Effect of acupuncture on gut microbiota in participants with subjective cognitive decline
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Abstract
A close relationship has recently been described between subjective cognitive decline (SCD) and gut microbiota disorders. Herein, we aim to investigate the effect of electroacupuncture (EA) on gut microbiota in participants with SCD.

We conducted a study of 60 participants with SCD. Sixty participants were allocated to either EA group (n=30) or sham acupuncture group (n=30). Both groups received 24 sessions of real acupuncture treatment or identical treatment sessions using the placebo needle. Global cognitive change based on a comprehensive neuropsychological test battery was evaluated to detect the clinical efficacy of acupuncture treatment at the baseline and the end of treatment. Faecal microbial analyses were carried out after collecting stools at T\textsubscript{0} and T\textsubscript{12} weeks. Microbiomes were analyzed by 16S ribosomal RNA gene sequencing. Correlation analyses were performed to investigate the relationships between the changes in gut microbiota and symptom improvement.

Age is a particularly important factor leading to the severity of dementia. Compared with sham acupuncture group, the number of Escherichia–Shigella in EA group decreased after treatment. The number of Escherichia–Shigella in EA group decreased after treatment compared with EA group before treatment. Bifidobacterium is positively correlated with clinical efficacy Z-score and Montreal Cognitive Assessment Scale (both P < .005).

Acupuncture could improve global cognitive change among SCD participants by regulating the intestinal flora. Dysbiosis was found in the gut microbiome in SCD and partially relieved by acupuncture. Our study suggests that gut microbiota could be a potential therapeutic target and diagnostic biomarker for SCD.

Abbreviations: DU20 = Baihui, DU24 = Shenting, ST36 = Zusanli, EA = electroacupuncture, MCI = mild cognitive impairment, OTU = operational taxonomic unit, rRNA = ribosomal RNA, SA = sham acupuncture, SCD = subjective cognitive decline, SCFA = short-chain fatty acid.

Keywords: acupuncture, effect, gut microbiota, subjective cognitive decline

1. Introduction
With an ageing population, concerns about cognitive decline are becoming an increasingly relevant topic that arises during medical consultations. Concerns about cognitive decline can be associated with different objective levels of cognitive and functional impairment, which are revealed by clinical and neuropsychological examinations. Dementia is the most severe level of impairment and is defined by cognitive deficits that impair daily functioning and lead to loss of independence.\textsuperscript{[1,2]} Similar to dementia, mild cognitive impairment (MCI) is also characterized by objective cognitive impairment. However, by contrast with dementia, the day-to-day functioning of individuals with MCI remains largely intact and independence is preserved.\textsuperscript{[3]} Some individuals experience a subjective decrease in cognitive function, but cognitive performance by neuropsychological testing and in daily functioning shows no evidence of objective cognitive impairment. In clinical practice, these individuals are generally considered healthy. Regardless of the absence of evidence for objective cognitive impairment, the subjective decline in cognitive function experienced by individuals might become increasingly important for clinicians, because the number of individuals with such concerns who seek medical help and advice is growing. In 2014, the term subjective cognitive decline (SCD) was conceived by researchers to describe this condition, which has received increasing attention because of evidence of its association with an increased risk of future objective cognitive decline, especially among maternal.\textsuperscript{[4–6]}

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Acupuncture, as a nondrug intervention, has been widely used in patients with cognitive impairment.\textsuperscript{7,8} Many clinical studies have provided evidence that acupuncture is beneficial for treatment of dementia or MCI.\textsuperscript{9-11} Animal studies have also shown that acupuncture has dual regulatory functions on the p38 MAPK pathway, and can regulate cytokines, ion channels, scar proteins and transcription factors (including TRPV1/4, Nav, BDNF, NADMR1, Bcl-2, NF-xB, Bax, glutamate receptors, and transporters, etc), produce antiapoptosis and anti-inflammatory effects, regulate cell communication, remodeling, regeneration, and gene expression, reduce the deposition of Aβ in the brain and improve learning and memory of MCI patients’ ability.\textsuperscript{12-14} It was found that acupuncture can improve the diversity of gut microbiome and the content of beneficial flora through different acupoints, so as to achieve the purpose of adjusting gut microbiome. It is also recognized that the gut microbiome can have a profound influence on neuroinflammatory and cognitive ageing.\textsuperscript{15} At present, there are few reports on whether acupuncture can improve cognitive decline and reduce inflammatory response by regulating the amount and structure of intestinal flora in SCD patients. In-depth discussion in this field has certain practical significance to reveal the internal mechanism of acupuncture to improve function and memory of SCD.

