J. Neurovirol. (2014) 20:627–635
DOI 10.1007/s13365-014-0293-z

Impact of the HIV Tat C30C31S dicysteine substitution on neuropsychological function in patients with clade C disease

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Received: 30 June 2014 / Revised: 25 August 2014 / Accepted: 18 September 2014 / Published online: 1 November 2014

Abstract Previous animal studies have identified a C31S residue substitution in the C30C31 dicysteine motif of the Tat protein that is associated with reduced neurovirulence in clade C human immunodeficiency virus (HIV). However, clinical studies of patients infected with clade C HIV have reported significant levels of cognitive impairment. To date, no study has specifically examined cognitive function in clade C-infected patients as a function of the presence or absence of the Tat C31 substitution. The present study investigated the impact of the Tat C30C31S genetic substitution among individuals residing in South Africa infected with clade C HIV that either exhibited the C30C31 motif (n=128) or the C31S motif (n=46). A control group of seronegative individuals was included to examine the overall impact of HIV on cognitive performance. All individuals completed a comprehensive neuropsychological battery consisting of tests sensitive to HIV. Results revealed that clade C-infected individuals performed significantly worse across cognitive tests compared to seronegative controls. However, there were no significant differences in cognitive performances between individuals with the C31S motif versus those without the C31S substitution. Proximal CD4 cell count and plasma viral load were unrelated to cognitive performances for either group. Results confirm that the C31S dicysteine motif substitution of the Tat protein does not appreciably moderate neuropsychological outcomes in clade C. Further, these findings highlight the importance of clinical management of cognitive symptoms among individuals infected with this viral clade worldwide.

Keywords HIV · Clade C · Tat protein · C31S dicysteine motif · Cognitive performance

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Introduction

The human immunodeficiency virus (HIV) is comprised of multiple genetic clades that are distributed throughout the world (Hemelaar et al. 2006). Clade C accounts for more than half of the known infections worldwide, with high prevalence in South Africa, India, and some regions of Brazil and China where large populations of infected patients reside (Wainberg 2004). Clade B is most common in North America and parts of Brazil (Gilbert et al. 2007; Thomson and Nájera 2005), whereas subtypes A and D are common in North Africa, and clade A/E is most common in Southeast Asia (Hemelaar et al. 2006). Recent studies have revealed that the frequency of clade recombination and the presence of multiple subtypes

within a region are becoming increasingly more common (Siemieniuk et al. 2012).

A number of previous investigations have identified functional differences between the HIV subtypes that are driven by unique biological properties. Specifically, significant differences in disease progression, replication rates, and resistance to treatment have been identified across clades (Constantino et al. 2011; for review, see Santoro and Perno 2013). Less consistent results have been reported regarding clade-specific outcomes associated with brain integrity among infected patients (Rao et al. 2008), particularly in the comparison between clade B and clade C (Ortega et al. 2013).

The neuropsychological deficits associated with clade B HIV have been well documented. Patients typically exhibit a "subcortical" pattern of impairment characterized by reduced verbal learning, psychomotor slowing, reduced information processing speed, and reduced motor speed (Basso and Bornstein 2003; Becker et al. 1997; Heaton et al. 1995; Marcotte et al. 2003; Martin et al. 1992). These deficits correlate with abnormalities in subcortical brain regions including the basal ganglia and subcortical white matter as well as cortical gray matter (Navia 1997). By contrast, early clinical reports of cognitive outcomes related to clade C identified very low rates of dementia among infected individuals in India versus individuals infected with clade B disease in the North America (Teja et al. 2005). The lower frequency of HIV-associated dementia (HAD) in India was interpreted as evidence that clade C HIV is less neurovirulent than clade B disease (Riedel et al. 2006).

