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The Characterization of the Th1/Th2 Ratio in Moderate-Severe Alzheimer's Disease Patients and Its Response to an Aloe Polymannose-Based Dietary Supplement

John E. Lewis[a,*], H. Reginald McDaniel[b], Judi M. Woolger[c] and Sher Ali Khan[d]
[a]*Department of Psychiatry & Behavioral Sciences, University of Miami Miller School of Medicine, Miami, FL, USA*
[b]*Wellness Quest, LLC, Grand Prairie, TX, USA*
[c]*Department of Medicine, University of Miami Miller School of Medicine, Miami, FL, USA*
[d]*Department of Family and Community Medicine, University of New Mexico School of Medicine, Albuquerque, NM, USA*

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Abstract.
Background: Alzheimer's disease (AD) is a leading killer of Americans, imparting a tremendous societal toll. Relationships between immune function and inflammation with cognition are well-established in AD, but the Th1/Th2 ratio of immune function is unknown. Describing the Th1/Th2 ratio and its relationship with cognition may shed light on the disease's clinical context. How the Th1/Th2 ratio responds to dietary supplementation is another unknown question in this population.
Objective: The objectives of the study were to: 1) characterize the Th1/Th2 ratio according to IL-2/IL-10, IFN-γ/IL-10, IL-2/IL-4, IFN-γ/IL-4, IL-2/TNF-α, and IFN-γ/TNF-α in subjects with moderate-to-severe AD and in comparison to healthy adults; 2) investigate the effect of an aloe polymannose multinutrient complex (APMC) dietary supplement on the Th1/Th2 ratios over 12 months; and 3) compare the changes in the Th1/Th2 ratios with the changes in cognition from baseline to 12 months.
Methods: Subjects consumed 2.5 g of the APMC four times per day for 12 months, and they were assessed on cognition and cytokines at baseline and 12 months.
Results: The Th1/Th2 ratios in AD patients were significantly higher than the healthy controls, and five of the six ratios decreased from baseline to 12 months follow-up (other than IL-2/TNF-α). Several significant relationships were noted between the changes in Th1/Th2 ratios with cognitive assessments.
Conclusions: Our results showed an overall rebalancing of the Th1/Th2 ratio in response to APMC, these changes were related to improved cognition in subjects with moderate-to-severe AD, and the APMC supplement was safely tolerated.

Keywords: Aloe, Alzheimer's disease, cognition, cytokines, dietary supplements, immunology, polysaccharides

*Correspondence to: John E. Lewis, PhD, Voluntary Associate Professor, University of Miami Miller School of Medicine, Department of Psychiatry & Behavioral Sciences, 1120 NW 14th Street, Suite #1474 (D21), Miami, FL 33136, USA. Tel.: +1 305 962 5286; E-mail: jelewis@miami.edu.

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INTRODUCTION

Alzheimer's disease (AD) continues to be a leading cause of death of Americans, and the number of people being diagnosed with the disease continues to rise [1]. The conventional treatment approach for people diagnosed with AD and other forms of dementia remains limited in its efficacy at best [2, 3]. Despite so much effort to determine what actually causes or contributes to AD, e.g., inconsistently documented relationships between the concentrations and distribution of amyloid-β (Aβ) deposits in the brain and AD pathology, such as disease severity and loss of synapses and neurons [4, 5], tau protein accumulation [6], *Helicobacter pylori* bacterium and other infectious pathogens [7, 8], neuroinflammation [9], oxidative stress [10], and the 'starving brain' theory related to necroptosis, hyperglycemia, and insulin resistance [11], among others, a lack of general consensus persists. Without common agreement on the etiology of the disease, it is no wonder that efficacious treatments are sorely lacking. Thus, the ominous prospect for the AD patient has not substantially improved, as researchers continue to look for effective solutions to both treat and prevent AD and other dementias.

While the primary focus of dementia research centers on the brain and cognitive function, the etiology of AD has known links to inflammation and immune function, just as in all other chronic diseases [12–14]. Chronic, dysregulated inflammation has multiple consequences for health, and neuroinflammation is a key feature of dementia, causing deleterious impacts on microglia, neurons, meninges, choroid plexus, and other brain structures [12, 15]. For example, it is hypothesized that reducing tumor necrosis factor-alpha (TNF-α) may be beneficial in improving the cognitive function and overall health status of AD patients [16].

One interesting aspect of immune function that theorizes with limitations the importance of the balance between pro-inflammatory and anti-inflammatory components is the CD4+ T helper (Th)1/Th2 ratio [17]. The Th1/Th2 paradigm evolved initially from mouse models of immune responses against pathogens to human defense mechanisms [18–20]. This paradigm has gone through considerable criticism and reconsideration since its inception for its limitations, as it is now known that the naïve T helper cells are much more involved than in just Th1 and Th2 cells [21]. Classically, this paradigm was constructed on the knowledge that the Th1 and Th2 cells secrete and are involved with specific cytokines (e.g., interferon-gamma [IFN-γ] and interleukin [IL]-2 in the former; IL-4, IL-5, and IL-10 in the latter; and TNF-α in both) [22, 23]. Despite what has more recently been discovered about immunity and the recognition that the Th1/Th2 paradigm is overly simplistic for comprehensively accounting for the many angles of how the immune system operates and how it is involved in the progression and expression of disease, the ratios of Th1/Th2 cytokines serve as useful benchmarks for patient evaluation. The production of Th1 and Th2 cytokines significantly determines T cell-mediated immune reactions, and maintaining their balance is associated with prevention of infectious and allergic diseases, immune-related disorders, and the development of cancers [24].

While it is relatively well-established that inflammation contributes considerably to the cause and course of AD [12], unlike multiple sclerosis that is associated with and worsened by an imbalanced Th1/Th2 ratio (i.e., Th1 dominant) [25–27], it is unknown how the Th1 and Th2 cytokines are compared in AD and how that functional balance may be linked to the clinical profile. To our knowledge, the Th1/Th2 ratio has not been characterized in persons with AD, nor has that ratio been compared to healthy individuals. If it can be shown that persons with AD are characterized by a Th1/Th2 ratio that is out of balance, and further that this imbalance is linked to cognition, then this would shed additional light on the interrelationships between the immune and central nervous systems and that of our own work linking inflammation, immune function, and cognition [28–30]. The all-important question lingers regarding how to effectively counteract inflammation in general and in AD specifically, as it is hypothesized that lowering inflammation is important for at least slowing the progression of disease, if not playing a role in reversing it. Among other findings, we have already shown that a dietary supplement called aloe polymannose multinutrient complex (APMC) lowered TNF-α and vascular endothelial growth factor (VEGF), hallmarks of inflammation, and increased the CD4+/CD8+ ratio in persons with moderate to severe AD [28]. Thus, taking the next step in the research process by characterizing the Th1/Th2 ratio, comparing the ratio between AD patients and healthy adults, assessing its change in response to the administration of APMC, and relating it to cognitive function enables several questions to be answered in the search for more effective ways to help people with AD.

