Dysregulation of the IL-23/IL-17 axis and myeloid factors in secondary progressive MS

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ABSTRACT

Objective: In the current exploratory study, we longitudinally measured immune parameters in the blood of individuals with relapsing-remitting multiple sclerosis (RRMS) and secondary progressive multiple sclerosis (SPMS), and investigated their relationship to disease duration and clinical and radiologic measures of CNS injury.

Methods: Peripheral blood mononuclear cells (PBMCs) and plasma were obtained from subjects with RRMS, SPMS, and from healthy controls on a monthly basis over the course of 1 year. MRI and Expanded Disability Status Scale evaluations were performed serially. PBMCs were analyzed by enzyme-linked immunosorbent spot assay to enumerate myelin basic protein-specific interleukin (IL)-17- and interferon (IFN)- γ -producing cells. Plasma concentrations of proinflammatory factors were measured using customized Luminex panels.

Results: Frequencies of myelin basic protein-specific IL-17- and IFN- γ -producing PBMCs were higher in individuals with RRMS and SPMS compared to healthy controls. Patients with SPMS expressed elevated levels of IL-17-inducible chemokines that activate and recruit myeloid cells. In the cohort of patients with SPMS without inflammatory activity, upregulation of myeloid-related factors correlated directly with MRI T2 lesion burden and inversely with brain parenchymal tissue volume.

Conclusions: The results of this exploratory study raise the possibility that Th17 responses and IL-17-inducible myeloid factors are elevated during SPMS compared with RRMS, and correlate with lesion burden. Our data endorse further investigation of Th17- and myeloid-related factors as candidate therapeutic targets in SPMS. *Neurology*® 2014;83:1500-1507

GLOSSARY

DMA = disease-modifying agent; **EDSS** = Expanded Disability Status Scale; **ELISPOT** = enzyme-linked immunosorbent spot; **HC** = healthy control; **IFN** = interferon; **IL** = interleukin; **MBP** = myelin basic protein; **MS** = multiple sclerosis; **PBMC** = peripheral blood mononuclear cell; **RRMS** = relapsing-remitting multiple sclerosis; **SP** = secondary progressive; **SPMS** = secondary progressive multiple sclerosis.

Multiple sclerosis (MS), an inflammatory disease of the CNS, typically presents with a relapsing-remitting course. During this early stage of disease, peripheral blood leukocytes cross the blood-brain barrier to drive the formation of demyelinating plaques. Disease-modifying agents (DMAs) that modulate or suppress the peripheral immune system provide therapeutic benefit in relapsing-remitting MS (RRMS).¹

The majority of individuals with RRMS ultimately enter a secondary progressive (SP) stage, characterized by gradual accumulation of neurologic disability independent of clinical relapses. The cellular and molecular basis for this transition is unclear, and the role of inflammation during the SP stage is a subject of considerable debate. DMAs lose efficacy after transition to SPMS,^{2,3} prompting the hypothesis that clinical deterioration in SPMS is driven by a neurodegenerative process, possibly unconnected to the autoimmune assault that predominates earlier.⁴ In contrast, a growing body of data suggests that immune dysregulation is a salient feature of progressive MS, although the proinflammatory pathways involved and consequent pattern of

Supplemental data
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injury differ from those that typify relapsing forms of the disease.^{5–13} In the current study, we performed immune assays on plasma and peripheral blood mononuclear cells (PBMCs) serially collected from individuals with RRMS, SPMS, and healthy controls (HCs). In parallel, patients with MS were monitored via the Expanded Disability Status Scale (EDSS) and cranial MRI scans to investigate associations between immune abnormalities and disease progression.

METHODS Subjects and clinical assessments. Patients with RRMS (n = 12) and SPMS (n = 26) were recruited from the Multiple Sclerosis Clinics at the University of Rochester and the University of Michigan. More complete demographic details are displayed in table 1. Patients were diagnosed with RRMS or SPMS based on the revised McDonald Diagnostic Criteria and clinical course.¹⁴ Secondary progression was defined by a progressive increase in disability (of at least 6 months' duration) in the absence of relapses. Based on our inclusion/exclusion criteria, subjects with SPMS had not experienced a clinical relapse or had an MRI scan demonstrating a gadolinium-enhancing lesion within 2 years of study enrollment. Blood was collected from study subjects and from 25 age- and sex-matched HCs on a monthly basis. Every patient with MS underwent EDSS scoring by a board-certified neurologist blinded to the results of MRI scanning and immune assays. Cranial MRI and clinical assessments were performed every other month and every third month, respectively, on all 38 patients with MS. None of the participants experienced a clinical relapse or received corticosteroids during the 1-year study. Six of 12 patients with RRMS had at least one enhancing lesion on one or more MRI scans. Three of the 12 had a significant increase in T2 lesion burden between initial and final MRI scans; all 3 had enhancing lesions on multiple MRI scans.

