INTRODUCTION

Significant evidence supports the view that the mechanisms contributing to brain alterations in human immunodeficiency virus (HIV) infection are due, at least in part, to neuroinflammatory processes (9). Brain autopsy reveals in HIV with acquired immune deficiency syndrome (AIDS) a characteristic neuropathology including macrophage infiltration, microglial nodules and multinucleated giant cells (44, 58, 64). Central nervous system (CNS) HIV infection appears to begin with the transmigration of peripheral HIV-infected cells (eg, monocyttes or macrophages) across the blood brain barrier (BBB) (38, 46, 47, 53, 57, 82, 89, 100, 104) and consequently infection, primarily of microglia, the resident macrophages of the brain (24, 32, 37, 40, 50, 55, 64). Activation of microglia and the release of chemokine, cytokines and neurotoxins in conjunction with secreted HIV protein can damage the brain (36, 56, 76, 85, 103).

In addition to diseases such as HIV wherein the primary pathological process has an inflammatory component, recent hypotheses suggest that “neuroinflammation” is also causative in the brain damage that occurs in neurodegenerative diseases, for example, Alzheimer’s and Parkinson’s diseases (4, 70, 91). These theories speculate that microglial activation results in the release of noxious stimuli (eg, reactive oxygen species, chemokines, cytokines) that cause activation of nearby microglia and other cell types (eg, astrocytes, neurons) to release additional toxic stimuli. This cascade results in a self-perpetuating cycle of sustained chronic neuroinflammation that can drive neurodegeneration (eg, 69, 86). That microglia have a low activation threshold, however, suggests that they may be ubiquitously involved even in subtle brain pathologies (42). Thus, in neurodegenerative diseases, pervasive microglial activation may reflect neuronal injury, dysfunction or loss, and is more likely a response to than the cause of neuronal damage (81, 90).

Regardless of the underlying primary pathology, it is clear that in a variety of CNS disorders microglial activation, neuroinflammation and neurodegeneration are intimately connected (43). Thus, the identification of markers that can distinguish between component processes of these independent mechanisms is important in elucidating a better understanding of brain pathology. In vivo brain magnetic resonance (MR) techniques are unique in their ability for longitudinal tracking of disease course. Longitudinal study can assess exacerbation/remission cycles of the natural course of disease as well as treatment efficacy. Quantitative MR assessment tools include structural MR imaging (MRI), which provides assessment of brain macrostructure; diffusion tensor imaging (DTI), which assesses microstructural white matter tissue integrity; and MR spectroscopy (MRS), which quantifies brain...
methods

participants

all subjects provided written, informed consent to participate in this study, which was approved by the institutional review boards of sri international and stanford university. mrs data were collected in 170 participants: 62 healthy controls (27 women, age 48.3 ± 20.4, range 20.0–86.3), 37 individuals with alcohol use disorders (13 women, age 48.5 ± 10.6, range 21.7–67.0), 33 individuals positive for hiv (11 women, age 50.0 ± 9.1, range 25.6–64.3) and 38 individuals comorbid for alcoholism + hiv (13 women, age 51.0 ± 6.6, range 31.9–64.1; table 2). controls, reported in a previous study on healthy aging (109), were recruited from the local community by flyers, advertisement and word of mouth. the alcoholic subjects were recruited from local treatment programs. the hiv-infected subjects were recruited and screened through the aids community research consortium by clinical research registered nurses or physicians. subjects in the four groups underwent a formal interview with clinical research psychologists, who used the structured clinical interview for the diagnostic and statistical manual iv. potential participants were excluded for axis i diagnoses of bipolar disorder or schizophrenia, or for any non-alcohol substance use (except cannabis) in the previous month or abuse in the previous 3 months. additional exclusion criteria for all subjects were diagnoses or medical conditions that can affect brain functioning (eg, diabetes, head injury, epilepsy, uncontrolled hypertension, radiation, cns opportunistic infection or chemotherapy) or preclude mr study (eg, pacemakers). at the start of each test day all subjects were given a breathalyzer test; subjects not achieving a score of 0.0 were not tested that day.

