Neurometabolic Remodeling in Chronic Hiv Infection: a Five-Year Follow-up Multi-Voxel Mrs Study

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There is a lack of data about the long-term follow-up changes in neurometabolic profile and neuropsychological performance of HIV-positive subjects under continuous antiretroviral therapy (cART). The aim of the study was to assess changes in neurometabolic profile in chronically-infected, HIV-positive subjects during a five-year follow-up period, using multi-voxel proton magnetic resonance spectroscopy (1H-MRS). Nineteen neurologically asymptomatic, aviremic, HIV-positive subjects, underwent multi-voxel 2D MRS on a 3T MR unit and synchronous neurocognitive assessment in a five-year follow-up period. Twelve voxels were placed in prefrontal cortices, anterior and posterior cingulate gyrus, intraparietal sulci, and frontal centrum semiovale white matter, to identify peaks of N-acetyl-aspartate (NAA), creatine (Cr), choline (Cho), and myoinositol (mI). Ratios of NAA/Cr, NAA/Cho, NAA/mI, mI/Cr, and Cho/Cr were analyzed. Longitudinal differences in ratios and neurocognitive scores were tested with the Wilcoxon signed-rank-test. Statistical significance was set at $p \leq 0.004$ significant, and $0.05 > p > 0.004$ trending toward significance. A significant longitudinal increase in NAA/Cr ratio was observed in 5/12 voxels, while there was a trend toward significance in an additional three. The increase in Cho/Cr reached statistical significance in one voxel. Changes in the mI/Cr ratio demonstrated a significant increase in 4/12 voxels. A progressive increase in NAA/Cr, followed by better neurocognitive performance, may be an indicator of brain plasticity in the setting of chronic HIV-related neuronal injury. A progressive mI/Cr increase could be partly explained by glial proliferation due to functional compartment remodeling and partly attributable to insufficient control of persistent neuroinflammation by cART.

Several longitudinal studies have shown changes in the biochemical profile of the brain in human immunodeficiency virus (HIV) infection, depending on the phase of infection and the introduction of combination antiretroviral therapy (cART)1-4. Although no signs of neuronal injury can be detected on magnetic resonance imaging (MRI) and magnetic resonance spectroscopy (MRS) in the early phase of HIV transmission, there are already prominent signs of ongoing inflammation. This inflammation is reflected by elevated levels of myoinositol (mI), a marker of microglial proliferation, and choline (Cho), a marker of membrane metabolism and gliosis. Soon after the initiation of cART, in the absence of neuronal dysfunction, normalization of inflammation markers can occur in several weeks1. The initial increase in inflammation markers will decline after cART initiation, although they do not reach the level of healthy controls5. Further in the course of disease, a decline in N-acetyl aspartate (NAA), a neuronal marker, occurs and becomes the dominant finding in chronically infected HIV-patients6.

In the first MRS study performed in our institution on 110 subjects (60 HIV-positive patients and 50 age- and gender-matched controls), the results clearly showed that HIV-associated neurodegeneration affects the whole volume of the brain. Furthermore, the results of that study, as well as other, smaller studies, confirmed ongoing inflammation under cART2. The neurodegenerative process associated with long-standing, well-controlled HIV infection results in progressive neurocognitive dysfunction6. The explanation for this process is thought

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to be due to chronic neuroinflammation and persistent immune activation, driven by an uneradicated pool of HIV-particles6.

In this longitudinal study, our aim was to assess longitudinal changes in the neurometabolic profile of neurologically asymptomatic HIV-positive individuals treated with cART during the follow-up period of five years, using multi-voxel proton magnetic resonance spectroscopy (1H MRS). In addition, a relationship between longitudinal changes in neurocognitive performance and MRS findings was investigated.

**Methods**

**Participants.** Baseline neuroimaging was performed in 31 HIV-positive patients, of whom 22 patients underwent follow-up imaging after five years. Three patients were excluded from the study based on the exclusion criteria. An overall total of 19 chronically infected HIV+ male patients on cART, mean age 45.16 ± 11.47, were included in this institutional review board-approved study (Ethical Committee of the Faculty of Medicine Novi Sad). Plasma viral load remained undetectable during the follow-up period in all patients. Participants underwent standard magnetic resonance imaging (MRI) and an MRS study on a 3 T MR unit (Trio Tim, Siemens, Erlangen, Germany), in the follow-up period of five years (e.g., baseline MRS studies were performed in 2011 and follow-up studies in 2016). Demographic and basic clinical data for both time points (including the nadir CD4 T-cell count, the current count of CD4+ T-cells, and cART duration) are summarized in Table 1.