Standard protocol approvals, registrations, and patient consents. The institutional review boards of the University of Michigan and the University of Rochester approved our study protocol. All study subjects provided written informed consent after the nature and possible consequences of the study were explained.

Cell preparation. PBMCs were isolated from the blood of patients and HCs using CPT Vacutainer tubes (BD, Fisher Scientific, Pittsburgh, PA). PBMCs were suspended in fetal bovine serum with 20% dimethyl sulfoxide and stored in liquid nitrogen until thawed for enzyme-linked immunosorbent spot (ELISPOT) assays. Plasma was stored at –80°C until thawed for Luminex assays.

ELISPOT assays. Ninety-six-well, filter-bottom plates (MSIPN4W; Millipore, Billerica, MA) were coated with anti-IFN-γ or anti-IL-17A antibodies at 3 μg/mL (eBioscience, San Diego, CA). PBMCs were plated at 1–5 × 10⁵ cells per well either alone or with whole human myelin basic protein (MBP) (50 μg/mL; Sigma-Aldrich, St. Louis, MO) or tetanus toxoid (1.5 μg/mL; Fisher Scientific). In preliminary studies, PBMCs were plated across serial dilutions to determine the optimal conditions for eliciting reproducible antigen-specific responses in the linear range of the dose-response curve. Plates were incubated for 24 hours (interferon [IFN]-γ) or 48 hours (interleukin [IL]-17) at 37°C with 5% CO₂ after which they were washed and incubated with biotinylated detection antibodies (eBioscience) for 1 hour at room temperature, followed by streptavidin-alkaline phosphatase (Southern Biotech, Birmingham, AL). Plates were developed with the Vector alkaline phosphatase substrate kit (SK5300; Vector, Burlingame, CA). Spots were counted and analyzed using the ImmunoSpot analyzer (Cellular Technology Limited, Shaker Heights, OH). All assays were performed in triplicate and data were normalized to 10⁶ cells/well. Technicians performing ELISPOT assays were blinded to the identity of patients.

Luminex assays. Plasma levels of cytokines and chemokines were measured with customized multiplex magnetic bead-based arrays (EMD Millipore, Billerica, MA) following the manufacturer's protocol. Data were collected using the Bio-Plex 200 system (Bio-Rad, Hercules, CA). Standards were run in parallel to allow quantification of individual factors. The data shown indicate levels that fell within the linear portion of the corresponding standard curve. Technicians performing Luminex assays were blinded to the identity of patients.

MRI protocol and image analysis. All patients were evaluated with cranial MRI examination on a 1.5-tesla-strength magnet using axial T2-weighted and axial and sagittal T1-weighted sequences. Brain parenchymal and CSF volumes and T2 lesion load were measured using commercially available software

Table 1 Demographic details of the study population

Variable	Healthy controls (n = 25)	Patients with RRMS (n = 12)	Patients with SPMS (n = 26)	p Value ^a
Sex				
Female	11 (44.0)	5 (41.7)	11 (42.3)	0.99
Male	14 (56.0)	7 (58.3)	15 (57.7)	
Age at enrollment, y	48.4 ± 11.0	46.3 ± 11.1	53.8 ± 8.3	0.062
Disease duration, y	NA	9.53 ± 7.04	21.6 ± 10.8	<0.01
Average EDSS score	NA	2.71 ± 0.80	5.20 ± 1.33	<0.01

Abbreviations: EDSS = Expanded Disability Status Scale; NA = not applicable; RRMS = relapsing-remitting multiple sclerosis; SPMS = secondary progressive multiple sclerosis. Data are mean ± SD or n (%).

