Magnetic resonance imaging of neuroinflammation in chronic pain: a role for astrogliosis?

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Abstract
Non-invasive measures of neuroinflammatory processes in humans could substantially aid
diagnosis and therapeutic development for many disorders, including chronic pain. Several
proton Magnetic Resonance Spectroscopy (1H-MRS) metabolites have been linked with
glial activity (i.e. choline and myo-inositol) and found to be altered in chronic pain patients,
but their role in the neuroinflammatory cascade is not well known. Our multimodal study
evaluated resting fMRI connectivity and 1H-MRS metabolite concentration in insula cortex
in 43 patients suffering from fibromyalgia, a chronic centralized pain disorder previously
demonstrated to include a neuroinflammatory component, and 16 healthy controls. Patients demonstrated elevated choline (but not myo-inositol) in anterior insula (p=0.03), with greater choline levels linked with worse pain interference (r=0.41, p=0.01). In addition, reduced resting functional connectivity between anterior insula and putamen was associated with both pain interference (whole brain analysis, p_corrected<0.01) and elevated anterior insula choline (r=-0.37, p=0.03). In fact, anterior insula/putamen connectivity statistically mediated the link between anterior insula choline and pain interference (p<0.01), highlighting the pathway by which neuroinflammation can impact clinical pain dysfunction.

In order to further elucidate the molecular substrates of the effects observed, we investigated how putative neuroinflammatory $^1$H-MRS metabolites are linked with ex-vivo tissue inflammatory markers in a nonhuman primate model of neuroinflammation. Results demonstrated that cortical choline levels were correlated with glial fibrillary acidic protein, a known marker for astrogliosis (Spearman r=0.49, p=0.03). Choline, a putative neuroinflammatory $^1$H-MRS-assessed metabolite elevated in fibromyalgia and associated with pain interference, may be linked with astrogliosis in these patients.

**Key words**: chronic pain, pain interference, fibromyalgia, neuroinflammation, astrogliosis

**Introduction**

Prior studies have suggested that magnetic resonance imaging (MRI) methods such as proton Magnetic Resonance Spectroscopy ($^1$H-MRS) [3; 13] may be promising for non-invasive evaluation of neuroinflammation. Our aim was to explicitly evaluate whether $^1$H-MRS metabolites previously associated with neuroinflammation [13], choline and myo-
inositol, are altered in chronic pain patients suffering from fibromyalgia (FM). Brain neuroinflammation has been noted in animal models of chronic pain [29], and FM is a chronic centralized pain disorder which has recently been shown to display a neuroinflammatory component assessed with more invasive positron emission tomography (PET) [2]. We further sought to evaluate possible mechanisms by which levels of the above mentioned $^1$H-MRS metabolites are linked with neuroinflammation and contribute to chronic pain pathology.

Several neuroimaging studies have suggested neuroinflammation, mediated by activation of microglia or astrocytes, is pronounced in chronic pain patients. For instance, positron emission tomography (PET) imaging using the glia-linked radioligand [$^{11}$C] PBR28 demonstrated increased binding for chronic low back pain patients and FM [2; 4; 31], with elevated levels linked with fatigue [2]. In contrast to PET, $^1$H-MRS is a non-invasive neuroimaging technique that has suggested neuroinflammation by quantifying levels of metabolites such as choline, which shows greater concentration in glial cells than neurons, and has been linked with glial cell membrane turnover [13]. Myo-inositol, another $^1$H-MRS metabolite, plays a role in regulating glial cell volume and may also be a marker for neuroinflammation [13]. Myo-inositol is believed to be an essential requirement for cell growth, an osmolyte, and a storage source of glucose [50]. Furthermore, this metabolite is thought to serve as marker of neuroinflammation/gliosis [10; 46], however, its role as glial marker has also been questioned [44]. Choline and/or myo-inositol are elevated in the anterior cingulate cortex in spinal cord injury patients with neuropathic pain [57] and
rheumatoid arthritis patients [18]. Choline has also been found to be elevated in the anterior cingulate cortex in chronic pelvic pain patients and associated with higher levels of negative affect [24]. However, the precise role that choline and myo-inositol play in neuroinflammation, and how they are linked with other markers and maladaptive brain changes in FM, are unknown.

In this study, analyses focused on whether purported $^1$H-MRS markers of neuroinflammation (i.e. choline and myo-inositol) are linked with functional brain connectivity and clinical variables for FM. Our prior neuroimaging studies with chronic pain patients demonstrated that anterior insula cortex (aIns) connectivity was increased to the default mode network [37] and, specifically, aIns to medial prefrontal cortex connectivity was associated with $^1$H-MRS Glx metabolite concentration and clinical pain intensity in pelvic pain patients [7]. In a separate study, FM patients’ response to pharmacotherapy was associated with reduced $^1$H-MRS Glx metabolite concentration and insula connectivity to default mode network brain regions [25]. Given prior evidence of physiological disruption in the insula cortex, we assessed $^1$H-MRS derived metabolite levels in both anterior and posterior insula, and functional connectivity between insula and the rest of the brain. An exploratory analysis evaluated the fractional amplitude of low frequency fluctuations (fALFF) of the resting functional MRI signal, which has also been associated with neuroinflammation in chronic pain [6; 26]. Finally, in order to better understand the mechanisms supporting altered choline or myo-inositol in neuroinflammation, we directly linked levels of these metabolites with tissue pathology.
markers for astrogliosis and microglia activation in a nonhuman primate model of widespread viral neuroinflammation [48].