Inclusion criteria for the follow-up study were: completion of two MR examinations and synchronous neuropsychological testing; chronic HIV infection at the baseline (over one year after transmission); stable on cART (over one year after initiation); no signs of manifest neurocognitive disorder at baseline; and a normal conventional MRI. Exclusion criteria were: detectable viral load over the follow-up period (>50 copies/mL), current or past confounding neurological disease (opportunistic infection, multiple sclerosis, vascular and non-vascular dementia, neurodegenerative disease); brain tumors; traumatic brain injury; major psychiatric disorders; cardi-vascular diseases (hypertension, chronic occlusive carotid disease, ischemic vascular dementia); active abuse of narcotic drugs (according to the Diagnostic and Statistical Manual of Mental Disorders); and hepatitis B or C coinfection at the baseline and over the follow-up period.

All selected patients were compliant with therapy, without any changes in the regimen of cART over the observed period of time. Three patients were excluded from follow-up imaging due to a change in the cART regimen (transient neuropsychiatric symptoms presented due to the introduction of efavirenz). A CONSORT study diagram showing patient selection in detail is shown in Fig. 1. For all subjects, the CNS penetrance efficacy score (CPE) was generated, with a mean value of 8.9 ± 1.3.

All subjects signed written, informed consent.

**Neuroimaging protocol.** Imaging was performed in 2011 and 2016, using an eight-channel head array coil. The MRI protocol consisted of a three-planar, multi-sequential study: sagittal T1-weighted spin echo (repetition time 440 ms, time of echo 3.8 ms, thickness of the slice 5 mm); axial T2-weighted turbo spin echo (5150 ms, 105 ms, 5 mm); FLAIR (FLuid Attenuation Inversion Recovery) (8000 ms, 101 ms, 5 mm); and coronal T2-weighted turbo spin echo (7150 ms, 111 ms, 5 mm) MR tomograms.

Proton multi-voxel intermediate- (1700 ms, 135 ms) and short-echo-time (1700, 30 ms) MRS data sets were acquired using a two-dimensional (2D) PRESS (Point RESolved Spectroscopy) technique, with automatic volume-selective shimming. Features of the spectroscopic slab were: volume of interest 80 × 80 × 10 mm; thickness 10 mm; and region of interest positioned in the supracallosal white and gray matter. The multi-voxel network consisted of 64 voxels, of which we chose 12 symmetrical voxels in both hemispheres:

1, 2: the grey matter of the prefrontal cortex (PFC);
3, 4: the ventral part of the anterior cingulate gyrus (ACG);
5, 6: the frontal centrum semiovale white matter (FCSWM);
7, 8: the dorsal part of the ACG;
9, 10: the intraparietal sulcus (IPS); and
11, 12: the posterior cingulate gyrus (PCG) (Fig. 1).

| Variable                      | N  | Mean  | SD   | Min  | Max  | 25th | 50th  | 75th |
|-------------------------------|----|-------|------|------|------|------|-------|------|
| Age (years)                   | 19 | 45.16 | 11.47| 25   | 66   | 34.00| 43.00 | 56.00|
| Education (years)             | 19 | 12.21 | 3.39 | 4    | 16   | 10.00| 10.00 | 16.00|
| Nadir CD4 count (cells/mL)    | 19 | 267.32| 208.62| 11 | 694 | 105.00| 200.00| 460.00|
| CPE                           | 19 | 8.92  | 1.23 | 7    | 10   | 8.50 | 9.00  | 10.50|
| CD4 count (cells/mL)          |    |       |      |      |      |      |       |      |
| 2011                          | 19 | 558.58| 296.28| 210 | 1122 | 261.00| 526.00| 801.00|
| 2016                          | 19 | 731.06| 283.78| 347 | 1195 | 450.00| 754.00| 984.00|
| cART (years)                  |    |       |      |      |      |      |       |      |
| 2011                          | 19 | 5.65  | 2.25 | 1.5  | 11   | 2.25 | 5     | 8.75 |
| 2016                          | 19 | 10.65 | 2.25 | 6.5  | 16   | 7.25 | 10    | 13.75|

**Table 1.** Demographic and basic clinical data for the study participants at baseline and after five-year follow up.
Post-processing was performed on the Leonardo workstation (Siemens, Erlangen, Germany) by two experienced neuroradiologists. The post-processing protocol consisted of baseline corrections, peak identification, and metabolite ratio calculations.

In each subject, two experienced neuroradiologists in consensus, selected the voxels that best covered the primary regions of interest (ROI), based on their location on the matching images (high-resolution). The investigators were blinded to the time of acquisition of the data. The mean of the peak area for observed metabolites were computed from fitted spectral data.

The peaks identified were: NAA; Cho; mI; and total creatine (Cr), used as a reference marker. Ratios of NAA/Cr and Cho/Cr on intermediate-echo time and mI/Cr on short-echo time were analyzed.

