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Intracellular cystatin B levels are altered in HIV-infected participants with respect to neurocognitive status and anti-retroviral therapy

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Abstract

With advances in HIV treatment, people with HIV (PWH) are living longer but experience aging-related comorbidities, including cognitive deficits, at higher rates than the general population. Previous studies have shown alterations in lysosomal proteins in blood from PWH with severe dementia. However, these markers have not been evaluated in PWH with milder neurocognitive impairment. We sought to determine if levels of the lysosomal cysteine protease cathepsin B (CatB), and its endogenous inhibitor cystatin B (CysB) were altered in PWH with neurocognitive impairment, and if antiretroviral therapy (ART) further influenced these levels.

Peripheral blood mononuclear cells (PBMCs) were obtained from the tenofovir arm of a multi-center clinical trial in which ART-naïve, HIV+ participants received treatment for 48 weeks (ACTG A5303, NCT01400412). PWH were divided by neurocognitive status (e.g., with or without neurocognitive impairment) prior to ART initiation. Intracellular levels of CatB and CysB were measured in T-cells and monocytes via flow cytometry.

Levels of CysB were significantly decreased in both CD4+ T-cells and CD8+ T-cells after 48 weeks of ART in HIV+ participants without neurocognitive impairment, but not in participants with neurocognitive impairment. Levels of CysB were increased in CD14+ monocytes from the participants with neurocognitive impairment post-ART. Levels of CysB and CatB were positively correlated regardless of HIV, neurocognitive status, or exposure to ART.

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These findings suggest CysB has potential to provide mechanistic insight into HAND or provide a molecular target for systemic monitoring or treatment of neurocognitive impairment in the context of ART and should be investigated further.

Keywords

cathepsin B; cystatin B; HAND; lysosome; T cell

Introduction

With advances in antiretroviral therapy (ART), people with HIV (PWH) now have life expectancies similar to the general population [1]. However, PWH experience aging-related comorbidities earlier and at higher rates than the general population. By age 50, almost 90% of PWH have at least one comorbidity versus 65% of seronegative individuals [2], partially attributed to accelerated aging. Neurocognitive function is often impacted, leading to HIV-associated neurocognitive disorders (HAND). HAND severity is assessed via neurocognitive function tests and ranges from asymptomatic impairment to HIV-associated dementia (HAD). In the early HIV epidemic, many patients presented with HAD, an AIDS-defining condition. Currently, HAD is less prevalent, due largely to the success of ART, yet many still present with milder neurocognitive deficits that compromise functional, independent living [3]. HAND currently affects an estimated 30–50% of PWH despite ART [4], underscoring the importance of identifying peripheral biomarkers to identify those at risk, monitor disease pathogenesis and progression, and delineate useful interventions.

Lysosomes are membrane-bound organelles containing over 50 different hydrolases, which function optimally at low pH to degrade macromolecules and organelles, in turn maintaining intracellular energy balance and organellar homeostasis. Cathepsin B (CatB) represents a class of lysosomal cysteine proteases, which are inhibited endogenously through interaction with cystatin proteins, such as cystatin B (CysB) [5]. Lysosome dysfunction is evidenced in normal aging as well as in age-related neurodegenerative diseases [6]. For example, alterations in lysosomal enzymes have been documented in murine models of Alzheimer's disease (AD), and in human AD peripheral blood mononuclear cells (PBMCs[6,7]. Previous studies have implicated cystatins B and C as markers of neurocognitive impairment (NI) in PWH [8]. Supplemental Digital Content Figure 1 illustrates the potential role of CatB and CysB in HAND pathogenesis. HIV has been shown to cause lysosomal permeabilization, precipitating secretion of lysosomal enzymes. Extra-lysosomal CatB has been shown to be neurotoxic, especially if its interaction with CysB is disrupted [6,7,9]. Previous work showed elevated CatB levels in post-mortem brain tissue and CSF from PWH with HAND [8,10]. Additionally, increased CysB and CatB have been reported in monocyte-derived macrophages (MDM) and plasma from women with HAD [8]. However, these markers have not been assessed in PWH with currently more prevalent milder NI. While ART is known to reduce viral load and systemic inflammation, PWH still experience chronic inflammation and immune activation [1,10]. Since CysB and CatB have been associated with inflammation and NI in PWH, we also sought to determine the longitudinal effects of ART on HAND severity and peripheral lysosomal markers.