Methods

Human Participants

We enrolled female patients with FM diagnosis for at least one year, meeting Wolfe criteria [59], who were recruited from the Chronic Pain and Fatigue Research Center Registry at the University of Michigan. At screening visits, all participants completed an informed consent interview and were screened for exclusion criteria: 1) presence of a known coagulation abnormality, thrombocytopenia, or bleeding diathesis; 2) presence of a concurrent autoimmune or inflammatory disease; 3) history of head injury with substantial loss of consciousness; 4) peripheral neuropathy, 5) routine daily use of narcotic analgesics, marijuana, or history of substance abuse; 6) stimulant medications; 7) pregnant or nursing; 8) severe psychiatric illnesses (current schizophrenia, major depression with suicidal ideation, consistent with current medications), or 9) MRI contraindications. Female healthy adults were also recruited to form an age-matched control group, with the following exclusion criteria: 1) diagnosis of FM, 2) exclusion criteria noted for patients. For all subjects, non-steroidal anti-inflammatory medications (NSAIDs) were allowed, as were FDA approved medications for fibromyalgia (pregabalin and duloxetine). However, use of over-the-counter pain medications (NSAIDs, etc.) on the day of MRI scan was excluded as was use of narcotic pain medications 48 hours prior to MRI scan. Subjects were also excluded for routine daily use of narcotic analgesics, marijuana, or history of substance
abuse. Subjects taking stimulant medications, such as those used to treat ADD/ADHD (e.g., amphetamine/ dextroamphetamine [Adderall®], methylphenidate, dextroamphetamine), or the fatigue associated with sleep apnea or shift work (e.g., modafinil), were also excluded. Medication usage for all subjects has been included in Supplementary Table 1 (available at http://links.lww.com/PAIN/A952).

We enrolled Forty-three (N=43) female FM patients and sixteen (N=16) health controls (HC). All subjects were right-handed and there was no statistically significant age difference between FM and HC groups (FM: 41.07 ± 11.64 years old, HC: 44.94 ± 16.29 years old, mean ± SD; p=0.31, two-sample Student’s t-test). Twelve patients reported using pregabalin or gabapentin for treatment, which was controlled for in analyses due to our prior publications demonstrating significant neuroimaging changes within the insula following pregabalin treatment [25].

**Image acquisition and preprocessing**

Proton magnetic resonance spectroscopy ($^1$H-MRS)

$^1$H-MRS acquisition was conducted on a 3.0T scanner (Philips, Ingenia) using a 15 channel receive head coil. Anatomical T1-weighted images (Axial 3D-MP-RAGE, TR/TE = 8.2/3.7ms, matrix = 240×240, number slices = 154) were acquired to guide region of interest (ROI) definition for $^1$H-MRS. PRESS single voxel spectra (TR/TE = 2000/35ms) were acquired from a 2×2×3cm sized voxel covering the right anterior insula (aIns) region. A second voxel was acquired in the right posterior insula (pIns) region. Values for different
metabolites were computed from the raw spectra data based on a linear combination of
individual spectra using the LCModel software [42] (Version 6.3, Stephen Provencher Inc.,
Oakville, ON, Canada). As our study focused on potential neuroinflammation, analyses
evaluated choline and myo-inositol concentration. For the sake of transparency, we wanted
to report both of the conventionally reported measures for $^1$H-MRS concentration: the
metabolite ratio values (dividing by total creatine, tCr) and absolute concentrations, using
the water peak as reference from a separate scan. For the former, tCr concentration did not
differ between FM and HC (aIns: p=0.15, pIns: p=0.95, ANOVA with age correction),
allowing for interpretable FM vs HC comparisons using ratios of choline and myo-inositol
to tCr. For the latter, the concentrations were adjusted by an estimate of cerebrospinal fluid
(CSF) content in the voxel (hence, reported as Choline:CSF in the Table 2), with volume
computed using statistical parametric mapping 8 (SPM8; Wellcome Trust Centre for
Neuroimaging) software.