the four groups were matched in age, ratio of women, body mass index (bmi), systolic and diastolic blood pressure, and heart rate (table 1). based on a quantitative handedness questionnaire (on which right-handed scores = 14–32 and left-handed scores = 50–70), all but 13 participants (5c, 3a, 2h, 3ha), who scored above 50, were right handed (average score = 23.5 ± 11.0) (25). analysis of variance (anova) and follow-up t-tests demonstrated that the control group relative to the other three groups had higher socioeconomic status \[f(3,166) = 17.61, p ≤ 0.0001\] (51), more education \[f(3,166) = 14.25, p ≤ 0.0001\] and higher estimated general intelligence \[f(3,165) = 11.27, p = 0.0001\], based on the national adult reading test (nart) (80). on the dementia rating scale (drs), all but one participant (an h man, score = 121) scored within the normal range for healthy individuals living in the community (cutoff for dementia ≤24 out of 144) (65, 101). lifetime consumption of alcohol averaged 1319 kg in the a group and 829 kg in the ha group. the two alcohol groups consumed significantly more alcohol in their lifetime than did the non-alcoholic groups, and the a group reported a higher total lifetime alcohol consumption than did the ha group. the two hiv

### Table 1. Magnetic resonance spectroscopy signals†.

| Metabolite       | Contains                          | Location               | Proposed roles/function(s)                                                                 |
|------------------|-----------------------------------|------------------------|------------------------------------------------------------------------------------------|
| N-acetyl aspartate | N-acetyl aspartate, N-acetyl aspartate glutamate | Gray matter, primarily neurons | Amino acid, osmolite, source of acetyl for myelin lipid synthesis, storage form of aspartate, energy metabolism; marker for neuronal density/integrity |
| Total creatine   | tCr                               | Neurons, astrocytes, oligodendrocytes | Reservoir for ATP generation, influenced by the state of high-energy phosphate metabolism, osmolyte |
| Choline-containing compounds | Cho | White matter | Essential nutrient, required for acetylcholine and phosphatidylcholine synthesis, osmolyte; marker for cell membrane synthesis and turnover |
| Myo-inositol     | ml                                | Gray matter, primarily glia | Osmolite, storage form of glucose, precursor of myelin phosphatidyl inositol, progenitor of the inositol polyphosphate messenger cascade, breakdown product of phosphatidyl inositol; glial marker |
| Glutamate        | Glu                               | Primarily neurons      | Amino acid, key molecule in cellular metabolism, most abundant excitatory neurotransmitter, principal neurotransmitter of cortical efferents |

†From references (41, 108). ATP = adenosine triphosphate.
groups were matched in CD4 count and viral load. All but four individuals in the H group and six individuals in the HA groups were on highly active antiretroviral HIV treatment (HAART). An AIDS-defining event was classified by the presence of one of the following: CD4 count <200, cytomegalovirus, *Pneumocystis carinii* pneumonia, *Mycobacterium avium* complex, *Mycobacterium tuberculosis*, cryptosporidiosis, toxoplasmosis or wasting syndrome attributed to HIV.

### Blood draws

Blood collected from 146 subjects (38 C, 37 A, 33 H, 38 HA) was processed by Quest Diagnostics for determination of complete blood count (CBC) with differential, comprehensive metabolic panel (CMP), HIV variables including CD4 and CD8 counts, HCV and hepatitis B (HBV) status, and quantification of HIV and HCV viral load. Thiamine diphosphate (vitamin B1) levels were determined in whole blood by ARUP Laboratories, Salt Lake City, UT, USA. Blood analysis was missing on 24 healthy control subjects because they were recruited for an initial aging MRS study that did not include blood draws (106).

### MRI acquisition

An axial fast spin echo (FSE) MRI [field of view = 24 cm, frequency encode = 256, average echo time (TE)/T2/repetition time (TR) = 17/102/7500 ms, phase encode = 192, echo train length = 8, slice thickness = 2.5 mm, spacing = 0 mm] was acquired on a GE 3T MR (GE Healthcare, USA) scanner and was used for MRS voxel placement.

### MRS acquisition

MRS was performed using constant time point-resolved spectroscopy (CT-PRESS) (30). Single voxels were manually positioned in left or right striatum (10.6 cc), left or right cerebellum (9.8 cc) and central pons (5.9 cc); hemisphere of voxel placement was balanced across subjects and groups (Figure 1A). The acquisition time was ~9 minute per voxel [TE = 139 ms, 129 chemical shift (CS) encoding steps, $\Delta t/2 = 0.8$ ms, TR = 2 s, 2 averages] (66). A scan without water suppression was acquired (17 CS encoding steps, $\Delta t/2 = 6.4$ ms, 2 averages) to measure the tissue water content used to normalize the metabolite signal intensities. Data acquired without water suppression were apodized in $t_z$ with a 5 Hz Gaussian line broadening and zero filled up to 4K points for each TE.