Further evidence in support of reduced neurovirulence in clade C was provided by the recognition of a natural polymorphism in the C30C31 dicysteine motif of the Tat protein that was highly conserved across samples from India (Ranga et al. 2004). The C31S substitution resulted in reduced monocyte chemotaxis, and was referenced as a potential biological explanation for the lower incidence of HAD reported in clade C (Ranga et al. 2004). Numerous subsequent laboratory studies revealed reduced astrogliosis (Gandhi et al. 2009), reduced activation of proinflammatory cytokines (Gandhi et al. 2009), decreased augmentation of tumor necrosis factor (TNF; Campbell et al. 2007), and less severe cognitive impairment in mice with the C31S substitution (Rao et al. 2008; Rao et al. 2013). Further, work by Li et al. (2008) revealed that the C30C31 motif is directly related to the NMDA-induced excitotoxicity, and the Tat C30C31S substitution resulted in reduced NMDA-mediated activity. Collectively, these studies provided initial evidence of reduced neuropathogenesis in clade C secondary to the C31S substitution.

By contrast, a number of studies utilizing formal cognitive testing protocols have demonstrated significant cognitive abnormalities among individuals infected with clade C HIV without consideration of Tat status. Yepthomi et al. (2006) administered a standardized battery of cognitive tests to a sample of patients residing in southern India and revealed significant impairments on tests of verbal and visual learning, motor speed, information processing speed, and response fluency. Additional research utilizing a larger independent sample in India revealed nearly identical results (Gupta et al. 2007). Further, recent work from South Africa where clade C is dominant suggests HIV-associated dementia (HAD) rates exceed 30 % among untreated individuals (Joska et al. 2011), and significant cognitive deficits across cognitive domains mirror the pattern of impairments common in clade B virus (Joska et al. 2012). Neuroimaging studies of HIV-infected adolescents and adults in South Africa (Heaps et al. 2012) also reveal abnormalities in the cortical gray and subcortical brain regions, suggesting that despite the reported presence of the Tat C31S substitution, individuals infected with clade C HIV exhibit significant brain abnormalities. Interestingly, Rao et al. (2013) recently reported that the C31S motif substitution of the Tat protein is not conserved ubiquitously in South Africa. These results help to explain the high level of cognitive impairment and neuroimaging abnormalities in South Africa but the findings do not explain the high rate of cognitive impairment previously reported in India where the C31S substitution is present in more than 90% of cases (Ranga et al. 2004).

To date, no study has directly examined cognitive status among individuals based on the presence or absence of the C31S substitution within the same cohort. The lack of complete conservation of the C30C31 substitution in South Africa as described by Rao et al. (2013) provides a unique opportunity to address this issue. The present study examined this important question among patients infected with clade C inclusive with or without the C31S Tat substitution.

Methods

Participants

A total of 224 Xhosa-speaking individuals were enrolled in the study including 174 seropositive individuals (C30C31 motif $n=128$, C31S motif $n=46$) and 50 seronegative individuals similar in ethnicity, age, language, and education. Inclusion criteria included the following: (1) age between the years of 18 and 45—this age band was selected to avoid age-related central nervous system (CNS) abnormalities; (2) Xhosa as the primary language; (3) HIV serostatus documented by ELISA and confirmed by Western blot (for the HIV patients), plasma HIV RNA, or a second antibody test for the HIV patient groups; (4) CDC stage B or C; (5) enrollment within 3 months of initiation of antiretroviral therapy (ART); (6) at least 5 years of formal education. Exclusion criteria included the following: (1) any major axis I psychiatric condition that could significantly affect cognitive status (e.g.,

schizophrenia or bipolar disorder); (2) confounding neurological disorders including multiple sclerosis and other CNS conditions; (3) head injury with loss of consciousness greater than 30 min; (4) clinical evidence of opportunistic CNS infections (toxoplasmosis, progressive multifocal leukoencephalopathy, neoplasms); (5) current substance abuse or alcohol abuse as defined by structure interview.

Subjects were recruited from primary care HIV clinics in Cape Town, South Africa. Patients who were in the pretreatment counseling phase were identified from clinic records. Interested participants completed a comprehensive consent process followed by a detailed medical and demographic history. HIV-positive subjects subsequently initiated treatment within 3 months of participation in the present study. Study participation was voluntary and individuals were free to withdraw from the study at any point. Subjects received compensation for transportation costs at each assessment period. Study procedures were approved by local University IRB committees.