Neurocognitive assessment. Synchronous follow-up neurocognitive testing was performed at the same time points as MRS. All participants underwent a 90-minute neuropsychological test battery that assessed multiple neurocognitive functions typically found to be impaired in HIV-positive subjects. This battery consisted of: the Mini Mental Score Examination (MMSE); the Trial making test form A and B (TmtA, TmtB); the Rey auditory-verbal learning test (RAVLT); the Rey-Osterrieth complex figure test (ROCF); the Verbal fluency test-phonemic and categorical fluency tests; the Verbal and visuospatial memory span; the Wisconsin Sorting Card test; and the Beck depression inventory scale II. The same experienced clinical psychologist conducted all the neuropsychological testing. The study was conducted in compliance with the Helsinki Convention principles, was ethically approved, and every subject signed a written consent to participate in the research.

Statistical analysis. Statistical analysis was performed using IBM SPSS software (version 21.0, Chicago, IL, USA). Descriptive statistics included mean values, standard deviation, median, minimum and maximum for continuous and median values, range (minimum-maximum), and interquartile range (IQR) for variables that did not follow a normal distribution.

Metabolite ratios of NAA/Cr, Cho/Cr, and mI/Cr that were obtained on the MRS scans in 2011 and in 2016 were compared using the Wilcoxon signed-rank test for repeated measurements. Due to the suspected instability of Cr, we performed longitudinal analysis calculations of NAA/Cho and NAA/mI ratios, in order to exclude the influence of Cr on metabolite ratios. The results of follow-up neurocognitive assessments performed at the same time as MRS were compared using the same test. We used this non-parametric test used because of the small sample size. For the same reason, we used Bonferroni corrections, designating p-values equal to or below 0.004 as significant (12 voxel locations, 0.05/12 = 0.004), and 0.05 > p > 0.004 as indicative of a trend toward significance.

Ethical approval. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent. Informed consent was obtained from all individual participants included in the study.

Figure 1. Multi-voxel network with analyzed voxel locations: prefrontal cortex in the right (1) and left (2) hemisphere; ventral anterior cingulate gyrus in the right (3) and left (4) hemisphere; dorsal anterior cingulate in the right (7) and left (8) hemisphere; centrum semiovale deep white matter in the right (5) and left (6) hemisphere; posterior cingulate gyrus in the right (11) and left (12) hemisphere; and intraparietal sulcus in the right (9) and left (10) hemisphere.
Results

NAA/Cr ratios. A significant rise in the NAA/Cr levels in 5/12 analyzed voxels was observed in the following locations (Table 2): in the PFC on the left and right; in the right ventral ACG; in the left FCSWM; and in the right PCG ($p < 0.001$). In the left dorsal ACG, the right IPS, and the left IPS, the increase trended toward significance ($p = 0.019$, $p = 0.015$ and $p = 0.027$, respectively).

Cho/Cr ratios. A significant increase in the Cho/Cr levels was detected in only one voxel, the right dorsal ACG ($p = 0.001$). However, in several additional voxels, we observed an increase in Cho/Cr levels that trended toward statistical significance in the left PFC ($p = 0.013$), in the left FCSWM ($p = 0.005$), and in the left ($p = 0.007$) and right IPS ($p = 0.009$) (Table 3).

mI/Cr ratios. Longitudinal changes in mI/Cr were heterogeneous. We observed an increase in the mI/Cr level in the left ventral ACG ($p = 0.002$), the right dorsal ACG ($p = 0.001$), the right IPS ($p < 0.001$), and the right PCG ($p = 0.001$). In the left IPS ($p = 0.029$), the increase trended toward significance. In the left dorsal ACG, and in the right and left FCSWM, we confirmed a decline in mI/Cr ($p = 0.033$, $p = 0.008$ and $p = 0.017$, respectively), but this only trended toward significance (Table 4).