After performing a fast Fourier transform (FFT) along $t_z$, water spectra were evaluated by peak integration. The amount of cerebrospinal fluid (CSF) and tissue water was estimated by fitting the spectra in the following: CD4 count $<200$, cytomegalovirus, *Pneumocystis carinii* pneumonia, *Mycobacterium avium* complex, *Mycobacterium tuberculosis*, cryptosporidiosis, toxoplasmosis or wasting syndrome attributed to HIV.
decoupled one-dimensional CT-PRESS spectra were obtained by integrating the two-dimensional spectrum in magnitude mode along $f_2$ within a ±13 Hz interval around the spectral diagonal. The quality of the spectra allowed evaluation of signals of the major proton metabolites: NAA (2.01 ppm), tCr (3.03 and 3.93 ppm), Cho (3.20 ppm), Glu (2.35 ppm) and mI (3.52 ppm) (Figure 1B). After baseline subtraction, three large singlet resonances (NAA, tCr and Cho) were fitted simultaneously, and the Glu and mI resonances fit independently, with a Gaussian function within a ±7.95 Hz window using a downhill simplex method (IDL AMOEBA). The integrated area under the fitted Gaussian was used for quantification.

**Motion evaluation**

Accuracy of voxel placement is potentially affected by subject motion in the time interval between voxel placement and acquisition as well as during acquisition itself, which can result in reduced spatial overlap between the prescribed and the actual voxel volume. To exclude voxels with significantly reduced overlap, subject motion was detected by comparison of the FSE images acquired before MRS acquisition and a three-plane anatomical localizer acquired thereafter.

To this end, binary masks of the prescribed voxel volumes were first generated in the space of the FSE images. The FSE images were then aligned with the post-MRS localizer by rigid image registration (http://nitrc.org/projects/cmlk). If there was no motion, the resulting image-to-image coordinate transformation should be identical to the ideal transformation, which is determined from the image coordinates in the scanner coordinate system as recorded in the DICOM image files. The deviation of the actual from the ideal transformation (ie, the difference transformation) represents the subject motion between the FSE used for voxel prescription and the post-MRS localizer scan. The effect of motion on a voxel depends not only on the detected motion but also on the location and size of that voxel (eg, rotation around the center of a voxel has a smaller effect than rotation by the same angle around a point outside the voxel). To quantify this effect, the aforementioned difference transformation was, therefore, applied to each prescribed voxel ROI in FSE space, yielding a reformatted voxel ROI, also in FSE space, which represented the voxel that was actually acquired. The overlap of prescribed and acquired voxel was then computed and expressed in percentage of the voxel volume. When there was no motion detected, the difference transformation was the identity mapping and both ROIs were identical, resulting in perfect 100% overlap. A threshold for partitioning of the overlap distribution at 62% for all three regions was used.

**Statistical analysis**

A total of seven striatal (five poor spectral quality, two low overlap; 3A, 3H, 1HA), 12 cerebellar (seven poor spectral quality,
four low overlap, one never collected; 5C, 5A, 2HA) and 18 pontine (nine poor spectral quality, six low overlap, three never collected; 6C, 8A, 1H, 3HA) voxels were excluded. Thus, data of adequate quality were analyzed for 163 striatal voxels, 158 cerebellar voxels and 152 pontine voxels. Metabolite values used for analysis were age-corrected $z$-scores. ANOVA was used to evaluate group-by-region effects on metabolites. Significant effects were followed up by simple regressions or paired $t$-tests, where appropriate.

RESULTS

Four-group analyses

ANOVA were used to determine group differences in the levels of five metabolites (NAA, tCr, Cho, Glu, mI) in three regions (striatum, cerebellum and pons). The only significant four-group ANOVA were for Cho [$F(162) = 2.92, P = 0.0360$] and mI [$F(162) = 4.19, P = 0.0069$] in the striatum (Figure 2). Follow-up $t$-tests showed that group differences in striatal Cho ($P = 0.0044$) and mI ($P = 0.0007$) levels were due to higher levels in HA compared with the C group.