Healthy controls were recruited from regional Voluntary Counseling and Testing Clinics in Cape Town, South Africa. Inclusion/exclusion criteria matched the criteria for the seropositive group with the exception that all individuals in the healthy control group were required to have a laboratory-confirmed seronegative status. Recruitment from these sites served to minimize differences in key demographic variables between the two groups (e.g., language, socioeconomic status).

Mood assessment

All participants completed the Centers for Epidemiologic Studies Depression Scale-Revised (CES-D; Radloff 1977). The CES-D is a 20-item self-report measure of depression that is widely utilized in the HIV literature. Total score served as the dependent variable. As self-reported depression was an exclusion criterion for healthy controls, the CES-D was administered only to the HIV sample. The total score was utilized as a dependent measure for statistical analyses.

HIV-1 viral load and CD4+ cell counts

EDTA blood samples were collected at the time of enrollment in the study and plasma and cell aliquots were stored at −70 °C. RNA was isolated from patient samples using the Abbott RealTime HIV-1 amplification reagent kit, according to the manufacturer's instructions. Viral load was determined using the Abbott m2000sp and the Abbott m2000rt analysers (Abbott laboratories, Abbott Park, IL, USA). For CD4 counts, analyses of cells from fresh blood samples were completed on the FACSCalibur flow cytometer in conjunction with the MultiSET V1.1.2 software (BD Biosciences, San Jose, CA, USA).

PCR amplification and sequencing of the tat exon 1 region

We amplified the tat exon 1 region (HXB2 position 5831–6045) by polymerase chain reaction (PCR) using the Promega GoTaq Flexi Kit (Promega, Madison, WI) according to the manufacturer's instructions. The primer pair, TAT-1_OF (5′-AAAGCCACCTYTGCCTAG)/TAT-1_OR (5′-CTCATTGC CACTGTCTTCTGC), and TAT-1_IF (5′-GTAGARGA TMGATGGAACRA)/TAT-1_IR (5′-CYCTAATTCTTTYA AYTAACC) were used for prenested and nested PCR respectively. Both prenested and nested amplification reactions were held at 94 °C for 2 min, followed by 40 cycles of denaturing (94 °C; 30 s), annealing (55 °C; 30 s), and extension (72 °C; 1 min). This was followed by a final extension step of 7 min at 72 °C. The PCR product was kept at 4 °C until visualized using agarose gel electrophoresis.

To purify the PCR products, single-stranded DNA and diphosphates were degraded using exonuclease 1 (Exo1) and shrimp alkaline phosphatase (SAP) (Amersham Pharmacia Biotech., NJ), respectively. All PCR products were sequenced on both strands using the BigDye Terminator Cycle Sequencing Ready Reaction Kit and analyzed on an ABI Prism 3130×l automated DNA sequencer (Applied Biosystems, Foster City, CA). Sequences were analyzed and the overlapping DNA fragments were assembled using Sequencher version 4.8 (Gene Codes Corporation, Ann Arbor, MI). Nucleotide sequences were translated into amino acid sequences and the C30C31 motif or C31S mutation for each patient was noted. The tat exon 1 subtype was determined using online subtyping tools COMET (http://comet.retrovirology.lu/) and jpHMM (http://jphmm.gobics.de/).

Neuropsychological evaluation

A battery of cognitive tests sensitive to deficits associated with HIV was administered to each group. Testing was completed by a highly trained research technician fluent in Xhosa and English. These tests have been utilized by our group in international studies in India and South Africa, with significant attention to cultural relevancy for language items.

Cognitive tests were administered to assess three domains of function. Each test was administered according to standard procedures. *Learning*: Verbal learning was tested with the Hopkins Verbal Learning Test-Revised (HVLT-R; Brandt and Benedict 2001). We replaced gemstones with vegetables on the list-learning task to ensure cultural relevancy. Visual learning was examined with the Brief Visual Memory Test-Revised (Benedict et al. 1996). Total correct on the learning trials were included as the dependent variables both the HVLT-R and BVMT-R. *Executive functions/visuospatial*: (1) Color Trails 2 (D'Elia et al. 1996), (2) verbal fluency (fruits and animals), and (3) Block Design from the WAIS-IV (Wechsler 1997). The dependent variable for Color Trails 2

was time to completion, and total correct served as the dependent variables for the letter fluency and Block Design. *Psychomotor speed*: (1) Color Trails 1 (D'Elia et al. 1996), (2) Grooved Pegboard Test (GPT; Klove 1963) nondominant hand, (3) Trail Making Test A (Reitan 1955), (4) Digit Symbol (Wechsler 2008), and (5) Symbol Search (Wechsler 2008). Time to completion was the dependent variable for Color Trails 1, Trail Making Test A, and GPT, and total correct was the dependent variable for Symbol Search and Digit Symbol.