The goals of the present study are to determine if there are differences in lysosome-associated markers in PBMCs from PWH with NI compared to HIV-seronegative controls and PWH without NI, and if marker levels change in response to ART using samples from the tenofovir arm of clinical trial ACTG A5303.

Methods

Samples:

PBMCs from PWH were obtained from the tenofovir arm of a clinical trial in which ART-naïve participants received treatment for 48 weeks (ACTG A5303, NCT01400412) [11]. We categorized PWH into the following participant groups: without NI at the time of entry into A5303 (HN; n=24) & with NI (NI; n=23). For HIV+ groups, specimens were collected at time 0, before ART initiation (HN0, NI0), and 48-weeks after initiating ART (HN48, NI48) (Supplemental Digital Content 2, cohort delineation flowchart). PBMCs from healthy, HIV-seronegative controls (C; n=22) were obtained through a separate study funded by the UAB CCTS, and did not receive neurocognitive testing. Inclusion and exclusion criteria, as well as neurocognitive test methodology previously performed by Robertson et al, are included in Supplemental Digital Content 4–6[11].

All PWH received once-daily combination ART with tenofovir disoproxil fumarate (TDF, 300mg) darunavir (DRV, 800mg), ritonavir (RTV, 100mg), and emtricitabine (FTC, 200mg). Demographic data was collected for all cohorts (Table, Supplemental Digital Content 3, demographics), and clinical data was collected for all HIV+ cohorts (Table, Supplemental Digital Content 7, clinical data).

Flow Cytometric Analysis and Reagents:

Isolated PBMCs where phenotyped by staining with the following cell surface mAbs: CD3-APC efluor780 (SK7, eBioscience, San Diego, CA), CD4-Qdot655 (S3.5, Invitrogen, Carlsbad, CA), CD8-V500 (RPA-T8, BD Biosciences, Franklin Lake, NJ), and CD14-Pecy7 (M-5E2, BD Biosciences). LIVE/DEAD- Fixable Aqua Stain (Invitrogen, Carlsbad, CA) was used to exclude dead cells from analysis. Intracellular staining for antibodies recognizing CysB-FITC (Assaypro, St. Charles, MO) and CatB-PE (Cell Signaling, Danvers, MA) was performed after permeabilizing and fixing cells with the BD Cytofix/Cytoperm kit according to the manufacturer's protocols. Supplemental Digital Content Figures 9 and 10 illustrate the gating strategy used. All antibody-stained cells were fixed in 1% formaldehyde (Sigma, St Louis, MO) prior to sample acquisition on a LSR II flow cytometer (BD Biosciences). We ran at least 100,000 gated lymphocytes/monocytes for each stained specimen. Gates for flow cytometric acquisition and analyses were based on isotype controls and single stain compensation controls. Antibody expression was measured via mean fluorescent intensity (MFI). Data were analyzed using FlowJo Version 9.9 software for Mac (TreeStar, San Carlos, CA). Cell viability was >70% across all participant groups.

Statistical Analysis:

Comparisons between groups (C vs HIV+; HN vs NI), were measured using Mann-Whitney test. Comparisons between paired groups (HN/NI0 vs 48-weeks) were performed using the

Wilcoxon-matched pairs signed rank test. Samples with negative or near-zero MFIs (n = 1–5 per data set), and those not virally suppressed at 48-weeks post-ART (n = 1), were removed before using the ROUT method (Q = 1%) to remove outliers (n ≤ 1 outlier per data set) [12]. Comparisons with p values ≤ 0.05 were considered statistically significant. Data analysis and graphing were performed using GraphPad Prism version 5.02.

Results

Both CD4+ count and CD4:CD8 ratio increased post-ART for all HIV+ participants regardless of neurocognitive status (p ≤ 0.0001). CD4:CD8 ratio was decreased in NI participants compared to participants without baseline NI (p = 0.0373). This same trend is seen post-ART, albeit without statistical significance (p = 0.2568) (Table, Supplemental Digital Content 7, clinical data).

HIV+ cohorts were analyzed for differences in neurocognitive function by comparing global deficit scores (GDS); a higher GDS indicates worsened neurocognitive function. (Figure 1; Table, Supplemental Digital Content 8, neurocognitive data). GDS decreased after 48-weeks of ART, regardless of neurocognitive status (HN p = 0.0382; NI p = 0.0006) (Figure 1A). Activities of daily living were increased in HIV+ participants with NI compared to those without NI (HN) both pre- and post-ART (NI0 vs HN0 p = 0.0395; NI48 vs HN48 p = 0.0144) (Figure 1B).

