Increased immune activation and signs of neuronal injury in HIV-negative people on pre-exposure prophylaxis (PrEP)

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Short title: Immune activation in people on PrEP

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Abstract

Objective

Persistent immune activation in the central nervous system and systemically are common in people living with HIV (PLHIV) despite antiretroviral therapy. It is not known whether this is generated by HIV replication or other components such as coinfections and lifestyle-related factors.

Design

To determine the importance of different factors, it is crucial to find well-matched HIV-negative controls. In this context, HIV-negative persons on pre-exposure prophylaxis (PrEP) may constitute a suitable control group to PLHIV with similar lifestyle-related factors.

Methods

Cerebrospinal fluid (CSF) and blood were collected from 40 HIV-negative persons on PrEP and 20 controls without PrEP. Biomarkers of immune activation, blood-brain barrier (BBB) integrity, and neuronal injury were analysed.

Results

CSF and serum β2-microglobulin, serum neopterin, and CSF neurofilament light protein were higher in persons on PrEP compared to controls. Furthermore, persons on PrEP had higher CSF/plasma albumin ratio, and matrix metalloproteinase-3 concentrations, indicating BBB dysfunction. Of persons on PrEP, 90% were CMV-positive compared to 65% of the controls. CMV-positive individuals as a group had higher levels of serum β2-microglobulin than CMV-negative individuals ($p < 0.05$). Drug users had higher serum β2-microglobulin compared to non-users ($p < 0.01$).

Conclusions

HIV-negative persons on PrEP had higher levels of biomarkers for immune activation, BBB impairment, and neuronal injury, compared to volunteers without PrEP. Moreover, serum β2-microglobulin was higher in CMV-positive compared to CMV-negative individuals and in drug users compared to non-users. These findings are important to consider when analyzing immune activation and CNS injury in PLHIV, and emphasize the importance of appropriate controls.

Introduction

Many people living with HIV (PLHIV) display a persistent systemic immune activation and inflammation despite successful antiretroviral therapy (ART) (1, 2). This inflammatory state is present also in the central nervous system (CNS) (3, 4), and has been found to be associated with neurocognitive impairment, known as HIV-associated neurocognitive disorder (5). The underlying cause of the inflammatory state remains unknown although several mechanisms have been proposed, including residual HIV replication in spite of effective ART, viral coinfections (*i.e.*, cytomegalovirus (CMV)), loss of immune regulatory responses, and unfavorable lifestyle factors (6). In a study of PLHIV on suppressive ART and lifestyle-matched controls, both groups showed higher levels of cellular monocyte activation compared to age-matched blood-bank donors, and the monocyte activation was strongly associated with high pro-inflammatory cytokine production and cerebrospinal fluid (CSF) inflammation (7). These findings emphasize the potential impact of lifestyle-related factors on persistent inflammation, as well as the importance of using appropriate controls to PLHIV when analyzing and interpreting inflammatory markers in blood and CSF in relation to treated HIV. In this context, HIV-negative people on pre-exposure prophylaxis (PrEP) constitute a potentially suitable control group to PLHIV, often sharing several lifestyle-related factors such as increased alcohol and drug use (8, 9), and high rates of sexually transmitted infections (10, 11). We therefore investigated whether HIV-negative people on PrEP have altered levels of immune activation and neuronal injury markers in blood and CSF compared to volunteers without PrEP.

Methods

Subjects

We included 40 HIV-negative persons on PrEP with tenofovir disoproxil fumarate (TDF)/emtricitabine (FTC), recruited from the Department of Infectious Diseases at the Sahlgrenska University Hospital, Gothenburg, Sweden. Confirmatory HIV tests were performed every third month throughout the study period and until recently to make sure that no participant was seroconverting. Exclusion criteria were pre-existing cerebrovascular disease or severe psychiatric or neurological disorders that may have a confounding impact on CSF biomarkers. Twenty volunteers without PrEP, comprising health care workers, students, and their relatives, were included as controls. Lumbar punctures and blood sampling were performed according to a previously described standardized protocol (12), and were collected between April 2018 and December 2019. All individuals completed self-reported questionnaires for alcohol and drug use (Alcohol use disorders identification test (AUDIT) and Drug use disorders identification test (DUDIT)). The study was conducted in accordance with the ethical principles set out in the declaration of Helsinki, and was approved by the Swedish Ethical Review Authority (Dnr: 060-18). Written informed consent was obtained from all participants.