^aThe comparisons of sex distribution and age are among the 3 groups, including healthy controls and patients with RRMS and SPMS, while the comparisons of disease duration and average EDSS score are between patients with RRMS and SPMS. Fisher exact test was used for comparison of sex distribution, 1-way analysis of variance was used for age at enrollment, and 2-sample t tests were used for comparison of disease duration, and average EDSS score.

developed by VirtualScopics (Rochester, NY). This involved coregistering each MRI to a presegmented anatomical atlas with manual refinement of automated brain boundaries by an expert analyst where necessary, as previously described.¹⁵ Lesion boundaries were identified in 3 dimensions using geometrically constrained region growth.^{15,16} T2 lesion volume was normalized to total brain parenchymal tissue volume.

Statistical analysis. Descriptive analyses were conducted to examine group differences among HCs and patients with RRMS and SPMS for demographic variables. Summary statistics were computed for continuous measures as mean $\pm$ SD, and for categorical variables as frequency and proportion (%). One-way analysis of variance was used to assess whether group differences existed among the 3 cohorts for continuous variables (age), and Fisher exact test was used for categorical variables (sex). EDSS and disease duration are not relevant to HCs, and were compared only between patients with RRMS vs SPMS using 2-sample *t* tests. Personal average and personal variation over a 1-year observation period were calculated for immune parameters of interest. We evaluated group differences in personal average and personal variance using Wilcoxon nonparametric tests, considering the limited group-specific sample sizes and the fact that some of the distributions are skewed. The group-specific mean longitudinal trajectories of plasma levels of chemokines and neutrophil elastase were assessed and compared using linear mixed models with random effects to account for within-subject correlations. When fitting longitudinal models, more observations were available because of repeated measurements for each patient, and we were able to adjust for the effect of age when appropriate. Comparisons were made between chemokine levels and disease duration, EDSS, T2 lesion volume, or brain parenchymal tissue volume using all available measurements. The analysis was performed using within-cluster resampling methodology, considering the concern of the intraclass correlation. Specifically, we randomly chose one observation per patient, and calculated Spearman correlations. This process was repeated 200 times, and we merged all 200 estimated correlations to obtain the final results using established methods.^{17,18} Statistical significance was set at $p < 0.05$. All statistical analyses were performed using the R 2.15.2 software program.

RESULTS MBP-specific IFN- γ - and IL-17-producing PBMCs are prevalent in RRMS and SPMS. Prior studies on the frequency of autoreactive T cells in MS are conflicting; some investigators report a significantly higher incidence of myelin-specific PBMCs in individuals with MS compared with age- and sex-matched HCs, while others report no significant difference.^{19–23} Most are cross-sectional studies that used proliferation or IFN- γ production as a measure of antigenic responsiveness.¹⁹ Because Th1 and Th17 cells have both been implicated in the pathogenesis of autoimmune demyelination,²⁴ we revisited this question by performing both IFN- γ and IL-17 ELISPOT assays on PBMCs collected longitudinally over the course of 1 year from individuals with RRMS or SPMS in comparison to HCs. None of the patients with SPMS were receiving treatment with a DMA or had received a DMA within the last year.

The same was true for 7 of 12 subjects with RRMS; the remainder had been treated with glatiramer acetate for 3 months or longer at the time of enrollment, and remained on this medication through the duration of the study. For each assay, personal average and variation were calculated across multiple blood samples from each subject, thereby minimizing the introduction of artifact produced by spurious background fluctuations in immune parameters.

We found that the mean frequencies of PBMCs that produced IL-17 or IFN- γ in response to challenge with MBP were higher in subjects with RRMS or SPMS than in HCs (figure 1, left panels). MBP-reactive IL-17-producing cells tended to be more abundant in SPMS than RRMS, although this difference fell short of statistical significance ($p = 0.114$). The mean frequency of MBP-reactive IL-17, but not IFN- γ , producers correlated with disease duration when the data were analyzed across all patients with MS ($R = 0.323$, $p = 0.05$). The same was true of the Th17 polarizing monokine, IL-23 ($R = 0.366$, $p = 0.026$). Personal variation of both the IL-17 and IFN- γ responses were greater in the MS cohorts (figure 1, right panels). There was no significant difference in cytokine responses between untreated patients with RRMS and those treated with glatiramer acetate, or between patients recruited in Rochester and those recruited in Michigan (data not shown). Our results appeared specific for MBP-reactive cells, because the mean frequency and variance of tetanus toxoid-reactive IL-17- and IFN- γ -producing PBMCs were comparable across all 3 cohorts (figure e-1 on the *Neurology*[®] Web site at Neurology.org).