**Resting state functional MRI (rs-fMRI)**

Resting, whole brain blood-oxygen-level dependent (BOLD) images were acquired on the
same 3.0T system using a T2*weighted echo-planar sequence (TR/TE = 2000/30ms, flip
angle = 90°, matrix = 80×80, number slices = 38, slice thickness = 3.5mm, voxel size =
2.75×2.75mm$^2$, number of volumes = 180, total scan time = 360s). Subjects were instructed
to rest and keep their eyes open during the 6-minute scan.
Respiratory artifacts were corrected using the RETOICOR [21] algorithm, with respiratory belt data collected during the fMRI scan. Head motion correction included linear realignment with FSL-MCFLIRT. Brain extraction was completed with FSL-BET, and cortical surface reconstruction was completed with T1-weighted anatomical images using FreeSurfer’s recon-all for co-registration between anatomical and functional images using FreeSurfer’s bbregister tool. Spatial smoothing with 5mm FWHM gaussian kernel was applied to fMRI data, and further motion/cardiorespiratory artifact correction was completed with ICA-AROMA [43]. High-pass filtering was completed for functional connectivity analysis (f_c = 0.008Hz). Additionally, the six translation/rotation motion parameters from FSL-MCFLIRT and motion outlier confound matrices (FSL_motion_outliers) were regressed out of the fMRI data using generalized linear model (GLM) analysis for additional motion censoring. Following preprocessing, we excluded five patients due to excessive head movement (over 1mm displacement between successive image volumes). Thus, a total thirty-eight (N=38) FM patients and sixteen HC (N=16) were included for analysis. There were no differences in head motion (framewise displacement RMS) between FM and HC groups (p=0.26, two-sample Student’s t-test).

Seed-based functional connectivity

The alns seed was defined as gray matter voxels extracted from the larger 1H-MRS alns voxel from each subject’s native space in conjunction with cortical surface reconstructions from the recon-all Freesurfer tool. These ROIs in native space were registered to the Montreal Neurological Institute (MNI) space, and common overlapping voxels across
subjects was defined as the aIns seed for functional connectivity and power analyses (Fig. 1a), regionally linked to $^1$H-MRS data. Preprocessed resting BOLD fMRI images in native space were also spatially normalized to standard MNI space using FSL-FNIRT. Time-series averaged over the seed ROI were used as regressor in a GLM with the head motion nuisance regressors noted above (FSL-FEAT). Estimated parameter weight maps and their variance were then passed up to a group level GLM (FSL-FEAT, mixed-effect model).

In order to link connectivity with clinical, imaging, and spectroscopy metrics, a multivariable regression model was performed with functional connectivity metrics and metabolites/behavioral measures, correcting for age and pregabalin usage (yes/no). Maps from this covariate analysis were threshold on the voxel level ($z > 2.81$) and cluster corrected for multiple comparisons (familywise error rate, $p < 0.01$).

**Fractional amplitude of low-frequency fluctuations (fALFF)**

The fractional amplitude of low frequency fluctuations (fALFF) of the resting functional MRI signal [64], which is increased in multiple brain areas in chronic pain patients, has been recently suggested to play a role in neuroinflammation and associated with calcium waves in activated astrocytes [6; 26]. As low frequency power of the fMRI time series has been tangentially linked with glial processes, we also included the fALFF metric in this study.
We assessed spectral power within regions of interest using fALFF [63], a measure of total power normalized ALFF. This metric has been subdivided into three frequency bands [12] (slow5 = 0.01-0.027 Hz; slow4 = 0.027-0.073 Hz; and slow3 = 0.073-0.192 Hz), thought to relate to different functional process, with slow4 possibly linked with neuroinflammation [6]. Computation of fALFF slow4 was carried out using MATALB (The Mathworks Inc., Natick, MA, USA). This metric was computed using the same resting state BOLD fMRI data as was used for connectivity analyses. The fALFF metric assessed the amplitude of the fluctuating BOLD fMRI signal within a specific frequency band. Preprocessing included motion censoring, which was performed using FSL_REGFIT in native space. Further preprocessing for fALFF analyses was similar to preprocessing for seed-based function connectivity analyses. However, instead of frequency-based temporal filtering, linear detrending was performed prior to fALFF calculation. Following preprocessing, the fMRI data were fast-Fourier transformed, and the sum of amplitudes within the slow4 frequency band was calculated and normalized by dividing by total power. Averaged fALFF values for the gray matter portion of the aIns $^1$H-MRS ROI were then calculated.

Clinical measures
Clinical pain was assessed with the short form of the Brief Pain Inventory [15] (BPI) which consists of separate pain severity and pain interference subscales. Patients also completed the Fibromyalgia Impact Questionnaire [11] (FIQ), while depression and anxiety severity were assessed using the Hospital Anxiety and Depression Scale [61] (HADS).
Statistical analysis

Clinical assessment scores, alns choline and myo-Inositol, which were normally distributed (Lilliefors test, p>0.05), were compared between FM and HC using an analysis of variance (ANOVA) controlling for age and pregabalin status (yes/no). Subsequent analysis with functional connectivity metrics used z-statistics averaged over a 4 mm diameter sphere centered on the peak voxel of the significant cluster. Correlations between $^1$H-MRS, alns functional connectivity, fALFF, and the clinical assessment scores noted above were calculated using a partial correlation analysis, controlling for age and pregabalin status.