Biomarker analyses

CSF and serum β2-microglobulin were measured using the N Latex β2M kit on the Atellica NEPH 630 System (Siemens Healthcare GmbH. Erlangen, Germany). Normal reference values were ≤ 1.8 mg/L in CSF and ≤ 2.1 mg/L in serum. CSF and serum neopterin were measured by a commercially available radio-immunoassay (BRAHMS, Berlin, Germany). Serum samples and standards were treated with Igepal (Sigma-Aldrich, Vienna, Austria; final concentration in serum or standards was 2% (v/v)). Normal reference values were ≤ 5.8 nmol/L in CSF and ≤ 9.1 nmol/L in serum (13, 14). CSF neurofilament light protein (NfL) concentrations were measured using an in-house sandwich ELISA as previously described (15). Age-adjusted (35 years) values of CSF NfL were calculated, since concentrations naturally increase with age in the uninfected population (16). The lower limit of quantification was 50 ng/L, and the upper normal age-adjusted reference limit was 622 ng/L. Immunoglobulin G (IgG) and albumin concentrations were measured by immunoturbidimetry on a Cobas instrument (Roche Diagnostics, Penzberg, Germany). IgG-index and CSF/plasma albumin ratio were calculated according to previous descriptions (17, 18). CSF levels of matrix metalloproteinase-3 (MMP-3) and matrix metalloproteinase-9 (MMP-9) were determined using the human MMP-3 and MMP-9 Ultra-Sensitive Kit from MSD. CSF soluble platelet-derived growth factor receptor-β (sPDGFRβ) concentration was measured by sandwich ELISA (Thermo Fisher Scientific, Loughborough, UK), as previously described (19). Peripheral blood CD4+ and CD8+ T-cell counts, as well as CSF leukocytes measurements were performed in the local clinical laboratories using routine methods. In individuals with pleocytosis, CSF PCR for herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), varicella zoster virus (VZV), enterovirus, human herpesvirus 6 (HHV-6), human paraechovirus, and CMV were performed. CMV and HSV-2 total antibody titers were measured using ELISA.

Next-Generation Sequencing (NGS) analysis

Total RNA was reverse transcribed using Omniscript Reverse Transcription Kit (Qiagen) as previously described (20). DNA and cDNA were amplified by nested PCR (20). PCR was purified with QIAquick PCR purification kit (Qiagen) and Illumina sequencing libraries were built by Nextera DNA Flex Library Prep Kit (Illumina, San Diego, CA, USA) according to manufacturers' protocol. The libraries were sent to Eurofins Genomics (Eurofins Genomics Germany GmbH, Ebersberg, Germany) for sequencing on a HiSeq 4000 platform (Illumina) to produce 150-bp paired-end reads. Raw data from Illumina sequencing was imported into CLC Genomic Workbench 12 (Qiagen, Hilden, Germany) for analysis. Sequences were trimmed and primer sequences were removed. Human sequences were removed, and remaining sequences were assembled using the built-in de novo assembler, where contigs were blasted using BLASTn (20).

AUDIT and DUDIT

The Alcohol use disorders identification test (AUDIT) was used to screen for harmful patterns of alcohol use. AUDIT is composed of ten self-rating questions, developed by the World health organisation, and has been internationally validated for use in primary health care (21). To identify recreational drug use, we utilized the Drug use disorders identification test (DUDIT), consisting of eleven questions (22).

Statistical analyses

Descriptive statistics were performed using Prism (GraphPad software version 8.0, La Jolla, California, USA). The Mann-Whitney test was used for comparisons between independent groups and Fishers exact test was used for comparisons of categorical variables. Correlations were explored with Spearman correlation. A significance level below 0.05 was considered as statistically significant. The effect size that can be detected with 80 % power is Cohen's $d = 0.8$.

Results

The median age of the HIV-negative persons on PrEP was 36 (interquartile range (IQR) 29–40) years compared to 35 (25–44) years in the group with volunteers. All but one on PrEP were males (97.5%), whereas 9 of 20 (45%) were males among the volunteers (Table 1).