Plasma levels of myeloid factors increase with disease duration and EDSS score. The above data suggest that autoreactive Th17 responses are prominent late in the course of MS. Innate immune dysregulation has been described in SPMS.^{7–11,25,26} A major biological function of IL-17 is to stimulate the production of chemokines and growth factors that activate, mobilize, and recruit myeloid cells to sites of inflammation, including CXCL1 and CCL11.^{24,27,28} We found that mean plasma levels of CXCL1 and CCL11 increased with disease duration when data were analyzed across all patients with MS (table 2). In contrast, there was no significant correlation between length of disease and expression of CCL3.

Participants in our longitudinal study underwent an EDSS assessment at the time of enrollment and every 3 months thereafter. Plasma levels of CXCL1 and CCL11 also directly correlated with EDSS score (table 2). Conversely, CCL3 inversely correlated with EDSS. Based on these results, we compared the expression of myeloid factors in RRMS and SPMS.

Figure 1 Frequencies of MBP-reactive IFN- γ - and IL-17-producing PBMCs are elevated in RRMS and SPMS compared with HCs

PBMCs were collected from individuals with RRMS ($n = 12$) and SPMS ($n = 26$) as well as from HCs ($n = 25$) on a monthly basis over the course of 1 year. PBMCs were analyzed using ELISPOT to measure the frequencies of MBP-reactive IFN- γ (A) and IL-17 (B) producers. To determine antigen specificity, the number of spots counted in wells without antigen was subtracted from the corresponding spots in MBP-pulsed wells. Mean frequency (left panels) and variation (right panels) were calculated for each subject from all blood samples collected during the course of the study. Wilcoxon nonparametric tests were used to determine statistical significance, in consideration of the limited group-specific sample sizes and the fact that some of the distributions are skewed. ELISPOT = enzyme-linked immunosorbent spot; HC = healthy control; IFN = interferon; IL = interleukin; MBP = myelin basic protein; PBMC = peripheral blood mononuclear cell; RR = relapsing-remitting; RRMS = relapsing-remitting multiple sclerosis; SP = secondary progressive; SPMS = secondary progressive multiple sclerosis.

Mean plasma levels of CXCL1 and CCL11, as well as neutrophil elastase (a marker of neutrophil activity), were all significantly higher in patients with SPMS compared to those with RRMS (figure 2). Similar to our results with MBP-specific PBMC responses, expression of CXCL1, CCL11, and neutrophil elastase did not differ significantly between untreated and

glatiramer acetate-treated patients with RRMS, or between patients recruited from different sites (data not shown).

Myeloid chemokine expression correlates with radiologic lesion burden in SPMS. We next investigated the relationship between plasma levels of innate immune

Table 2 Relationship between chemokine levels and disease duration or EDSS

Immune parameter	Disease duration	EDSS
CXCL1	0.443/<0.001	0.394/<0.001
CCL11	0.522/<0.001	0.323/<0.001
CCL3	-0.093/0.542	-0.281/0.006

Abbreviation: EDSS = Expanded Disability Status Scale.

Data represent correlation (*R*)/significance (*p* value). Spearman correlation coefficients were calculated, and the corresponding exact tests were used, to evaluate statistical significance. Data were combined from the relapsing-remitting multiple sclerosis and secondary progressive multiple sclerosis cohorts.

molecules and radiologic measures of CNS damage in subjects with SPMS who had no evidence of inflammatory activity over the 1-year observation period. Plasma CXCL1, CCL11, granulocyte colony-stimulating factor, and neutrophil elastase correlated with normalized T2 lesion volume, a radiologic measure of global tissue damage (table 3). Consistent with this finding, plasma IL-23, CXCL1, CCL11, and

neutrophil elastase were negatively associated with total brain parenchymal tissue volume (table 3).