We next determined if CysB, an inhibitor of CatB, was altered in CD4+, CD8+, and CD14+ cells (Figure 2A; Table, Supplemental Digital Content 11, CysB data). Compared to HIV-seronegative participants, CysB levels were significantly increased in CD8+ T-cells from ART naïve participants without (HN0, p = 0.0153) and with (NI0, p = 0.0377) NI (Figure 2A-middle). In addition, among participants without NI, CysB levels were significantly reduced in CD4+ (p = 0.0024) and CD8+ (p = 0.0035) T-cells after ART (HN48) compared to before ART (HN0). In contrast, CysB levels post-ART were unchanged in T-cells from participants with NI (NI0 vs NI48) (Figure 2A-left/middle). In CD14+ monocytes, CysB levels were significantly increased in participants with NI post-ART (NI48) compared to levels obtained at baseline (NI0, p = 0.0035) (Figure 2A-right).

We next analyzed CatB levels to determine if this lysosomal enzyme relevant to CysB was similarly altered among cohorts (Figure and Table, Supplemental Digital Content 12 and 13, CatB levels by cell type & CatB data, respectively). CatB levels were decreased in CD8+ T-cells from participants in the NI0 cohort compared to controls (p = 0.0338); other significant differences were not observed.

Knowing CysB is an endogenous inhibitor of CatB, we sought to determine if levels of CysB correlated with CatB (Figure 2B). Regardless of cell type, HIV status, neurocognitive status, or ART status, CysB and CatB levels maintained a significant positive correlation (Table, Supplemental Digital Content 14, p-values).

Discussion

We identified differences in T-cell CysB levels between ART-naive PWH and HIV-seronegative controls. With ART, levels in T-cells only decreased in participants without NI. These findings suggest CysB may play a role in HAND pathogenesis and in the response to ART. T-cells have been implicated in the pathogenesis of several neurocognitive disorders, including HAND, via traversing the blood-brain-barrier and/or secreting pro-inflammatory cytokines [13]. Previous studies have also associated a reduced CD4:CD8 ratio with increased risk of HAND, which is supported by our findings [13]. Conversely, CysB levels increased post-ART in CD14+ monocytes from PWH with NI. Previous work indicated CysB, while normally neuroprotective, may contribute to HAND pathogenesis, and through its contribution to HIV-replication has been classified as an HIV-determining factor (HDF) [14]. Increased CysB and CatB have been observed *in vitro* in HIV-infected MDM and in PWH with HAD [8–10,14]. Future studies should explore the potential for cell type-specific differences in CysB in PWH, with respect to neurocognitive and ART-status. If subsequent analysis of CysB further implicates its role in HAND pathogenesis, modulation of its levels and/or function should be considered as a therapeutic strategy.

Interestingly, reductions in T-cell CysB levels corresponded with improved GDS in PWH without NI. Thus, CysB, along with GDS, may represent candidate factors to discriminate PWH at risk for HAND despite ART. GDS improved post-ART in participants with NI but remained higher than for those without NI. Since ART has been shown to decrease systemic inflammation [1,10], this could also contribute to reduced CNS inflammation, thus improving GDS in those with NI. However, chronic inflammation persists regardless of viral suppression, which could partly explain why GDS are still higher after ART than in PWH without NI.

While CSF was not collected from participants for this study, prior work has indicated increased CysB in both plasma and CSF of PWH with HAD [8]. Knowing immune cell trafficking contributes to the CNS as an HIV reservoir, and in turn persistent CNS neuroinflammation[8], it would be useful in future studies to determine if ART temporally regulates levels of peripheral and CNS CysB and other inflammatory markers. This would provide important information on how these factors may regulate the pathogenesis, onset/progression, and effective management of HAND.

While minimal changes in CatB were observed in our study, robust changes were observed previously in specimens from participants with the more severe HAD classification of HAND [8,10]. Also, HIV+ participants with NI had elevations in one or both proteins compared to HIV-seronegative controls but not compared to HIV+ participants without NI. Thus, it is possible CatB and CysB may not be sensitive enough to discriminate milder neurocognitive deficits. Prior analysis of CatB in HAD also indicated increased enzyme activity and extracellular secretion, and a decreased interaction between CatB and CysB [8,10], endpoints that were not investigated in this present study. Also, as only baseline and 48-week measurements were obtained, we were unable to measure lysosomal molecule kinetics in relationship to ART and subsequent viral suppression. Our study did indicate positive correlations between CysB and CatB in all cell types regardless of HIV, ART, or

neurocognitive status, which suggest confidence in our experimental design and analysis. Future studies of NI would benefit from a more rigorous investigation of CatB and CysB function, to further determine their relative role in the pathogenesis of milder forms of HAND.