MATERIALS AND METHODS

Study participants

The AD participants ($n = 34$) were recruited from referrals to the Miami Jewish Health Systems outpatient facility. The study was conducted with the approval of the Stein Gerontological Institute Institutional Review Board for human subjects research, which operates within the standards set forth by the Helsinki Declaration of 1975, and each subject (and/or the primary caregiver) signed informed consent before participating in the study. Subjects were not required to stop or change their medication regimen for entry into the study and continued taking their drugs as ordered by the treating physician. Additionally, subjects had to be diagnosed with moderate-to-severe AD for at least one year prior to entering the study. Our participants were typically not eligible for other trials due to the severity of their condition and/or other co-morbid conditions. Each participant was evaluated by the study psychiatrist prior to enrollment in the study to verify the diagnosis of AD.

The second set of participants ($n = 20$) in this analysis was recruited by referrals to another dietary supplement study at the University of Miami Miller School of Medicine. Healthy subjects (i.e., no known heart, liver, or kidney disease, type 2 diabetes, active infections or cancer, mental illness, or obesity) completed a similar protocol to assess the effects of a dietary supplement on the same panel of cytokines and growth factors as those subjects in the AD study. The study was conducted with the approval of the University of Miami Institutional Review Board for human subjects research, and all participants signed informed consent and HIPAA forms before participating in the study.

Intervention

The powdered formula used in the AD study was the APMC dietary supplement composed of the following constituents in a fixed combination by weight: stabilized rice bran, golden flax seed, sunflower lecithin, dioscorea (yam), BiAloe® (15% acetylated aloe polymannose), tart cherry, N-acetyl cysteine, UltraTerra® calcium silicate clay, citric acid, natural vanilla, and inositol hexaphosphate. The product is packaged in 300 g containers, and the powder dissolves readily in any liquid. All participants orally consumed 2.5 g of the APMC four times per day (with three meals and before bedtime). The primary caregiver was shown how to administer the APMC at the baseline assessment, and the first dose was given to the participant at our facility to ensure compliance with the protocol and to monitor for any complications or adverse reactions.

Outcomes and assessments

Each AD participant and caregiver completed a basic demographics and medical history questionnaire at baseline. In addition to a neuropsychological battery to measure changes in cognitive functioning, activities of daily living, and quality of life, a standard assessment at 12 months was conducted to monitor: (a) adverse reactions and compliance to the intervention, (b) basic medical and health status, and (c) current medications. We asked the caregiver if the AD participant was taking any type of anti-inflammatory medication ("yes" or "no" response to "Are you currently taking an anti-inflammatory medication?") to use as a covariate in the analyses, given the potential effect the use of this drug class might have on the Th1/Th2 ratios [31].

The neuropsychological battery consisted of four measures to assess changes in disease severity, overall cognitive function, and activities of daily living. The AD Assessment Scale (ADAS) [32] is a sensitive and reliable psychometric scale and is considered the gold standard to assess cognition in dementia studies [33]. It has 11 subscales that evaluate memory, orientation, attention, language, reasoning, and constructional and ideational praxis that are summed to create a total cognition score [34]. The total score can range from zero (no impairment) to 70 (severe impairment). The ADAS assessment included an additional concentration score with values ranging from zero (no impairment) to five (severe impairment). Different, counterbalanced word lists were used at the follow-up visit to ensure that practice and carry-over effects would not confound our results. The Severe Impairment Battery (SIB) is a 40-item questionnaire designed to assess the severity of cognitive dysfunction in AD and is divided into nine domains: memory, language, orientation, attention, praxis, visuospatial, construction, orientation to name, and social interaction [35, 36]. The total score on the SIB ranges from zero (greatest impairment) to 100 (no impairment). The Mini-Mental State Examination (MMSE) is one of the most widely utilized and popular brief cognitive assessments to provide a rapid screen of orientation, registration, attention and calculation,

recall, and language domains [37]. The score can range from zero to 30 (25+ is normal) and can indicate severe (≤9 points), moderate (10–20 points), or mild (21–24 points) cognitive impairment [38]. The modified 19-item AD Cooperative Study-Activities of Daily Living (ADCS-ADL) is a structured measure originally designed to assess functional capacity over a wide range of dementia severity [39]. Each statement includes a series of hierarchical questions designed to determine the patient's ability to perform one of the ADL, ranging from total independence to total inability. A total score of 54 signifies optimal performance, and lower scores indicate worse performance. Caregivers were asked to assess a patient's activities during the preceding four-week interval.

Sample collection and processing

Venous blood was obtained at baseline and 12 months from all AD participants and at baseline from the healthy subjects. Blood samples were collected in EDTA tubes and delivered to the laboratory within 2 h of collection. All specimens were subjected to complete blood cell counts and auto 5-part differential count determinations by a fully-automated Coulter AcT5 hematology analyzer (Beckman Coulter, Fullerton, CA).

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque gradient centrifugation. PBMC were recovered from the gradient interface and washed in phosphate buffered saline. Blood was diluted with 1:1 RPMI 1640 (Gibco, Grand Island, NY), layered over Ficoll-Hypaque solution (Pharmacia, Piscataway, NJ), and centrifuged for 30 minutes at 1,500 rpm at ambient temperature. The PBMC were collected, washed with RPMI 1640, and counted and assessed for viability in trypan blue dye. Plasma for cytokine detection was separated and stored at –80°C until used.