Statistical analyses

Following descriptive analyses, most hypotheses were addressed using multivariate analysis of covariance (MANCOVA). Before MANCOVAs were fitted, each pair of outcome variables was combined in the same model to ensure the relationship was approximately linear and not redundant (e.g., less than $r=0.6$). Similar MANCOVA models were fitted to compare HIV-positive individuals with and without the C31S defect and to test whether viral factors and immune status significantly predicted each multivariate neuropsychological construct.

Results

HIV-seropositive versus seronegative individuals

HIV-seropositive individuals differed significantly from seronegative individuals on average years of age ($t=6.42$, $p<0.001$, $\beta=5.42$, SE=0.84), years of education ($t=2.50$, $p=0.013$, $\beta=0.60$, SE=0.24), and sex ($\chi^2(1)=94.05$, $p<0.001$). The means (and percentage female) for each group are provided in Table 1. The magnitude of the group difference in education was less than 1 year and therefore not of significant magnitude to include as a covariate in subsequent analyses. By contrast, age and gender were included as covariates in the subsequent MANCOVA models. Sample sizes for the models below differed slightly from those given in Table 1 because model-specific listwise deletion eliminated a small number of cases from inclusion in particular models.

Age and gender were used as covariates in the learning domain ($n=224$), and results of the MANCOVA revealed a significant difference on the multivariate outcome variable between HIV-seropositive and seronegative groups (Wilk's $\Lambda=0.90$, $F(4217)=12.04$, $p<0.001$) (Table 2). Univariate analyses revealed that HIV-seropositive individuals performed significantly more poorly on both the HVLT-R ($F(1220)=7.83$, $p<0.01$) and the BVMT-R total learning ($F(1200)=21.45$, $p<0.01$).

For the executive function domain ($n=219$), results of the MANCOVA revealed a significant difference on the multivariate outcome variable between HIV-seropositive and seronegative groups (Wilk's $\Lambda=0.89$, $F(3217)=8.96$, $p<0.001$). Univariate analyses revealed that HIV-seropositive individuals performed significantly more poorly on all measures of executive function: Color Trails 2 ($F(1219)=10.45$, $p<0.01$), verbal fluency ($F(1219)=14.46$, $p<0.001$), and Block Design ($F(1219)=15.80$, $p<0.001$).

Group contrasts for the psychomotor speed domain ($n=219$) also revealed a significant difference on the multivariate outcome variable between HIV-seropositive and seronegative groups (Wilk's $\Lambda=0.73$, $F(5211)=27.57$, $p<0.001$, $\eta^2=0.395$). Univariate analyses revealed that HIV-seropositive individuals performed significantly more poorly on the Trail Making Test A ($F(1215)=123.5$, $p<0.001$) and Color Trails 1 ($F(1215)=7.33$, $p<0.01$) compared to seronegative controls (Fig. 1). There were no significant differences between groups on the measures of GPT ($F(1,215)=0.68$, $p=0.41$), Symbol Search ($F(1215)=0.03$, $p=0.88$) or Digit Symbol ($F(1215)=0.69$, $p=0.41$).

C30C31S Tat comparison

Among HIV-seropositive individuals for whom data were available pertaining to the presence of the Tat C31S substitution ($n=179$), there were no significant differences between those with and without the substitution with respect to average years of age ($t=0.79$, $p=0.427$, $\beta=0.66$, SE=0.93), years of education ($t=1.47$, $p=0.144$, $\beta=0.37$, SE=0.27), and sex ($\chi^2(1)=0.81$, $p=0.369$). The means (and percentage female) for each group are given in Table 1. Additionally, the Tat C31C and C31S groups did not differ significantly in terms of CD4 count ($t=-1.68$, $p=0.096$, SE=24.47), Log_{10} viral load ($t=0.331$, $p=0.741$, SE=0.179), or time since diagnosis ($t=0.394$, $p=0.694$, SE=4.47).