In summary, our results indicate CysB in combination with GDS have potential predictive value in assessing HAND risk and progression. Future examination of these markers in conjunction with other molecules known to be altered in PWH with NI, including HDFs or inflammatory cytokines [14,15] could further bolster their predictive value. Future mechanistic studies of CysB function would help further delineate its role in regulating HAND pathogenesis, and its potential utility as a HAND biomarker.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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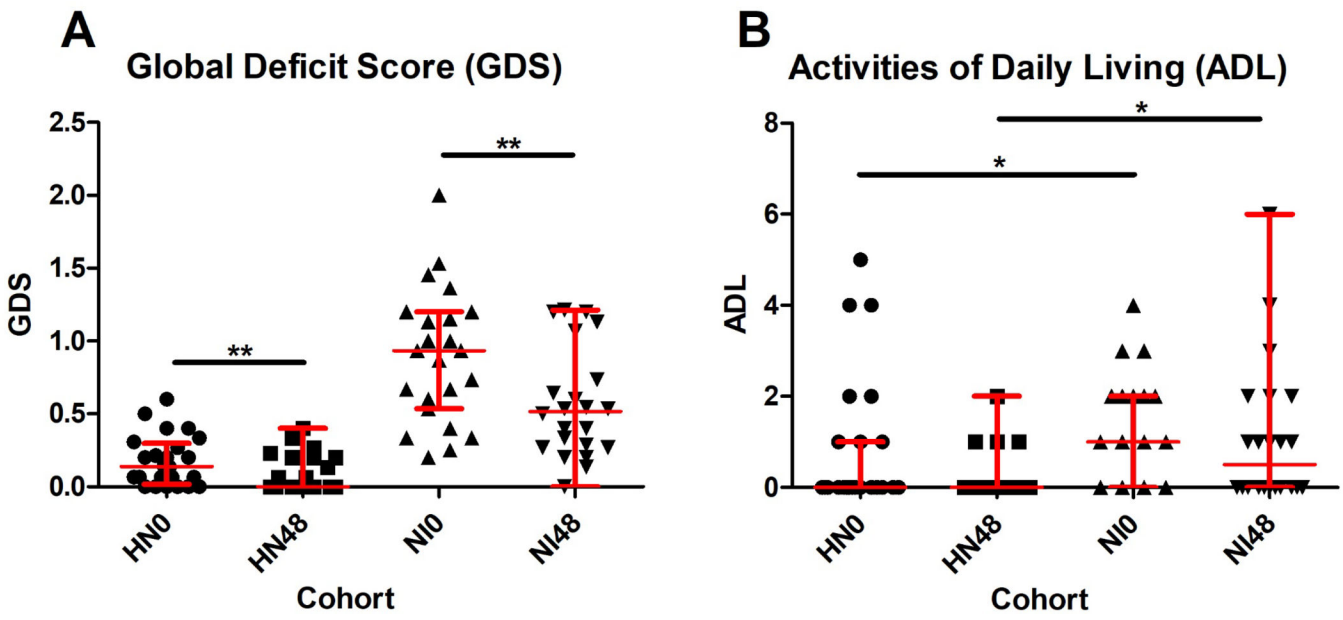

Figure 1. ART improved global deficit scores (GDS) in PWH, regardless of neurocognitive status.

(A) GDS and (B) activities of daily living (ADL) (median & IQR; **SDC 8**) were evaluated in HIV cohorts. HIV cohorts were defined a priori as either without [HN] or with NI [NI]. Specimens were collected from HN and NI cohorts at time 0 (ART naïve; HN0 or NI0) or after 48 weeks of ART (HN48 or NI48). * $p \leq 0.05$ via Mann Whitney test for unpaired analysis (HN vs NI); ** $p \leq 0.05$ via Wilcoxon rank sums test for paired analysis (0 vs 48 weeks ART).