| NAA/Cr     | N  | Mean  | SD   | Min  | Max  | 25th | 50th | 75th | Z*   | p*   |
|------------|----|-------|------|------|------|------|------|------|------|------|
| Right prefrontal cortex | 2011 | 19   | 1.636 | 0.229 | 1.21 | 2.16 | 1.480 | 1.690 | 1.800 | −3.059 | 0.002 |
|             | 2016 | 19   | 2.017 | 0.330 | 1.54 | 2.69 | 1.750 | 2.010 | 2.230 | −3.622 | <0.001 |
| Right ventral ACG | 2011 | 19   | 1.402 | 0.252 | 0.83 | 1.91 | 1.220 | 1.350 | 1.600 | −3.622 | <0.001 |
|             | 2016 | 19   | 1.890 | 0.173 | 1.58 | 2.24 | 1.740 | 1.870 | 1.980 | −0.644 | 0.520 |
| Left prefrontal cortex | 2011 | 19   | 2.235 | 0.422 | 1.63 | 3.20 | 1.900 | 2.200 | 2.520 | −3.159 | 0.002 |
|             | 2016 | 19   | 2.141 | 0.294 | 1.63 | 2.56 | 1.890 | 2.210 | 2.380 | −0.762 | 0.446 |
| Left ventral ACG | 2011 | 19   | 1.587 | 0.169 | 1.29 | 1.93 | 1.430 | 1.580 | 1.690 | −3.341 | 0.001 |
|             | 2016 | 19   | 1.955 | 0.351 | 1.27 | 2.53 | 1.680 | 2.030 | 2.150 | −2.354 | 0.019 |
| Right centrum semiovale WM | 2011 | 19   | 2.514 | 0.503 | 1.74 | 3.52 | 2.020 | 2.490 | 2.880 | −2.435 | 0.015 |
|             | 2016 | 19   | 2.392 | 0.539 | 1.62 | 3.53 | 1.990 | 2.150 | 2.790 | −3.824 | <0.001 |
| Right dorsal ACG | 2011 | 19   | 1.993 | 0.291 | 1.58 | 2.67 | 1.760 | 1.970 | 2.110 | −1.503 | 0.133 |
|             | 2016 | 19   | 1.769 | 0.300 | 1.18 | 2.16 | 1.540 | 1.760 | 1.990 | −2.354 | 0.019 |
| Left dorsal ACG | 2011 | 19   | 1.483 | 0.282 | 0.72 | 1.92 | 1.370 | 1.530 | 1.680 | −3.341 | 0.001 |
|             | 2016 | 19   | 1.781 | 0.830 | 1.07 | 2.67 | 1.530 | 1.720 | 2.020 | −2.435 | 0.015 |
| Left centrum semiovale WM | 2011 | 19   | 1.440 | 0.405 | 1.00 | 2.94 | 1.240 | 1.330 | 1.510 | −3.824 | <0.001 |
|             | 2016 | 19   | 2.176 | 0.407 | 1.59 | 3.08 | 1.840 | 2.100 | 2.460 | −0.806 | 0.420 |
| Right intraparietal sulcus | 2011 | 19   | 2.057 | 0.350 | 1.62 | 2.89 | 1.780 | 1.980 | 2.200 | −2.214 | 0.027 |
|             | 2016 | 19   | 2.376 | 0.380 | 1.59 | 3.01 | 2.160 | 2.400 | 2.690 | −3.824 | <0.001 |
| Right PCG | 2011 | 19   | 1.516 | 0.189 | 1.03 | 1.85 | 1.420 | 1.480 | 1.620 | −0.806 | 0.420 |
|             | 2016 | 19   | 1.947 | 0.236 | 1.62 | 2.45 | 1.740 | 1.920 | 2.140 | −2.214 | 0.027 |
| Left PCG | 2011 | 19   | 1.998 | 0.352 | 1.39 | 2.78 | 1.790 | 1.960 | 2.170 | −2.214 | 0.027 |
|             | 2016 | 19   | 1.908 | 0.212 | 1.39 | 2.17 | 1.740 | 1.980 | 2.080 | −2.214 | 0.027 |
| Left intraparietal sulcus | 2011 | 19   | 1.902 | 0.316 | 1.47 | 2.85 | 1.670 | 1.910 | 2.010 | −0.806 | 0.420 |
|             | 2016 | 19   | 2.163 | 0.329 | 1.65 | 2.93 | 1.940 | 2.030 | 2.420 | −2.214 | 0.027 |

Table 2. Longitudinal changes in NAA/Cr levels in named locations in the brain obtained on long-echo time MRS ($p \leq 0.004$ significant, $0.05 > p > 0.004$ trending toward significance). *Wilcoxon signed-rank test. ACG- anterior cingulate gyrus, WM-white matter, PCG-posterior cingulate gyrus.
In the right frontal ACG ($p < 0.001$), the left PFC ($p < 0.001$), the left FCSWM ($p < 0.001$), the right IPS ($p = 0.001$), and the right PCG ($p = 0.001$), we observed a significant increase in the NAA/Cho ratio, while, in the right PFC and the left PCG, the increase trended toward significance ($p = 0.011$ and $p = 0.009$, respectively). However, in the right FCSWM and the dorsal ACG on the same side, a significant decline in this ratio was observed ($p = 0.001$ and $p = 0.001$, respectively), as well as a decrease in the left PCG ($p = 0.009$) that trended toward significance.

