G-395A polymorphism in the promoter region of the KLOTHO gene associates with reduced cognitive impairment among the oldest old

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Abstract This study aimed to examine the possible association between G-395A polymorphism in the promoter region of the KLOTHO gene and cognitive impairment among Chinese nonagenarians and centenarians. This study is a secondary analysis of the Project of Longevity and Aging in Dujiangyan (PLAD) study. Community-dwelling Chinese people aged 90 years or older were included. G-395A (rs1207568) genotyping in the promoter region of the KLOTHO gene was performed using the TaqMan allelic discrimination assay. Cognitive function was assessed with the mini-mental status examination (MMSE). A total of 706 participants (68.0 % female; mean age 93.5 ± 3.6 years) were included. The KLOTHO G-395A polymorphism genotype frequencies for the whole sample were 2.0 % AA, 30.3 % GA, and 67.7 % GG. The GG genotype frequencies for the cognitive impairment and control groups were 70.2 and 62.7 %, respectively. Cognitive impairment prevalence was significantly lower in the GA+AA group than in the GG genotype group (61.4 vs. 69.0 %, $p=0.044$). GA+AA genotype subjects had a significantly lower risk of cognitive impairment (odds ratio 0.66; 95 % confidence interval 0.44 to 0.98) than GG genotype individuals after adjusting for age, gender, and other relevant risk factors. KLOTHO G-395A polymorphism associates with reduced cognitive impairment in a sample of Chinese nonagenarians and centenarians.

Keywords KLOTHO gene · Cognitive impairment · Single nucleotide polymorphism

Introduction

Aging, which is defined as the age-related decline in physiological functions (Wang and Sun 2009), is inevitable in multicellular organisms. Genetic influences are critical factors in the aging process. KLOTHO is a new anti-aging gene discovered by Kuro-o and colleagues in 1997 (Kuro-o et al. 1997). KLOTHO-deficient mutant mice have shortened life spans and exhibit many phenotypes resembling those found in human aging (e.g., arteriosclerosis, osteoporosis, and hypoactivity) (Kuro-o et al. 1997).

Cognitive deterioration is one of the most common consequences of aging in animals and humans (Deary et al. 2005). The prevalence of cognitive impairment increases with advancing age and difficulties in daily living activities and reduces in the quality of life of elderly adults (Ward et al. 2012). The relationship between the KLOTHO gene and cognitive impairment is an interesting line of research. A previous study found that KLOTHO-deficient mice exhibited memory impairment and hippocampal damage earlier than wild-type mice (Nagai et al. 2003). Recent studies showed that the KLOTHO gene, KL-VS, is associated with improved cognition, and increases in klotho expression can decrease premature mortality and network dysfunction (key aspects of Alzheimer's disease) in human amyloid precursor protein transgenic mice (Dubal et al. 2014, 2015). The human KLOTHO protein shares

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86 % identity with the mouse protein (Nabeshima 2002). The serum level KLOTHO protein also declines with age in humans (Xiao et al. 2004). Dubal and colleagues also found that KLOTHO KL-VS heterozygosity was associated with better executive function and a greater right dorsolateral prefrontal cortex volume in mice (Yokoyama et al. 2015). Based on these findings, we hypothesize that the KLOTHO gene is significantly correlated with cognitive impairment in humans.

The KLOTHO gene is located on chromosome 13q12. More than ten mutations or single nucleotide polymorphisms (SNPs) have been found in the human KLOTHO gene, and some of these SNPs are associated with aging-related disease (Wang and Sun 2009). For example, G-395A (rs1207568) is one SNP of the KLOTHO gene located in the promoter region. GG represents the wild-type genotype, whereas GA+AA represents the mutant (A is the mutant allele) for the G-395A polymorphism (Wang et al. 2010). G-395A polymorphism has been reported to be associated with age-related diseases. For example, the A allele increased the risk of essential hypertension (Wang et al. 2010) and cardioembolic stroke (Kim et al. 2006), whereas the G allele increased the risk of osteoarthritis (Tsezou et al. 2008; Zhang et al. 2007). The associations of the KLOTHO gene allele with bone mineral density (Kawano et al. 2002; Yamada et al. 2005), coronary artery disease (Imamura et al. 2006; Rhee et al. 2006), and cognitive impairment (Shimokata et al. 2006) remain controversial. However, the relationship between G-395A polymorphism and cognitive impairment has only been studied in humans aged 60–79 years old. Furthermore, cognitive function differences assessed using the mini-mental status examination (MMSE) have not been significant (Shimokata et al. 2006). Therefore, the relationship between G-395A polymorphism and cognitive impairment remains unclear in very old populations (80+ years old).