When analyzing CSF and blood immune activation markers, we found that compared to controls, persons on PrEP displayed significantly higher median (IQR) concentrations of β2-microglobulin in both CSF [1.2 (1.0–1.4) vs. 1.0 (0.9–1.1) mg/L, $p < 0.01$] and serum [1.9 (1.7–2.2) vs. 1.6 (1.4–1.6) mg/L, $p < 0.0001$], whereas neopterin was only found to be higher in serum [8.1 (5.8–11.3) vs. 7.0 (6.1–7.2) nmol/L, $p < 0.05$] (Figure 1, Table 1). Moreover, when analyzing the neuronal injury marker NfL in CSF, we found that persons on PrEP had significantly higher age-adjusted concentrations compared to controls [324.5 (264.0–433.0) vs. 228.0 (206.8–339.8) ng/L, $p < 0.01$] (Figure 2). Five out of 40 on PrEP, compared to none of the non-PrEP volunteers, had CSF NfL levels above the age-specific upper normal reference value of 622 ng/L. CSF/plasma albumin ratio was also found to be higher in persons on PrEP (Table 1, Figure 2), indicating affected BBB integrity. The albumin ratio was above the upper normal reference level in six out of 40 persons on PrEP (15%) compared to 1 out of 20 without PrEP. MMP-3 was significantly higher in persons on PrEP [304.8 (250.0–428.9) vs. 229.8 (179.8–356.5) pg/L, $p < 0.05$], giving further support to BBB dysfunction (Figure 2). MMP-9 and sPDGFRβ were numerically higher but did not reach statistical significance (Table 1).

To explore the cause of the neuronal injury (elevated NfL levels), we performed correlation analyses and found that albumin ratio, β2-microglobulin, and sPDGFRβ correlated to CSF NfL (CSF NfL vs albumin ratio, r = 0.298, p = 0.022; CSF NfL vs CSF β2-microglobulin, r = 0.428, p = 0.001; CSF NfL vs CSF sPDGFRβ, r = 0.294, p = 0.024), suggesting that BBB

impairment and immune activation may both be involved in the neuronal injury found in persons on PrEP. Taken together, our findings show that persons on PrEP have elevated levels of several important markers for immune activation, neuronal injury, and BBB impairment.

To test the potential impact of viral coinfections, we analyzed CMV and HSV-2 seroprevalences, as well as CSF pleocytosis. In persons on PrEP, 90% were positive for CMV compared to 65% among the volunteers without PrEP ($p < 0.05$). Corresponding rates for HSV-2 were 20% versus 5% ($p = 0.25$). CSF pleocytosis (CSF WBC ≥ 5 per μL) was present in 5 out of 40 persons on PrEP, and in 1 out of 20 controls. Among the five individuals with CSF pleocytosis, PCR analyses of CSF were negative for HSV-1 and -2, VZV, enterovirus, HHV-6, human paraechovirus, and CMV, suggesting that the pleocytosis was not caused by a viral coinfection in the CNS. The two persons with highest CSF WBC count, who also had increased CSF NfL concentrations, were analyzed with NGS, in which no viral sequences were detected. In one individual, low amounts of 819 nucleotides matching *Corynebacterium accolens* were identified, which was, however, considered as contamination. In a separate analysis comparing CMV-positive and CMV-negative individuals, we found significantly higher levels of β2-microglobulin in serum among the CMV-positive ($p < 0.05$), while the difference observed for serum neopterin did not reach statistical significance ($p = 0.08$) (Figure 3). CSF β2-microglobulin, CSF neopterin, CSF MMP-3, albumin ratio, and CSF NfL did not differ between groups (data not shown). Apart from viral coinfections, we also performed syphilis tests every third month throughout the study period, and none of the participants had an ongoing infection.