Multiplex cytokine and growth factor testing

Cytokine levels in plasma specimens were measured using a biochip array system, Evidence Investigator™ (Randox Laboratories Ltd., Crumlin, UK) as reported previously [40]. The testing platform consisted of biochips secured in the base of a well placed in a carrier holding nine biochips in a 3×3 format. Each biochip was coated with the capture antibodies specific for each of the Th1 (IL-2 and IFN-γ) and Th2 (IL-4, IL-10, and TNF-α) proteins on a particular test region. A sandwich chemiluminescent assay was performed with 100 µl plasma using reagents (including the calibrators and controls) and protocols supplied by the same manufacturer. The light signal generated from each of the test regions on the biochip was detected using a charge-coupled detector camera and imaging system and compared with a calibration curve generated with known standards during the same run. All specimens were run in duplicate, and the concentration of each cytokine present in each plasma specimen was calculated from the standard curve and reported in pg/ml.

Statistical analysis

Data were analyzed using SPSS 28 for Windows (IBM Inc., Chicago, IL). Frequency and descriptive statistics were calculated on all variables. We calculated the following ratios, IL-2/IL-10, IFN-γ/IL-10, IL-2/IL-4, IFN-γ/IL-4, IL-2/TNF-α, and IFN-γ/TNF-α, to represent Th1/Th2 at baseline (for AD patients and healthy subjects) and 12 months follow-up (for AD patients only). We compared the different ratios between AD patients and healthy subjects with independent samples *t*-tests assuming heterogeneous variances between groups. We compared the ratios and the scales and subscales of the cognitive function battery measured at baseline and 12 months with paired-samples *t*-tests. We utilized linear mixed modeling (LMM) to assess the fixed effect of time on changes in these ratios from baseline to 12 months follow-up in the AD patients. If the type III test of the fixed effect of time was significant, then we evaluated the parameter estimate between baseline and 12 months follow-up. We used the dichotomous variable for anti-inflammatory drug use as a covariate and also assessed its fixed effect for significance in each model. LMM with heterogeneous compound symmetry covariance allowed us to account for subject attrition, inter-correlated values between time points, and non-constant variability. We calculated the difference in each ratio from baseline to 12 months follow-up. We also calculated the differences in all scales and subscales of the cognitive function battery from baseline to 12 months follow-up. We utilized Spearman's rank correlation coefficients to determine the relationships between the changes in the Th1/Th2 ratios and the various measures of cognition from baseline to 12 months. The criterion for statistical significance for all analyses was $\alpha = 0.05$.

Table 1
Descriptive statistics of the Th1/Th2 ratios at baseline for Alzheimer's patients and healthy adults

Variable	Alzheimer's Patients	Healthy Adults
IL-2/IL-10^	423.2 ± 413.4 (0.02, 1,577)	1.9 ± 4.3 (0.01, 16.2)
IFN-γ/IL-10*	69.4 ± 169.2 (0.01, 737)	0.32 ± 0.78 (0.01, 2.4)
IL-2/IL-4^	434.0 ± 488.9 (0.01, 1,910)	1.5 ± 2.3 (0.01, 9.0)
IFN-γ/IL-4*	54.9 ± 150.9 (0.01, 737)	0.19 ± 0.37 (0.01, 1.1)
IL-2/TNF-α*	147.5 ± 347.7 (0.01, 1,348)	2.2 ± 2.8 (0.01, 12.8)
IFN-γ/TNF-α^	31.4 ± 13.6 (0.01, 737)	0.24 ± 0.45 (0.01, 1.3)

Values are significantly different ($p < 0.001$^ or $p < 0.05$*) between groups, and mean ± standard deviation (minimum, maximum).

RESULTS

Safety and tolerability

During the 12-month study period, one AD subject's caregiver reported an initial three-day period of loose stool that was remedied by halving the amount of APMC given per day and then increased to four 2.5 g scoops/day amount in one week. A second AD subject's caregiver reported elevations in blood pressure and pulse, which were remedied by reducing the daily amount to one 2.5 g scoop/day and increased by one 2.5 g scoop/day/week until achieving the desired dose of four 2.5 g scoops/day. No other adverse events were reported in this study. Three AD participants died during the course of the intervention, which were deemed by the medical team to be unrelated to the study: one male due to myocardial infarction and two females due to stroke. Five other AD participants dropped out of the study due to non-compliance with the protocol according to the caregivers (e.g., the participant was unwilling to take the APMC four times per day), leaving 26 subjects who completed the 12-month intervention.

Demographics of each sample

The AD patient sample comprised of 82% females ($n = 28$) and 18% males ($n = 6$) with a mean age of 79.9 years (SD = 8.4; R = 60, 98). The racial/ethnic distribution of the subjects was as follows: 62% Hispanic ($n = 21$), 29% white, non-Hispanic ($n = 10$), and 9% black, non-Hispanic ($n = 3$). Subjects had been diagnosed with AD for an average of 3.2 years (SD = 2.0; R = 1, 11). The healthy subjects sample comprised of 60% females ($n = 12$) and 40% males ($n = 8$) with a mean age of 33.6 years (SD = 13.2; R = 20, 66). The racial/ethnic distribution of the subjects was: 55% white, non-Hispanic ($n = 11$), 35% Hispanic ($n = 7$), and 10% Asian/Pacific Islander ($n = 2$). Eight AD subjects reported taking some type of anti-inflammatory medication.

Comparisons of the Th1/Th2 ratios between AD patients and healthy subjects

Table 1 shows the descriptive values of the IL-2/IL-10, IFN-γ/IL-10, IL-2/IL-4, IFN-γ/IL-4, IL-2/TNF-α, and IFN-γ/TNF-α ratios at baseline for the AD patients and healthy subjects. For the IL-2/IL-10 ratio, a significant difference was found between the two groups ($t[33] = 5.9$, $p < 0.001$). For the IFN-γ/IL-10 ratio, a significant difference was found between the two groups ($t[33] = 2.4$, $p = 0.02$). For the IL-2/IL-4 ratio, a significant difference was found between the two groups ($t[33] = 5.2$, $p = 0.001$). For the IFN-γ/IL-4 ratio, a significant difference was found between the two groups ($t[33] = 2.1$, $p = 0.04$). For the IL-2/TNF-α ratio, a significant difference was found between the two groups ($t[33] = 2.4$, $p = 0.02$). For the IFN-γ/TNF-α ratio, a significant difference was found between the two groups ($t[33] = 13.3$, $p < 0.001$). Without exception, all ratios were significantly higher in the AD patients compared to the healthy subjects. Interestingly, the ratios for the healthy subjects were near unity for all values.