In 2005, our team performed a cross-sectional study of 870 adults aged 90 years and older. Most participants had lived in their homelands for their entire lifetime and had never been exposed to immigrants (Yue et al. 2010; Zhou et al. 2012). Thus, the participants in this study were stable and represented the Chinese population well. The prevalence of cognitive impairment in this population is approximately 66 %. This high rate allowed us to examine the relationship between the KLOTHO G-395A SNP and cognitive impairment. Thus, we detected this relationship in this specific cohort.

Material and methods

Study population

Data were collected from the Project of Longevity and Aging in Dujiangyan (PLAD), a cross-sectional study conducted in the small southwestern Chinese town of Dujiangyan in April 2005. The methods of the PLAD study have been previously reported (Yue et al. 2010; Zhou et al. 2012). Briefly, a total of 1115 community members aged 90 years and older were screened via face-to-face interviews. Of these community members, 870 agreed to participate in the study. Trained medical staff then collected anthropometric measurements and blood samples from all participants in their homes or community centers. Written informed consent was obtained from all subjects or their legal proxies. The Research Ethics Committee of Sichuan University approved the study protocol. Subjects without blood samples, MMSE scores (129 cases), or the results of relevant clinical covariates (35 cases) were excluded from the current analyses, which resulted in a study sample of 706 participants (226 males, 480 females).

KLOTHO genotype analysis

Genomic DNA was isolated from whole blood samples using commercial DNA isolation kits from QIAGEN (Chatsworth, CA, USA) based on standard procedures. The KLOTHO promoter region G-395A (rs1207568) was genotyped using the TaqMan allelic discrimination assay, as previously described by Wang and colleagues (Wang et al. 2010). The following primers and probes (Takara, Dalian, China) were used:

- Forward primer 5'-TAGGGCCCGGCAGGAT-3'
- Reverse primer 5'-CCTGGAGCGGCTTCGTC-3'
- Probe A 5'-(FAM) CCCCAAGTCGGGAAA AGTTGGTC (TAMRA)-3'
- Probe G 5'-(HEX) CCCCAAGTCGGGGA AAGTTGGTC (TAMRA)-3'

The PCR reaction was performed in 20 µl reaction volumes containing 10 µl of Premix Ex Taq, 1.5 µl of each primer, 0.5 µl of probe A, 1 µl of probe G, 1 µl of genomic DNA, and 4.25 µl of sterile, double-distilled water. The reaction conditions were an initial denaturation step at 95 °C for 30 s, 40 cycles of denaturation at 95 °C for 5 s, and annealing at 60 °C for 30 s. This

reaction was performed and analyzed on a Thermal Cycler Dice Real Time System (Takara, Dalian, China). We also randomly selected 10 % of the samples for forward and reverse sequencing to confirm promoter region KLOTHO G-395A genotype. The results were identical to those of the TaqMan allelic discrimination assay.

Evaluation of cognitive function

Cognitive function was assessed with the 30-item MMSE. This widely used test evaluates orientation, attention, calculation, language, and recall. Most items of the MMSE rely on visual and auditory abilities (Holtzman et al. 1995), which may be a challenge in very old populations. Specifically, 100 participants (28 men and 72 women) in our very old populations did not complete the MMSE test due to visual or hearing impairment. To address this problem, we excluded these subjects when the data were analyzed.