2. Method

2.1. Study cohort

We first conducted a placebo-controlled study with 2 parallel groups involving elderly adults. The study was designed to examine the effect and mechanism of acupuncture treatment on gut microbiome before and after 12 weeks intervention period. Facial samples were collected twice before and after treatment by signing an informed consent. Sixty SCD participants were enrolled in a 1:1 allocation ratio to electroacupuncture (EA) group or sham acupuncture (SA) group from Dongcheng, Fengtai, and Shunyi District in Beijing. Participants were recruited from the community through media, outpatient and poster paper advertisements in those districts (January 2019–December 2019). The inclusion criteria were as follows: age from 55 to 75; native Chinese speakers who were right-handed and had at least 8 years of education; self-reported persistent memory decline compared with a previous normal status within the last 5 years, which was confirmed by caregivers; no or minimal impairment in activities of daily living. The exclusion criteria included history of alcohol or drug abuse/addiction; presence of serious heart, kidney, liver, gastrointestinal, infectious, endocrine disease or cancer; presence of significant psychiatric history (eg, bipolar disorder, schizophrenia) and/or severe anxiety and depression (a score of >24 on the Hamilton depression rating scale or a score of >29 on Hamilton anxiety scale); presence of a positive neurologic history (eg, traumatic brain injury, stroke, Parkinson disease, multiple sclerosis). Participants were also not included if they had had acupuncture treatment that affected cognitive function in the preceding month.

After a brief telephone screening, participants were scheduled to visit one of the 3 participating districts to sign an informed consent statement. All those who met the inclusion criteria received a study information sheet including the design, procedure, benefits, and risks of the study. Eligibility to participate was determined initially by the research investigators at each district involved. They were responsible for completion of the medical assessment and to check eligibility criteria. Eligibility data were entered on a secure online database and were monitored centrally before confirmation of study participation. The subject’s demographic data and medical history were obtained at baseline.

Prior to the study, the study process was explained to participants during recruitment. Participants were informed that participation in the trial was absolutely voluntary and that they could withdraw from the trial at any time. In the event of their withdrawal, collected data would not be deleted and were used in the final analyses. Otherwise, research investigators had to comply with Good Clinical Practice guidelines in the study. No participant was recruited without full, written informed consent being first obtained. The study was approved by the medical ethical review committee of Beijing Hospital of Traditional Chinese Medicine affiliated to Capital Medical University (2018BL-061-02) and was prospectively registered at Clinical-Trials.gov, prior to recruitment of the first participant.

2.2. Study treatment

All acupuncturists participant in the study had Chinese medicine practitioner licenses. They have at least 3 years clinical experience. Andi brand disposable, sterile steel needles (size 0.30 × 40 mm, manufactured by Suzhou Medical Appliance in Jiangsu, China) were used. Pragmatic placebo needles (size 0.30 × 25 mm) were also used.

2.2.1. EA group. For the EA group (Fig. 1), acupuncture needles were placed at acupoints Baihui (DU20), Shenting (DU24), Fengfu, Fengchi, Danzhong, Zhongwan, Qihai, Neiguan, Tongli, Xuehai, Zusanli (ST36), Zhaohai, Xinshu, and Yixi. After skin disinfection in patient in the supine position, the adhesive pads were pasted on the acupoints surface except for DU20 and DU24. Then, the acupuncture needles were inserted through the adhesive pads approximately 5 to 15 mm into the skin depending on the location of the needle. Manual acupuncture by acupuncturists using a small, equal manipulations of twirling, lifting, and thrusting performed on all needles to reach “Deqi”. The patients felt the “Deqi” sensation, such as soreness, numbness, distention, heaviness, and other sensations. Paired electrodes from the EA apparatus (HANS-200A acupuncture nerve stimulator, Nanjing Jisheng Medical Co, Ltd. production, density wave with frequency of 2/100 Hz) were attached to the needle holders of the DU20 and DU24 with alligator clips. The fixed current intensity was uniformly 0.2 mA. The needles were extracted after 20 minutes for each treatment. The acupoints of Fengfu, Xinshu, and Yixi achieved “Deqi” in participants with sitting position without retaining needle.

