Journal of Cerebral Blood Flow & Metabolism (2015) 35, 917–921

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BRIEF COMMUNICATION

The neuroprotective agent CNTF decreases neuronal metabolites in the rat striatum: an *in vivo* multimodal magnetic resonance imaging study

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Ciliary neurotrophic factor (CNTF) is neuroprotective against multiple pathologic conditions including metabolic impairment, but the mechanisms are still unclear. To delineate CNTF effects on brain energy homeostasis, we performed a multimodal imaging study, combining *in vivo* proton magnetic resonance spectroscopy, high-performance liquid chromatography analysis, and *in situ* glutamate imaging by chemical exchange saturation transfer. Unexpectedly, we found that CNTF expression through lentiviral gene transfer in the rat striatum significantly decreased the levels of neuronal metabolites (*N*-acetyl-aspartate, *N*-acetyl-aspartyl-glutamate, and glutamate). This preclinical study shows that CNTF remodels brain metabolism, and suggests that decreased levels of neuronal metabolites may occur in the absence of neuronal dysfunction.

Journal of Cerebral Blood Flow & Metabolism (2015) **35,** 917–921; doi:10.1038/jcbfm.2015.48; published online 1 April 2015

Keywords: brain imaging; glutamate; magnetic resonance; *N*-acetyl-aspartate; reactive astrocytes; striatal metabolism

INTRODUCTION

The cytokine ciliary neurotrophic factor (CNTF) exhibits well-established neuroprotective effects against a large variety of pathologic conditions, including excitotoxicity and metabolic impairment.[1,2] It has been tested in clinical trials for Huntington's disease,[3] amyotrophic lateral sclerosis,[4] and diseases of the retina.[5] However, the mechanisms involved in CNTF neuroprotection remain largely uncharacterized.

Ciliary neurotrophic factor is known to directly activate catabolic pathways in peripheral organs such as the liver, muscle, and adipose tissues, and to regulate food intake and energy expenditure in the hypothalamus.[6] Besides these metabolic effects, CNTF is a potent activator of astrocytes. We previously reported that astrocyte activation by CNTF alters their metabolic profile, leading to protective effects on neurons exposed to glycolytic inhibition.[7]

Here, we aimed to further delineate CNTF metabolic effects on the intact brain. We performed a multimodal imaging study combining proton magnetic resonance spectroscopy (^{1}H-MRS), high-performance liquid chromatography (HPLC) analysis and brain mapping of glutamate (Glu) with Chemical Exchange Saturation Transfer (gluCEST) on rats injected with a lentiviral vector encoding CNTF.

MATERIALS AND METHODS

Injection of Lentiviral Vectors

We used self-inactivated lentiviral vectors that encode either the human CNTF gene ('lenti-CNTF') or the β-galactosidase gene ('lenti-LacZ').[8]

Two-month-old male Sprague Dawley rats (Charles River, France) were injected with lentiviral vectors as described previously,[9] and analyzed between 1.5 and 3 months after injection (CNTF effects being stable for at least 6 months[8]). To avoid bias associated with lateralization, both combinations of injections (lenti-LacZ in the left striatum and lenti-CNTF in the right striatum, or the opposite) were performed.

Housing and experiments were performed in strict accordance with the European Community regulations (Directive 2010-63/EEC) and French regulations (Code Rural R214/87-130). Experimental procedures were approved by a local ethics committee registered by the French Research Ministry (committee #44, approval #10-057).

Proton Magnetic Resonance Spectroscopy

Proton magnetic resonance spectroscopy (^{1}H-MRS) experiments were performed on a horizontal 7 T Agilent scanner (Palo Alto, CA, USA). We used a volume coil for radiofrequency transmission and a quadrature surface coil for reception.