To assess the use of alcohol and drugs, study participants were asked to fill out the questionnaires AUDIT and DUDIT. The answer rate was 73% (29/40) in the PrEP group, compared to 70% (14/20) of the controls. Among persons on PrEP, 9 reported regular drug use, whereas none of the controls reported use of any drugs ($p < 0.05$). Four of the users stated that they consumed drugs once a month or less often, 4 that they consumed drugs 2–4 times a month, and one reported usage at the second highest level, meaning 2–3 times a week. All participants confirmed that they were alcohol consumers, except for 2 persons on PrEP ($p = $ ns). Several participants in both groups reported alcohol consumption 2–4 times a month (16 (55%) in the PrEP group, and 7 (50%) in the control group). Four (14%) of the persons on PrEP and 5 (36%) of the controls answered that they used alcohol 2–3 times a week. None of the participants reported alcohol consumption 4 or more times a week. In a subgroup analysis of biomarkers in drug users, serum β2-microglobulin was significantly higher than among non-users ($p < 0.01$). None of the other serum or CSF biomarkers differed significantly between groups (data not shown).

To investigate the potential influence of recreational drug use and CMV seroprevalence on level of serum β2-microglobulin, we performed a multivariable regression analysis with serum β2-microglobulin as the dependent variable and group (PrEP versus controls), CD8 count, CMV, and drug use as predictors (variables that significantly correlate with serum β2-microglobulin). Group was the only variable that significantly affected serum β2-microglobulin levels (data not shown).

Discussion

In the present study, we compared levels of immune activation in serum and CSF, as well as neuronal injury markers in CSF in HIV-negative people on PrEP and volunteers without PrEP. We found that several important markers for inflammation and impaired BBB function were elevated among individuals on PrEP. We also demonstrated that the PrEP group had higher seroprevalences of CMV and HSV-2, as well as higher rates of drug use, which may contribute to increased immune activation. These findings are important to consider when analyzing biomarkers in PLHIV.

It is well known that many PLHIV suffer from a systemic inflammatory state (1, 2), including the CNS (3, 4, 5), in spite of effective ART with apparent suppression of systemic and CSF viral replication. The underlying cause of this inflammation is not fully understood. Apart from the HIV-infection, PLHIV have higher rates of lifestyle-related risk factors compared to the general population, which may contribute to immune activation (9, 23). To decide whether the inflammatory state is attributable to the HIV-infection *per se* or to other factors associated with HIV, it is crucial to find appropriate controls when interpreting clinical biomarkers in PLHIV. Interestingly, it was recently reported that both PLHIV on ART and lifestyle matched controls had higher levels of cellular monocyte activation compared to age-matched blood-bank donors (7). These findings support that lifestyle factors contribute to the persistent inflammation. In this context, HIV-negative persons on PrEP may constitute suitable controls to PLHIV, since they often share several lifestyle-related factors. For instance, alcohol and drug use (8, 24), increased sexual risk behaviors including multiple partners and unsafe sexual practices (25, 26), as well as high rates of sexually transmitted bacterial infections (10) have been reported among both PLHIV and individuals on PrEP. Based on this, we investigated whether persons on PrEP display an altered inflammatory activation in blood and CSF compared to volunteers without PrEP.

In persons with PrEP, we found higher levels of several markers for immune activation (CSF and serum β2-microglobulin, serum neopterin), neuronal injury (CSF NfL), and BBB impairment (CSF/plasma albumin ratio, CSF MMP-3) compared to controls. This triad – intrathecal immune activation, neuronal injury, and disturbed BBB integrity – has previously been reported by us in untreated, as well as in neuroasymptomatic HIV-infected individuals on ART (27). Thus, both PLHIV and some persons on PrEP display an inflammatory state with signs of neuronal injury and BBB impairment. This indicates that factors shared by PLHIV and persons on PrEP, other than the HIV-infection, may drive the persistent inflammation seen in both groups.

A potential contributor to the immune activation and inflammation seen in persons on PrEP are coinfections, such as human CMV or other herpesviruses. In line with this, we found a 90% seroprevalence of CMV among people on PrEP, which was considerably higher than among controls, and at similar levels as earlier seen in HIV-infected individuals (28). Coinfections have previously been suggested to fuel the inflammatory state observed in HIV disease (29), and accordingly, we found that CMV-seropositive individuals had higher

concentrations of β2-microglobulin and neopterin in blood. HIV-infected patients who received valganciclovir treatment were shown to have a significantly reduced CD8 activation compared to placebo controls (30), further supporting CMV coinfection as an important contributor to persistent immune activation.