Correlations between Cho levels and blood markers

Correlations between various blood markers and brain Cho and mI levels were evaluated in only the H + HA groups. Although all laboratory results (eg, CBC, CMP) were evaluated for their relationship with regional brain Cho and mI levels, correlational relationships were limited. After examination of all potentially contributing factors, only four variables contributed to explaining the variance in regional Cho levels: the presence of an AIDS-defining event (yes/no), HCV status (yes/no), medication status (yes/no—on efavirenz or atipla, an HIV medication including efavirenz, emtricitabine, tenofovir) and levels of thiamine (ie, TDP) in whole blood. Of the HIV subjects, 15 H and 18 HA individuals had a history of an AIDS-defining event, 8 H and 9 HA individuals were on efavirenz, 10 H and 18 HA individuals tested positive for HCV. Whole-blood TDP levels (quantified in 15 H and 24 HA individuals) were lower in the HA than A group [$F(39) = 4.33, P = 0.0443$].

In the striatum, these four variables together explained 40% of the variance in Cho levels [$F(35) = 5.31, P = 0.0022$]. Indeed, just medication status (yes/no—on efavirenz or atipla) and thiamine
levels together explained 40% of the striatal Cho variance \([F(35) = 11.17, P = 0.0002]\). Figure 3 presents parametric and non-parametric results of the effects of each of these four variables independently on Cho levels in the three regions. In the striatum, having HCV was associated with higher striatal Cho levels \([t(64) = 2.3, P = 0.0234; \chi^2 = 8.57, P = 0.0034]\), but being on efavirenz was associated with lower striatal Cho levels \([t(65) = 4.2, P \leq 0.0001; \chi^2 = 14.14, P = 0.0002]\). These competing effects on Cho levels (ie, HCV and an AIDS-defining event associated with higher Cho, being on efavirenz and low thiamine levels associated with lower Cho) were also seen in the cerebellum and pons, and likely contribute to the variance remaining relatively consistent whether two or four variables are included in the multiple regressions.

Examination of all potentially contributing factors to the variance in striatal mI showed that total lifetime alcohol consumed was associated with higher mI \((r = 0.28, P = 0.0230; \rho = 0.24, P = 0.0493)\), whereas being on HAART medication was associated with lower striatal mI levels \([t(65) = 1.9, P = 0.0573; \chi^2 = 4.2, P = 0.0395]\) (Figure 4).

**DISCUSSION**

Here, we attempted to identify a pattern of differences in MRS-detectable metabolites (ie, elevated mI, Cho and tCr) in HIV observed elsewhere (eg, 21, 61, 71, 98, 99) that might reflect neuroinflammation (22). In the current study, Cho and mI were elevated in the striatum of individuals comorbid for HIV and alcoholism compared with controls, but not in subjects with HIV alone. The other MRS metabolites (ie, NAA, tCr, Glu) measured in the striatum, cerebellum and pons were not different among the four groups.

One explanation for the discrepancy between current and previous findings is the status of HIV subjects included in the studies with respect to control of viral load. Although treatment-naïve HIV infection may be associated with elevated mI and Cho levels that correlate with viral load (20, 35), concentrations of these metabolites normalize with treatment (19, 96). Such an interpretation is supported by the current findings showing that HIV-positive individuals taking efavirenz have lower striatal Cho levels, and those...
on HAART have lower striatal mI levels compared with those not on such a medication regime. Another interpretation, however, is that HAART medications, particularly efavirenz, rather than counteracting neuroinflammation in HIV are themselves toxic (1, 23), thereby causing reductions in Cho levels (34). Indeed, there is evidence that efavirenz may induce neurotoxicity by increasing brain proinflammatory cytokines (29).

Patterns of abnormal metabolite levels must be interpreted within the context of the disease examined. In addition to neuroinflammation, elevated levels of Cho have been interpreted as reflecting energy failure, membrane degradation, demyelination, angiopathy or edema in neurodegenerative disorders such as MS (14, 62). Elevated Cho levels have also been reported in pathologies including tumors (15), stroke (95), epilepsy (31), traumatic brain injury (13, 18, 93) and acute disseminated encephalomyelitis (3, 7). Relevant to the current study, HCV is associated with elevated Cho levels (45, 68, 102). By contrast, Wernicke’s encephalopathy, a neurological disorder caused by thiamine deficiency, is associated with abnormally low Cho levels prior to treatment (63, 79). Our animal models have revealed similar