The cutoff point of MMSE is highly variable and ranges from 17/30 to 29/30 in Asian people (Rosli et al. 2015). Because the majority of participants were illiterate in our study, the subjects were categorized into two groups based on these scores: cognitive impairment (scores of 0–18) and free of cognitive impairment (scores of 19–30). This cutoff point has been shown to be 80 to 90 % sensitive and 80 to 100 % specific for cognitive impairment diagnosis in the Chinese population (Cui et al. 2011; Katzman et al. 1988; Tombaugh and McIntyre 1992; Zhu et al. 2006). MMSE administrators were trained by experienced geriatricians to ensure high-quality assessment and methodological reliability. The following contents were included: (1) reviewing MMSE procedures and grading system, as outlined in a short booklet and video; (2) observing tutors administering the MMSE to standardized patients; and (3) receiving supervision while administering the MMSE to standardized patients.

Evaluation of potential confounders

The following information were included: age, gender, educational levels (illiteracy, primary school, or secondary school and advanced), cigarette smoking status (smoking or not), alcohol drinking status (drinking or not), and exercise habits (yes or no). Height and weight were measured using a wall-mounted stadiometer and

digital floor scale to the nearest 0.1 cm and 0.1 kg, respectively. The body mass index (BMI) was calculated by dividing weight in kilograms by the height in meters squared (kg/m^2). The waist circumference (WC) was measured to the nearest 0.1 cm using a steel measuring tape. This measurement was performed in a standing position on naked skin at the end of light exhalation. Diabetes was diagnosed according to the American Diabetes Association criteria (Mellitus 2003) or if the subjects had diabetes history and were currently using antidiabetic agents. Hypertension was diagnosed according to the JNC VII criteria (Chobanian et al. 2003) and characterized by a systolic BP (SBP) of ≥ 140 mmHg and/or a diastolic BP (DBP) of ≥ 90 mmHg. Hypertension was also diagnosed if subjects had confirmed clinical diagnoses of hypertension according to formal medical records and were currently taking anti-hypertension medications. A history of stroke or coronary artery disease (CAD), which may be linked to cognitive impairment, was also included in the analyses. Laboratory analyses included the total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and serum uric acid (SUA) levels.

Statistical analysis

All statistical analyses were performed using the SPSS for Windows software package, version 11.5 (SPSS Inc., Chicago, IL, USA). Two-tailed p values of <0.05 were considered to indicate significant differences. A chi-square test was used to evaluate the allelic and genotypic frequencies calculated from the observed genotypic counts. A chi-square test and an unpaired Student's t test were used for categorical and continuous variables, respectively. The baseline characteristics were compared between subjects with and without cognitive impairment and the KLOTHO gene -395A allele. Binary logistic regression was used to estimate the odds ratio (OR) and 95 % confidence interval (CI) of the G-395A allele as a function of increased cognitive impairment. We adjusted for age, gender, educational levels, smoking, alcohol consumption, and exercise habit as general covariates in model 1. We further adjusted for BMI, WC, hypertension, diabetes, stroke history, serum lipid/lipoprotein, and SUA as potential confounders in model 2.

Results

Characteristics of the study sample

We included 706 participants in the current analyses. The mean age of participants was 93.5 ± 3.6 years, and age ranged from 90 to 108. The prevalence of cognitive impairment was 66.6 %. All subjects were of Han Chinese descent.

Table 1 shows the characteristics of subjects with or without cognitive impairment. The cognitive impairment

Table 1 Characteristics of the study population according to cognitive function

	Cognitive impairment		<i>p</i> value
	No (<i>n</i> = 236)	Yes (<i>n</i> = 470)	
Age (years)	93.2 ± 3.3	93.6 ± 3.1	0.082
Female (%)	49.6	77.2	<0.001
BMI (kg/m ²)	19.7 ± 3.8	18.9 ± 3.6	0.012
WC (cm)	77.3 ± 10.0	76.7 ± 8.8	0.422
Diabetes (%)	6.8	6.8	0.989
Hypertension (%)	61.9	61.1	0.837
History of stroke (%)	2.1	1.7	0.698
Education level (%)			
Illiteracy	51.7	84.7	
Primary school	42.7	13.8	
Secondary school or advanced	5.6	1.5	<0.001
Smoking (%)	44.7	43.2	0.742
Alcohol drinking (%)	32.2	23.6	0.015
Having exercise habit (%)	41.9	39.6	0.544
TG (mmol/l)	1.3 ± 0.7	1.3 ± 0.7	0.998
TC (mmol/l)	4.1 ± 0.8	4.9 ± 1.5	0.428
HDL-C (mmol/l)	1.6 ± 0.7	1.6 ± 0.8	0.693
LDL-C (mmol/l)	2.2 ± 0.6	2.3 ± 0.6	0.152
SUA (μmol/l)	332.6 ± 86.2	310.5 ± 89.6	0.004
Genotype distribution			
GG (%)	62.7	70.2	
GA (%)	33.9	28.5	
AA (%)	3.4	1.3	0.042