The alcohol and drug use surveys showed similar alcohol habits between groups, but only use of recreational drugs and chemsex among persons on PrEP, of which most were men who have sex with men (MSM). The reported recreational drug use among PrEP persons (31%) is somewhat lower than in previous studies on PrEP and MSM (43%) (31, 32). Since drug use is also common in PLHIV and MSM (9), a more liberal consumption may contribute to the immune activation seen both in persons on PrEP and PLHIV.

The small sample size and sex differences between the groups are two main limitations of the present study, however, there is no reason to believe that sex has a direct impact on the measured biomarker levels (33, 34). Furthermore, information about alcohol and drug use was collected by questionnaires, which introduces a potential risk of information bias with underestimation of the true consumption. Unfortunately, measurements of alcohol and drug use markers were not available.

In conclusion, the present study of HIV-negative persons on PrEP shows higher levels of several markers for immune activation, neuronal injury, and BBB impairment compared to volunteers without PrEP. Moreover, serum β2-microglobulin was higher in CMV-positive compared to CMV-negative individuals and in drug users compared to non-users. These findings are important to consider when analyzing biomarkers for immune activation and CNS injury in PLHIV, and emphasize the importance of appropriate controls.

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Competing interests

Acknowledgements -

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Figure 1. Levels of β2-microglobulin (mg/L) and neopterin (nmol/L) in serum and cerebrospinal fluid (CSF) in persons on PrEP (n = 40) and controls without PrEP (n = 20).
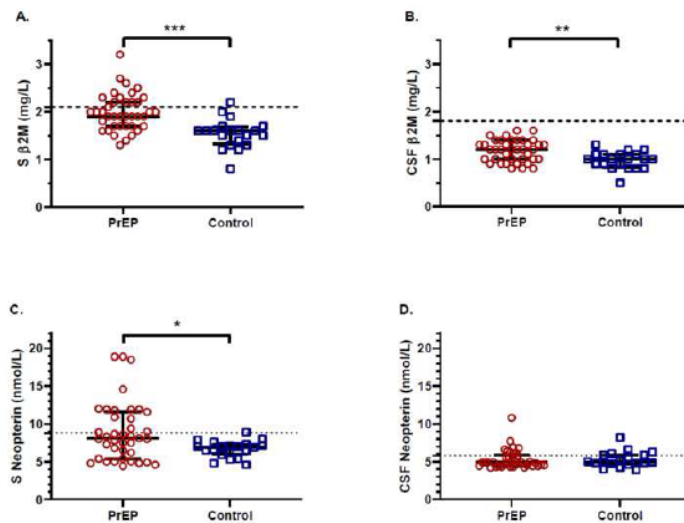

Figure 2. Levels of cerebrospinal fluid (CSF) neurofilament light protein (NfL, ng/L), CSF/plasma (P) albumin ratio, and CSF matrix metalloproteinase 3 (MMP-3, pg/L) in persons on PrEP (n = 40) and controls without PrEP (n = 20).
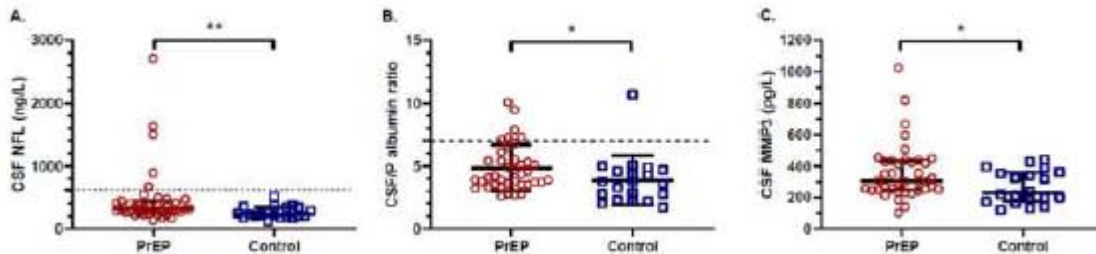