Figure 4. Striatal, cerebellar and pontine mI levels in H + HA individuals grouped by total lifetime alcohol consumed and medication status (HAART, yes/no). a.u. = arbitrary units; H = human immunodeficiency virus (HIV) positive; HA = HIV positive + alcoholic individuals; HAART = highly active antiretroviral HIV treatment; mI = myo-inositol; a.u. = arbitrary units.
competing effects on Cho levels: high alcohol exposure is associated with elevations in Cho (107, 108, 110, 112), whereas thiamine deficiency, a model for Wernicke’s encephalopathy, is accompanied by reductions in Cho (111).

ml is an organic osmolyte that participates in volume regulatory mechanisms in astrocytes. Specifically, ml is reduced in hypo-osmolar conditions but elevated in hyper-osmolar states such as hypernatremia, renal failure and diabetes (10, 49). ml is also elevated in acute MS during active myelin breakdown (27, 54) and in HCV (8) but reduced in anorexia nervosa (17) and hepatic encephalopathy (6, 59, 73, 92), a condition that has neuroinflammatory components (77, 113). Acute alcohol given to humans (5) or rats (2, 12) results in reduced ml, but treatment seeking alcohol-dependent patient show elevated ml (94). It is thought that alcohol-induced hyper-osmolarity may trigger ml accumulation in chronic alcoholism in order to stabilize the intracellular environment. These later results support the current finding of higher ml in individuals who have consumed high levels of alcohol. As mentioned, lower levels of ml in individuals on HAART medications may reflect efficacy of treatment.

Few MRS studies have conducted histopathological analysis to confirm that the interpretations of changes to MRS-detectable metabolites are selective, robust and reliable (75). As an initial example, numerous MRS studies reference an in vitro NMR spectroscopy experiment conducted on rat brain tissue extracts to support the claim that ml is a glial marker (eg, 22, 41, 48, 87). However, even the original study (11) cautioned against over-interpretation because “these results (were) obtained from juvenile primary cells (and) cannot necessarily be extrapolated to the situation in the adult brain.” Furthermore, a similar study comparing ml levels in extracts from neuronal and a glialoma cell lines found that the concentration of ml from the two cultures was similar (78).

Studies in which correlations between MRS-detectable metabolites and markers for inflammation were conducted expand on this point. In an MRS study of HIV, CSF levels of neopterin, a compound synthesized by macrophages and indicative of a pro-inflammatory immune status, were found to correlate modestly with higher ml in the posterior cingulate cortex ($P = 0.1$) and frontal white matter ($P = 0.09$) (26). In a similar MRS study in HIV, however, CSF neopterin levels did not correlate with MRS-detectable metabolites. This latter study instead showed that early HIV is associated with decreased NAA and Glu indicating neuronal injury (83).

In a macaque model of HIV (ie, simian immunodeficiency virus, SIV), lower NAA/tCr levels correlated with the presence of perivascular histiocytic infiltrates and multinucleated giant cells in brain parenchyma (60). Similarly, other SIV models found coincident increases in the percentage of CD14+CD16+ monocytes and decreases in NAA/tCr (16) with no effects on the infection of the ratios of Cho/tCr or ml/tCr (105). Such results suggest a relationship between NAA levels and inflammation, but NAA is clearly a non-specific marker of neuroinflammation. In a more recent MRS study of SIV, changes in tCr levels did not correlate with glial fibrillary acidic protein (GFAP) (ie, astrocytic) or ionized calcium binding adaptor molecule 1 (Iba-1) (ie, microglial) staining, but there was a modest relationship between higher tCr levels and histological evidence (ie, increased cellularity) for gliosis (88). Similarly, although marked gliosis was observed in postmortem tissue from SIV monkeys, this did not correlate with Cho levels quantified using in vitro MRS (39).

Thus, given the current results and the limited histopathology supporting interpretations of in vivo MRS findings, it is challenging to support the hypothesis that MRS-detectable elevations in ml and tCr represent a unique and robust pattern of metabolite changes that reflects neuroinflammation. Further considerations include the absence of standardized MRS methodology. For example, even though a number of studies have shown that tCr levels can change with age or disease (eg, 72, 84), tCr is still frequently used as a referent with the assumption that its concentration is stable. Indeed, the techniques currently available for MRS acquisition and analysis vary and are complex, thus making it difficult to achieve consistency across studies (28).

In conclusion, in vivo MRS markers alone are insufficient for identifying neuroinflammation. Indeed, it is unlikely that any single imaging modality will permit discrimination between neuroinflammatory and neurodegenerative mechanisms. A multimodal approach including postmortem histological confirmation will be necessary to achieve selective, robust and reliable markers for neuroinflammation.

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