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Impact of minocycline on cerebrospinal fluid markers of oxidative stress, neuronal injury, and inflammation in HIV seropositive individuals with cognitive impairment

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Abstract

Elevated cerebrospinal fluid (CSF) levels of markers of oxidative stress, neuronal injury, and inflammation, and decreased neurotransmitter levels have been reported in HIV-associated neurocognitive disorders (HAND). Minocycline may have a neuroprotective effect by inhibiting inducible nitric oxide synthase, which produces nitric oxide, a compound that induces oxygen free radical production. In A5235, “Phase II, Randomized, Placebo-Controlled, Double-Blind Study of Minocycline in the Treatment of HIV-associated Cognitive Impairment”, minocycline was not associated with cognitive improvement, but the effect on the above CSF measures was not examined previously. The objective of this study was to examine the effect of minocycline on markers of oxidative stress, neuronal injury, neurotransmitter levels, and inflammation from CSF in participants in A5235. 107 HIV+ individuals received either minocycline 100 mg or placebo orally every 12 hours for 24 weeks. 21 HIV+ individuals received the optional lumbar punctures. Lipid and protein markers of oxidative stress (*e.g.*, ceramides and protein carbonyls), glutamate, neurotransmitter precursors, kynurenine metabolites, neurofilament heavy chain and inflammatory cytokines were measured in the CSF before and after treatment. The 24-week change in ceramides was larger in a beneficial direction in the minocycline group compared to the placebo group. The

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two groups did not differ in the 24-week changes for other markers. These results suggest that minocycline may decrease lipid markers of oxidative stress (ceramides) in individuals with HAND; however, an effect of minocycline on other CSF markers was not observed. A larger sample size is needed to further validate these results.

Keywords

minocycline; HIV; cognitive; oxidative; stress; biomarker

Cognitive impairment remains as an important neurological manifestation of HIV-1 infection in 40–50% of HIV-1 seropositive (HIV+) individuals despite the introduction of combination antiretroviral therapies (ART) (McArthur, Steiner, *et al* 2010). HIV-associated neurocognitive disorders (HAND) are associated with chronic inflammatory processes and oxidative stress induced neuronal injury within the central nervous system (CNS). Oxygen free radicals can attack lipid membranes and proteins resulting in cellular dysfunction. HAND is associated with elevated cerebrospinal fluid (CSF) levels of lipid markers of oxidative stress (*e.g.*, ceramides), protein markers of oxidative stress (protein carbonyls), markers of neuronal injury (neurofilament heavy chain), excitotoxic neurotransmitters and metabolites (*e.g.*, glutamate, quinolinic acid) and markers of inflammation [TNF-α, IL-6, CXCL8, CXCL12, hepatocyte growth factor (HGF), osteopontin (OPN), and soluble FAS (sFAS), sFAS ligand], while concomitantly displaying deficiencies in neuroprotective neurotransmitters (serotonin and its precursor tryptophan, and dopamine) (Bandaru, McArthur *et al*, 2007; Nath, Li, *et al* 2006; Graham, Zeger *et al* 1992; Brown, Islam *et al* 2011; Burdo, Ellis *et al*, 2008; Letendre, Zheng *et al*, 2011; Yadav and Collman, 2009). An adjunctive therapy that interferes with the cascade of inflammatory events triggered by the HIV virus within the CNS is likely to play an important role in the treatment of HAND (Epstein and Gendelman 1993). Minocycline may have a neuroprotective effect by inhibiting inducible nitric oxide synthase, which produces nitric oxide, a compound that induces oxygen free radical production (Amin, Attur, *et al*, 1996). We conducted a phase II, randomized double-blind, placebo-controlled study within the Adult AIDS Clinical Trials Group (A5235) of minocycline to assess the safety, tolerability and efficacy of minocycline for the treatment of HIV-associated cognitive impairment (Sacktor, Miyahara *et al* 2011). In this study (Sacktor, Miyahara *et al* 2011), minocycline was safe and well-tolerated in individuals with HAND, but cognitive improvement was not observed. However, it is possible that CSF measures may be more sensitive of CNS injury than clinical measures of cognition. The objective of this study was to examine the effect of minocycline on CSF markers of oxidative stress, neuronal injury, neurotransmitter levels, and inflammation in A5235.