Comparisons of the Th1/Th2 ratios for AD patients from baseline to 12 months follow-up

Table 2 shows the descriptive values of the IL-2/IL-10, IFN-γ/IL-10, IL-2/IL-4, IFN-γ/IL-4, IL-2/TNF-α, and IFN-γ/TNF-α ratios at baseline and 12 months follow-up for the AD patients. For the IL-2/IL-10 ratio, a significant fixed effect was found for time ($F[1,29.4] = 9.8$, $p = 0.004$), revealing that the IL-2/IL-10 ratio significantly decreased at 12 months. The fixed effect for anti-inflammatory use was non-significant ($F[1,30.5] = 1.3$, $p = 0.27$). For the IFN-γ/IL-10 ratio, non-significant fixed effects

Table 2
Descriptives for the Th1/Th2 ratios at baseline and 12 months for Alzheimer's patients

Variable	Baseline	12 Months
IL-2/IL-10*	423.2 ± 413.4 (0.02, 1,577)	166.2 ± 253.7 (0.02, 836)
IFN-γ/IL-10	69.4 ± 169.2 (0.01, 737)	27.0 ± 74.5 (0.01, 267)
IL-2/IL-4*	434.0 ± 488.9 (0.01, 1,910)	289.4 ± 412.3 (1.0, 1,728)
IFN-γ/IL-4	54.9 ± 150.9 (0.01, 737)	16.7 ± 55.2 (0.71, 234)
IL-2/TNF-α	147.5 ± 347.7 (0.01, 1,348)	167.3 ± 401.2 (0.01, 1,728)
IFN-γ/TNF-α	31.4 ± 13.6 (0.01, 737)	6.8 ± 31.7 (0.01, 159)

Values are significantly different ($p < 0.05$) * from Baseline to 12 Months, mean ± standard deviation (minimum, maximum), and decreasing values indicate more balanced Th1/Th2 ratios.

Table 3
Linear mixed model results with and without anti-inflammatory use as a covariate

Linear mixed model without anti-inflammatory use as a covariate				
Variable	Parameter Estimate for Time	Standard Error	95% Confidence Interval	*p*
IL-2/IL-10	255.9	83.7	85.0, 426.8	0.005
IFN-γ/IL-10	42.6	33.9	–26.2, 111.5	0.22
IL-2/IL-4	151.3	77.2	–6.8, 309.4	0.06
IFN-γ/IL-4	38.2	28.9	–20.4, 96.9	0.19
IL-2/TNF-α	–25.7	103.7	–236.8, 185.5	0.81
IFN-γ/TNF-α	24.5	24.5	–25.2, 74.2	0.32
Linear mixed model with anti-inflammatory use as a covariate				
Variable	Parameter Estimate for Time	Standard Error	95% Confidence Interval	*p*
IL-2/IL-10	265.0	84.5	92.3, 437.7	0.004
IFN-γ/IL-10	45.6	34.1	–23.6, 114.9	0.19
IL-2/IL-4	159.9	77.3	1.4, 318.4	0.048
IFN-γ/IL-4	40.6	28.8	–18.0, 99.2	0.17
IL-2/TNF-α	–10.7	103.7	–221.9, 200.5	0.92
IFN-γ/TNF-α	25.3	24.5	–24.4, 75.1	0.31

were found for time ($F[1,34.3] = 1.8$, $p = 0.19$) and anti-inflammatory use ($F[1,29.4] = 1.2$, $p = 0.28$). For the IL-2/IL-4 ratio, a significant fixed effect was found for time ($F[1,27.6] = 4.3$, $p = 0.048$), revealing that the IL-2/IL-4 ratio decreased at 12 months. The fixed effect for anti-inflammatory use was non-significant ($F[1,34.5] = 1.8$, $p = 0.18$). For the IFN-γ/IL-4 ratio, non-significant fixed effects were found for time ($F[1,34.4] = 2.0$, $p = 0.17$) and anti-inflammatory use ($F[1,28.2] = 1.3$, $p = 0.27$). For the IL-2/TNF-α ratio, non-significant fixed effects were found for time ($F[1,32.2] = 0.01$, $p = 0.92$) and anti-inflammatory use ($F[1,37.4] = 3.1$, $p = 0.09$). For the IFN-γ/TNF-α ratio, non-significant fixed effects were found for time ($F[1,34.2] = 1.1$, $p = 0.31$) and anti-inflammatory use ($F[1,25.6] = 0.45$, $p = 0.51$). Table 3 shows the parameter estimate for time, the standard error, the 95% confidence interval, and the p value for each LMM result with and without controlling for anti-inflammatory use.

The relationships between changes in Th1/Th2 ratios and cognitive functioning

Table 4 shows the descriptive values of the ADAS, SIB, MMSE, and ADCS-ADL at baseline and 12 months follow-up for the AD patients. We calculated changes in IL-2/IL-10, IFN-γ/IL-10, IL-2/IL-4, IFN-γ/IL-4, IL-2/TNF-α, IFN-γ/TNF-α, ADAS, SIB, MMSE, and ADCS-ADL from baseline to 12 months follow-up and then correlated the Th1/Th2 ratios with the cognition battery on their difference scores. We found multiple significant relationships between changes in the Th1/Th2 ratios and changes in the cognition assessments. Table 5 shows the Spearman ρ correlations for all relationships between the cognitive assessments and the Th1/Th2 ratios.

Correlations with ADAS subscales

The change in IFN-γ/IL-10 was correlated with the change in Orientation ($\rho = 0.49$, $p < 0.02$) and

Table 4
Descriptives for the ADAS, MMSE, ADCS-ADL, and SIB at baseline and 12 months follow-up