DISCUSSION A controversial topic in MS is whether accumulation of disability during progressive stages is primarily mediated by an autoimmune or a degenerative process.⁴ Our findings contribute to a growing body of data indicating that inflammation is still relevant, and associated with CNS damage, in at least some patients with SPMS. We found increased frequencies of circulating MBP-reactive Th1 and Th17 cells in individuals with SPMS, as well as with RRMS, compared with age- and sex-matched HCs. Moldovan et al.,²¹ studying an independent cohort of patients with RRMS, detected high frequencies of PBMCs producing IFN- γ in response to challenge with libraries of overlapping 9-mer peptides spanning the length of MBP. In contrast, other investigators found no difference in myelin-specific T-cell responses between patients

Figure 2 IL-17-inducible, myeloid-related factors are expressed at significantly higher levels in SPMS compared with RRMS

The levels of the indicated immune parameters were averaged over all plasma samples collected from each subject with RRMS or SPMS during the course of the study. The box plots show the mean personal average of patients in each cohort (A). Longitudinal expression of CXCL1, neutrophil elastase, and CCL11 is shown on a month-to-month basis (B). In A, *p* values were calculated using Wilcoxon nonparametric tests, in consideration of the limited group-specific sample sizes and the fact that some of the distributions are skewed. In B, *p* values were calculated using longitudinal mixed-effects models, adjusting for age. IL = interleukin; RRMS = relapsing-remitting multiple sclerosis; SPMS = secondary progressive multiple sclerosis.

Table 3 Relationship between immune parameters and T2 lesion volume or brain parenchymal tissue volume

Immune parameter	T2 lesion volume ^a	Brain parenchymal tissue volume
IL-23	NS	−0.646/<0.001
CXCL1	0.305/0.029	−0.415/0.001
CCL11	0.466/<0.001	−0.370/0.017
G-CSF	0.349/0.011	NS
Neutrophil elastase	0.325/0.026	−0.297/0.051

Abbreviations: G-CSF = granulocyte colony-stimulating factor; IL = interleukin; NS = not significant.

Data represent correlation (R)/significance (p value). Spearman correlation coefficients were calculated, and the corresponding exact tests were used, to evaluate statistical significance. Data are from the cohort of patients with secondary progressive multiple sclerosis with no inflammatory activity.

^aT2 lesion volume was normalized to total brain parenchymal tissue volume for this analysis.

with MS and controls.^{19,22} These discrepancies could be attributed, at least in part, to technical and methodologic inconsistencies between studies. Hence, discordant results have been obtained when cytokine production and proliferation were used as measures of myelin antigen-specific responses in the same cohort of patients with MS.²⁹ We chose IFN- γ and IL-17 production as our primary outcome measures because of emerging data implicating both Th1 and Th17 cells in MS pathogenesis.³⁰ It has been suggested that IFN- γ /IL-17 double-producing CD4⁺ T cells are the critical effectors in MS.^{30,31} We used single-color ELISPOT assays to measure MBP-specific cytokine responses and therefore could not enumerate the frequency of IFN- γ /IL-17 double-producing cells in comparison to single producers.

Recent publications attest to the plasticity of Th17 cells and their propensity to acquire Th1-like properties over time.^{32,33} Consequently, we anticipated that IFN- γ -producing autoreactive PBMCs would preferentially accumulate, and that IL-17-producing PBMCs would contract, in patients with MS who had longer disease duration. Contrary to our expectations, the mean frequency of MBP-reactive IL-17, but not IFN- γ , producers correlated directly with disease duration. This might be driven by IL-23, the expression of which also increased with disease duration. IL-23 stabilizes Th17 cells, resulting in their preferential accumulation *in vivo*.³⁴ In support of our results, independent studies have found elevated frequencies of circulating IL-23 receptor⁺ CD4⁺ T cells (consistent with Th17 cells) and high serum IL-17 levels in patients with progressive MS compared to patients with RRMS or HCs.^{35–37} Furthermore, IL-17 messenger RNA and protein have been detected in autopsy brain specimens from patients with progressive MS.³⁸ These findings, in conjunction with our data, are particularly intriguing in light of the discovery of lymphoid-like follicles in the sulci of brain specimens from patients with SPMS, but not RRMS.⁵ Myelin-reactive Th17 cells induce

ectopic follicles in the CNS during experimental autoimmune encephalomyelitis.³⁹ By extension, increased Th17 frequencies in individuals with SPMS might facilitate the formation of meningeal follicles. It remains to be demonstrated whether differences in the immune response are a cause, or consequence, of the transition from an RR to an SP clinical course. A clinical trial of a monoclonal antibody specific for the IL-12p40 subunit (which neutralizes both IL-12 and IL-23) showed no beneficial effect in RRMS.⁴⁰ A monoclonal antibody specific for IL-17 is under investigation in RRMS. However, IL-23 and IL-17 neutralizing agents have yet to be assessed as therapeutic agents in progressive MS.