Mediation analysis was then performed with three modal variables using the R mediation package [54]. We evaluated whether the direct association between dependent and independent variables was significantly reduced by including a mediator variable, using nonparametric bootstrap estimation with 5000 iterations and random sampling. Confidence intervals for a causal mediation effect, direct effect, and total effect were computed at the 95% confidence level (age and pregabalin status corrected). All statistical analyses were performed with the R Statistical Package (R-Core-Team, 2012).

Non-human primates and neuroinflammation model

In order to gain insights into the cellular contributions to the imaging changes observed in the FM patients, and in particular to further validate the status of choline and myo-inositol as neuroinflammatory markers, we directly linked these $^1$H-MRS metabolites with immunohistochemical measures in an animal model of widespread viral neuroinflammation.
Because no universally accepted animal model for FM exists, we used the Simian immunodeficiency virus (SIV) infection model, which reliably produces extensive neuroinflammation. Evidence that HIV infection results in neuroinflammation originates predominantly from patients with HIV-associated neurocognitive disorders (HAND) and from animal models of NeuroAIDS [62]. HAND have been linked to altered $^1$H-MRS metrics, by our group and others [1; 45], as well as in animal models of NeuroAIDS [49]. Specifically, neuroinflammation with activated astrocytes and microglia is often associated with elevated myo-inositol and choline containing compounds.

The SIV model of NeuroAIDS was also specifically chosen because it exhibits dynamic changes in choline and myo-inositol during SIV progression, allowing for excellent sensitivity for correlating $^1$H-MRS markers of neuroinflammation with established tissue and CSF markers. These associations for widespread viral neuroinflammation may then be extended to better understand the role of $^1$H-MRS markers of neuroinflammation for conditions that may not have well established animal models, such as fibromyalgia.

Data from nineteen adolescent/adult (4- to 5-year old) rhesus monkeys (Macaca mulatta) were included for analysis. Detailed methods for our model have been previously published [47; 48]. Briefly, all animals were infected with SIVmac251 virus. To accelerate AIDS progression, CD8+ T lymphocytes were depleted using an anti-CD8 antibody which permits the virus to replicate unchecked, leading to neuroinflammation and SIV encephalitis in 85% of animals [58]. As we were interested in linking neuroinflammation...
with MRS metabolites, dynamic range in neuroinflammation was experimentally increased. Specifically, 12 animals remained untreated and were sacrificed at 4 weeks (4 animals), 6 weeks (4 animals) and 8 weeks (4 animals) post inoculation, respectively. Seven SIV infected CD8-depleted animals were treated with an agent known to reduce neuroinflammation, minocycline, orally starting at 4 weeks post inoculation, and at daily dosage of 4 mg/kg b.i.d for 4 weeks. The studies were approved by the Massachusetts General Hospital (MGH) Subcommittee on Research Animal Care (SRAC) and by the Institutional Animal Care and Use Committee of Harvard University. All studies were performed in accordance with federal laws and regulations, international accreditation standards, and institutional policies.

MRI and MRS was performed pre-inoculation and biweekly until sacrifice. During MRI sessions, each animal was tranquilized with 15–20 mg/kg intra-muscular ketamine hydrochloride and intubated to ensure a patent airway. Atropine (0.4 mg/kg injected intravenously) was administered to prevent bradycardia. A continuous infusion of propofol (0.25 mg/kg/min) was maintained throughout the scans via a saphenous vein catheter. Heart rate, oxygen saturation, end-tidal CO2, and respiratory rate were monitored continuously. A heated water blanket was used to prevent hypothermia. At study endpoints animals were anesthetized with ketamine-HCl and sacrificed by intravenous pentobarbital overdose.

All MRI/MRS scans were performed on a 3 Tesla whole-body imager (Magnetom TIM Trio, Siemens AG, Erlangen, Germany). As neuroinflammation is known to be diffuse with this
model, single voxel $^1$H MR spectroscopy was performed in an accessible cortical region, precuneus within the medial parietal cortex, using a point-resolved spectroscopy sequence (PRESS) with water suppression enhanced through T1 effects (WET), and the following parameters: voxel size $1.2 \times 1.2 \times 1.2 \text{ cm} = 1.7 \text{ cm}^3$, $\text{TE}=30 \text{ ms}$, $\text{TR}=2500 \text{ ms}$, spectral bandwidth = 1.2 kHz, 1024 complex data points and 192 acquisitions resulting in an acquisition time of 8 minutes. All spectra were processed offline using the LCModel software package [42] to determine the quantities of the brain metabolites choline and myo-inositol. Absolute metabolite concentrations in institutional units were derived from the same voxel using the water signal as reference. We could not report the metabolite ratio values (dividing by total creatine) as total creatine was significantly variable over the longitudinal timeframe for H-MRS scans [47]. To guide placement of the $^1$H-MRS volumes of interest, sagittal and axial turbo spin echo (TSE) images were obtained using the following parameters: $140 \times 140 \text{ mm}^2$ field of view, $512 \times 512$ matrix, $\text{TE (echo time)}$ of 16ms; slice thickness was 2mm for sagittal images and 1.2mm for axial images; $\text{TR (repetition time)}$ was 4500ms for sagittal and 7430ms for axial images.