Methods

Recruitment, enrollment, randomization, therapy, and follow-up

The recruitment, enrollment, randomization, therapy, and follow-up details have been reported previously (Sacktor, Miyahara *et al* 2011). In brief, from March 2007 to September 2009, 107 HIV+ individuals with progressive neurocognitive impairment defined by either

objective or subjective criteria (Sacktor, Miyahara *et al* 2011) and on a stable antiretroviral regimen for at least 16 weeks prior to study entry were enrolled across 16 US sites. Participants were excluded if they were <18 or >65 years of age, had an estimated premorbid IQ <70 [as determined by the vocabulary section of the Wechsler Adult Intelligence Scale-Revised (WAIS-R)], or had a Karnofsky Functional Performance score <60. Participants were also excluded if they were pregnant or breast-feeding or had concurrent conditions including an active symptomatic AIDS-defining opportunistic infection within 45 days prior to entry, a current neoplasm, severe premorbid psychiatric illness, confounding neurologic disorder, CNS infection, active drug or alcohol use or dependence, or serious illness requiring systemic treatment that in the opinion of the investigator would interfere with study requirements.

At entry, stratified randomization, generated by the statistical and data management center at the Harvard School of Public Health and Frontier Science Technology Research Foundation, was utilized to assign treatment: minocycline 100mg orally every 12 hours or matching placebo orally every 12 hours (Sacktor, Miyahara *et al* 2011).

Participants received the study drug daily for 24 weeks during the double-blind phase. Participants were re-evaluated at 2, 4, 8, 12, 18, and 24 weeks after randomization for safety measures, and at baseline and 24 weeks after randomization for CSF measures. The safety measures, neuropsychological test results, and other clinical measures have been reported previously (Sacktor, Miyahara *et al* 2011).

Standard protocol approvals, registrations, and patient consents

The protocol was reviewed and approved by all appropriate committees of the AIDS Clinical Trials Group (ACTG) and the Institutional Review Board (IRB) at all participating sites. The study is registered in clinical trials.gov (NCT 0036 1257). Informed consent was obtained from all participants.

Outcome measures

Lipid markers of oxidative stress

Total lipids from CSF samples were prepared as previously described (Nath, Li et al 2006) by diluting 500 μL of CSF in three volumes of 100% methanol containing 30 mM ammonium acetate and then vortexing. Four volumes of chloroform were then added, and the mixture was vortexed and then centrifuged at 1,000*g* for 10 minutes. The bottom (chloroform) layer was removed and analyzed by direct injection into a tandem mass spectrometer. Electrospray ionization tandem mass-spectrometry (ESI/MS/MS) analyses were performed using methods similar to those used in our previous studies (Cutler, Kelly *et al* 2004). Samples were injected using a Harvard Apparatus pump at 15 μL/min into an electrospray ionization (*i.e.,* Turbo Ion Spray module) Sciex API 3000 triple-stage quadruple tandem mass spectrometer from Sciex Inc. (Thornhill, Ontario, Canada) operated in the positive mode. The ion spray voltage (V) was 5,500 at a temperature of 80 °C with a nebulizer gas of 8 psi, curtain gas of 8 psi, and the collision gas set at 4 psi. The declustering potential was 80 V, the focusing potential 400 V, the entrance potential −10 V, the collision energy 30 V, and the collision cell exit potential was 18 V. The MS/MS scanned from 300 to

2,000 atomic mass units (amu) per second at a step of 0.1 amu. Each species of ceramide initially was identified by a Q1 mass scan and then by precursor ion scanning or neutral loss scanning of a purified standard. Samples were injected into the ESI/MS/MS for 3 minutes, where the mass counts accumulated and the sum of the total counts under each peak was used to quantify each species. Ceramides C16:0, C18:0, C20:0, C22:0, C24:0, and C24:1, were purchased from Avanti Polar Lipids (Alabaster, AL). Palmitoyl-lactosyl ceramide C16:0 to C16:0, stearoyl-lactosyl ceramide C16:0 to C18:0, lignoceryl-glucosyl ceramide C16:0 to C24:0, lignoceryl-galactosyl ceramide C16:0 to C24:0, and stearoyl-galactosyl ceramide sulfate C18: to C24:0 were purchased from Matreya Inc. (Pleasant Gap, PA).

