low level CSF HIV by SCA showed that neopterin, a marker of monocyte activation, was higher in individuals with detectable CSF HIV.(Dahl *et al*, 2014) It would be interesting to include even more markers in future studies to evaluate if a composite biomarker panel could be more reflective of HIV CNS persistence than individual biomarkers by themselves.

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Variable	All participants (n=66)	CSFHIV+ (n=12)	CSFHIVneg (n=54)	P value for difference
Age	45 (41-50)	42 (39-49)	46 (42-50)	0.15
Male Sex	60 (90.9%)	10 (83.3%)	50 (92.6%)	0.3
Race **White** **Black** **Other**	 35 (53%) 17 (25.8%) 14 (21.2%)	 8 (66.7%) 3 (25.0%) 1 (8.3%)	 27 (50%) 14 (25.9%) 13 (24.1%)	0.82
Estimated years with HIV	14 (8-18)	13 (9-16)	15 (7-18)	0.7
Current CD4+	483 (356-718)	476 (359-752)	500 (351-717)	0.98
Nadir CD4+	98 (21-231)	88 (34-259)	104 (14-226)	0.61
Months on current ART regimen	19 (9-36)	15 (5-23)	19 (10-37)	0.19
Proportion with plasma HIV+ RNA by SCA	33 (50%)	10 (83%)	23 (43%)	0.023*
CSF results **WBC[a]** **Protein[b]** **CXCL10[c]** **sCD30[d]** **NFL[c]** **NSE[c]**	 2 (1-4) 40 (33-51) 341 (218-541) 7.6 (4.2-11.6) 572 (407-706) 11039 (8805-14178)	 4 (2-7) 39 (34-55) 514 (353-800) 7.5 (4.2-10.0) 586 (433-669) 12094 (9780-13623)	 2 (1-3) 41 (32-51) 317 (213-480) 7.6 (4.1-12.4) 572 (402-732) 10918 (8716-14701)	 0.035* 0.99 0.019* 0.78 0.96 0.8

Table 1: Variables are reported as either median (interquartile range), mean [standard deviation] or number (%). *Denotes p value <0.05. a=measurement in cells/milliliter; b= measurement in milligrams/deciliter; c= measurements in picograms/milliliter; d= measurement in nanograms/milliliter.

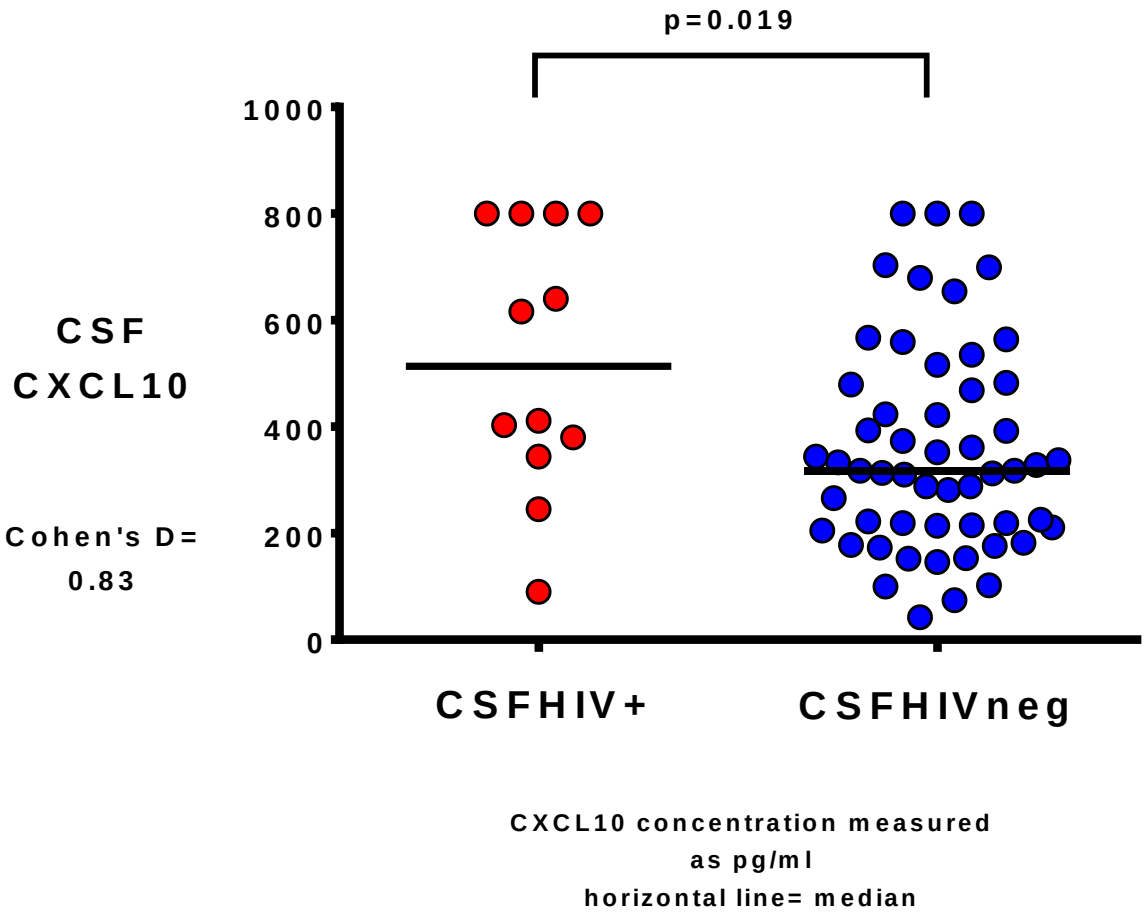

Figure 1: CSF CXCL10 concentrations based on CSF HIV RNA positivity. Pg= picograms; ml= milliliters