T_2-weighted images were acquired with a 2D fast spin-echo sequence, and were then used to position two voxels of $4 \times 3.4 \times 3$ mm^3 in each striatum for ^{1}H-MRS analysis (Figure 1B). Excellent shimming (9 to 11 Hz) was achieved in these voxels. A LASER (Localization by Adiabatic Selective Refocusing) sequence with echo time/repetition time = 25/5,000 ms was used for ^{1}H-MRS acquisition (384 repetitions for metabolite spectrum). Pulse bandwidth of the hyperbolic secant pulses in LASER was equal

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The authors thank N Dufour, C Joséphine, Pr. N Déglon, and Dr A Bémelmans for viral vector production. We are grateful to P Gipchtein and Dr C Jan for their help with immunostainings. The authors thank Dr V Lebon and C Marchadour for valuable inputs on NMR acquisition.
This work was supported by the grants ANR 2010-JCJC-1402-1 (to CE), by a grant 'Investissement d'Avenir–ANR-2011-INBS-0011'–NeurATRIS (to PH), CEA and CNRS.
Received 21 November 2014; revised 23 February 2015; accepted 25 February 2015; published online 1 April 2015

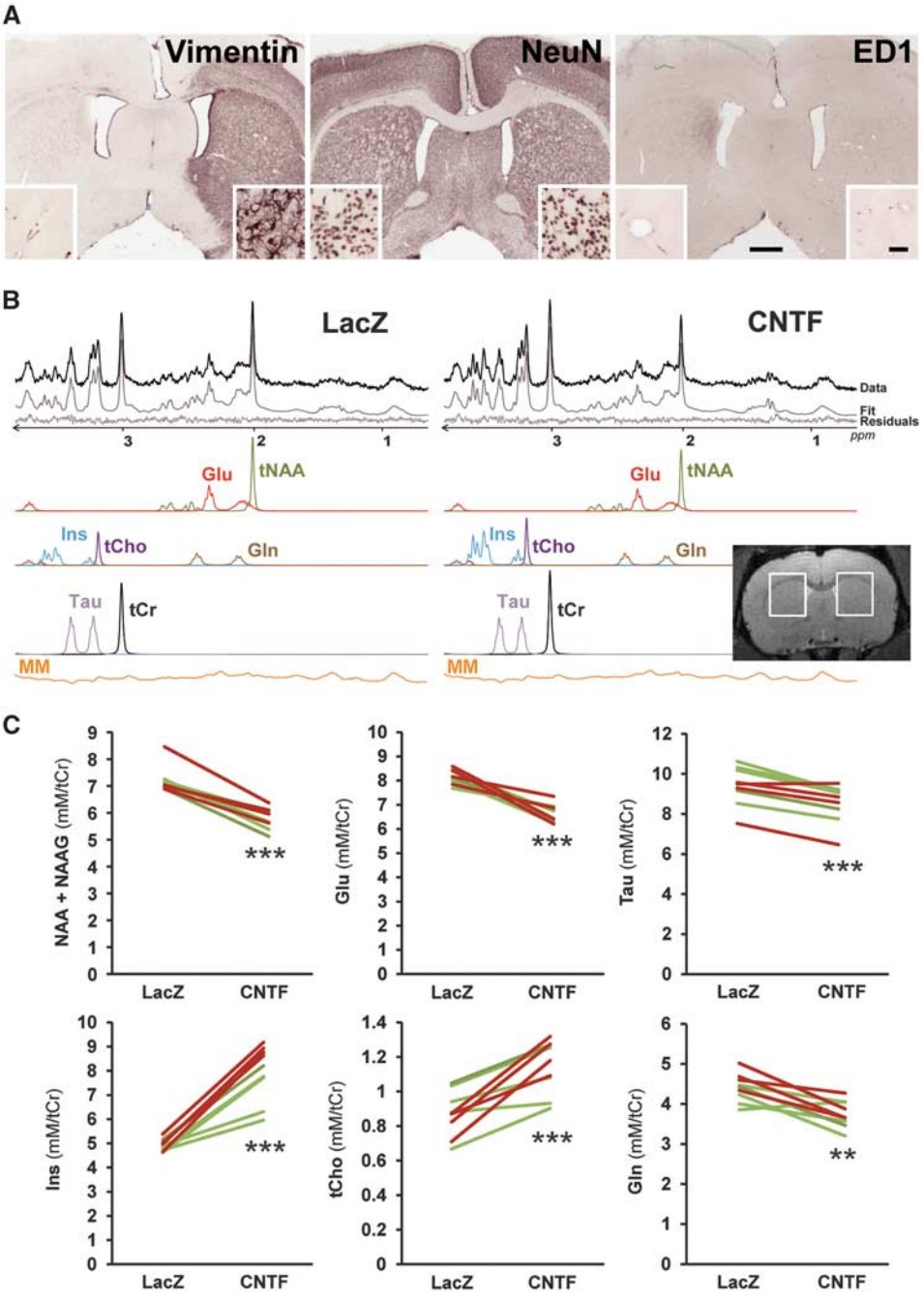

Figure 1. Ciliary neurotrophic factor (CNTF) remodels striatal metabolite concentrations as measured by proton magnetic resonance spectroscopy (^{1}H-MRS). (**A**) Representative images of immunostaining for vimentin, NeuN, and ED1/CD68. Vimentin is strongly induced in the lenti-CNTF injected striatum (right), but not in the lenti-LacZ striatum (left). At higher magnification, multiple reactive astrocytes are observed. There is no difference in the density or the morphology of neuronal cell bodies between the two striata. There is no positive staining for ED1/CD68, a selective marker of reactive microglia. Scale bar: 1 mm (large panels), 50 μm (small panels). (**B**) Representative *in vivo* ^{1}H-MRS spectra obtained from the two striata, 2 months after lentiviral injection. Analysis by LCModel allows spectra decomposition into (1) neuronal metabolites (total NAA (tNAA = N-Acetyl-Aspartate (NAA)+ N-Acetyl-Aspartyl-Glutamate (NAAG)) and Glutamate (Glu)); (2) glial metabolites (Inositol (Ins), total Choline (tCho) and Glutamine (Gln)) (3) other metabolites (Taurine (Tau), total creatine (tCr), and macromolecules (MM)). *Inset,* representative T_2-weighted image showing the position of the voxel (total volume = 40.8 μL). There is no visible difference in T_2 contrast between striata. (**C**) Metabolite concentrations measured in both voxels. Green lines correspond to rats injected with lenti-LacZ in the left striatum and lenti-CNTF in the right striatum; red lines correspond to rats injected with lenti-CNTF in the left striatum and lenti-LacZ in the right striatum. $N = 9$; $^{**}P < 0.01$, $^{***}P < 0.001$, paired *t*-test.

to 10 kHz, ensuring less than 6% chemical shift localization artifact over the spectral range of the metabolites of interest (2 to 4 ppm).

Metabolite concentrations were quantified with LCModel.[10] A macromolecule spectrum was acquired by metabolite nulling and included in LCModel basis set. Seven metabolites were reliably quantified (Cramér-Rao lower bounds $\leqslant$5%). Metabolite concentrations were normalized to total creatine, set at 8 mmol/L.

Chemical Exchange Saturation Transfer Imaging of Glutamate

The gluCEST imaging was performed on a horizontal 11.7 T Bruker scanner with a volume coil for radiofrequency transmission and a quadrature surface coil for reception (Bruker, Ettlinger, Germany). The 'Mapshim' routine was applied in a voxel encompassing the slice of interest and a B_1 map was also acquired to correct for inhomogeneities of the saturation pulse. A gluCEST image centered on the injection sites was acquired with a 2D fast spin-echo sequence preceded by a frequency-selective continuous-wave saturation pulse ($300 \times 300\ \mu m^2$ in-plane resolution, 1 mm slice thickness) with echo time/repetition time = 6/5,000 ms, 10 echoes and effective echo time = 30 ms. The saturation pulse was applied during 1 second with a 5-μT B_1 amplitude. Two images were acquired with saturation pulse applied either at +3 ppm (M_{sat}(+3 ppm), the center of CEST peak for Glu) or at a symmetrical frequency relative to bulk water (M_{sat}(−3 ppm)). The gluCEST contrast was calculated pixel by pixel, as a percentage, by the equation: $100 \times (M_{sat}(-3\ ppm) - M_{sat}(+3ppm))/M_{sat}(-3\ ppm)$.