Protein carbonyl detection

Relative protein carbonyls were quantitated using the Oxyblot TM Protein Oxidation Detection Kit (Millipore cat# S7150). The kit was adapted to measuring protein carbonyls in CSF by using a larger volume for the derivatization reaction. The derivatized products were then quantitated by applying them to PVDF-FL transfer membrane by using a BioRad Slot-Blot apparatus. The blots were exposed to the Rbt X DNP in the Millipore kit and Licor's donkey anti-rabbit before scanning blots on Licor's Odyssey infrared imaging system. Semi-quantification of the relative densities was done by using the equipment software. The density readings were normalized between blots so there was a comparative measure of protein carbonyls in pixels/mm^2 for 20μl volume of CSF used in the assay.

Neurofilament heavy chain measurement

The neurofilament heavy chain (NFH) was measured by an electrochemiluminescent assay. An electrochemiluminescence (ECL) based sandwich ELISA was used to measure levels of NFH in CSF as previously described (Kuhle, Regeniter *et al* 2010). SMI35 antibody was used to capture the NFH after which Rabbit anti-NFH (cat#N4142 Sigma) was used as the secondary antibody. Sulfa Tag™ goat anti-Rabbit antibody was then used for detection (Mesoscale Discovery cat#R32AB-5) and read in a Sector2400 Reader (MesoScale Discovery) Our analytical sensitivity (background plus 3 SD) was 19.2pg/ml.

Neurotransmitters, neurotransmitter precursors, and kynurenine metabolites

For tryptophan metabolites, 25 μl of CSF were mixed with 25 μl of 0.2% ascorbic acid on ice. For amino acids (*e.g.*, glutamate, tryptophan, and tyrosine), 0.5 μl of CSF were mixed with 49.5 μl of 0.1% ascorbic acid. All samples were then spiked with 50 μl of a solution containing heavy standards: [$^2H_5$] glutamate, [$^2H_5$] tryptophan, [$^2H_2$] tyrosine (all from CDN Isotopes, Quebec, Canada); [$^{13}C_6$] anthranilic acid (Sigma Aldrich), [$^2H_6$] kynurenine (custom synthesis from Sigma Aldrich Isotec), and [$^2H_3$] quinolinic acid (custom synthesis from Synfine, Ontario, Canada). Heavy standards were at a concentration of 1 μM in the 50 μl volume with the exception of kynurenine (15 μM) and quinolinic acid (0.2 μM). 50 μl of −20°C acetone was added and samples were spun at >10,000 g for 5 minutes to precipitate protein. Eighty percent (120/150 μl) of the acetone supernatant was transferred to glass vials. Samples were derivatized as previously described (Notarangelo, Wu *et al* 2012) and were dried down at 50°C in a speedvac and stored at −80°C until analyzed by GC/MS.

GC/MS/MS was performed as previously described (Notarangelo, Wu *et al* 2012) with the addition of transitions for glutamate and tyrosine based on the methods described previously (Eckstein, Ammerman *et al* 2013). Briefly, the new transitions for glutamate and tyrosine are: glutamate Q1 = 537.2, Q3 = 313.0, CE = 15V, RT = 8.4 min; [$^2H_5$] glutamate Q1 = 542.2, Q3 = 314.0, CE = 10V, RT = 8.4 min; tyrosine Q1 = 585.2, Q3 = 268, CE = 20, RT = 10.95 min; [$^2H_2$] tyrosine Q1 = 587.2, Q3 = 436.9, CE = 20, RT = 10.95.

Data were analyzed with Agilent MassHunter software, Build B.04. Samples were injected twice and the average of the peak areas were normalized to the average of the heavy standards for each respective compound. These values were fit to the matrix-spiked light standard curves for each compound based on the standard addition method and multiplied by the appropriate dilution factor.