In the right ventral ACG, the left FCSWM, the right IPS, and the right PCG ($p = 0.003$, $p < 0.001$, $p = 0.001$ and $p < 0.001$, respectively), a significant increase in NAA/mI was observed. A decrease in the NAA/mI ratio was significant in the right FCSWM ($p = 0.003$) and in the right dorsal ACG that trended toward significance ($p = 0.009$).

In the five-year follow-up period, there was a trend toward a better performance for functions of delayed recall and recognition of verbal material (RAVLT A7, RAVLT recognition list A), for tests of visuospatial orientation abilities (ROCF), and for visuospatial span (WMS-R visual span: backward and forward span) (Table 5). The results of the other neurocognitive tests showed no significant longitudinal differences.
Discussion

Short-term longitudinal changes on MRS in the HIV-infected brain are well documented, and show a reduction of immune activation and inflammation in the brain parenchyma after the introduction of cART. Despite the clear signs of neuroinflammation in the early phase of HIV infection, no signs of a neurodegenerative process have been observed. Prior to the cART era, Chong et al. were the first to report a progressive reduction of the NAA level in HIV-positive subjects in a short follow-up period (three to eight months). Furthermore, based on later longitudinal studies, long-term control of both processes, neuroinflammation and consequent neurodegeneration, seemed to be suboptimal despite cART and good peripheral viral suppression.

To the best of our knowledge, this is the first five-year follow-up longitudinal MR spectroscopic study on chronic, neurologically asymptomatic, aviremic HIV-positive patients, stable on cART (≥ one year at the baseline), with a normal MRI. According to the published data on neuropathogenesis of HIV infection and the suboptimal efficacy of cART in the brain, we expected to find signs of further, steady HIV-associated neuronal damage despite the reduction of an ongoing inflammatory process in patients with undetectable plasma viremia. Surprisingly, the results showed the expected, ongoing, low-level inflammation (reflected by a mI/Cr increase), and a clear long-term increase in NAA/Cr, suggesting the possibility of partial functional brain remodeling under cART.

Table 4. Longitudinal changes in mI/Cr levels in named locations in the brain obtained on short-echo time MRS (p ≤ 0.004 significant, 0.05 > p > 0.004 trending toward significance). *Wilcoxon signed-rank test. ACG—anterior cingulate gyrus, WM—white matter, PCG—posterior cingulate gyrus.

