Presence of EBV DNA in Cerebrospinal Fluid is Associated with Greater HIV RNA and Inflammation

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Lupia T[1#], Milia MG[2], Atzori C[3], Gianella S[4], Audagnotto S[1], Imperiale D[3], Mighetto L[5], Pirriatore V[1], Gregori G[2], Lipani F[1], Ghisetti V[2], Bonora S[1], Di Perri G[1], Calcagno A[1].*

1 Unit of Infectious Diseases, Department of Medical Sciences, University of Torino, Torino, Italy

2 Laboratory of Virology and Molecular Biology, Ospedale Amedeo di Savoia, ASL "Città di Torino", Torino, Italy

3 Unit of Neurology, Ospedale Maria Vittoria, ASL "Città di Torino", Torino, Italy

4 University of California San Diego, La Jolla, California, USA

5 Laboratory of Immunology, Ospedale Maria Vittoria, ASL "Città di Torino", Torino, Italy.

Running Head: EBV and HIV in CSF

#Address correspondence to Tommaso Lupia, tommaso.lupia89@gmail.com

*Present address: Tommaso Lupia, Unit of Infectious Diseases, Department of Medical Sciences, University of Torino, Amedeo di Savoia Hospital, C.so Svizzera 164, 10149 Torino, Italy

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Abstract

Epstein-Barr virus (EBV) and Cytomegalovirus (CMV) can infect several cells, replicate in the central nervous system and affect blood-brain barrier (BBB) integrity. This study aimed to investigate whether cerebrospinal fluid (CSF) EBV or CMV DNA was associated with viral, inflammatory and neuronal damage biomarkers in people living with HIV (PLWH).

EBV, CMV DNA and HIV RNA were measured on CSF, through RT-PCR, from PLWHs undergoing lumbar punctures for clinical reasons (excluding oncho-haematological comorbidities). Immune-enzymatic assays evaluated BBB inflammation and damage. Patients were stratified according to plasma HIV RNA levels in viremic (≥50 copies/mL) and aviremic (<50 copies/mL). We included 298 participants. Among 167 viremic patients CSF EBV and CMV DNA were detectable in 42 (25.1%) and 10 (6.3%) participants; among 130 aviremic subjects CSF EBV and CMV DNA were detectable in 12 (9.2%) and 0 (0%) participants, respectively. In viremic group detectable CSF EBV DNA was associated with CSF pleocytosis (p<0.001), higher CSF HIV RNA (p<0.001) and neopterin levels (p=0.002). In aviremic participants detectable EBV DNA was associated with pleocytosis (p=0.056), higher neopterin (p=0.027) and immune globulins (p=0.016) in the CSF; CSF escape was more common in those with detectable EBV DNA (50% vs 21.2%, p=0.036).

EBV DNA was frequently detected in the CSF of viremic and fewer aviremic patients on antiretroviral treatment. In PLWH without clinical evidence of encephalitis CSF EBV DNA was associated with higher levels of HIV RNA and biomarkers of neuronal damage/inflammation. The role of EBV reactivation in HIV-associated CNS disorders warrants further studies.

ha eliminato:

ha eliminato: The aim of this study was

ha eliminato: We examined CSF samples participants undergoing lumbar punctures for clinical reasons (excluding those with lymphoproliferative disorders): we measured EBV, CMV DNA and HIV RNA (by PCR), markers of neuronal damage and inflammation (by immune-enzymatic assays).¶

ha eliminato: (with plasma HIV RNA <50 copies/mL)

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ha eliminato: EBV DNA was frequently detected at low levels in the CSF of viremic participants and in a minority of aviremic patients on antiretroviral treatment.

Background:

The central nervous system (CNS) is a clinically relevant target of HIV and severe forms of encephalitis and dementia have been described since the beginning of the epidemics [1]. Even in the current era of antiretroviral therapy (ART), chronic CNS involvement is a significant issue for people living with HIV (PLWH) [2]. In fact, HIV persists in the brain tissue of people living with HIV (PLWH) despite systemic viral control and its detection in the cerebrospinal fluid (CSF) has been associated with acute/subacute neurological symptoms, worse neurocognitive performances and immune activation [3]. While the clinical relevance of HIV RNA escape within the CSF is controversial, symptomatic cases have been described and ART optimizations have led to clinical/radiological/virologic improvements, thus supporting a pathogenetic role of HIV RNA active replication in the CNS [4]. The factors associated with HIV RNA escape in the CSF are poorly understood with several identified risk factors, such as low nadir CD4 cell count, dementia, poor adherence to ART, low level viremia in blood plasma and the presence of drug resistance associated mutations [5-6]. Another possible driver of compartmentalized HIV RNA replication in the CSF might be co-infection with other chronic viruses ("secondary escape") but this has not been systematically investigated [7].