Variable	Baseline	12 Months
ADAS Word Recall	8.0 ± 2.4 (0, 10)	7.2 ± 2.3 (2.3, 10)
ADAS Objects and Fingers	3.1 ± 1.7 (0, 5)	3.1 ± 1.9 (0, 5)
ADAS Commands	3.1 ± 1.6 (0, 5)	3.2 ± 1.7 (0, 5)
ADAS Constructional Praxis	3.0 ± 1.8 (0, 5)	3.3 ± 1.8 (0, 5)
ADAS Ideational Praxis	2.9 ± 2.1 (0,5)	3.3 ± 2.0 (0, 5)
ADAS Orientation	6.3 ± 1.7 (0, 8)	6.3 ± 2.0 (1, 8)
ADAS Word Recognition	10.5 ± 2.5 (3, 12)	9.1 ± 4.0 (1, 12)
ADAS Word Recognition Reminders*	9.2 ± 7.9 (0, 24)	3.9 ± 4.0 (0, 14)
ADAS Remembering Test Instructions	3.8 ± 2.2 (0, 7)	3.1 ± 2.3 (0, 8)
ADAS Spoken Language Ability*	1.7 ± 2.2 (0, 5)	2.4 ± 2.1 (0, 5)
ADAS Word-Finding Difficulty*	2.6 ± 1.9 (0, 5)	3.4 ± 1.5 (0, 5)
ADAS Comprehension	2.1 ± 1.8 (0, 5)	2.2 ± 2.0 (0, 5)
ADAS Total Cognition Score*	42.0 ± 14.0 (13, 69)	37.8 ± 12.9 (8, 70)
ADAS Concentration	1.6 ± 1.6 (0, 5)	1.2 ± 1.6 (0, 5)
SIB Social Interaction*	4.8 ± 2.1 (0, 6)	4.0 ± 2.2 (0, 6)
SIB Memory*	4.4 ± 3.9 (0, 12)	3.2 ± 3.9 (0, 11)
SIB Orientation	2.9 ± 1.7 (0, 6)	2.6 ± 1.8 (0, 6)
SIB Language*	29.5 ± 14.9 (0, 46)	24.5 ± 17.4 (0, 46)
SIB Attention*	3.6 ± 2.3 (0, 6)	4.1 ± 2.3 (0, 6)
SIB Praxis	4.8 ± 3.4 (0, 8)	4.0 ± 2.3 (0, 6)
SIB Visuospatial Ability	3.9 ± 3.4 (0, 8)	2.9 ± 3.4 (0, 8)
SIB Construction*	2.4 ± 1.8 (0, 4)	1.9 ± 1.9 (0, 4)
SIB Orienting to Name*	1.6 ± 0.7 (0, 2)	1.3 ± 0.8 (0, 2)
SIB Total Score*	58.1 ± 31.4 (0, 96)	48.9 ± 35.3 (0, 97)
MMSE	10.7 ± 6.5 (0, 23)	9.2 ± 8.0 (0, 26)
ADCS-ADL*	17.7 ± 12.6 (0, 47)	13.2 ± 10.9 (0, 49)

*Values are significantly different ($p < 0.05$) from Baseline to 12 Months, mean ± standard deviation (minimum, maximum), higher scores indicate better performance on the MMSE, ADCS-ADL, and SIB, and lower scores indicate improvement on the ADAS, other than Word Recognition Reminders. When utilizing the Holm-Modified Bonferroni Correction to counteract the family-wise error rate for each cognition test, none of the scales on the ADAS remains significant, and only the SIB Total Score and Construction and Language scales remain significant. The results for the ADCS-ADL and MMSE remain the same.

negatively correlated with the change in Word Recognition Reminders ($\rho = -0.74$, $p = 0.01$). The change in IL-2/IL-4 was correlated with the change in Word Recognition Reminders ($\rho = 0.63$, $p = 0.04$). The change in IFN-γ/IL-4 was correlated with the changes in Orientation ($\rho = 0.46$, $p = 0.02$) and Comprehension ($\rho = 0.42$, $p = 0.04$). No significant correlations were found with the ADAS-cognition total score.

Correlations with SIB subscales

The change in IL-2/IL-10 was negatively correlated with the change in Social Interaction ($\rho = -0.40$, $p = 0.05$). The change in IFN-γ/IL-10 was negatively correlated with the change in Social Interaction ($\rho = -0.50$, $p = 0.01$). The change in IFN-γ/IL-4 was negatively correlated with the change in Attention ($\rho = -0.43$, $p = 0.03$). No significant correlations were found with the SIB total score.

Correlations with MMSE and ADCS-ADL

The change in IFN-γ/TNF-α was negatively correlated with the change in the MMSE ($\rho = -0.45$, $p = 0.02$). No significant correlations were found with the ADCS-ADL.

DISCUSSION

To our knowledge, we have characterized for the first time the Th1/Th2 ratio according to six different values, i.e., IL-2/IL-10, IFN-γ/IL-10, IL-2/IL-4, IFN-γ/IL-4, IL-2/TNF-α, IFN-γ/TNF-α, in persons with moderate-to-severe AD. The Th1/Th2 ratio has been investigated more so for its significance and relationship to health status and clinical characteristics in other conditions, e.g., autoimmunity, HIV, cancer, type 2 diabetes, and allergies [41–44]. For example, Th1 dominance (i.e., imbalance between Th1 and Th2 cytokines) has been shown to be linked to the pathogenesis and disease severity of rheumatoid arthritis [45–47]. However, the Th1/Th2 ratio has apparently not been considered as a useful contextual factor of disease severity for people with AD, given the lack of information about it in the literature. Nonetheless, the crosstalk between immune

Table 5
Spearman ρ correlations for the ADAS, SIB, MMSE, and ADCS-ADL with the Th1/Th2 ratios

Variable	IL-2/IL-10	IFN-γ/IL-10	IL-2/IL-4	IFN-γ/IL-4	IL-2/TNF-α	IFN-γ/TNF-α
ADAS Word Recall	0.26	0.08	0.19	0.21	0.34	0.30
ADAS Objects and Fingers	0.01	–0.03	0.06	0.05	–0.03	–0.13
ADAS Commands	–0.27	–0.20	–0.12	–0.04	0.14	0.23
ADAS Constructional Praxis	–0.14	–0.02	–0.14	0.02	–0.25	–0.18
ADAS Ideational Praxis	–0.15	–0.14	–0.33	–0.32	–0.09	0.02
ADAS Orientation	–0.16	0.49*	–0.25	0.46*	–0.16	0.27
ADAS Word Recognition	0.26	–0.32	–0.02	–0.49	0.24	0.04
ADAS Word Recognition Reminders	–0.06	–0.74^	0.63*	–0.02	0.17	–0.41
ADAS Remembering Test Instructions	0.12	–0.43	0.52	–0.015	0.35	–0.04
ADAS Spoken Language Ability	–0.20	–0.04	–0.10	–0.04	–0.08	–0.13
ADAS Word-Finding Difficulty	0.16	–0.04	0.24	0.05	0.33	0.21
ADAS Comprehension	–0.29	0.14	–0.11	0.42*	–0.21	0.02
ADAS Concentration	0.24	0.18	0.10	0.001	–0.13	–0.23
ADAS Total Cognition Score	0.09	–0.01	0.003	–0.15	0.11	0.02
SIB Social Interaction	–0.40*	–0.50^	–0.28	–0.27	–0.13	0.05
SIB Memory	–0.07	0.33	–0.18	0.26	–0.23	0.03
SIB Orientation	–0.21	–0.35	–0.06	–0.05	–0.24	–0.18
SIB Language	0.06	0.11	–0.07	–0.01	–0.09	–0.002
SIB Attention	0.16	–0.04	–0.06	–0.43*	–0.06	–0.26
SIB Praxis	0.06	0.18	0.10	0.28	0.22	0.26
SIB Visuospatial Ability	–0.02	0.08	0.05	0.27	–0.30	–0.16
SIB Construction	0.10	–0.16	0.26	0.01	–0.003	–0.19
SIB Orienting to Name	0.09	–0.33	0.24	–0.21	0.20	–0.07
SIB Total Score	0.03	0.04	–0.08	–0.02	–0.20	–0.11
MMSE	0.24	–0.02	0.08	–0.28	–0.19	–0.45*
ADCS-ADL	–0.25	–0.25	–0.16	–0.02	–0.18	0.02