High-Performance Liquid Chromatography Analysis

Six rats were killed with a lethal dose of pentobarbital 4 months after lentiviral vector injection. Each striatum was rapidly dissected out, weighted, and frozen in isopentane. Samples were stored at − 80°C until HPLC analysis coupled with UV detection of *N*-Acetyl-Aspartate (NAA) and *N*-Acetyl-Aspartyl-Glutamate (NAAG), according to a protocol adapted from.[11]

ELISA Measurement of Human Ciliary Neurotrophic Factor

The striatum of five lenti-CNTF rats and four sham-operated rats was rapidly dissected out on ice, homogenized in PBS with 1% Triton X-100 and protease inhibitors (Roche, Basel, Switzerland). After centrifugation (20,000 *g*, 15 minutes, 4°C), the supernatant was collected and after fivefold dilution, analyzed by ELISA with the Quantikine ELISA Human CNTF immunoassay kit in reference to standards, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).[12]

Immunohistochemistry

Immunohistochemistry was performed on the brain of paraformaldehyde-perfused rats as described previously,[8] with the following antibodies: ED1/CD68 (1:500; Serotec, Raleigh, NC, USA), NeuN (1:2,000; Millipore, Billerica, MA, USA), and vimentin (1:2,000; Calbiochem, La Jolla, CA).

Statistical Analysis

Results are expressed as mean ± SEM. Paired *t*-tests were performed with Statview. *N* indicates the number of animals. No blinding was performed but rats were injected randomly with lenti-CNTF in either the left or right striatum.

RESULTS

Lentiviral Vector-Mediated Expression of Ciliary Neurotrophic Factor in the Rat Striatum

We first measured the levels of human CNTF by ELISA, 5 weeks after infection with lenti-CNTF in the rat striatum. Ciliary neurotrophic factor levels were 2.39 ± 0.53 ng/mg total protein in lenti-CNTF injected rats, while it was below 3.10^{-4} ng/mg total protein in sham-operated rats.

To further evidence CNTF production in the rat striatum, we performed immunostaining for vimentin, a marker of reactive astrocytes known to be induced by CNTF.[8,9] Astrocytes over-expressed vimentin in a large part of the striatum injected with lenti-CNTF (28.47 ± 4.80 mm^3, Figure 1A).

We also checked that lentiviral injection or transgene expression had no detrimental effect on striatal cells. There was no detectable change in NeuN staining and no immunoreactivity for ED1/CD68, a marker for activated microglia (Figure 1A), as already reported.[8,9]

Ciliary Neurotrophic Factor Induces Multiple Changes in Metabolite Concentrations

Rats injected with lenti-LacZ in one striatum and lenti-CNTF in the contralateral striatum were imaged using a 7 T magnet, 1.5 to 3 months after infection. There was no detectable change in T_2-weighted signal in the striatum for any of the nine rats studied (Figure 1B). Proton magnetic resonance spectroscopy was performed on two voxels positioned over each striatum (Figure 1B). High-quality spectra were acquired (Figure 1B), allowing the reliable quantification of seven metabolites, using total creatine as a reference.

There was a significant increase in the concentrations of two metabolites enriched in glial cells: myo-inositol (Ins, +61 ± 7%, $P < 0.001$) and total choline (glycerophosphocholine+phosphocholine+choline, tCho, +33 ± 7%, $P < 0.001$), in the CNTF striatum compared with LacZ (Figure 1C). On the contrary, the astrocyte metabolite glutamine (Gln) was decreased in the CNTF striatum (−14 ± 3%, $P < 0.01$). Surprisingly, CNTF also decreased the levels of the neuronal metabolites NAA+NAAG (tNAA, − 19 ± 2%, $P < 0.001$) and glutamate (Glu, − 18 ± 2%, $P < 0.001$, Figure 1C). Taurine (Tau) concentrations were also significantly decreased by CNTF (−9 ± 1%, $P < 0.001$). The same pattern was observed independently of the side of lenti-CNTF injection (Figure 1C), ruling out a possible bias due to voxel position, chemical shift artifact, or intrinsic differences between hemispheres. The same metabolic profile was observed in three independent cohorts of rats.

The Decrease in *N*-Acetyl-Aspartate and *N*-Acetyl-Aspartyl Glutamate Concentrations Is Confirmed by High-Performance Liquid Chromatography Analysis

To measure NAA concentrations by an alternative method, we performed HPLC analysis coupled with UV detection on striatal samples. This method allows the separate measurement of NAAG, which is otherwise combined with the main NAA peak on the ^{1}H-MRS spectrum.