As part of this study, we focused on the effects of Cytomegalovirus (CMV) and Epstein-Barr virus (EBV) since PLWH have a higher risk of acquiring and incompletely controlling both viruses; both EBV [8-10] and CMV [11-13] have been associated with neurological disorders as well as vascular inflammation, thus suggesting the potential for chronic CNS and endothelial involvement (well recognized for CMV) [11-13]. In particular, EBV can infect macrovascular endothelial cells in human tissue [14-17], human brain micro-vessels [18] and human umbilical vein endothelial cells [19]. Endothelial cells with lytic reactivation of EBV present increase

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production of pro- inflammatory molecules (CCL-2 and CCL-5) and also hyper-expression of surface adhesion molecules (ICAM-1 and VCAM-1) with a potential creation of an inflammatory breach through the Blood Brain Barrier (BBB) [12, 20-21]. This may be relevant because a key factor in chronic HIV RNA CNS involvement seems to be the alteration of the BBB: the latter has been recently described as being part of the neurovascular unit where endothelial cells (and perycites) co-operate with astrocytes and neurons [22]. Thus, CMV and EBV may potentially cause a sub-clinical chronic infection and facilitate inflammatory cells' trafficking through the BBB increasing migration of HIV into the CNS. [7, 15, 23]

Material and Methods

Cohort and Samples

We enrolled adult PLWH undergoing lumbar punctures for clinical reasons, in a cross-sectional design study, including late presentation with <100 $CD4^+$ T lymphocytes/mm3 in peripheral blood, opportunistic infections, new or persistent neurological symptoms (including headache), worsening cognitive impairment, need of lumbar puncture in case of syphilis or white mater hyperintensities at brain MRI. Patients with primary central nervous system lymphomas, lymphoproliferative diseases and autoimmune disorders were excluded from this study. We also excluded HIV controllers without ART. Demographic, immunovirological, clinical and therapeutic data were recorded as well as CSF characteristics. The protocol was approved by our Ethics Committee (Comitato Etico Inter-aziendale di Orbassano, n. 103/2015). Study partecipants signed a written informed consent at enrollment.

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Viral Measures

Levels of HIV RNA were measured by real time Polymerase Chain Reaction (RT-PCR) assay CAP/CTM HIV-1 vs. 2.0 (CAP/CTM, Roche Molecular System, Branchburg, NJ, HIV RNA detection limit: 20 copies/mL). Levels of EBV DNA and CMV DNA were measured through the RT-PCR (detection limit: 100 copies/mL). CSF escape was defined as CSF HIV RNA >50 copies/mL with plasma HIV RNA <50 copies/mL and CSF/plasma discordance as CSF HIV RNA 0.5 Log_{10} higher than plasma HIV RNA (if both were detectable) [24].

Immunological Measures and BBB Damage

Quantitative determination of albumin in serum and CSF was measured by Immunoturbidimetric methods (AU 5800, Beckman Coulter, Brea, CA, USA). CSF to serum albumin ratio (CSAR) was calculated as albumin in CSF (mg/L)/albumin in serum (g/L), and was used to evaluate BBB permeability. Impaired BBB was defined according to age-adjusted Reibergrams (normal if below 6.5 in patients aged <40 years and below 8 in patients >40 years) [25]. The presence of Immune globulins (Ig)G produced inside the CNS was calculated according to Tibbling index [26]. CSF pleocytosis was defined as ≥5 cells/mm^3.

CSF total tau (t-tau), phosphorylated tau (p-tau) and 1-42 β-amyloid ($A\beta^{1-42}$) were measured by immunoenzymatic methods (Fujirebio diagnostics, Malvern, U.S.A.) with limits of detection of 57, 20 and 225 pg/ml, respectively. Neopterin and S100B were measured through ELISA [DRG Diagnostics (Marburg, Germany) and DIAMETRA S.r.l. (Spello, Italy), respectively]. Upper limits of normality in HIV-negative individuals were as follows: t-tau [<300 pg/mL (in participants aged 21–50), <450 pg/mL (in participants aged 51–70) or <500 pg/mL in older participants], p-tau (<61 pg/mL), 1–42 beta amyloid (>500 pg/mL), neopterin (<1.5 ng/mL) and S100B (<380 pg/mL) [27].

Statistical Analysis

We performed descriptive statistics on the entire study population and then stratified between study participants with suppressed plasma HIV RNA (<50 copies/mL) and those with detectable HIV RNA.