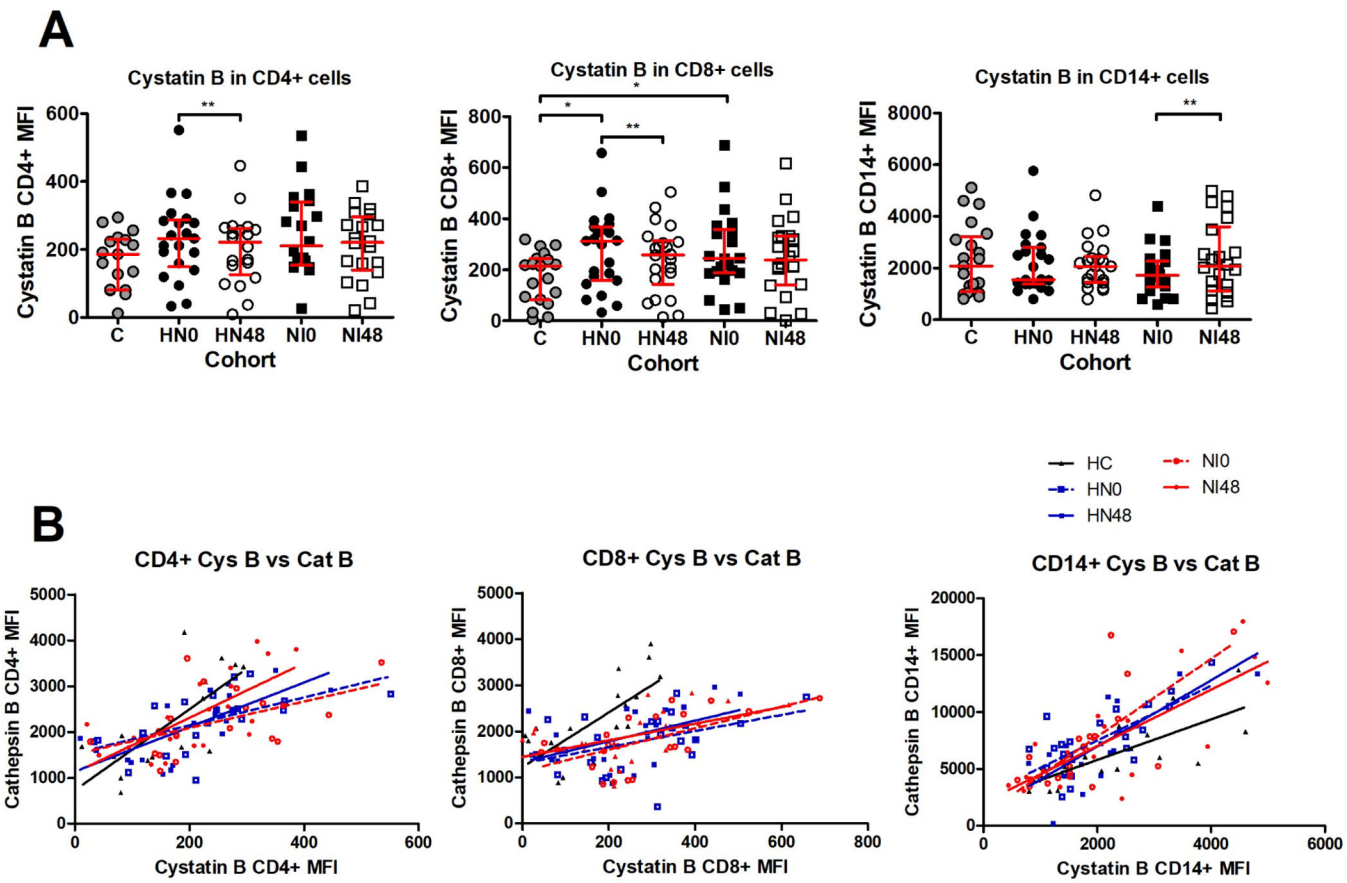

Figure 2. Cystatin B levels by cell type & Correlations between cystatin B and cathepsin B.

(A) Levels of cystatin B (median & IQR MFI, **SDC 11**) and (B) correlations between cystatin B and cathepsin B were evaluated in CD4+ (left), CD8+ (middle), and CD14+ (right) cells isolated from seronegative controls [C] and HIV cohorts **(SDC 14)**. HIV cohorts were defined a priori as either without [HN] or with NI [NI]. Specimens were collected from HN and NI cohorts at time 0 (ART naïve; HN0 or NI0) or after 48 weeks of ART (HN48 or NI48). * $p \leq 0.05$ via Mann Whitney test for unpaired analysis (HN vs NI); ** $p \leq 0.05$ via Wilcoxon rank sums test for paired analysis (0 vs 48 weeks ART). MFI = mean fluorescent intensity