Multivariate contrasts revealed no significant differences between the two HIV groups based on Tat status in regards to learning ($n=171$), (Wilk's $\Lambda=0.99$, $F(4171)=0.42$, $p=0.797$), executive function ($n=175$), (Wilk's $\Lambda=0.99$, $F(3171)=0.43$, $p=0.73$), or psychomotor speed ($n=171$), (Wilk's $\Lambda=0.95$, $F(5165)=1.79$, $p=0.118$), domains (Fig. 2).

Relation between CD4, viral load, depression scores, and neuropsychological outcomes

Neither CD4 count nor viral load ($n=171$) was associated with any of the neuropsychological measures. Considering only the HIV-positive group, the C31C group had a significantly higher mean score on the CES-D ($n=118$ $m=5.81$ sd=5.81) compared to the C31S group ($n=42$ $m=4.79$ sd=3.65). However, the scores for both groups did not meet the cutoff to identify clinical depression in any of the participants.

Table 1 Subject characteristics

	HIV+ (n=201)	HIV− (n=50)	p value
Age (years), M (SD)	31.44 (5.44)	26.02 (4.93)	0.000*
Education (years), M (SD)	10.24 (1.55)	10.84 (1.33)	0.013
Sex, % female	81 %	46 %	0.000*
HIV+ TAT defect (n=46)		HIV+ no TAT defect (n=128)	
Age (years), M (SD)	31.17 (5.46)	31.79 (5.47)	0.427
Education (years), M (SD)	9.98 (1.44)	10.35 (1.61)	0.144
Sex, % female	76.60 %	82.60 %	0.369
LogVL M (IQR)	4.22 (3.46–4.99)	4.16 (3.40–4.86)	0.741
CD4 mmHg M (IQR)	260 (170–454)	219 (115–315)	0.096
Time since diagnosis (months) M (SD)	11.69 (21)	13.46 (25)	0.694

*p<0.001, statistically significant

Discussion

The present study is the first direct examination of the C30C31S Tat substitution on cognitive performance among individuals infected with clade C HIV. Results from the present study suggest that the C31S substitution does not confer decreased risk of cognitive impairment among seropositive individuals. HIV-seropositive individuals with and without the Tat dicysteine motif substitution exhibited significant impairments in cognitive performance when compared to seronegative individuals. While the group with the intact motif performed more poorly on tests of psychomotor speed, the overall group differences were not significantly different. Further, neither CD4 cell count nor viral load covaried with cognitive status in either group of HIV patients.

Results from the present study are consistent with the outcomes of studies revealing substantial cognitive impairment in patients residing in India, where the C31S substitution is reportedly dominant (Gupta et al. 2007; Yepthomi et al. 2006), and also South Africa (Ortega et al. 2013), where the C31S substitution is present but with reduced frequency (Rao et al. 2013). Results from studies in both countries revealed significant impairments among HIV-positive individuals on tests of information processing speed, executive function, verbal and visual learning, and memory (Gupta et al. 2007; Ortega et al. 2013; Yepthomi et al. 2006). Recent work conducted in Brazil where distinct populations of clade B and clade C exist supports these findings. De Almeida et al. (2013) reported a similar rate of cognitive impairment in patients with clade B compared to clade C when individuals completed a comprehensive assessment of cognitive function. The diagnostic frequency of mild to moderate cognitive impairment was nearly identical between clades, with 50 % of clade B individuals and 48 % of clade C individuals meeting