Inflammatory marker measurements

Inflammatory markers (TNF-α IL-6, CXCL8, CXCL12, HGF, OPN, sFAS, and sFAS ligand,) were measured by multiplexed bead assays (Luminex®). Multiplexed assay kits and beads were obtained from commercial sources (*e.g.*, Millipore ®) and procedures followed manufacturers' recommendations. Blinded samples were measured in duplicates and blank values subtracted from all readings. Measurements and data analysis of all assays were performed with the Luminex-200® system in combination with Luminex manager software (Bioplex manager 5·0, Bio-Rad, Hercules, CA). The laboratory quality assurance program used standard operating procedures to review results for unexpected or unacceptable variance (evidence of bead clumping; coefficients of variation greater than 20%; unusual distributions of values; outliers more than 4 SD from the mean) (Pardo, Buckley *et al* 2013).

Statistical analysis

All analyses were conducted based on observed data and as-treated. All markers were compared between the minocycline and placebo groups by the Wilcoxon test without adjustment for multiple comparisons. A total of 21 tests were conducted with 5% Type I error for each test, and thus the chance of having at least one false positive is 66% even if there is no minocycline effect. All analyses were conducted based on the selected participants who were willing to receive the lumber punctures at week 0 and 24, and there may be a possibility of sampling bias. All 24 week changes of biomarkers are defined as baseline value minus 24 week value.

RESULTS

Baseline characteristics

We examined potential selection bias by comparing the baseline characteristics of participants with a CSF marker (and thus included in this study) vs. those A5325 participants without a CSF marker. None of these differences in baseline demographics were statistically significant. Differences were not detected with respect to baseline demographics (data not shown).

Twenty-one HIV+ individuals received the optional lumbar puncture for CSF measures at baseline and 24 weeks after baseline. All HIV+ individuals were on combination antiretroviral drug therapy (ART). Differences were not detected in age, education, gender, plasma HIV RNA levels, non nucleoside reverse transcriptive inhibitor (NNRTI) based ART, protease inhibitior (PI) based ART, or the central nervous system penetration effectiveness (CPE) of the ART as defined by the CPE scale (Letendre, Marquie-Beck *et al* 2008) between the minocycline and placebo groups. The minocycline group had a higher baseline CD4 lymphocyte count [median (IQR) = 772 (595–910) cells/mm^3], compared to the placebo group [420 (356–647) cells/ mm^3], (p=0.033) (see Table 1).

CSF ceramide changes over 24 weeks

CSF was not available for all of the markers examined in this study. Fourteen HIV+ individuals (minocycline arm = 6, placebo arm = 8) had CSF ceramides measured at baseline and at the week 24 visit. Table 2 describes the 24 week changes in ceramide, monohexoysylceramides, dihydroglycosyl galactosylceramides (dihydroG/c galactosylceramides), and dihexosylceramides over 24 weeks stratified by treatment arm.

The ceramide median 24 week change in the minocycline group was 0.0007 counts per second (cps) suggesting a beneficial decrease in oxidative stress for ceramide compared to the placebo group (median 24 week change = −0.0051 cps), (p=0.017). Improvements in CSF ceramide measures were noted both as a summary measure of the median ceramide change for 8 individual ceramides of varying carbon lengths as well as the median ceramide change for specific carbon lengths, *e.g.*, C16:1 (p=0.020), C22:0 (p=0.020), C24:0 (*p*=0.039), and C26:0 (p=0.0045), (data not shown). The monohexosylceramide median 24 week change in the minocycline group was 0.0013 cps compared to −0.0066 in the placebo group (*p*=0.024) suggesting a beneficial decrease in the minocycline arm. The dihydro Glc galactosylceramide median 24 week change in the minocycline group was 0.0201 cps compared to −0.0048 in the placebo group (p=0.002), suggesting a beneficial decrease in the minocycline arm. Differences were not detected in dihexosylceramides between the minocycline and placebo groups.

CSF protein carbonyl changes over 24 weeks

Differences were not detected in a protein marker of oxidative stress, protein carbonyls, median 24 week changes among the two treatment groups (see Table 2).

CSF neurofilament heavy chain changes over 24 weeks

Differences were not detected in the marker of neuronal injury, neurofilament heavy chain, median 24 week changes among the two treatment groups (see Table 2).