At necropsy, animals were exsanguinated and perfused with 4L of chilled saline. A complete set of brain and peripheral tissues were collected in 10% neutral buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin (H&E). The degree of reactive astrogliosis was assessed in 16 animals with the monoclonal anti-glial fibrillary acidic protein (GFAP, 1:1000; Boehringer Mannheim, Indianapolis, IN). Paraffin sections (5mm-thick) from the precuneus were immunolabeled overnight with the monoclonal
antibody followed by biotinylated horse anti-mouse immunoglobulin G, avidin-horseradish peroxidase (Vectastain Elite kit; Vector, Burlingame, CA), and reacted with diaminobenzidine tetrahydrochloride and peroxide (0.03%). Levels of GFAP were estimated by means of computer-aided image analysis, as previously described [48]. The units of all measurements for GFAP are in arbitrary optical density units and range from 0 to 500.

Microglial activation was assessed by quantifying ionized calcium binding adaptor protein 1 (IBA-1) in 8 animals. Parietal cortical brain sections were incubated with rabbit anti-IBA-1 (Wako Corp. Japan). Images of tissue sections were captured without manipulation using an Olympus 3-CCD T60C color video camera mounted on an Olympus Vanox-SI microscope and analyzed using NIH Image J software (see [48] for more detail).

Additionally, CSF (≤1.0 ml) was collected at 4 time points from the cerebellomedullary cistern or lumbosacral junction from 8 animals while they were anesthetized for MRI/MRS scans. CSF samples were frozen immediately until assay. Levels of monocyte chemoattractant protein-1 (MCP-1), a chemokine biomarker of response to inflammation [16], was determined with ELISA (R and D Systems using the manufacturers protocol).

All statistical analyses for non-human primate data were performed using the R Statistical Package. Associations between neuroinflammatory markers (GFAP, MCP-1, and IBA-1) and both choline and myo-inositol levels were assessed with the non-parametric Spearman
correlation coefficient, due to non-normal distribution of the data.

Results

Pain-related clinical assessments

For demographic and clinical assessments, FM patients reported significantly higher BPI pain severity, pain interference, as well as HADS depression, and anxiety scores (all p<0.001) compared to HC (Table 1). Within the patient group, the FIQ score was highly correlated with BPI pain interference (r=0.76, p<0.001), whereas there was no correlation between the FIQ and pain severity scores (r=0.21, p=0.21).

$^1$H-MRS metabolite levels in aIns and pIns

Choline levels in aIns were significantly higher in FM patients compared to HC (Choline/tCr: p=0.03; trending for Choline/CSF: p=0.09) (Table 2, Fig. 1b), adjusted for age. Furthermore, pain interference was correlated with aIns choline/tCr (r=0.41, p=0.01), demonstrating that FM patients with high aIns choline also reported high pain interference. HADS depression and anxiety scores were not significantly correlated with aIns choline (Choline/tCr vs. depression: r=0.14, p=0.42; Choline/tCr vs. anxiety: 0.08, p=0.64). In contrast, aIns myo-inositol was not different between FM and HC (myo-inositol/tCr: p=0.41; myo-inositol/CSF: p=0.19) and there was no correlation between choline and myo-inositol in aIns (tCr: r=0.12, p=0.48; CSF: r=0.01, p=0.56). Posterior insula showed no significant metabolite level difference between FM and HC (Table 2). There were no outliers (Grubbs's test) for aIns choline/tCr, pain interference, pain severity, depression, or
anxiety levels. We have also included results for concentrations of other relevant metabolites, such as N-acetylaspartate (NAA), which has been previously linked with neurodegeneration and/or neuroinflammation (Supplementary Table 2, available at http://links.lww.com/PAIN/A952).

**Functional aIns connectivity**

While resting aIns connectivity was not different between FM and HC, within FM patients, aIns connectivity to left putamen (peak voxel MNI coordinates: -28, 10, -2 mm) was inversely correlated with pain interference score (Fig. 2b). Thus, greater pain interference was associated with weaker connectivity between aIns and left putamen. Connectivity between aIns and left putamen was also anti-correlated with aIns choline/tCr (r=-0.37, p=0.03) and FIQ score (r=-0.39, p=0.02). A whole brain analysis within HC found no significant correlation between aIns functional connectivity and aIns choline/tCr levels.