| Location                  | mI/Cr | N  | Mean  | SD   | Min  | Max  | 25th | 50th | 75th | Z*  | p* |
|---------------------------|-------|----|-------|------|------|------|------|------|------|-----|----|
| Right prefrontal cortex   |       |    |       |      |      |      |      |      |      |     |    |
| 2011                      | 19    |    | 0.483 | 0.184 | 0.22 | 0.87 | 0.320 | 0.480 | 0.650 | −1.699 | 0.089 |
| 2016                      | 19    |    | 0.708 | 0.448 | 0.32 | 1.91 | 0.440 | 0.560 | 0.750 |    |    |
| Right ventral ACG         |       |    |       |      |      |      |      |      |      |     |    |
| 2011                      | 19    |    | 0.674 | 0.238 | 0.34 | 1.19 | 0.520 | 0.650 | 0.840 | −1.894 | 0.058 |
| 2016                      | 19    |    | 0.553 | 0.151 | 0.28 | 0.90 | 0.430 | 0.560 | 0.650 |    |    |
| Left prefrontal cortex    |       |    |       |      |      |      |      |      |      |     |    |
| 2011                      | 19    |    | 0.587 | 0.121 | 0.39 | 0.80 | 0.510 | 0.600 | 0.650 | −0.926 | 0.354 |
| 2016                      | 19    |    | 0.649 | 0.160 | 0.43 | 1.05 | 0.530 | 0.640 | 0.790 |    |    |
| Right ventral ACG         |       |    |       |      |      |      |      |      |      |     |    |
| 2011                      | 19    |    | 0.465 | 0.148 | 0.20 | 0.66 | 0.320 | 0.460 | 0.580 | −3.081 | 0.002 |
| 2016                      | 19    |    | 0.601 | 0.109 | 0.32 | 0.77 | 0.570 | 0.620 | 0.660 |    |    |
| Left prefrontal cortex    |       |    |       |      |      |      |      |      |      |     |    |
| 2011                      | 19    |    | 0.587 | 0.121 | 0.39 | 0.80 | 0.510 | 0.600 | 0.650 | −0.926 | 0.354 |
| 2016                      | 19    |    | 0.649 | 0.160 | 0.43 | 1.05 | 0.530 | 0.640 | 0.790 |    |    |
| Right centrum semiovale WM|       |    |       |      |      |      |      |      |      |     |    |
| 2011                      | 19    |    | 0.402 | 0.154 | 0.20 | 0.81 | 0.250 | 0.400 | 0.470 | −2.657 | 0.008 |
| 2016                      | 19    |    | 0.556 | 0.160 | 0.23 | 0.83 | 0.420 | 0.560 | 0.680 |    |    |
| Right dorsal ACG          |       |    |       |      |      |      |      |      |      |     |    |
| 2011                      | 19    |    | 0.391 | 0.147 | 0.21 | 0.89 | 0.310 | 0.350 | 0.430 | −3.220 | 0.001 |
| 2016                      | 19    |    | 0.614 | 0.122 | 0.30 | 0.78 | 0.550 | 0.590 | 0.720 |    |    |
| Left dorsal ACG           |       |    |       |      |      |      |      |      |      |     |    |
| 2011                      | 19    |    | 0.725 | 0.218 | 0.30 | 1.09 | 0.540 | 0.750 | 0.850 | −2.134 | 0.033 |
| 2016                      | 19    |    | 0.598 | 0.131 | 0.35 | 0.81 | 0.470 | 0.590 | 0.720 |    |    |
| Left centrum semiovale WM |       |    |       |      |      |      |      |      |      |     |    |
| 2011                      | 19    |    | 0.712 | 0.180 | 0.31 | 1.09 | 0.610 | 0.710 | 0.830 | −2.392 | 0.017 |
| 2016                      | 19    |    | 0.575 | 0.209 | 0.15 | 1.20 | 0.460 | 0.570 | 0.620 |    |    |
| Right intraparietal sulcus|       |    |       |      |      |      |      |      |      |     |    |
| 2011                      | 19    |    | 0.444 | 0.143 | 0.19 | 0.83 | 0.360 | 0.420 | 0.500 | −3.543 | <0.001 |
| 2016                      | 19    |    | 0.650 | 0.146 | 0.34 | 0.85 | 0.540 | 0.680 | 0.770 |    |    |
| Right PCG                 |       |    |       |      |      |      |      |      |      |     |    |
| 2011                      | 19    |    | 0.382 | 0.105 | 0.16 | 0.59 | 0.320 | 0.360 | 0.450 | −3.315 | 0.001 |
| 2016                      | 19    |    | 0.607 | 0.148 | 0.32 | 0.92 | 0.500 | 0.610 | 0.720 |    |    |
| Left PCG                  |       |    |       |      |      |      |      |      |      |     |    |
| 2011                      | 19    |    | 0.415 | 0.101 | 0.27 | 0.61 | 0.350 | 0.390 | 0.470 | −0.095 | 0.240 |
| 2016                      | 19    |    | 0.418 | 0.103 | 0.22 | 0.67 | 0.370 | 0.400 | 0.420 |    |    |
| Left intraparietal sulcus |       |    |       |      |      |      |      |      |      |     |    |
| 2011                      | 19    |    | 0.395 | 0.165 | 0.07 | 0.66 | 0.230 | 0.390 | 0.550 | −2.179 | 0.029 |
| Test      | Year | N  | Mean  | SD    | Min | Max  | 25th | 50th | Median  | 75th^a | Z* | p* |
|----------|------|----|-------|-------|-----|------|------|------|---------|--------|----|----|
| MMSE     | 2011 | 19 | 27.36 | 2.014 | 24  | 30   | 25.00| 28.00| 29.00   | 29.00  | −1.179 | 0.238 |
| MMSE     | 2016 | 19 | 28.36 | 2.335 | 24  | 30   | 27.00| 29.00| 30.00   | 30.00  | −0.460 | 0.654 |
| TmtA     | 2011 | 19 | 37.27 | 14.51 | 15  | 64   | 25.00| 35.00| 32.00   | 52.00  | −0.059 | 0.953 |
| TmtA     | 2016 | 19 | 54.18 | 26.17 | 22  | 113  | 36.00| 42.00| 73.00   | 58.00  | −1.513 | 0.130 |
| RAVLT A1-A5 | 2011 | 19 | 49.00 | 7.823 | 38  | 59   | 42.00| 47.00| 58.00   | 58.00  | −0.148 | 0.137 |
| RAVLT A1-A5 | 2016 | 19 | 44.64 | 9.811 | 32  | 62   | 34.00| 46.00| 50.00   | 50.00  | −2.661 | 0.008 |
| RAVLT list A6 | 2011 | 19 | 10.00 | 3.493 | 5   | 15   | 8.00 | 9.00 | 14.00   | 14.00  | −0.955 | 0.339 |
| RAVLT list A6 | 2016 | 19 | 8.36  | 3.472 | 2   | 15   | 6.00 | 9.00 | 10.00   | 10.00  | −1.487 | 0.137 |
| RAVLT list A7 | 2011 | 19 | 12.30 | 4.06  | 4   | 18   | 10.5 | 12.00| 15.5    | 15.5   | −1.542 | 0.123 |
| RAVLT list A7 | 2016 | 19 | 11.45 | 4.72  | 6   | 20   | 7.00 | 11.00| 15.0    | 15.0   | −0.358 | 0.720 |
| Verbal fluency S | 2011 | 19 | 11.80 | 4.76  | 5   | 20   | 8.50 | 11.00| 14.5    | 14.5   | −0.298 | 0.765 |
| Verbal fluency K | 2011 | 19 | 10.91 | 4.66  | 4   | 18   | 8.00 | 10.00| 16.0    | 16.0   | −0.358 | 0.720 |
| Verbal fluency L | 2011 | 19 | 10.80 | 3.53  | 6   | 18   | 7.75 | 10.5 | 12.5    | 12.5   | −1.542 | 0.123 |
| Verbal fluency animals | 2011 | 19 | 12.20 | 6.29  | 16  | 34   | 17.75| 19   | 29      | 29     | −1.368 | 0.171 |
| WCST-cat  | 2011 | 19 | 5.45  | 1.29  | 2   | 8    | 3.00 | 6.00 | 6.00    | 6.00   | −0.756 | 0.450 |
| WCST-cat  | 2016 | 19 | 5.00  | 1.73  | 2   | 6    | 3.00 | 6.00 | 6.00    | 6.00   | −0.756 | 0.450 |
| WCST-nos  | 2011 | 19 | 1.18  | 0.67  | 0   | 2    | 0.00 | 0.00 | 1.00    | 1.00   | −1.289 | 0.197 |
| WCST-pers.err | 2011 | 19 | 12.55 | 10.32 | 3   | 38   | 5.00 | 9.00 | 19.0    | 19.0   | −0.153 | 0.878 |
| Digit span forward | 2011 | 19 | 8.91  | 0.539 | 6   | 8    | 7.00 | 7.00 | 7.00    | 7.00   | −1.586 | 0.113 |
| Digit span backward | 2011 | 19 | 6.18  | 1.250 | 4   | 8    | 5.00 | 7.00 | 7.00    | 7.00   | −0.997 | 0.319 |
| Digit span backward | 2016 | 19 | 5.55  | 1.440 | 4   | 8    | 4.00 | 5.00 | 7.00    | 7.00   | −1.926 | 0.044 |
| Digit span backward | 2016 | 19 | 5.09  | 0.944 | 4   | 7    | 4.00 | 5.00 | 6.00    | 6.00   | −1.978 | 0.040 |
| Digit span backward | 2016 | 19 | 5.91  | 1.044 | 5   | 8    | 5.00 | 6.00 | 7.00    | 7.00   | −1.978 | 0.040 |
| ROCF copy | 2011 | 19 | 5.36  | 0.924 | 4   | 7    | 5.00 | 6.00 | 6.00    | 6.00   | −1.926 | 0.044 |
| ROCF copy | 2016 | 19 | 6.18  | 1.250 | 4   | 8    | 5.00 | 6.00 | 7.00    | 7.00   | −0.997 | 0.319 |
neurodegeneration. Microglial cells are not equally distributed in the brain, with a variable density in the cerebral cortices, the brain stem, and the fiber bundles. Banati observed that persistent subtle microglial activity suggests a suboptimal performance of cART in the brain, especially in the regions that have a significant number of microglial cells. This might explain the stable prevalence and incidence of milder forms of HIV-associated cognitive disorder in patients with cART.