Data were analyzed using standard statistical methods: variables were described with medians [interquartile ranges (IQR)], groups were compared using non-parametric tests (Mann–Whitney, Kruskal-Wallis and Spearman's tests as specified in the text). Linear logistic regressions were used for estimating the association between detectable EBV/CMV DNA, HIV RNA, as well as biomarker of CNS damage and inflammation. Models were adjusted for $CD4^+$ cell counts and CSF HIV RNA. Data analysis was performed using PASW software version 22.0 (IBM).

Results

Two hundred and ninety-seven PLWH were included in this study, of whom 118 (39.4 %) were naïve for ART. Baseline and immune-virological characteristics, stratified by plasma HIV RNA (below or above 50 copies/mL) are shown in **Table 1**.

EBV DNA was detected in the CSF of 42 (25.1%) and 12 (9.2%) participants with detectable and undetectable HIV RNA in plasma, respectively (p<0.001). Similarly, higher levels of EBV DNA were observed in the CSF of participants with detectable plasma HIV RNA (152 vs. <100 copies/mL, p<0.001). Virological, neuronal damage and inflammation biomarkers stratified by plasma HIV RNA and CSF EBV DNA detection are shown in **Table 2.**

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ha spostato in basso [1]: CMV DNA was detected only in the CSF of participants with plasma HIV RNA >50 copies/mL [10 (6.3%) vs. 0 (0%), p=0.006]; participants with detectable CSF CMV DNA presented lowed $CD4^+$ T lymphocytes (25 vs. 91/mm^3, p=0.005), higher plasma and CSF HIV RNA (5.8 vs. 5.1 Log_{10} copies/mL, p=0.039 and 5.0 vs. 3.6 Log_{10} copies/mL, p=0.016), higher CSAR (9.0 vs. 5.6, p=0.046) and neopterin (5.4 vs. 2.2 ng/mL, p=0.012) and they were more often diagnosed with opportunistic infections (40% vs. 17.3%, p=0.093).¶

CMV DNA was detected only in the CSF of participants with plasma HIV RNA >50 copies/mL [10 (6.3%) vs. 0 (0%), p=0.006]; participants with detectable CSF CMV DNA had lower $CD4^+$ T lymphocytes (25 vs. 91/mm^3, p=0.005), higher plasma and CSF HIV RNA (5.8 vs. 5.1 Log_{10} copies/mL, p=0.039 and 5.0 vs. 3.6 Log_{10} copies/mL, p=0.016), higher CSAR (9.0 vs. 5.6, p=0.046) and neopterin (5.4 vs. 2.2 ng/mL, p=0.012) and they were more often diagnosed with opportunistic infections (40% vs. 17.3%, p=0.093).

In PLWH with detectable plasma HIV RNA, presence of detectable EBV DNA was associated with a significantly higher number of cells, greater CSAR, as well as increased HIV RNA and neopterin in the CSF. In participants with plasma HIV RNA <50 copies/mL, presence of detectable EBV DNA was associated with significantly higher number of cells and greater neopterin in the CSF. The presence of IgG produced within the CNS was more common in aviremic participants with detectable CSF EBV DNA (38% vs. 0%, p=0.036). Additionally, CSF HIV RNA escape was more common in ART-suppressed participants with detectable EBV DNA (50% vs. 21%, p=0.036) (**Figure 1**).

No correlation was observed between EBV DNA concentrations and the studied biomarkers.

Linear logistic analysis (adjusted for CD4 cell count and CSF HIV RNA) suggested that a detectable EBV DNA was independently associated with CSF pleocytosis in PLWH with CSF HIV RNA <50 copies/mL (p<0.001) and non-controllers (p<0.001), with neopterin in HIV non-controllers (p=0.015) and with the production of IgG within the CNS in HIV-controllers (p=0.008).

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Discussion

To better understand the role of EBV and CMV in the CNS on HIV RNA replication, BBB damage and biomarkers of neuronal damage/inflammation, we measured levels of CMV and EBV DNA in CSF of 298 PLWH.

Overall, we observed that EBV DNA was detectable at low levels in 18% of all PLWH and it was associated with higher levels of HIV RNA in the CSF and up to three-time higher rate of pleocytosis. Compared to viremic subjects (25.1%), EBV DNA was found less frequently in study participants with undetectable HIV RNA in plasma (9.2%), and its presence was associated with pleocytosis, IgG production within the CNS and presence of CSF HIV RNA escape. CMV DNA, on the contrary, was found only in HIV viremic participants and was associated with low $CD4^+$ cell count, high plasma HIV RNA and opportunistic disorders.