CSF neurotransmitter and neurotransmitter metabolic changes over 24 weeks

Differences were not detected in the following neurotransmitter and neurotransmitter metabolite median 24 week changes [glutamate, tryptophan, tyrosine, anthranilic acid, quinolinic acid, kynurenine or 3-hydroxykynurenine (3HK)] among the two treatment groups (see Table 2).

Inflammatory marker changes over 24 weeks

Differences were not detected in the following inflammatory marker median 24 week changes (TNF-α IL-6, CXCL8, CXCL12, HGF, OPN, sFAS, and sFAS ligand) among the two treatment groups (see Table 2).

DISCUSSION

Treatment for HAND with adjunctive agents is likely to be most effective when initiated early in the disease process during the chronic period of persistent low-level neuroinflammation when there is neural injury with synaptic and dendritic damage (Mielke, Bandaru *et al* 2010). Although cognitive improvement was not observed in our randomized, double-blind, placebo controlled study of minocycline for the treatment of HAND, CSF markers of neural injury may be more sensitive markers of CNS injury than neuropsychological testing results or temporally predate cognitive changes. In this study, we examined as outcome measures several markers of neural injury including lipid metabolite markers of oxidative stress (*i.e.*, ceramides, monohexosylceramides, and dihydro Glc galceramides) and protein markers of oxidative stress (protein carbonyls) (Bandaru, McArthur *et al* 2007). In addition, we examined a marker of neuronal injury, (neurofilament heavy chain) which is released into the extracellular space and CSF when neuroaxonal damage occurs in HAND (Giovannoni and Nath 2011). Also, we examined markers of excitotoxicity associated with HAND pathogenesis including the neurotransmitter glutamate, amino acid precursors of serotonin and dopamine (tryptophan and tyrosine respectively), and central neuro-modulatory or immune activation markers (*i.e.*, anthranilic acid, quinolinic acid, kynurenine, and 3HK). In addition, we examined several inflammatory cytokines/chemokines (TNF-α IL-6, CXCL8, CXCL12, HGF, OPN, sFAS, and sFAS ligand) which are increased in HAND.

Minocycline treatment for 24 weeks was associated with improvement in several lipid metabolite measures including ceramides, monohexosylceramides, and dihydro Glc galactosylceramides compared to placebo in HIV+ individuals in this study suggesting a possible beneficial decrease in neural injury and CNS oxidative stress. However, no improvement was observed in another marker of oxidative stress, protein carbonyls, other markers of neuroaxonal damage and excitotoxicity, and markers of inflammation. These findings are consistent with previous studies in which we found that ceramides accumulated early in the course of HAND (Bandaru *et al.* 2007, Bandaru *et al.* 2013). However, in these previous studies we were able to identify specific changes in individual ceramide species (based on acyl chain length and saturation) that were prognostic indicators for cognitive impairment. This level of detail allowed us to make inferences regarding the underlying pathology of HAND. Although the current study also found that ceramides accumulated in HIV+ patients with progressive neurocognitive impairment, the study was not sufficiently powered to identify changes in individual ceramide species. While these findings suggest that ceramides may be sensitive indicators of neurological dysfunction, and that minocycline may have beneficial effects, limitations of the current study temper this interpretation and make it difficult to identify a biological explanation for why ceramides were the only marker improved by minocycline treatment in this study.

Although it is likely that CSF ceramides originated from brain, it is not possible to determine the exact cellular source of these ceramides. Current data suggest that ceramides are enriched in lipoprotein complexes known as microparticles or exosomes (Trajkovic *et al*, 2008), and these particles can be released from glial cells such as astrocytes (Haughey, 2014). It is thus possible that minocycline dampened CNS glial activation, and this was reflected in a reduced release of ceramide enriched microparticles.

There are limitations to this study which should be noted and may explain the results observed. The sample size of individuals receiving the optional CSF measures at both baseline and the week 24 visit was small since the lumbar puncture was an optional procedure in the study in order to facilitate recruitment into the study (resulting in low power and wide confidence intervals). The small sample size could be a reason why we did not see an effect in some CSF markers. Also, there may have been a sampling bias due to informative censoring as participants receiving the CSF markers were not randomly selected. In addition, because of the small sample size and using a non-parametric method, we could not adjust for potentially significant clinical variables in the treatment comparisons. Furthermore, each result was not adjusted for multiple comparisons and with a Type 1 error of 5%, the change of having at least one false positive is 66% by chance. Also, the treatment period of 24 weeks may have been too brief to observe a neuroprotective effect from minocycline. In addition, HIV enters the CNS shortly after infection and may cause neuronal dysfunction that is unresponsive to minocycline treatment given many years after the disease process has commenced.