Table 2 HIV+ and HIV− neuropsychological performance

	HIV+ Mean (SD)	HIV− Mean (SD)	p value	η^2
Learning				
HVLT-R	23.06 (3.99)	24.88 (3.78)	0.01*	0.03
BVMT-R	12.95 (7.58)	21.16 (6.53)	0.00**	0.09
Executive function/visuospatial				
Color Trails 2	155.54 (73.98)	113.63 (39.42)	0.001*	0.05
Verbal fluency	14.18 (3.79)	16.84 (4.04)	0.00**	0.06
Block Design	10.95 (6.79)	17.49 (8.87)	0.000**	0.07
Psychomotor speed				
Grooved Pegboard	74.80 (19.55)	68.75 (7.82)	0.41	0.00
Trail Making Test A	123.20 (45.47)	38.02 (12.62)	0.00**	0.37
Digit Symbol	37.71 (12.91)	41.12 (12.27)	0.41	0.00
Color Trails 1	68.18 (25.37)	53.55 (15.79)	0.00*	0.03
Symbol Search	19.44 (6.09)	20.80 (5.66)	0.88	0.00

*p<0.01, statistically significant; **p<0.001, statistically significant

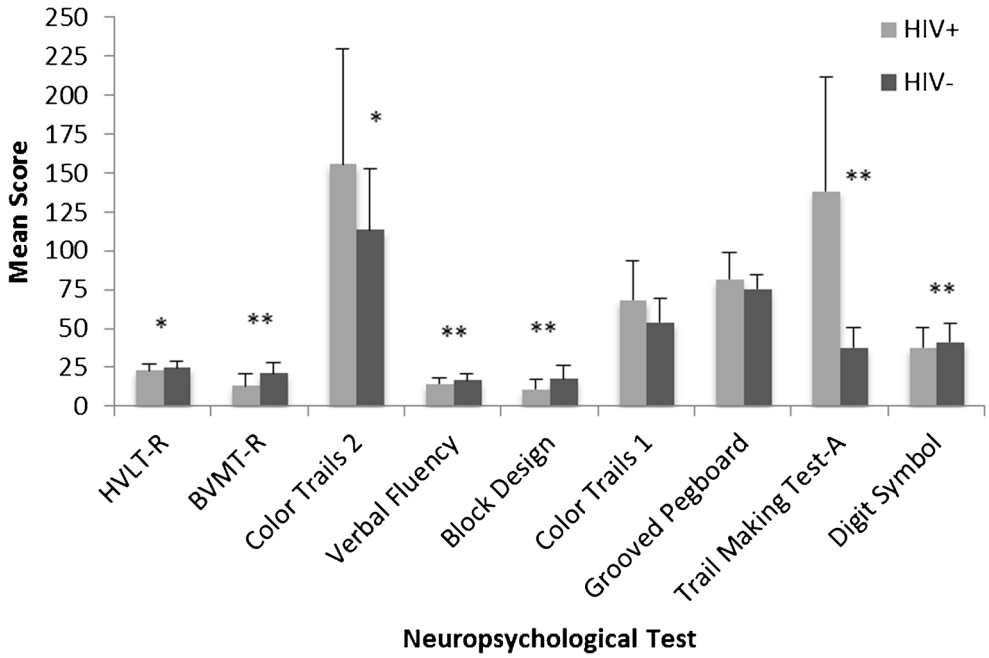

Fig 1 HIV+ and HIV− neuropsychological performance. *HVLT-R* Hopkins Verbal Learning Test-Revised, *BVMT-R* Brief Visuospatial Memory Test-Revised. *$p<0.01$, statistically significant; **$p<0.001$, statistically significant

criteria for asymptomatic neurocognitive impairment. Interestingly, the frequency of HAD was greater in clade B than C, albeit the difference did not reach statistical significance (de Almeida et al. 2013). The neuropsychological patterns of deficits observed in the present study and in previous studies suggest that clade C is neurotoxic regardless of the Tat C30C31 dicysteine motif.

A similar conclusion regarding the neurological impact of the C31S substitution in patients with clade C HIV can be drawn from neuroimaging studies. Data from South Africa have revealed significant reductions in total white matter volume, total gray matter volume, and thalamic volume compared to seronegative healthy controls as defined by structural magnetic resonance imaging (MRI; Heaps et al. 2012; Ortega