Mediation analysis demonstrated a significant mediation effect of aIns connectivity to putamen on the association between aIns choline/tCr and pain interference score (mediation effect: $\beta=15.78$, CI: 2.64 to 35.72, p=0.02; total effect: $\beta=28.72$, CI: 11.55 to 47.59, p<0.01) (Fig. 3). Thus, the direct effect of aIns choline on pain interference was significantly reduced (becoming non-significant) by aIns connectivity to left putamen ($\beta=12.94$, CI: -7.4 to 30.06, p=0.22).
Metabolite associations with blood-based and tissue pathology markers in non-human primates

The non-human primate neuroinflammation model demonstrated that GFAP in precuneus was positively correlated with the choline level change in this same brain region induced by SIV infection ($r=0.49$, $p=0.03$, Fig. 4a). Furthermore, MCP-1 demonstrated a trending correlation with the change in choline level ($r=0.32$, $p=0.07$, Fig. 4b). In contrast, IBA-1 was not correlated with choline level change in the same brain area ($r=0.34$, $p=0.27$). Moreover, change in myo-inositol levels was not correlated with GFAP ($r=0.34$, $p=0.14$), IBA-1 ($r=0.14$, $p=0.65$), nor MCP-1 ($r=0.10$, $p=0.57$).

Linking aIns fALFF with choline levels in FM patients

The slow4 band of fALFF was negatively correlated to choline/tCr levels in aIns ($r=-0.36$, $p=0.03$, Fig. 5), and a trending correlation was found for aIns myo-inositol/tCr levels ($r=-0.32$, $p=0.06$). Furthermore, aIns fALFF slow4 was also inversely correlated with HADS depression scores ($r=-0.37$, $p=0.03$), but not pain interference scores ($r=-0.13$, $p=0.44$). Interestingly, in the left putamen (the brain region implicated in aIns functional connectivity analyses above), fALFF slow4 power was inversely correlated with pain interference scores ($r=-0.35$, $p=0.04$) and HADS depression scores ($r=-0.44$, $p<0.01$). There was also trending negative correlation between putamen fALFF slow4 and aIns choline/tCr ($r=-0.32$, $p=0.06$), and putamen fALFF slow4 was correlated with aIns fALFF slow4 ($r=0.46$, $p=0.004$).
Discussion

Neuroinflammation is an important target in many disease pathologies including pain, and non-invasive measures of neuroinflammatory processes in humans could substantially aid diagnosis and therapeutic development. MRI-based metrics have been suggested in the past, and our study assessed both $^1$H-MRS metabolites (i.e. choline and myo-inositol) and resting fMRI-based metrics previously linked with glial activity. Our study found elevated choline (but not myo-inositol) levels in anterior insula for FM compared to healthy adults, with greater choline levels linked with worse pain interference. In addition, reduced connectivity between anterior insula and putamen was associated with both elevated anterior insula choline and pain interference. In fact, anterior insula/putamen connectivity mediated the link between anterior insula choline and pain interference.

Choline levels, assessed by $^1$H-MRS, have been associated with neuroinflammation, as elevated choline levels have been linked to glial activation or proliferation leading to abnormal phospholipid metabolism and accelerated cell membrane turnover [13; 22]. In previous studies, FM patients showed higher choline in right dorsolateral prefrontal cortex compared with healthy controls, and choline was also correlated with pain levels [40]. Urologic chronic pelvic pain syndrome patients have demonstrated elevated choline levels in anterior cingulate cortex [24], while other chronic pain disorders, including temporomandibular disorder, have also showed higher choline in posterior insula cortex [23]. However, we should note that increases in choline are commonly seen with diseases that have alterations in membrane turnover [9] in addition to inflammatory and gliotic
processes [34; 35]. Interestingly, we did not find altered myo-inositol levels in FM suggesting that this marker may not be as important as choline in tracking neuroinflammation in FM.

How neuroinflammation is linked with pain and pain-related morbidity also needs more clarification. Our mediation analysis revealed that while anterior insula choline was associated with pain interference, this linkage was in fact mediated by functional anterior insula connectivity to the putamen. Pain interference is a function of motivation and reduced bodily activity rather than the sensory aspect of clinical pain itself. The putamen is closely interconnected with other basal ganglia regions such as caudate nucleus, globus pallidus, and substantia nigra, as well as somatosensory, and motor areas. Such connections strongly influence the planning, learning, preparation, and execution of motor behaviors [5; 32]. Indeed, FM patients have shown prior evidence of disruptions in putamen structure and signaling, such as reduced gray matter volume and functional connectivity with insula cortex [14; 51]. Other FM studies have found reduced cerebral blood flow in putamen, which was correlated with pain disability [52], and reduced putamen response to a motor inhibition task [20], suggesting that attenuation of putamen activity in the motor initialization process. The results of these previous studies are supported by our findings linking neuroinflammation and reduced anterior insula / putamen connectivity in FM with pain interference, a measure that compared to pain intensity is more closely related to motor activity in these patients.
In fact, our study also found a relationship between fALFF slow4, another putative MRI marker for neuroinflammation, and choline levels in anterior insula. However, these two markers were inversely related – greater choline was linked with reduced fALFF slow4 power. Furthermore, reduced slow4 power was also correlated with depression, corroborating previous studies [30; 53]. The connection between fALFF slow4 and neuroinflammation is based on studies suggesting that astrocytic Ca\(^{2+}\) oscillations occur in both the slow4 and slow5 frequency range in the thalamus [38; 39]. In fact, a microglial mediator has also been linked with Ca\(^{2+}\) oscillations, and modulates synaptic neurotransmission [28]. Moreover, astrocytic Ca\(^{2+}\) waves have also been shown to regulate blood flow via a glio-vascular interaction [8; 41], leading to BOLD fMRI signal changes [56]. Such previous studies support the linkage between fALFF slow4 power and astrocytic Ca\(^{2+}\) oscillations. However, the inverse correlation in our study between slow4 power and choline was surprising and needs further clarification, which might come from a clarification of the role for choline in astrocyte and microglia activity. These results suggest that while neuroinflammation may be linked with both choline and fALFF slow4, the specific pathways reflected by these two metrics may be distinct, and may be due to different phases of the waxing and waning neuroinflammatory state in FM.