Our findings are similar to those reported by Weinberg et al. in HIV-negative individuals where the presence of pleocytosis was associated with detectable CSF EBV DNA but also presence of EBV related-mRNA, supporting the hypothesis that EBV DNA is not just carried by latently infected inflammatory cells (e.g. B cells) but the consequence of actively replicating virus [28]. Furthermore, EBV affects the immune system and it may enhance neuronal degeneration in chronic inflammatory conditions [29-30]. Our data are in line with this hypothesis since both viremic and ART-suppressed PLWH showed higher CSF HIV RNA levels (and CSF to plasma HIV RNA ratios) and increased white blood cells when EBV DNA was detectable. Additionally, PLWH with detectable HIV RNA and EBV DNA in CSF also showed higher CSAR supporting a potential role of chronic EBV infection in BBB damage, which in turn is associated with

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neurocognitive impairment, and with neuronal damage and inflammation [31-33]. In vitro experiments suggest that hosting EBV astrocytes and microglia may enhance cell-to-cell crosstalk and favoring migration of monocytic/macrophagic line cells into the CNS [34-36]. This effect may be independent from HIV control and immune system improvement: these conditions have been associated with the absence of neuronal damage and with the lowest CSF concentrations of neopterin [37-39].

Despite the low level or absent plasmatic EBV and CMV replication can be found at higher concentrations in tissues and organs. In a recent study that analyzed 108 gut biopsies collected from 19 HIV-infected and 22 HIV-uninfected participants, CMV and EBV were detected in more than 70% of samples but more commonly in HIV-positive subjects [40]. While the negative effects of sporadic or continuous CMV replication are well-known, there is still uncertainty on the role of EBV in favoring chronic immune activation. Neuroinflammation, neurodegeneration and its drivers are widely studied in MS and Late EBV infection seemed to be one of the risk factors involved in promoting the initial events and the relapses of this chronic neurological condition [41-42]. In most CSF of MS patients were founded elevated antibody levels against the entire EBV nuclear antigen (EBNA), and EBNA-1, a protein expressed during latent EBV-infection [43-44]. Anti-EBNA-1 IgG antibodies were correlated also with CSF oligoclonal bands and in some patients oligoclonal bands include anti-EBV antibodies [45]. Reactivation of EBV in the central nervous system (CNS) has been proposed as a possible cause of MS although the virus has not been consistently found in MS lesions [41].

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From a broader perspective the lifelong presence of most Herpesviridae in the organism may produce in some hosts alterations in neuronal cellular processes [46-47]. These observations were suggested by several studies suggesting an association between EBV, human

279 herpesvirus-1 and 6 (HSV-1 and HHV-6) with Alzheimer's (AD) and other neurodegenerative
280 diseases [48-50]. Very recently HHV-6A and HHV-7 produced disruption of molecular, genetic,
281 and clinical networks was reported in autoptic brains from patients suffering of AD along with
282 [51-52].

283 Additionally, EBV may play a role in suppressing the CNS immune system and therefore
284 maintain an incomplete T-cell mediated inflammatory response, through the expression of viral
285 genes encoding for proteins with immunoevasion-like function. This may translate into higher
286 rates of pleocytosis but with less inflammatory activity [53-54]. On the other hand, ART-
287 suppressed study participants with detectable EBV DNA showed lower $CD4^+$ T cell counts thus
288 suggesting that immune control may be needed in order to restore a partial control on EBV
289 replication.

290 CMV DNA was detected in naïve patients only and, specifically, in patients presenting with
291 advanced immune depletion and opportunistic infections and with high viral replication both in
292 plasma and CSF. Additionally, BBB damage and monocyte-derived inflammation were
293 significantly higher in participants with detectable CSF CMV DNA. Further speculations on the
294 role of CMV are limited by the low number of participants in this subgroup; yet it may be peculiar
295 of individuals with very severe immunedeficit and, as already shown by several reports, at
296 higher risk of poor survival. [55,56]

297 Some limitations of this study should be acknowledged including the low sample size, the cross-
298 sectional design, the lack of a control group and the lack of plasma EBV DNA measurements.
299 We also did not collect neurocognitive data in all participants. Importantly, in this observational
300 study we cannot assess the causal relationship between presence of EBV, inflammation and
301 HIV RNA escape. Additionally, our cohorts include several patients with very low nadir $CD4^+$ T

cell counts and heterogeneous clinical conditions: the same effect may not be observed in individuals with less advanced disease.

In conclusion we reported for the first time the presence of detectable EBV DNA in the CSF of PLWH without lympho-proliferative disorders and with no evidence of viral encephalitis. We observed that ART-naïve subjects with detectable EBV DNA in the CSF had a higher HIV RNA viral load and also higher markers of neuronal damage and inflammation; Similarly, in ART-suppressed individuals we observed increased HIV RNA and also evidence of BBB damage, greater pleocytosis and immune activation. Further studies are warranted for understanding the contribution of EBV to HIV-associated CNS disorders.

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