Data are the mean ± SD unless otherwise indicated

Abbreviations: BMI body mass index, CAD coronary artery disease, HDL-C high-density lipoprotein cholesterol, LDL-C low-density lipoprotein cholesterol, MMSE mini-mental status examination, SUA serum uric acid, TC total cholesterol, TG triglycerides, WC waist circumference

group contained more females than the control group (77.2 vs. 49.6 %, respectively, $p < 0.001$). Subjects with cognitive impairment had significantly lower BMIs and educational levels. The mean MMSE score was significantly lower in the cognitive impairment group than in the control group. Alcohol consumption was also less common in the cognitive impairment group.

KLOTHO G-395A genotype distribution

The KLOTHO G-395A polymorphism genotype frequencies were 2.0 % AA, 30.3 % GA, and 67.7 % GG in the entire sample. These frequencies were in compliance with Hardy-Weinberg equilibrium ($p = 0.074$). The G and A allele frequencies were 0.828 and 0.172, respectively.

The G-395A polymorphism genotype distribution significantly differed between the hypertension and control group (Table 2). Specifically, the GG, GA, and AA genotype frequencies were 70.2, 28.5, and 1.3 % in the cognitive impairment group, respectively. These respective frequencies were 62.7, 33.9, and 3.4 % for the control group (Table 1). The G and A allele frequencies for the cognitive impairment group were 0.845 and 0.155, respectively. The control group frequencies for G and A alleles were 0.797 and 0.203, respectively ($p = 0.042$).

Table 2 shows the GG and GA+AA genotype group characteristics. The mean MMSE score was significantly higher in the GA+AA genotype than in the GG genotype group (17.0 vs. 15.2, respectively, $p = 0.001$). The prevalence of cognitive impairment was significantly lower in the GA+AA genotype group than the GG genotype group (61.3 vs. 69.6 %, respectively, $p = 0.044$). Fewer GA+AA group participants had hypertension than participants in the GG genotype group. The mean WC was also significantly higher in the GA+AA genotype group than in the GG genotype group. Age, gender, BMI, diabetes, education level, history of stroke, smoking, alcohol consumption, exercise habit, TG, TC, HDL-C, LDL-C, or SUA did not significantly differ between the GA+AA and GG genotype groups.

The association between cognitive impairment and the KLOTHO G-395A polymorphism

Table 3 shows the results from the unadjusted and adjusted logistic regression models of cognitive

Table 2 Characteristics of the study population according to the G-395A polymorphism in the KLOTHO gene

	The G-395A polymorphism		<i>p</i> value
	GA+AA genotype (<i>n</i> = 228)	GG genotype (<i>n</i> = 478)	
Age (years)	93.3 ± 3.0	93.6 ± 3.2	0.892
Female (%)	72.8	65.7	0.058
BMI (kg/m ²)	19.5 ± 3.1	19.0 ± 3.9	0.190
WC (cm)	78.0 ± 7.6	76.4 ± 9.8	0.025
Diabetes (%)	6.6	6.9	0.873
Hypertension (%)	55.3	64.2	0.022
History of stroke (%)	2.6	1.5	0.281
Education level (%)			
Illiteracy	73.2	73.8	
Primary school	23.7	23.4	
Secondary school or advanced	3.1	2.7	0.961
Smoking (%)	41.7	44.6	0.468
Alcohol drinking (%)	27.6	25.9	0.634
Having exercise habit (%)	41.7	39.7	0.627
MMSE score	17.0 ± 5.9	15.2 ± 6.3	0.001
Cognitive impairment (%)	61.4	69.0	0.044
TG (mmol/l)	1.2 ± 0.5	1.3 ± 0.8	0.233
TC (mmol/l)	4.1 ± 0.8	4.9 ± 1.5	0.462
HDL-C (mmol/l)	1.6 ± 0.7	1.6 ± 0.8	0.448
LDL-C (mmol/l)	2.2 ± 0.6	2.3 ± 0.6	0.967
SUA (μmol/l)	320.9 ± 84.2	316.3 ± 91.2	0.554