2.2.2. SA group. Twelve nonacupoints that are separate from conventional acupoints or meridians were used for the SA group. The location of sham acupoints is showed in Table 1. Patients received noninertive acupuncture using the pragmatic placebo needles (Huatu brand, size 0.35 × 25 mm). As same as acupuncture group, the adhesive pads were firstly pasted on the sham acupoints surface except for sham acupoint 1. The pragmatic placebo needles with a blunt tip were placed on the adhesive pads. In order to minimize the physiological effect, acupuncturists were instructed to lightly place the placebo needle on the adhesive pads with no manipulation. The acupuncture needle was inserted to a shallow depth at the sham acupoint 1,
which did not penetrate below the skin, and needle manipulation for “Deqi”. Sham acupoint 11 and Sham acupoint 12 were inserted with placebo needles without retaining needle. Paired electrodes were attached the needle holders of the bilateral sham acupoint 2 but with no electricity output.

2.3. Outcome measures

2.3.1. Clinical outcome assessments. Global cognitive function based on a composite score were used to evaluate the clinical efficacy of acupuncture treatment at baseline, and at the end of the treatment. It was computed by averaging Z-scores from a comprehensive neuropsychological test battery that included 6 tests. Animal fluency test that assessed the language and executive function by examining categorical verbal fluency. Trail making test part A and B examined graphomotor speed, attention, and executive function. Digit symbol substitution test was used to assess processing speed, attention, and concentration. Stroop color word test was a classic instrument for the assessment of selective attention, cognitive flexibility, cognitive inhibition, and information processing speed. Auditory verbal learning test was a powerful neuropsychological test to assess episodic memory.

All patients did a SCD questionnaire to assess their degree of SCD. The Santa Barbara Sense of Direction Scale was used to measure the ability in facial recognition and direction.

2.4. Sample collection and DNA extraction

Faecal samples were collected at the hospital and frozen at −80°C within 3 hours after sampling. DNA extraction was performed using a QIAamp Fast DNA Stool Mini Kit (Qiagen, CA). The concentration of bacterial DNA was measured using Nano drop 2000 (Thermo Scientific, MA). Demographics and clinical variables were collected during the clinic visits.

| Sham Acupoint | Location |
|---------------|----------|
| Sham acupoint 1 | Midpoint of Shuaigu (GB8) and Touwei (ST8) |
| Sham acupoint 2 | Midpoint of Touwei (ST8) and Yangbai (GB14) |
| Sham acupoint 3 | Midpoint between Tianyou (SJ16) and Tianrong (SI17) |
| Sham acupoint 4 | 4 cun above the umbilicus and 1 cun right of the umbilical midline |
| Sham acupoint 5 | 2 cun below the umbilicus and 1 cun right of the umbilical midline |
| Sham acupoint 6 | 1 cun outside the point 1/4 of the line between Shenmen (HT7) and Shaohai (HT3) |
| Sham acupoint 7 | 1 cun outside the midpoint of Shenmen (HT7) and Shaohai (HT3) |
| Sham acupoint 8 | 6 cun above mediosuperior border of the patella |
| Sham acupoint 9 | 3 cun below the Yanglingquan (GB34) and in the middle of the gallbladder and bladder channels |
| Sham acupoint 10 | Midpoint between Jixi (ST 41) and Quan (GB40) |
| Sham acupoint 11 | 2 cun from the lower border of the spine of the fifth thoracic vertebra |
| Sham acupoint 12 | 2 cun from the lower border of the spine of the sixth thoracic vertebra |
2.5. 16S ribosomal RNA gene sequencing

The V3 to V4 region of the bacteria’s 16S ribosomal RNA (rRNA) gene was amplified by PCR with barcode-indexed primers (338F and 806R), using FastPfu Polymerase. Amplicons were then purified by gel extraction (AxyPrep DNA Gel Extraction Kit, Axygen Biosciences, Union City, CA) and were quantified using QuantiFluor-ST (Promega, WI). The purified amplicons were pooled in equimolar concentrations, and paired-end sequencing was performed using an Illumina MiSeq instrument (Illumina, San Diego, CA).

2.6. Microbial analysis

The 16S rRNA sequencing data were processed using the Quantitative Insights into Microbial Ecology platform (V.1.9.1). Sequencing reads were demultiplexed and filtered. Operational taxonomic units (OTUs) were picked at 97% similarity cutoff, and the identified taxonomy was then aligned using the Green genes database (V.13.8). Chimeric sequences were identified and deleted. OTUs with a number of sequences < 0.005% of the total number of sequences were removed from the OTU table. After filtering, an average of 34,661 reads per sample was obtained (min: 23,994; max: 42,940). In addition, rarefaction was performed on the OTU table to prevent methodological artefacts arising from varying sequencing depth. Alpha-diversity was measured by species richness and richness percentage. Mean-between-group differences and two-sided 95% confidence interval are also presented to assess superiority.