Values are significantly different ($p = 0.01$^ or $p < 0.05$*).

system components and other major organ systems, particularly the brain and central nervous system, is increasingly recognized for its importance, given that a balanced, surveillant, and responsive immune system is necessary for ensuring that all other organ systems function properly [48–53]. In fact, we have previously published data supporting such importance by identifying significant associations between various components of the immune system with cognition (a primary indicator of brain function) [28] and brain-derived neurotrophic factor [30].

In addition to reporting the Th1/Th2 ratios in people with AD for the first time in this study, we compared their values to those of a sample of healthy subjects, and the differences were substantial. Whereas the average values for the healthy subjects were mostly near unity, suggesting appropriate balance between the Th1 and Th2 domains of the immune system, the AD patients' values were much more elevated, suggesting that their immune function was Th1 dominant. Even as five of six of the ratios declined over the 12-month intervention period (only IL-2/TNF-α slightly increased at 12 months), the values were still indicative of Th1 dominance.

As we believe we are reporting Th1/Th2 values perhaps for the first time in people with moderate-to-severe AD, we were forced to review other branches of the medical literature to determine how our findings compare to other diseases and disorders. For example, scientists in Bénin cross-sectionally compared middle-aged adults with type 2 diabetes being treated with insulin to healthy controls on several Th1/Th2 values [54]. In assessing IL-2/IL-4, IL-2/IL-10, IFN-γ/IL-4, and IFN-γ/IL-10, their healthy controls had values of 2.16, 1.49, 5.91, and 4.07, respectively, and their type 2 diabetics had values of 0.99, 0.15, 3.95, and 0.61, respectively. Their data show several interesting findings compared to our study. Compared to the diabetic subjects, the healthy subjects had higher Th1/Th2 ratios for all four values; the diabetic subjects had values closer to our healthy subjects; the values of their diabetic subjects compared to our AD subjects helps to emphasize the disparity in immune imbalance in different disease states; and their ratios suggest that type 2 diabetes is shifted toward a Th2 phenotype. A group of scientists in China compared the Th1/Th2 ratio among four separate groups of subjects: acute coronary syndrome with normal glucose tolerance, acute coronary syndrome with impaired glucose tolerance, acute coronary syndrome with type 2 diabetes, and healthy controls [55]. The IFN-γ/IL-4 ratio was the only one

from their study that was directly comparable to our study, and as with the previously mentioned study, the values from this study were closer to our healthy adults than our AD patients, i.e., acute coronary syndrome with normal glucose tolerance (mean = 0.07), acute coronary syndrome with impaired glucose tolerance (mean = 0.17), acute coronary syndrome with type 2 diabetes (mean = 0.25), and healthy controls (mean = 0.05). Compared to AD patients, these IFN-γ/IL-4 values are quite low, and once again, may be indicative of how imbalanced the Th1/Th2 ratio is for patients with this disease compared to others who have some combination of a coronary disturbance with or without diabetes. Another group of investigators in China cross-sectionally compared IFN-γ and TNF-α in brucellosis patients (average age 46.7 years) versus healthy subjects (average age 39.0 years) [56], and their data allowed us to calculate the IFN-γ/TNF-α ratio, which showed similar values in our current study, 22.1 compared to 31.4 in AD patients and 3.25 to 0.24 to our healthy subjects, respectively. Overall, it appears that other disease states, such as coronary and metabolic issues, are closer to Th1/Th2 balance and/or slightly Th2 shifted, compared to our AD patients, which are Th1 dominant.

Our results of Th1 dominance in all six of the calculated ratios are consistent with previous research in multiple sclerosis indicating that Th1 dominance results in sequential and overlapping steps of pro-inflammation, disease progression, and worsening of symptoms [57–60]. While currently underappreciated if not completely unknown in AD, it has been previously suggested that shifting the ratio from a Th1 to a Th2 phenotype may deter autoimmunity and stave off disease progression in multiple sclerosis [61]. For example, glatiramer acetate treatment was used for one year in a study of relapse remitting multiple sclerosis patients, showing a Th2 anti-inflammatory shift response by raising IL-4 and IL-10 in relation to IFN-γ [25]. Unfortunately, no functional data were collected in this study, so it is unknown if the Th2 shift resulted in any clinical improvement. In addition, adverse effects were not reported, so it is unclear how this medication treatment compares to our approach using a dietary supplement regarding risk:efficacy, as the risk of side effects of APMC is exceptionally low, while its efficacy is notable. Nonetheless, this study in multiple sclerosis serves as a useful framework to help shed light on the implications of our results, where APMC was able to shift five of six Th1/Th2 ratios toward Th2 expression (two of them statistically significantly), along with associating these physiological changes with improved cognitive function (i.e., the clinical outcomes). Thus, APMC may be not only an effective dietary supplement for inducing a shift toward a Th2 cytokine anti-inflammatory response phenotype, but enabling an accompanying improvement in the clinical outcome; in this case cognitive function.