In addition to harboring myelin-reactive Th17 responses, patients with SPMS expressed elevated plasma levels of chemokines and growth factors that activate, mobilize, and recruit myeloid cells. A number of these factors, including CXCL1, CCL11, and granulocyte colony-stimulating factor, can be induced by IL-17,^{27,28} providing a hypothetical causative link between their upregulation and enrichment of circulating Th17 cells. Such a pattern of immune dysregulation suggests the existence of a vicious cycle whereby IL-23 produced by activated myeloid cells induces IL-17, and IL-17 induces chemokines and growth factors that mobilize and activate myeloid cells. Our study did not reveal a significant correlation between plasma levels of IL-17 and myeloid-related factors. It is possible that such associations exist, but our study was underpowered to detect them. Alternatively, IL-17 could induce production of innate chemokines and growth factors in a sequestered microenvironment such as the bone marrow and/or CNS, not reflected in plasma expression patterns.

A potential weakness of this exploratory study is its relatively small sample size. This is a reflection, in part, of stringent selection criteria and the extensive resources necessary to rigorously monitor each subject (with serial clinical and radiologic evaluations, as well as immunologic profiles) over the course of 1 year.

Despite that limitation, we were able to detect statistically robust correlations between clinical and immune parameters. Our results will need to be validated in independent cohorts of patients with larger sample sizes and a wider diversity of clinical courses and treatment histories. Although we found no statistically significant differences in the levels of immune parameters when comparing untreated patients with RRMS to those treated with glatiramer acetate, the inclusion of the latter group might have contributed to the differences we observed between the RRMS and SPMS cohorts. In addition, we did not observe a significant difference in age between cohorts, but subjects with SPMS had significantly higher EDSS scores and longer disease duration than subjects with RRMS (table 1). Hence, it is possible that expression of IL-17 is more closely associated with degree of disability and/or disease duration than with disease stage. Future studies could be helpful in clarifying these issues.

Pathologic engagement of the innate arm of the immune system, and particularly cells of the myeloid/granulocyte lineage, has previously been recognized as a distinctive feature of SPMS. Other investigators have found enhanced expression of costimulatory molecules and cytokines by peripheral blood monocytes from patients with SPMS compared to those from patients with RRMS or HCs.^{7–11,26} More recently, it was shown that myeloid dendritic cells isolated from patients with SPMS secrete greater quantities of IL-12 than the same cells from patients with RRMS or HCs.¹⁰ Collectively, these data are consistent with our finding of elevated plasma levels of myeloid-related factors in SPMS, and their association with MRI metrics of CNS damage. However, such associations do not definitively demonstrate cause and effect. It is possible that some of the immune abnormalities in SPMS represent an epiphenomenon, or are even part of a counter-regulatory pathway. Furthermore, cytokine profiles in the periphery may differ from, and even oppose, those in the CNS, which might explain the negative correlation between EDSS and proinflammatory mediators such as CCL3 (table 2). To resolve these and related matters, we advocate that further efforts be directed at documenting how the autoimmune response evolves during the course of MS and at identifying stage-specific biomarker, neuropathologic, and therapeutic response profiles. The goal is that such investigations will lead to the development of novel pharmaceutical approaches that ameliorate SPMS, possibly by targeting Th17 cells, myeloid factors, and/or the interactions between them.

AUTHOR CONTRIBUTIONS

A.H. performed immunologic assays and critically reviewed the manuscript. L.W. supervised the statistical analyses, interpreted data, and

critically reviewed the manuscript. P.H. performed statistical analyses. X.Z. performed statistical analyses. S.E. supervised MRI studies and critically reviewed the manuscript. A.S. supervised MRI studies and critically reviewed the manuscript. D.I. critically reviewed the manuscript. B.M.S. designed the study, supervised analyses, and wrote the manuscript.

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DISCLOSURE

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