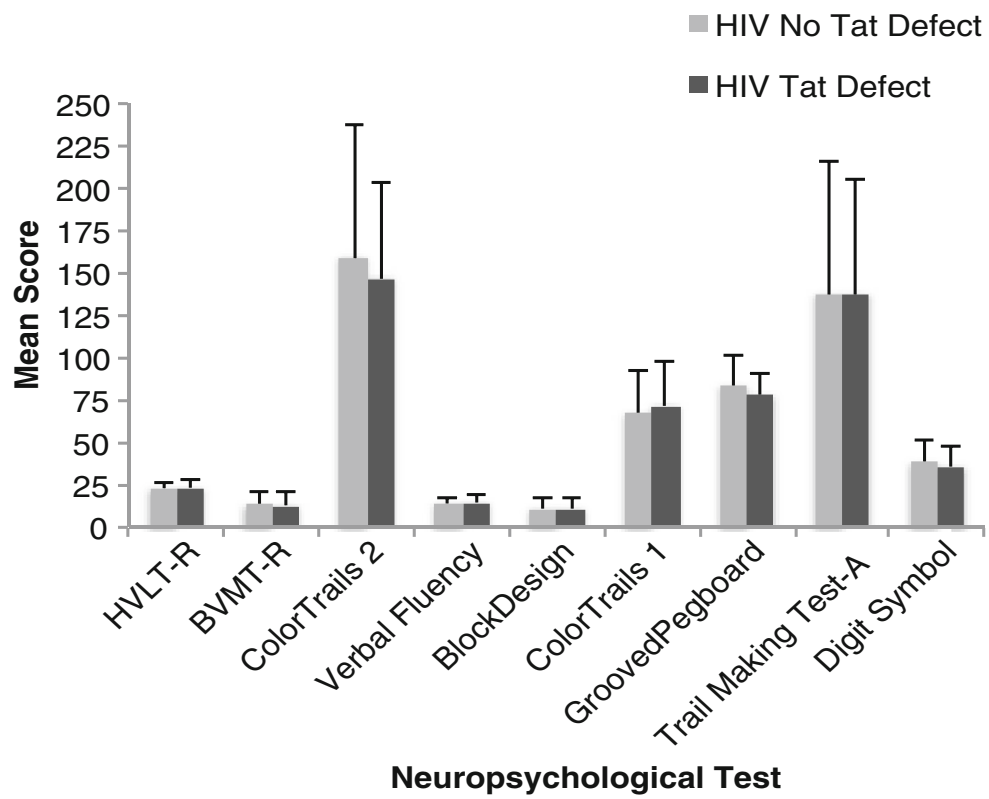

Fig 2 HIV Tat status—neuropsychological performance. *HVLT-R* Hopkins Verbal Learning Test-Revised, *BVMT-R* Brief Visuospatial Memory Test-Revised

et al. 2013). It is of note that caudate atrophy, which has commonly been reported in HIV clade B (Ances et al. 2006; Ances et al. 2012; Chiang et al. 2007; Harezlak et al. 2011), has not been observed among individuals with clade C, raising the possibility that the viral impact of clade C differentially affects brain systems. However, the literature is inconsistent regarding volumetric differences in HIV, particularly in mild to moderate stages of disease severity with some studies showing no caudate atrophy (Ortega et al. 2013) and other studies demonstrating hypertrophy of the putamen (Castelo et al. 2007). Studies that have directly compared brain integrity between clade B and clade C have also reported no significant differences between groups. For example, a MRI brain volumetric study conducted by our group revealed nearly identical outcomes in terms of reduced brain volumes across individuals infected with clade C in South Africa and individuals with clade B in the USA (Ortega et al. 2013). The similar neuroimaging signatures of HIV between the two clades provide some confidence that cultural factors or concerns with test validity artificially amplified the magnitude of deficits on cognitive tests among individuals with clade C HIV previous studies conducted in South Africa. These results provide objective evidence of brain disruption in the context of clade C HIV.