N-Acetyl-Aspartate levels were significantly decreased by 18 ± 3% in the CNTF striatum compared with the LacZ striatum ($P < 0.01$, Figure 2A), which is very similar to the difference measured by ^{1}H-MRS. There was also an important decrease in NAAG levels with CNTF (−31 ± 3%, $P < 0.001$, Figure 2A).

Chemical Exchange Saturation Transfer of Glutamate Imaging Reveals a Decrease in Glutamate Levels that Matches the Volume of Astrocyte Activation

To confirm the decrease in Glu levels with CNTF and to map its spatial extension *in vivo*, we took advantage of the recently developed NMR imaging technique called gluCEST.[13] The gluCEST contrast is correlated with Glu concentrations, allowing *in vivo* Glu mapping with good spatial and temporal resolution.[13] A lower gluCEST contrast was observed in the CNTF striatum (Figure 2B). The brain area displaying vimentin-positive reactive astrocytes matched precisely the zone exhibiting lower gluCEST contrast (Figure 2B). To compare gluCEST with ^{1}H-MRS results, we calculated the average gluCEST contrast in the same striatal voxels analyzed by ^{1}H-MRS. A significant reduction in gluCEST contrast was measured in the voxel positioned over the CNTF striatum compared with the LacZ striatum (−14 ± 1%, $P < 0.001$, Figure 2C), which is consistent with ^{1}H-MRS measurements.

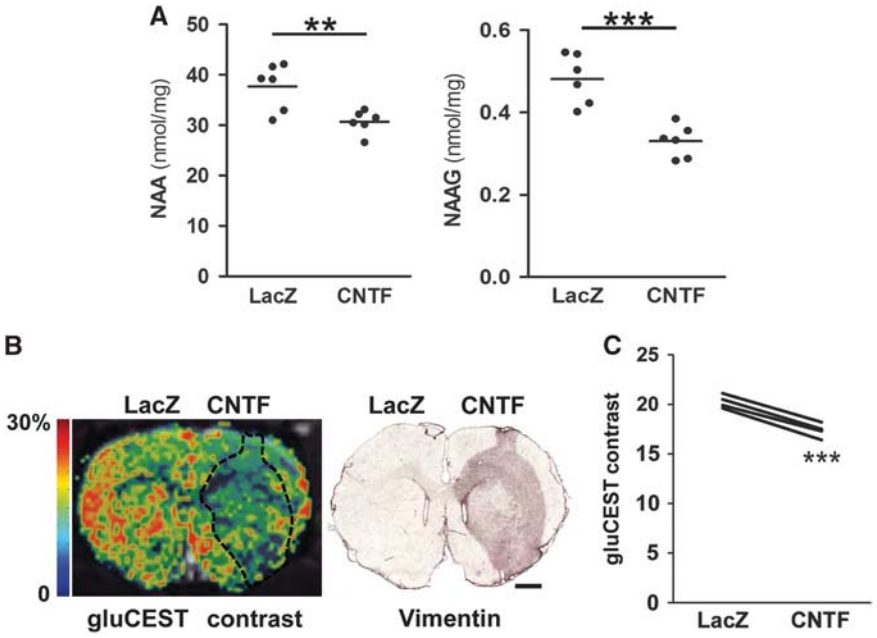

Figure 2. High-performance liquid chromatography (HPLC) analysis confirms decreased NAA and NAAG levels with ciliary neurotrophic factor (CNTF) and chemical exchange saturation transfer of glutamate (gluCEST) imaging reveals lower Glu levels in the striatum displaying CNTF-activated astrocytes. (**A**) Quantification of NAA and NAAG by HPLC coupled with UV detection on postmortem samples. Data are expressed as nmol/mg of tissue (wet weight). $N = 6$; $^{**}P < 0.01$, $^{***}P < 0.001$, paired *t*-test. (**B**) A representative gluCEST image exhibiting a lower gluCEST contrast in the white matter, e.g., in the corpus callosum and anterior commissure, as described previously.[13] GluCEST contrast is decreased in the hemisphere injected with lenti-CNTF. The region with vimentin$^+$ reactive astrocytes (black dots) closely matches the region exhibiting lower gluCEST contrast. (**C**) Quantification of the average gluCEST contrast in voxels similar to the ones used for proton magnetic resonance spectroscopy (^{1}H-MRS). $N = 4$, $^{***}P < 0.001$, paired *t*-test. Scale bar: 1 mm.