In addition to reporting the Th1/Th2 ratios, the next objective of the current study was to measure their changes over the 12-month period in response to consuming APMC. We previously reported that APMC was able to lower TNF-α, IL-2, and IL-4 and increase the CD4+/CD8+ ratio in AD patients, thus demonstrating net improvements in immune function and inflammation [28]. As noted above, five of the six Th1/Th2 ratios decreased over the 12-month period, and the IFN-γ/TNF-α ratio declined closer to unity than any other value, although non-statistically significant. In addition, the IL-2/TNF-α ratio non-significantly increased at 12 months. As TNF-α is secreted by both Th1 and Th2 components of the immune system [62], the values and subsequent changes over time in the two TNF-α ratios may not accurately represent a true Th1/Th2 ratio. Despite those considerations, the results of the current study suggest that APMC favorably improved the Th1/Th2 imbalance overall. Given that we believe we are reporting these data for the first time in people with moderate-to-severe AD, it is unknown how APMC compares to other dietary supplements or medications for attempting to restore the balance within these two components of the immune system in this patient population. One recent review of 26 animal studies found that flavonoid subclasses, i.e., flavones, flavonols, flavanones, flavanonols, isoflavones, and anthocyanins, were generally able to modulate and balance the Th1/Th2 cytokine ratios through various immunoregulatory mechanisms, but it is unknown how these results translate to humans [63].

In elderly patients (~77 average years of age) with chronic obstructive pulmonary disease (COPD), a group of Chinese scientists looked at the effect of six months of 1,200 mg/day of N-acetyl cysteine intake on CD4+ cells, IL-4, and IFN-γ [64]. Dementia or AD was not listed as an exclusion criterion, and it was not mentioned if caregivers were needed for the study participants, so it would appear that this group of subjects did not have any type of neurodegeneration. At baseline, the average IFN-γ/IL-4 ratio was 2.6 for the treatment and placebo groups, and after six months of treatment the average IFN-γ/IL-4 ratio was

1.1 for the treatment group and 2.5 for the placebo group. N-acetyl cysteine was effective in lowering the IFN-γ/IL-4 ratio after six months, but the values for the COPD subjects in this study were much closer to our healthy subjects versus our AD patients. The COPD subjects were of similar average age to our AD patients, but nonetheless their IFN-γ/IL-4 ratio was observationally different, lending credence to how immunocompromised people with AD are.

The third objective of this study was to determine how the changes in the Th1/Th2 ratios related to the changes in the various cognition assessments. We previously documented that the intake of APMC resulted in clinically and statistically significant improvements in cognition at 12 months follow-up according to the ADAS Total Cognition Score in AD patients [28], so documenting the relationships between the Th1/Th2 ratios and cognition was warranted in the current study. The current results are consistent with some of our previous findings relating mutually beneficial changes in immune function and inflammation with cognition [28–30]. Most of the significant relationships in the present study showed that the rebalancing of various Th1/Th2 ratios were associated with better performance on the cognitive measures. For example, on the ADAS, although the rebalancing of IL-2/IL-4 was associated with needing more Word Recognition Reminders (poorer performance), the rebalancing of IFN-γ/IL-10 was associated with a better score on the Orientation subscale and needing fewer Word Recognition Reminders. The rebalancing of IFN-γ/IL-4 was correlated with better performance on the Orientation and Comprehension subscales. Unfortunately, no relationships were demonstrated with the ADAS Total Cognition Score. On the SIB, the Social Interaction score improved along with rebalancing of both IL-2/IL-10 and IFN-γ/IL-10. The Attention score improved with rebalancing of IFN-γ/IL-4. The SIB Total Score was not correlated with any Th1/Th2 ratio. The MMSE improved with a rebalancing of IFN-γ/TNF-α, but the ADCS-ADL was not correlated with any Th1/Th2 ratio. Thus, several cognitive function scores showed corresponding improvement along with rebalancing of some of the Th1/Th2 ratios, particularly IFN-γ/IL-10 and IFN-γ/IL-4.

The results of the present study on the relationships between the changes in Th1/Th2 ratios and cognition are not readily comparable to other research due to a paucity of data in humans. However, the results of a preclinical study using a mouse model of AD may shed some light at least on the importance of IFN-γ and Th1 cell brain infiltration and the effects on cognitive function [13]. A group of investigators from Ireland showed that Aβ-specific Th1 cells increased Aβ deposition and microglial activation in transgenic mice that overexpress amyloid precursor protein and presenilin 1. The brains of these mice had significant T cell infiltration, some of which were secreting IFN-γ or IL-17. After immunization with Aβ and a toll-like receptor agonist, the mice demonstrated 5 weeks later that Th1 cells, but not Th2 or IL-17-producing CD4+T cells, increased microglial activation and Aβ deposition. These effects were related to deficits in spatial learning. The impacts of the Th1 cells were attenuated by treatment of the mice with anti-IFN-γ anti-β-galactosidase. Their results indicated that brain-infiltrating Th1 cells, and not Th2 cells, releasing IFN-γ significantly hasten typical hallmarks of AD (Aβ deposition and cognitive dysfunction) in this transgenic mouse model of the disease. Although this study did not evaluate the Th1/Th2 ratio, it does identify the importance of Th1 cells secreting IFN-γ and their relationship to worse cognitive function, which is consistent with our study showing that rebalancing of all the ratios involving IFN-γ was associated with improved cognition. In addition, this transgenic mouse model study suggests that the effects may be mediated by IFN-γ accompanying microglial activation that stimulates a problematic inflammatory cascade of ongoing pathology, which is consistent with other research that previously showed the presence of T cells [65] and activated microglia [66] in the brains of people with AD. It is difficult to speculate exactly how all this information relates to the current study given the differences in studies and data collected, but we can speculate the rebalancing of the IFN-γ-related ratios in response to consuming APMC was important for restoring some aspects of cognitive function, perhaps through lowering neuroinflammation and some as of yet undetermined effect on microglia. However, it is worth mentioning that other data suggest that IFN-γ is not just pro-inflammatory, as it can directly or indirectly counteract inflammatory pathways through different mechanisms, such as through neutrophil trafficking, Treg activity, and its involvement with Th17 cells [67]. Thus, IFN-γ is a bidirectional immunoregulatory cytokine, and its net effect requires consideration of its importance within the particular context.