Figure 3. Levels of β2-microglobulin (mg/L) and neopterin (nmol/L) in serum in study participants divided into CMV-positive (n = 49) and CMV-negative (n = 11) individuals.
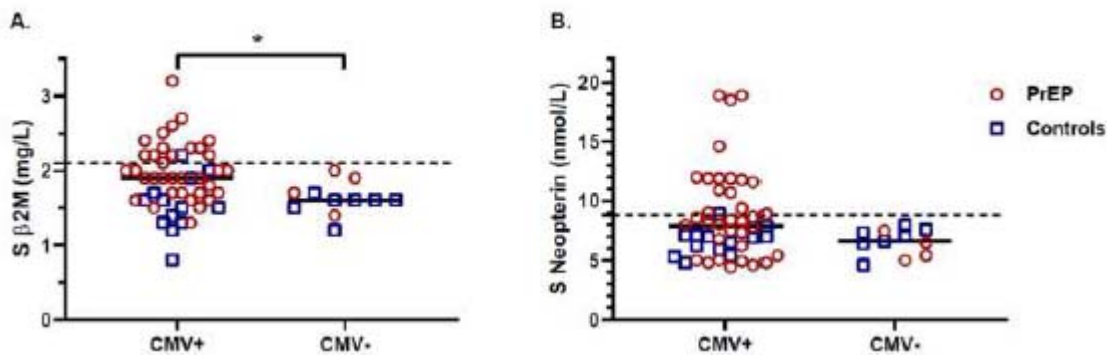
Table 1. Demographic data and concentrations of cerebrospinal fluid and serum markers from 40 HIV-negative persons on pre-exposure prophylaxis (PrEP) and 20 controls without PrEP.

	PrEP (n = 40) Median (IQR)	Controls (n = 20) Median (IQR)	p-value
Sex (M/F)	39/1	9/11	**p < 0.0001**
Age, years	36 (29–40)	35 (25–44)	p = 0.71
CMV (%)	36/40 (90)	13/20 (65)	**p < 0.05**
HSV-2 (%)	8/40 (20)	1/20 (5)	p = 0.25
Alcohol use, n (%)[a]	27 (93)	14 (100)	p > 0.99
Recreational drug use, n (%)[a]	9 (31)	0 (0)	**p < 0.05**
CD4 count, cells*10^6/L	825 (615–1025)	810 (670–1100)	p = 0.84
CD8 count, cells*10^6/L	635 (498–843)	495 (440–618)	p = 0.06
CD4/CD8 ratio	1.3 (1.0–1.7)	1.5 (1.4–1.8)	p = 0.07
CSF ß2-microglobulin (mg/L)	1.2 (1.0–1.4)	1.0 (0.9–1.1)	**p < 0.01**
Serum ß2-microglobulin (mg/L)	1.9 (1.7–2.2)	1.6 (1.4–1.6)	**p < 0.0001**
CSF neopterin (nmol/L)	4.9 (4.4–5.7)	4.9 (4.7–5.9)	p = 0.63
Serum neopterin (nmol/L)	8.1 (5.8–11.3)	7.0 (6.1–7.2)	**p < 0.05**
CSF/plasma albumin ratio	4.2 (3.6–5.5)	3.7 (2.7–4.6)	**p < 0.05**
IgG index	0.5 (0.5–0.5)	0.5 (0.5–0.5)	p = 0.53
Age-adjusted NfL (ng/L)	324.5 (264.0–433.0)	228.0 (206.8–339.8)	**p < 0.01**
MMP-3 (pg/L)	304.8 (250.0–428.9)	229.8 (179.8–356.5)	**p < 0.05**
MMP-9 (pg/L)	90.7 (56.1–139.2)	74.4 (41.9–84.8)	p = 0.09
sPDGFRβ (pg/L)	1108.8 (797.5–1272.5)	915.7 (809.9–1190.9)	p = 0.53

Abbreviations: IQR, interquartile range; CMV, Cytomegalovirus; HSV-2, Herpes simplex virus type 2; CSF, cerebrospinal fluid; NfL, neurofilament light protein; MMP, matrix metalloproteinase; sPDGFRß, soluble platelet-derived growth factor receptor-ß.

[a] *Percentages are calculated on the 43 participants that answered the questionnaires [29/40 in the PrEP group (73%), and 14/20 among controls without PrEP (70%)].*