Although we observed a transient increase in mI/Cr in acute (up to eight weeks after transmission) and primary HIV infection (up to six months after transmission) by highly penetrative cART. In addition, a neurotoxic effect from long-standing cART might also be implied, concordant with a recent study by Winston et al.22. The increase in both Cho/Cr and ml/Cr ratios in our study may represent ongoing low-level inflammation and cell proliferation triggered by the presence of uneradicated HIV virus and its particles in the brain. This further suggests a suboptimal performance of cART in the brain, especially in the regions that have a significant number of microglial cells. This might explain the stable prevalence and incidence of milder forms of HIV-associated cognitive disorder in patients with cART.

Since the first studies on HIV-related brain injury, the diffuseness of the neurodegenerative process has been emphasized42. A rather global increase in NAA/Cr was observed in our five-year follow-up study. Nevertheless, the increase in ml/Cr was seen only in some locations (PFC, IPS, and FCSWM). This raises the question of whether certain brain regions are more susceptible to HIV injury due to complex pathways of HIV-associated neurodegeneration. Microglial cells are not equally distributed in the brain, with a variable density in the cerebral cortices, the brain stem, and the fiber bundles. Banati observed that persistent subtle microglial activity modulates neuronal functioning directly or indirectly, via astrocytic interaction. Microglial activation mobilizes functional compartments in which signaling molecules (neurokines, etc.) imply a more open cell-to-cell communication, not present in the healthy adult brain. These communications occur in neuronal pathways where this glial activation is under the influence of specific brain pathology, independent of disease etiology. This forms the framework for functional remodeling in the brain43.