Overall, the results obtained to date suggest that altered chemotactic properties of the C30C31S Tat dicysteine motif do not translate into measureable differences in brain dysfunction among patients. It is possible that viral factors unrelated to the C30C31 motif underlie the expression of cognitive impairments in this population. Tilghman et al. (2014) recently reported that specific sequences of HIV Tat generated from blood plasma of individuals with clade C HIV in India identified signature residues unique to cognitively impaired participants. Specifically, exon 1 of Tat at codons 29 (arginine) and 68 (proline) covaried with severity of neuropsychological impairment, and the effects remained after correcting for low CD4 count (Tilghman et al. 2014). Further, increased levels of interleukin (IL)-1 alpha, IL-6, TNF-alpha, beta(2)-microglobulin, and neopterin in the cerebrospinal fluid have been reported among neurologically symptomatic individuals infected with the clade C virus (Gandhi et al. 2009). These findings reinforce the concept that clade C HIV exhibits neurotoxic properties independent of the NMDA-mediated excitotoxicity previously linked to Tat (Li et al. 2008).

The animal and laboratory studies clearly define a dose-response curve demonstrating most significant neurotoxicity from clade B followed by clade C (Rao et al. 2013). It is possible that clade C is less neurovirulent in highly sensitive assays, yet the virus remains sufficiently pathogenic to produce both cognitive and neuroimaging abnormalities among infected patients. As such, while the dose-response curve can be observed in animal and laboratory settings, the degree of differentiation is not of sufficient magnitude to determine unique behavioral and neuroimaging outcomes in patient populations. This position is supported by the similar neuropsychological outcomes in Brazil (de Alemeida et al. 2013) and the similar neuroimaging outcomes in South Africa and the USA among individuals with clade C compared to clade B (Ortega et al. 2013).

Some limitations of the current study merit discussion. First, the study did not include laboratory measures of Tat neurotoxicity. Future studies are needed to determine if the chemotactic properties and reduced excitotoxicity associated with clade C are present among individuals with the C30C31S substitution. The lack of group differences on the cognitive tests suggests these laboratory factors would have limited explanatory power, though confirmation in future studies would be beneficial. Second, the current study focused only on the C30C31 dicysteine motif and it is possible that significant new information would be gained from sequencing the complete genome of the virus. As demonstrated in the recent study revealing increased cognitive impairment associated with specific codons of Tat in clade C (de Almeida et al. 2013), additional sequencing studies may elucidate mechanisms of neuropathogenesis across all clades of HIV. It is also worth noting that HIV-positive individuals had initiated antiretroviral treatment within 3 months of study participation. It is possible that the highly dynamic period in the brain following initial treatment for HIV masked group differences due to Tat. This can be explored in future studies by examining individuals on stable treatment containing a protease inhibitor as recent work has revealed that Tat secretion is not blocked in this treatment context (Mediouni et al. 2012). Since protease inhibitors are not included as frontline treatment in South Africa, this could not be readily explored in the current cohort. Evidence that the HIV-positive individuals in the current study were within one or two standard deviations from the healthy controls on the cognitive tests suggests that pretreatment status did not result in an artificially low ceiling on cognitive tests due to uncontrolled methodological factors. Nevertheless, we recommend further studies among individuals on stable treatment to explore this issue further. Finally, the study is cross-sectional in nature and it will be important to understand the long-term outcome of individuals infected with clade C who do and do not exhibit the C31 substitution. Interestingly, Gopukumar et al. (2008) reported no significant decline in cognitive function over a two-year and one half-year period among individuals infected with clade C HIV residing in India. Since the Tat C31 substitution is more conserved in this population than in South Africa (Rao et al. 2013), it is unclear how cognitive impairment progresses among individuals with and without the Tat substitution in South Africa and therefore, additional studies are needed to address this concern.

Overall, the results of this cross-sectional study suggest that individuals with clade C HIV exhibit significant cognitive

impairment relative to seronegative healthy controls. Further, the results indicate that individuals that express this polymorphism do not perform differently on cognitive tests compared to individuals that do express this polymorphism. Longitudinal studies are needed to more fully determine long-term outcomes related to Tat status. Nevertheless, these results highlight the importance of clinical care involving brain health and cognitive outcomes among individuals infected with the clade C strain of HIV similar to other viral clades.

Acknowledgments There are no actual or potential conflicts of interest for any of the authors on this manuscript. The excellent technical assistance of Graeme Jacobs and Shahieda Isaacs for PCR and gene sequencing of the tat exon 1 region is acknowledged. Funding was supported by the National Institute of Mental Health (MH085604). Dr. Stein is supported by the Medical Research Council of South Africa.

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