All statistical analyses were performed using R packages (version 4.0.3; R Foundation for Statistical Computing, Vienna, Austria). For the comparison of continuous variables, Mann–Whitney U test (Kruskal–Wallis test for more than 2 groups) and one-way ANOVA test were used. For correlation analysis, Spearman rank test was performed. Multiple hypothesis tests were adjusted using Benjamin and Hochberg false discovery rate, and significant association was considered below a false discovery rate threshold of 0.05.

3. Result

Characteristics of treatment groups at baseline are presented in Table 2. There were no significant differences among the treatment conditions on any baseline demographic and clinical characteristics (all \( P > 0.05 \)).

3.1. Primary outcome in SCD patients at the end of week 12

After 12 weeks of treatment, compared with the SA group, the comprehensive Z-score of the acupuncture treatment group was significantly improved in Table 3. Analysis showed a statistically significant difference in Z-score between the 2 groups (\( P < 0.05 \)).

3.2. Secondary outcomes in SCD patients

After 12 weeks of treatment in Table 3, Montreal Cognitive Assessment Scale (MoCA) scale score of acupuncture treatment group was significantly improved (\( P = 0.035 \)), and the difference was statistically significant. The short-term recall of auditory verbal learning test in acupuncture group was improved compared with that in sham group. However, SCD questionnaire and other cognitive scales did not significantly improve patients’ subjective complaints. Compared with SA group, the scores of Pittsburgh Sleep Scale and Geriatric Depression Scale...
were not significantly improved after acupuncture treatment neither (all $P > .05$).

Adverse events were uncommon and did not occur more frequently in either group. There was no significant bleeding in either group.

During this trial, no participants took rescue medicine in both groups.

### 3.3. Changes of faecal microbial diversities in SCD

As shown in the Figure 2, the Sob index calculation at the genus level showed that there was no significant difference in Alpha-diversity of intestinal flora between SCD groups. The composition analysis chart of intestinal bacteria group manager in the groups of SCD participants shows that the horizontal axis represents the first principal component (contribution rate is 15.77%) and the vertical axis represents the second principal component (contribution rate is 9.21%). By comparing the distance of each point, SCD participants were arranged and clustered in the PCA diagram, indicating that there were obvious differences between the intestinal bacteria of SCD participants in pre–post-treatment.

### 3.4. Correlation analysis of environmental factors

According to Figure 3, compared with healthy subjects in Phylum level, the ranking of environmental factors affecting the incidence of SCD participants was: A (age), >D (duration), >X (gender), >T (body mass index). The horizontal axis represents the first principal component (contribution rate is 2.52%) and

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**Table 3**

Comparisons of acupuncture treatment group and sham acupuncture group.

| Variables       | Acupuncture group [post–pre] (95% CI)          | Sham acupuncture group [post–pre] (95% CI)          | $P$ (group × time) |
|-----------------|-----------------------------------------------|-----------------------------------------------|-------------------|
| Primary outcome | Composite Z score 0.314 (0.195 to 0.433)       | 0.135 (0.022 to 0.248)                         | .035              |
| Secondary outcomes | AVLT-S 1.516 (0.847 to 2.185)                 | 0.500 (–0.336 to 1.336)                        | .045              |
|                 | AVLT-L 1.774 (1.036 to 2.512)                 | 1.233 (0.363 to 2.103)                         | .300              |
|                 | AFT –0.613 (–2.054 to 0.829)                  | 0.433 (–0.973 to 1.840)                        | .264              |
|                 | BNT 1.161 (0.498 to 1.825)                    | 0.333 (–0.619 to 1.286)                        | .169              |
|                 | TMT-A –3.893 (–11.034 to 3.357)               | –3.467 (–11.529 to 4.586)                      | .946              |
|                 | TMT-B 0.097 (–16.040 to 16.233)               | –1.667 (–20.905 to 17.571)                     | .893              |
|                 | SCWT-C –8.780 (–13.5581 to –3.839)           | –4.067 (–9.483 to 1.350)                       | .178              |
|                 | DSST 1.323 (–0.568 to 3.213)                 | 1.733 (–1.078 to 4.545)                        | .524              |
|                 | DST 0.484 (–0.136 to 1.104)                  | 0.200 (–0.461 to 0.861)                        | .572              |
|                 | CDT 2.284 (0.068 to 4.900)                   | –0.233 (–1.108 to 0.215)                      | .068              |
|                 | SCDQ –1.677 (–2.345 to –1.010)               | –0.983 (–1.729 to –0.237)                      | .135              |
|                 | MoCA 2.290 (1.406 to 3.175)                  | 0.000 (–1.286 to 1.286)                        | .002              |
|                 | MMSE 0.774 (0.134 to 1.415)                  | 0.533 (–0.205 to 1.272)                        | .536              |
|                 | GDS –0.129 (–0.783 to 0.525)                 | –0.433 (–1.081 to 0.215)                      | .520              |
|                 | PSQI –0.226 (–1.686 to 1.234)                | –0.333 (–1.909 to 1.243)                      | .943              |