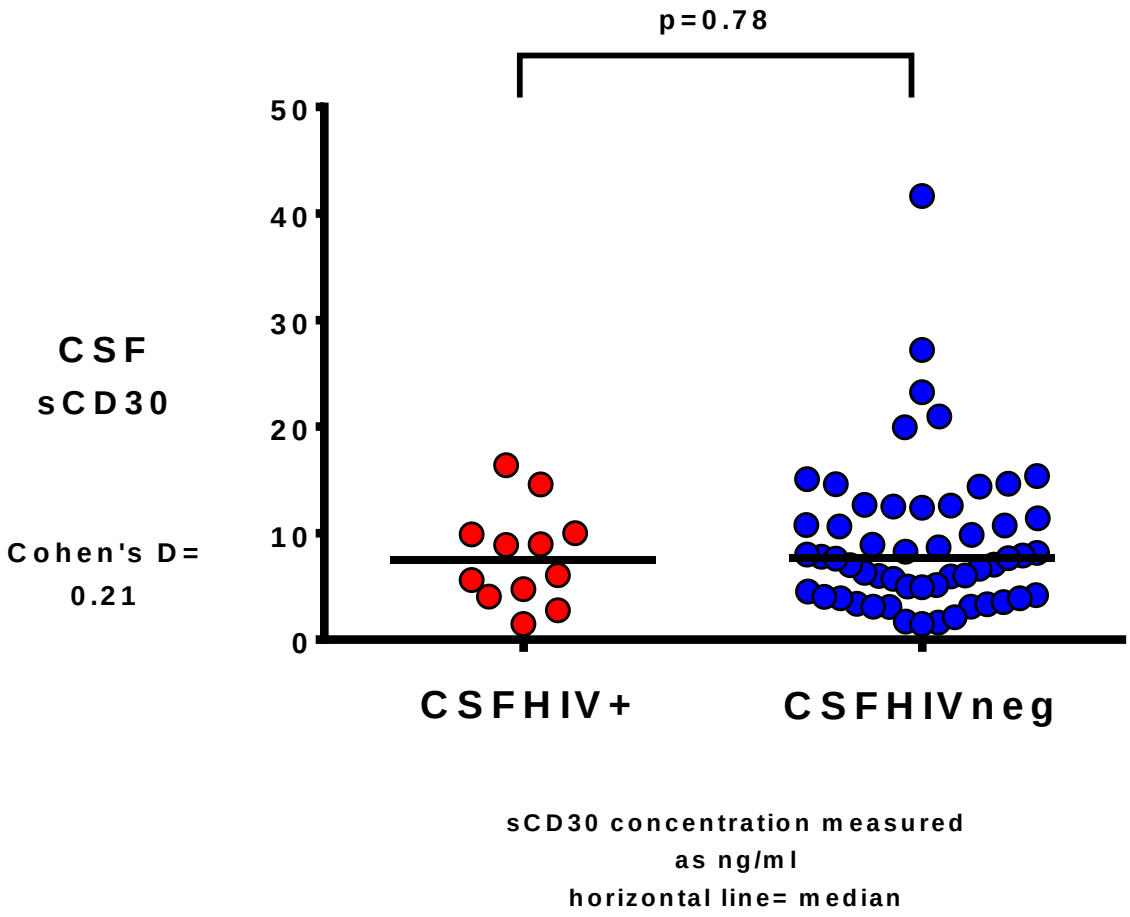

Figure 2: CSF sCD30 concentrations based on CSF HIV RNA positivity. Pg= picograms; ml= milliliters

Variable	Odds ratio	95% Confidence Interval	P value
Univariate Models			
Age per year increase	0.984	0.909-1.064	0.683
Male Sex	0.4	0.064-2.489	0.326
Current CD4+	1.0	0.997- 1.002	0.779
Nadir CD4+	1.0	0.995-1.007	0.766
Months on current ART	0.97	0.931-1.010	0.135
Plasma HIV RNA detectable by SCA	6.74	1.346-33.753	0.020*
CSF WBC	1.064	0.954-1.186	0.266
CSF total protein	0.996	0.957-1.035	0.822
CSF sCD30	0.964	0.861-1.078	0.517
CSF CXCL10	1.004	1.001-1.007	0.017*
Multivariate Model			
Plasma HIV RNA detectable by SCA	5.91	1.129-30.977	0.035*
CSF CXCL10	1.003	1.0002-1.007	0.038*

Table 2: Results of logistic regression models for the outcome of detectable CSF HIV RNA by SCA. Top part shows the results from univariate modeling, while bottom part shows the results for the multivariate model when univariables with $p<0.1$ only are included.