To better understand the mechanisms supporting altered choline or myo-inositol levels in neuroinflammation, we directly linked levels of these metabolites with blood-based and tissue pathology markers for neuroinflammation (i.e. astrogliosis and microglial activation). We used data collected from a previously reported nonhuman primate model of
neuroinflammation caused by SIV [48]. Our non-human primate model demonstrated that 
$^1$H-MRS choline levels were linked with glial fibrillary acidic protein (GFAP) and 
monocyte chemoattractant protein 1 (MCP-1). GFAP is the principal intermediate filament 
in mature astrocytes. Astrocytes rapidly synthesize GFAP in response to a neurologic insult, 
and GFAP is commonly used as marker of astrogliosis [19]. MCP-1 is a chemokine that 
plays a role in the recruitment of monocytes from the blood into the brain and is involved in 
the activation processes of inflammatory and neural cells that lead to development of 
degenerative changes in the neuronal population [17]. Interestingly, our primate model 
demonstrated no linkage between choline levels and IBA-1, which is an 
immunohistochemical marker involved in membrane ruffling of microglia – an essential 
morphological change for microglial activation [27]. Thus, our results suggest that $^1$H-
MRS-assessed choline levels do indeed track with neuroinflammation and are more likely 
associated with astrogliosis than microglial activation.

Furthermore, the nonhuman primate results found a positive correlation between choline 
and both GFAP and MCP-1, but not between myo-inositol and these inflammatory markers. 
The closer link between choline and neuroinflammation is also supported by our recent 
analysis showing that choline but not myo-inositol correlated with $^1$H-MRS-assessed brain 
temperature in the same regions [36]. While myo-inositol has been implicated in other 
studies as a potential neuroinflammatory marker, these data do not support its role in either 
FM neuroinflammation nor SIV-induced neuroinflammatory processes.
Several limitations of our study should be noted. For instance, previous studies have noted that anxiety and depression can also impact $^1$H-MRS metabolites [24; 33]. In our study, although anxiety and depression demonstrated marked differences between FM and HC, we did not find any association between aIns choline and mood measures suggesting that choline levels were more associated with pain-related neuroinflammatory processes. Additionally, alcohol consumption, which can also impact $^1$H-MRS metabolites in the brain [55; 60], was not considered in the exclusion criteria. Future studies should further consider alcohol consumption in exclusion criteria and as regressors in the general linear models being tested. Also, our ROI for $^1$H-MRS was limited to anterior insula and metabolite levels from the putamen would, in retrospect, have shed more light on the mechanisms by which neuroinflammation impacts pain variables in FM. However, our mediation analysis did reveal the importance of functional connectivity in mediating the link between choline levels and pain interference. Finally, our widespread viral neuroinflammation non-human primate model was not specific to fibromyalgia or chronic pain, and mechanisms underlying neuroinflammation in viral SIV (i.e. macrophage infiltration leading to glial activation), may differ from those that underlie neuroinflammation in fibromyalgia. However, we contend that the association between choline and astrogliosis tissue markers (i.e. GFAP) would generalize across neuroinflammatory states irrespective of mechanistic pathways.

In summary, our results suggest that neuroinflammation can be assessed non-invasively by $^1$H-MRS and resting BOLD fMRI-based metrics. Our non-human primate data suggest that
choline, which was an important metabolite in differentiating FM from healthy adults and linked with pain interference, may be specifically associated with astrogliosis.

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Conflict of Interest Disclosures: None

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**Fig. 1.** Metabolite levels in anterior insula (aIns). (A) $^1$H-MRS voxel placement for aIns (red mask represents overlapping voxels across all subjects). (B) Choline ratio to creatine was increased in FM compared to HC (p=0.02, red circle denotes mean). * indicates significant level of p < 0.05, age corrected (mean + residual), error bars denote SD.

**Fig 2.** Seed-based functional connectivity for the $^1$H-MRS voxel placed in right anterior insula (aIns) in FM patients. (A) A group map of aIns connectivity in FM patients (corrected p<0.01) demonstrated expected connectivity to other Salience Network regions such as mid-cingulate cortex, as well as posterior insula, visual cortex, basal ganglia, and cerebellum. (B) Correlation between aIns connectivity and pain interference score yielded a cluster in left putamen which was negatively correlated with pain interference (age and pregabalin status corrected).