AFT = animal fluency test; AVLT = auditory verbal learning test; BNT = Boston Naming Test; CDT = Clock Drawing Test; DSST = digit symbol substitution test; DST = digital span test; GDS = Geriatric Depression Scale; MMSE = mini-mental state examination; PSQI = Pittsburgh Sleep Quality Index; SBDSDS = Santa Barbara Sense of Direction Scale; SCDQ = Subjective Cognitive Decline Questionnaire; SCWT = Stroop color word test; TMT = trail making test.

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Figure 2. Alpha-diversity and Beta-diversity in SCD patients. SCD = subjective cognitive decline.
the vertical axis represents the second principal component (contribution rate is 1.23%) on RDA/CCA: Redundancy analysis/Canonical correspondence analysis analysis. We know that the main environmental factor affecting the incidence of SCD participants was the age.

3.5. Electroacupuncture treatment partially ameliorates gut dysbiosis of SCD

After treatment, the amount of *Escherichia–Shigella* (*P* = .049) in acupuncture group had statistically decreased. No differences were observed in SA group (all *P* > .05; Fig. 4A and B). Compared with SA on the genus level, the amount of *Escherichia–Shigella* decreased after acupuncture treatment (*P* = .022; Fig. 4C).

3.6. Correlations between the gut microbe and SCD clinical indices

At the genus level, *Bifidobacterium* was positively correlated with Z-score of clinical efficacy (*P* = .019), while *Bifidobacterium* was
positively correlated with clinical efficacy MoCA ($P = .044$). *Collinsella* was positively correlated with MoCA ($P = .022$; Fig. 5).

### 3.7. Kyoto Encyclopedia of Genes and Genomes pathway analysis

Kyoto Encyclopedia of Genes and Genomes function prediction shows that the top 10 metabolic functions of SCD patients were biosynthesis of amino acids, ABC transporters, carbon metabolism, ribosome, purine metabolism, pyrimidine metabolism and quorum sensing, two-component system, starch and sucrose metabolism, amino sugar and nucleotide sugar metabolism, amino sugar and nucleotide sugar metabolism. The gene abundance after acupuncture in 2 groups was higher than that before acupuncture, and the gene abundance after acupuncture in 2 groups was higher than that after SA (Fig. 6).

### 4. Discussion

In this randomized clinical trial, the results suggested that EA was more effective than SA for pain associated with SCD in 12 weeks. SCD, as the initial phase of AD, could serve as a window of opportunity for interventions at early disease stages. This study was planned as a randomized, assessor-blind, placebo-controlled trial to evaluate the efficacy and mechanism of acupuncture treatment on SCD compared with the SA group. This trial is also the first study to investigate the effect of acupuncture on SCD patients through gut microbiota.

In our current study, we applied a 16S rRNA sequencing approach to a unique SCD cohort in addition to a study of a subset of participants analyzed before and after acupuncture therapy, demonstrating that SCD was associated with altered composition and function of gut microbiota. In the prospective study, 3 months of acupuncture use was found to partially mitigate the microbial dysbiosis. The gut microbiome of SCD reflected a significant shift in the overall microbial diversity. No obvious differences were found within-individual or between-individual on Alpha-diversity in study of microbiomes due to a small cohort of SCD participants whereas differences could be found on Beta-diversity, which was different from before treatment. Therefore, our findings might optimize a large cohort to ensure adequate statistical power for capturing microbial diversities.

Interestingly, the SCD-enriched genera were relatively rare in the normal human gut. It is recognized that potentially pathogenic bacteria that are normally present in low abundance can thrive and contribute to inflammation or autoimmunity under inflammatory conditions. An uncommon genus in the family of *Proteobacteria* revealed the most significant association with SCD patients, especially enrichment of *Escherichia-Shigella* abundance could be found in SCD patients. However, the major genera in the samples of patients with SCD after 12 weeks of acupuncture treatment belong to *Firmicutes* family, indicating that gut microbiota signature could be used to discriminate pre–postacupuncture treatment in SCD patients.