One interesting aspect of our study that is not readily explainable is why our sample of AD patients had such high Th1 dominant ratios for all six val-

ues, particularly compared to our healthy subjects and of the subjects from the other aforementioned studies. Given the lack of understanding of the multifactorial etiology of AD, a more recent hypothesis to help explain the risk of neurodegeneration leading to this disease lies in the incidence of infections, i.e., virus, bacteria, and parasites. Multiple recent reviews document information that suggests that microorganisms are involved in pathological changes related to Aβ and tau protein deposition and accumulation, among others, which increase the risk of AD, and these types of infections impact acute and systemic inflammatory responses [68–70]. In the milieu of this inflammation and overactivation of the immune system, complex bidirectional positive feedback occurs as microglia and astrocytes are activated, they release IFN-γ, and the Th1 cells continue releasing their pro-inflammatory effector molecules that keep the microglia and astrocytes stimulated [68, 71]. Thus, one explanation for why we noted such high Th1 dominance in our sample could be that these subjects had significant microorganism infection, given that Th1 cells direct cellular immunity to fight viruses and other intracellular pathogens [17]. Unfortunately, our medical history and assessment protocol did not include collecting information about infections at baseline or during the course of the study, so we can only speculate if this may explain such high Th1/Th2 values. Otherwise, we have shown that AD might also fall into the category of a dominant Th1 immune response similar to rheumatoid arthritis and multiple sclerosis [43], but for as of yet undiscovered reasons.

Limitations

We note several limitations of the current study. In general, utilizing the Th1/Th2 ratio as a marker of immune balance is a gross oversimplification for how the immune system works and how much bidirectionality and shifting polarization occurs that can readily change its response from one component to another. Nonetheless, it is obvious that this paradigm still remains a useful tool to discuss these intricate immune component relationships and to use it to determine how these data are related to clinical outcomes. As noted previously, we recognize that collecting infection information would have been particularly useful to perhaps help to explain why our AD subjects have such remarkable Th1 dominance. We can only theorize based on other research that perhaps current, lasting infections may explain the values we are reporting. It would have been ideal to conduct more blood draws to assess more frequent immune responses over the course of the 12-month intervention, but, unfortunately, we did not have the budget to do that. Thus, we have a large gap in time between baseline and 12 months and can only speculate what may have occurred within the Th1/Th2 ratios during that period. In addition to only being able to draw blood at two time points, we also would have preferred to enroll more subjects in our clinical trial, but once again, we were limited with our study budget. A larger sample size might have resulted in even more significant findings for the changes in the Th1/Th2 ratios and their relationships to the changes in cognitive functioning. Although it is not accepted as a certainty, our cytokine and growth factor assays may have been unduly changed by the medication regimens of our AD patients [72]. Additionally, we noted that INF-γ and TNF-α are not just pro-inflammatory or anti-inflammatory and have bi-directional feedback and influence with both Th1 and Th2 components, which is also a limitation of this paradigm overall. Nonetheless, it is important to note that a fine line exists between protection and inflammation, within the context of trying to fully understand how the immune system works so intricately [73]. Ultimately, next steps in our research program will hopefully account for more frequent assessments in a larger number of subjects to help us more thoroughly explain and understand how APMC impacts immune function and at least somewhat attempts to rebalance the Th1/Th2 ratio.

Conclusions

AD is a rapidly expanding sad conundrum for patients, their caregivers and families, and the medical system, seemingly without any noticeable abatement. AD continues to lack consensus about its etiology and how to accurately identify it. One thing that is known about the disease is that neurodegeneration comes with an array of inflammation and dysregulated immune function. Keeping inflammation in check and the immune system properly modulated are just as important for AD patients as for any other patient population.

Our current analysis was conducted to document for the first time to our knowledge the Th1/Th2 ratio in AD patients, how it compared to a sample of healthy people, how it changed in response to taking APMC for 12 months, and how it changed in relationship to the change in cognitive function over

that time. Interestingly, we have reported remarkably high ratios in the six Th1/Th2 values compared to healthy adults, suggesting that our sample of AD patients had a Th1 dominant immune system profile. The Th1/Th2 ratio favorably rebalanced in five of six values in response to APMC treatment, and multiple assessments of cognition improved over the 12-month intervention in relation to the changes in the Th1/Th2 values. Since the Th1/Th2 paradigm emphasizes a balance between protection and inflammation, our results demonstrate that our AD patients are significantly Th1 dominant, which may help to explain the severity of their condition. APMC seems to have helped shift five of the six ratios more toward a Th2 phenotype, as the Th2 cells help to at least partially downregulate Th1 pro-inflammatory activation.

Despite our interesting findings, questions remain as to their significance in the greater context of explaining the immune system/central nervous system crosstalk. Did the Th1 dominance play a role in leading to either AD itself and/or greater severity of disease, as is noted in our sample, or did the progression of AD cause the rise in the Th1 dominance? Were underlying infections or other comorbidities that were unaccounted for in our protocol contribute to the Th1 dominance? Were unknown polypharmacy effects contributing to the results we documented here as well? These questions require additional research to help explain the high Th1/Th2 values found in the current study.

Meanwhile, the prevalence of AD and its associated financial costs are a significant drain on an already overburdened U.S. health system, are spiraling out of control, and are predicted to worsen due to the aging U.S. population. Unfortunately, the conventional approach to treatment continues to provide little efficacy for these patients. Thus, any intervention that demonstrates promise for improving the condition of the AD patient is urgently needed. Once again, we have shown that APMC has the capacity to effectively improve immune function, in this case by rebalancing the Th1/Th2 ratio. Further, this rebalancing of the immune system was related to improved cognitive function, which is perhaps the most important clinical indicator for the average AD patient's quality of life. APMC is safe, has negligible risk of adverse side effects, and can help to offset the burden of this tragic disease to the patient, the family, and society through its immunomodulatory activity that impacts clinical outcomes. We have documented, perhaps for the first time, in patients with moderate-to-severe AD that the Th1/Th2 ratio can be improved and rebalanced through the use of APMC, a well-studied dietary supplement. This novel information is important for stimulating new research in dietary supplementation and immune function in AD, a patient population desperate for help.

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CONFLICT OF INTEREST

H. Reginald McDaniel is the owner of Wellness Quest, LLC and the manufacturer of the New Eden dietary supplement. John E. Lewis is the owner of Morris Formulations, LLC and the manufacturer of the Daily Brain Care dietary supplement. Judi M. Woolger and Sher Ali Khan have no conflicts of interest to report.

DATA AVAILABILITY

The data supporting the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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