**Fig. 3.** Mediation modeling found that aIns functional connectivity to left putamen mediates the association between aIns choline and pain interference. For mediation modeling, (A) the correlation between aIns choline/tCr and pain interference (total effect) and (B) the correlation between aIns choline/tCr and aIns connectivity to left putamen (path a) were both significant (p<0.05). (C) A significant mediation effect of aIns connectivity to putamen was noted (CME: $\beta$=15.78, CI: 2.64 to 35.72, p=0.02; proportions mediated: $\beta$=0.55, CI: 2.11 to 1.37, p=0.02). The model corrected for the effects of age and pregabalin status (mean + residual).
**Fig. 4.** In the non-human primate neuroinflammation model, post-infection choline level change in precuneus was correlated with (A) GFAP (Spearman r=0.49, p=0.03), and (B) trending for MCP-1 (Spearman r=0.32, p=0.07). (C) Medial parietal cortex (precuneus) voxel placement for the non-human primate model.

**Fig. 5.** Fractional amplitude of low frequency fluctuations (fALFF) show correlations with choline and HADS depression score in FM patients. fALFF slow in aIns was inversely correlated with (A) aIns choline ratio to total creatine and (B) HADS depression scores. (C) fALFF show in left putamen was also inversely correlated with HADS depression scores. N.b. data are adjusted for age and pregabalin status.
Table 1. Clinical and demographic data on the study subjects

<table>
<thead>
<tr>
<th>Domains</th>
<th>Group means (standard error)</th>
<th>Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FM</td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>(n=38)</td>
<td>(n=16)</td>
</tr>
<tr>
<td>Age(years)</td>
<td>42.11(11.67)</td>
<td>44.94(16.29)</td>
</tr>
<tr>
<td>Symptom (FIQ)</td>
<td>50.18(18.55)</td>
<td>NA</td>
</tr>
<tr>
<td>Pain</td>
<td>Pain severity (BPI)</td>
<td>5.37(1.50)</td>
</tr>
<tr>
<td></td>
<td>Pain interference (BPI)</td>
<td>5.06(2.05)</td>
</tr>
<tr>
<td>Mood</td>
<td>Depression (HADS)</td>
<td>7.26(3.55)</td>
</tr>
<tr>
<td></td>
<td>Anxiety (HADS)</td>
<td>7.68(4.26)</td>
</tr>
</tbody>
</table>

Note: FM = fibromyalgia; FIQ = Fibromyalgia Impact Questionnaire; BPI = Brief Pain Inventory; HADS = Hospital Anxiety and Depression Scale; * indicates statistical significance.
Table 2. Metabolite differences between fibromyalgia patients and healthy control

<table>
<thead>
<tr>
<th>Regions</th>
<th>Metabolites</th>
<th>Group means (standard error)</th>
<th>ANOVA statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Fibromyalgia</td>
<td>Control</td>
</tr>
<tr>
<td>Anterior insula</td>
<td>Choline:tCr</td>
<td>0.273 (0.006)</td>
<td>0.249 (0.010)</td>
</tr>
<tr>
<td></td>
<td>Choline:CSF</td>
<td>1.655 (0.038)</td>
<td>1.506 (0.065)</td>
</tr>
<tr>
<td></td>
<td>Myo-Inositol:tCr</td>
<td>0.647 (0.017)</td>
<td>0.623 (0.029)</td>
</tr>
<tr>
<td></td>
<td>Myo-Inositol:CSF</td>
<td>4.804 (0.149)</td>
<td>4.458 (0.255)</td>
</tr>
<tr>
<td>Posterior insula</td>
<td>Choline:tCr</td>
<td>0.248 (0.007)</td>
<td>0.261 (0.012)</td>
</tr>
<tr>
<td></td>
<td>Choline:CSF</td>
<td>1.432 (0.044)</td>
<td>1.151 (0.076)</td>
</tr>
<tr>
<td></td>
<td>Myo-Inositol:tCr</td>
<td>0.645 (0.017)</td>
<td>0.638 (0.029)</td>
</tr>
<tr>
<td></td>
<td>Myo-Inositol:CSF</td>
<td>4.089 (0.113)</td>
<td>3.986 (0.193)</td>
</tr>
</tbody>
</table>

Note: Choline:tCr = choline ratio to total creatine; Choline:CSF = CSF adjusted choline concentration; Myo-Inositol:tCr = myo-inositol ratio to total creatine; Myo-Inositol:CSF = CSF adjusted myo-inositol concentration; Age corrected in ANOVA, * indicates p<0.05.
A. alns choline vs. pain interference

B. alns choline vs. alns/putamen connectivity

C. Mediation effect (path ab): $\beta=15.78$, $p=0.02$

$\beta=28.72$, $p<0.01$

$\beta=-25.98$, $p=0.03$

$\beta=-0.68$, $p<0.01$

$c': \beta=12.94$, $p=0.22$
A. Change in choline vs. GFAP in parietal cortex  B. Change in choline in parietal cortex vs. MCP-1  C. Voxel placement for parietal cortex