According to our study, age is a particularly important factor leading to the severity of dementia. In aged people, evidence has been provided that reduction of butyrate levels is depending on the decreased number of *Faecalibacterium prausnitzii*, *Eubacterium hallii*, and *Eubacterium rectal* and the increased number of *Escherichia*. Obesity-mediated gastrointestinal-microbiome changes are postulated to affect low-grade systemic and local inflammation in aging. Our observation is in line with the previous study that *Bifidobacterium* abundance was positively associated with Z-score and MoCA in SCD patients. This leads to believe that *Bifidobacterium* might also be involved in SCD and other inflammation disorders. Many anaerobic intestinal microbiota such as species in *Ruminococcus*, *Bacteroides*, *Blautia*, *Bifidobacterium*, *Faecalibacterium*, and *Collinsella* are known to produce short-chain fatty acids (SCFA) by fermentation of dietary fibres. SCFA is known to exert a beneficial effect on health through the anti-inflammatory effects. Decreased production of SCFA by microbiota raises luminal oxygen concentration in mice, resulting in the expansion of facultative anaerobes. Acupuncture is considered as a secure and powerful tool for improving memory.

![Figure 5. Correlations between the gut microbe and SCD clinical indices. SCD = subjective cognitive decline.](image-url)
the possibility that improvements in EA may through shaping the structure of gut microbiome, especially through reducing *Escherichia—Shigella* abundance, thereby achieving rapid reduction in signs and symptoms of SCD. Moreover, mechanisms on regulating intestinal flora may be explained that stimulation of EA increases the content beneficial bacterium of *Ruminococcus*, *Collinsella*, *Bacteroides*, *Bifidobacterium*, and *Agathobacter*, which microbiota have been shown to produce different amounts and profiles of SCFA from the same carbohydrate substrates. SCFA-producing bacteria with elevated fecal SCFA concentrations may promote the energy intake from fibers, inhibit opportunistic pathogens and protect the hosts against inflammation and colonic diseases.

According to TCM theory, acupuncture produces therapeutic effects by the retention of needles at acupoints through acquiring “Deqi” manually. Deqi is a specific needle sensation, referring to the response to stimulations such as the thrusting, lifting, or rotating of the needle after insertion. It has been asserted to be a criterion to determine the appropriateness of acupuncture stimulation. In the past decade, studies reported the potential of acupuncture at ST36 for infectious diseases due to its numerous effects, such as anti-inflammatory, improving micro-circulatory disturbance and accelerating the recovery of various gastrointestinal disorders. This study suggested that acupuncture at ST36 may increase the number of beneficial bacteria. After beneficial adjustment, intestinal flora improved intestinal barrier function, reduces inflammation and oxidative stress, and then inhibited the occurrence of chronic inflammation, which indirectly reduced the degree of cognitive decline.

The strength in our study is that we assessed patients with SCD on multiple levels, including comprehensive neuropsychological tests, functional brain alterations, cerebrovascular risk factors, and gut microbiome. This comprehensive assessment was used to identify possible biomarkers involved in the effects of acupuncture in SCD.
Although our investigations attempt to provide a comprehensive insight into the potential contribution of the gut microbiome in SCD, there are several limitations to be addressed in future studies. First, we were not able to observe the differentiations between SCD patients and health controls, however, we only compare difference between pre and postacupuncture treatment. Second, our pathway analysis was based on the inferred metagenome from 16S rRNA sequence. Although inference of a metagenome approach (PICRUSt) has been commonly used in 16S rRNA studies, short-gun sequencing for metagenomics and meta transcriptomics may reveal more accurate microbial community composition and function. Third, the small sample size in this exploratory trial increases the possibility of a type II error (ie, a real effect of acupuncture being missed because of insufficient power). For future trials, sample size estimation could be calculated, for example, using PASS software (NCSS_PASS2011), based on the data derived from this trial. Fourth, we did not validate the characteristics of the microbiota in an independent set of patients with SCD, and further investigation of insufficiency in this exploratory trial increases the possibility of a randomized controlled trial. 

**5. Conclusion**

EA may improve the mild cognitive level of dementia patients by reducing the number of pathogenic bacteria (Escherichia–Shigella), and age may be the primary influencing factor in dementia patients.

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