The observation of an improvement in CSF lipid metabolites with minocycline treatment in the absence of improvement in neuropsychological testing suggests that either this finding does not translate into a clinical benefit in neurocognitive performance or that a longer treatment period was needed. The lack of effect on other CSF markers of oxidative stress, inflammation, or neuronal injury suggests that the effect was very modest. Our results also suggest that CSF lipid metabolites may be sensitive markers of CNS injury which should be further explored and coupled with other markers such as neuroimaging to evaluate for evidence of neuroprotection in future clinical trials of HAND.

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Table 1

Baseline demographic characteristics of HIV+ individuals with cerebrospinal fluid markers at baseline and the week 24 visit

	Placebo n=13	**Minocycline n=8**	**p value**
Age, Median (IQR), years	50.0 (48–53)	49.5 (44.5–52.5)	0.66
Education, Median (IQR), years	14(12–16)	14.5 (13–17)	0.61
Gender, % male	62	88	0.34
Race, % Caucasian % African American	62 38	38 62	0.39
CD4 count, Median (IQR), cells/mm^3	420 (356–647)	772 (595–910)	0.033
Plasma HIV RNA, %≤30 copies/mL	23	13	1.00
Protease inhibitor (PI) based ART (%)	69	50	0.38
CPE score, Median (IQR)	7.0 (7–8)	7.5 (7–10)	0.69

IQR= interquartile range

CPE= CNS Penetration effectiveness scale

Table 2

Median changes in cerebrospinal fluid marker by treatment arm

	Placebo		Minocycline		95% Confidence		* p value
	n	Median 24 wk change	n	Median 24 wk change	Location parameter	shift	
Lipid markers of oxidative stress							
Ceramides	8	−0.0051	6	0.0007	0.001	0.029	0.017
Monohexosylceramides	8	−0.0066	6	0.0013	0.001	0.065	0.024
Dihydro glucosylceramide and galactosylceramide	8	−0.0048	6	0.0201	0.009	0.057	0.002
Dihexosylceramides	8	−0.0043	6	0.0005	−0.001	0.013	0.081
Protein marker of oxidative stress							
Protein carbonyls	7	13.2	3	4.0	−49.2	87.2	0.65
Marker of neuronal injury							
Neurofilament heavy chain	5	−3.04	5	−59.3	−58.4	168.2	0.14
Excitoxic neurotransmitters and metabolites							
Glutamate	6	−1.08	4	5.09	−21.7	17.7	0.92
Tryptophan	5	0.07	4	−0.21	−1.4	0.2	0.39
Tyrosine	6	0.29	4	−0.60	−6.2	1.3	0.24
Anthranilic acid	6	0.0	4	0.0	−8.4	21.7	0.75
Quinolinic acid	6	0.0	4	−0.55	−14.8	13.5	0.24
Kynurenine	6	7.0	4	6.9	−23.9	79.8	0.59
3-Hydroxykynurenine	6	−0.83	4	−4.09	−14.8	8.3	1.0
Markers of Inflammation							

	Placebo		Minocycline		95% Confidence		* p value
	n	Median 24 wk change	n	Median 24 wk change	Location parameter	shift	
TNF-α	13	0.14	8	0.04	−0.6	0.1	0.58
IL-6	13	0.95	4	−0.29	−0.28	3.0	0.80
CXCL8	13	−0.61	8	0.34	−6.9	16.5	0.29
Hepatocyte growth factor	13	90.5	7	16.1	−234.3	148.5	0.43
Osteopontin	13	673.6	8	−5208.9	−34308	15160	0.59
sFAS	13	134.1	8	0.68	−271.0	55.8	0.18
sFAS Ligand	13	0.11	8	−0.27	−2.1	0.8	0.14
CXCL12	13	1071	8	204	−1519	343	0.37

* Wilcoxon nonparametric test was used for all statistical comparisons