During pathological process in the brain, the proliferation of microglia forms a network with regional differences that are not random, but functionally dependent43. Thus, microglial proliferation and membrane metabolism may be increased in those regions of the brain that constitute the brain circuits involved in attention and working memory, immediate and delayed recall, and visuospatial abilities, as a consequence of functional compartment remodeling in response to HIV-related chronic neuronal injury. The demonstrated increase in

| Test      | N | Mean | SD  | Min  | Max  | 25th | Median | 75th | Z*  | p*   |
|-----------|---|------|-----|------|------|------|--------|------|-----|-----|
| 2011      | 19| 31.09| 3.254| 25.50| 36.00| 28.50| 31.50  | 34.00| −2.456| 0.014|
| 2016      | 19| 33.86| 2.491| 28.00| 36.00| 34.00| 34.00  | 36.00| −0.494| 0.621|

**Table 5.** Longitudinal trends in specific neuropsychological tests obtained on the Wilcoxon signed-rank test (statistical significance set at p < 0.05). *Wilcoxon signed-rank test. MMSE—Mini Mental Score Examination, TmtA—Trial making test A (time in seconds), Tmt B—Trial making test B (time in seconds), RAVLT—Rey auditory-verbal learning test, verbal fluency S, L, K, animals—Serbian adaptation of verbal fluency FAS test, WCST—Wisconsin card sorting test, cat—categories, pers. err. –perseverative errors, ROCF—Rey-Osterrieth complex figure, BDI-II—Beck Depression Inventory II.
neurobiochemical markers could represent proliferation of radial glial cells that lead neuronal migration to the site of injury. The dynamics of neurobiochemical changes observed in the locations analyzed in this study were supported by the results obtained in some neurocognitive tests. Namely, we observed better performance for the functions of immediate and delayed recall, regulated mostly by the dorsal ACG and parts of the PFC. Dysfunction in connections to the ACG results in apathy, impulsivity, and disinhibition - behavioral changes that occur in neurodegenerative diseases. Finally, we observed better achievement in visual attention and working memory, regulated by many networks in the brain, mainly PCG. It comes as no surprise that previous studies on HIV-positive subjects confirmed the most prominent reduction in NAA/Cr levels exactly in this region. In light of these findings, a question could be raised about the functional remodeling of the brain pathways involved in cognition, predominantly in attention/working memory, visuospatial abilities, and delayed recall and recognition of verbal material. Sanford et al., in a recently published two-year follow-up study, showed better interval performance on several neurocognitive tasks, especially TmtA, concordant with our study. The authors commented on the result as a probable beneficial effect of the on-time introduction of cART and long-standing stable aviremia.

Limitations. The major limitation of the present study is the small number of participants, partially due to strict inclusion criteria. However, we performed a power analysis calculation for all three metabolite ratios. For NAA/Cr levels, the power analysis showed a satisfactory sample size (0.84–0.99), so that differences in NAA/Cr...
levels can be considered relevant despite a small sample size. The results of the power analysis for Cho/Cr and mI/Cr levels also showed a satisfactory sample size (0.98–0.99 and 0.86–0.99, respectively). Additional limitation is the partial volume effect of the cerebrospinal fluid, white matter or blood vessels) that can be present in some voxels that include mostly grey matter, such as voxels placed in circulate gyrus. However, investigators that performed the MRS data processing controlled for this contaminating and selected only spectra that best covered the ROIs with the grey matter. Finally, a significant issue might be the use of metabolite ratios instead of absolute values, given that the level of Cr is unstable in chronic HIV infected population, due to decreased uptake in the gut38, or changes in the brain synthesis of Cr.

Conclusions
In summary, an unexpected longitudinal increase in NAA/Cr in neurologically asymptomatic HIV-positive patients on cART, observed on multivoxel MRS, followed by better performance on neurocognitive tests with regard to attention/work memory, delayed recall, recognition and visuospatial abilities, suggests functional remodeling of brain circuits in HIV-related neurodegeneration. Increased microglial proliferation and membrane metabolism might be implied in cell-to-cell communication and functional remodeling as a response to chronic neuronal injury. However, the latter may also reflect persistent inflammation and immune activation, probably due to the suboptimal efficacy of cART in the brain. Regional differences in mI/Cr increases suggest that some brain regions are more prone to inflammation, either due to anatomical or neurophysiological features.

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Competing interests
The authors declare no competing interests.

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