DISCUSSION

Our study shows that CNTF triggers complex changes in striatal metabolism, affecting metabolites enriched in astrocytes as well as those enriched in neurons. The significant decrease in Gln levels shows that CNTF does not globally increase the concentrations of glial metabolites, but rather modulates the relative abundance of specific brain metabolites. By alternating the side of lentiviral vector injections, we ruled out possible bias due to spectroscopic localization or lateralization. In addition, ^{1}H-MRS results were validated by two alternative methods. The decrease in NAA+NAAG concentrations was confirmed *ex vivo* by HPLC analysis, providing an independent measurement of these two metabolites. The decrease in Glu levels was also observed by gluCEST imaging and closely matched the volume displaying CNTF-activated astrocytes.

Ciliary neurotrophic factor is a neurotrophic cytokine with demonstrated protective effects against multiple brain insults including excitotoxicity[1,12] and metabolic impairment.[2,7] Ciliary neurotrophic factor neuroprotective effects were reproduced in nonhuman primates, opening the path to clinical trials for amyotrophic lateral sclerosis,[4] diseases of the retina[5] and Huntington's disease.[3] Therefore, it is rather unexpected that CNTF reduces neuronal metabolite levels in the rat striatum. Indeed, decreased tNAA levels are classically interpreted as reflecting neuronal death or at least dysfunction in ^{1}H-MRS studies.[14] However, using the same strategy to overexpress CNTF in the rat striatum, we previously showed that CNTF does not alter the expression of many neuronal proteins,[8] does not modify the spontaneous electrophysiologic activity of striatal neurons,[15] and confers significant neuroprotection against excitotoxicity.[15] This was consistent with two independent studies based on lentiviral vectors,[12,16] in which intrastriatalal CNTF levels were in the ng/mg protein range, as measured in the present study. Other delivery modes (adenoviral gene transfer[2] and encapsulated cells releasing CNTF[17]) confirmed the protective effects of CNTF against striatal lesions. The levels of CNTF achieved with these different strategies are not directly comparable to our ELISA measurement. Encapsulated cells are known to produce CNTF less efficiently due to their low survival rate after implantation.[18] This may explain the limited beneficial effects observed in a phase I clinical trial for HD patients,[3] although this strategy was very efficient in primates.[19] Considering the strong evidence for CNTF neuroprotective effects in the striatum, our results show that decreased tNAA and Glu levels may occur independently of neuronal dysfunction, and may instead be associated with significant neuroprotective effects.

Overall, CNTF induces a complex reorganization of striatal metabolism. Accordingly, we previously showed that CNTF activates the AMP kinase, a master regulator of energy metabolism,[7] and that CNTF alters the metabolic profile of astrocytes.[7] Given the importance of energy homeostasis for proper neuronal function, it is tempting to speculate that such metabolic plasticity may participate in CNTF neuroprotective effects, as demonstrated in an *in vitro* model of metabolic impairment.[7]

The molecular mechanisms mediating CNTF effects on neuronal metabolites remain to be determined; they may be direct or indirect. Indeed, both NAA and Glu metabolism involve complex interactions between neurons, astrocytes but also oligodendrocytes.[20] The facts that (1) CNTF acts primarily on astrocytes and (2) there is a strong spatial correlation between reactive astrocytes and decreased Glu levels observed by gluCEST, suggest that astrocytes may be involved in the remodeling of striatal metabolism.

Overall, we show that the neurotrophic cytokine CNTF induces significant metabolic plasticity in brain cells. Such metabolic signature would be classically interpreted as neuronal dysfunction or death in ^{1}H-MRS clinical studies. Instead, our results raise the intriguing possibility that it may indicate ongoing compensatory mechanisms.

AUTHOR CONTRIBUTIONS

MACS, JF, YB, LBH, MG, AB, GA, DH, JV, and CE performed experiments, MACS, JF, JV, and CE designed research, analyzed data and wrote the manuscript, EB, GB, and PH contributed reagents/materials and edited the manuscript.

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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