Data are the mean ± SD unless otherwise indicated

Abbreviations: BMI body mass index, CAD coronary artery disease, HDL-C high-density lipoprotein cholesterol, LDL-C low-density lipoprotein cholesterol, MMSE mini-mental status examination, SUA serum uric acid, TC total cholesterol, TG triglycerides, WC waist circumference

impairment and KLOTHO G-395A polymorphism. Compared with subjects harboring the GG genotype, the -395A allele carriers (GA+AA genotype) had a significantly lower risk of cognitive impairment (OR 0.63, 95 % CI 0.41 to 0.93), whereas the GA+AA genotype had a lower cognitive impairment risk (OR 0.66, 95 % CI 0.44 to 0.98) after adjusting for age, gender, and other potential risk factors.

Table 3 Estimate of the effect of the G-395A polymorphism on cognitive impairment modeled with logistic regression

	GG OR (95 % CI)	GA+AA OR (95 % CI)
Unadjusted	1 (reference)	0.63 (0.41, 0.93)
Adjusted model 1 ^a	1 (reference)	0.65 (0.44, 0.96)
Adjusted model 2 ^b	1 (reference)	0.66 (0.44, 0.98)

^a Adjusted for age, gender, educational levels, smoking, alcohol drinking, and exercise habit

^b Adjusted for age, gender, educational levels, smoking, alcohol drinking, exercise habit, BMI, WC, hypertension, diabetes, history of stroke, TG, TC, HDL-C, LDL-C, and SUA

Discussion

To gain a better understanding of how genetic risk factors influence cognitive impairment later in life, we studied the relationship between KLOTHO G-395A polymorphism and cognitive function in individuals aged 90–108 years in Dujiangyan, Sichuan province, China. To the best of our knowledge, this study is the first to investigate the relationship between KLOTHO G-395A polymorphism and cognitive function in this specific population. The results showed that the GA and AA genotypes (A carriers) were associated with a lower risk of cognitive impairment than the GG genotype, which indicates that KLOTHO G-395A polymorphism is associated with reduced cognitive impairment among very old people.

Age is an independent risk factor for cognitive impairment. Age-related cognitive decline is inevitable, even in people free of these diseases or environmental conditions. Our study shows that the prevalence rates of stroke and diabetes are approximately 2 and 7 %,

respectively, in Chinese nonagenarians and centenarians. Conversely, the prevalence of cognitive impairment is 66.6 %, but the reasons for cognitive impairment are not clear. Therefore, studies focusing on genetic factors in the development of cognitive impairment are important, and the specific genetic factors of dementia vary by race (Tsolaki 2014). Our data show that the A allele frequency is 0.172 in older Chinese individuals. The A allele frequency in this sample was similar to those in the Japanese (0.146; 40–79 years) (Shimokata et al. 2006) and Korean (0.155; 60±11 years) populations (Kim et al. 2008). These Asian population values are lower than those reported in Caucasians (0.201; 53.69±8.09 years) (Zhang et al. 2007). Data from this study also suggest that the KLOTHO gene promoter A allele prevalence is similar in very old and younger populations. Although functional KLOTHO variants influence secreted protein trafficking and catalytic activity, and they favor healthy aging and longevity (Di Bona et al. 2014), this gene's A allele may not prolong life span.

This study identified a potential regulatory SNP in the KLOTHO gene (G-395A polymorphism) promoter associated with reduced cognitive function in individuals aged 90 years or older. This finding is consistent with that of Shimokata et al. (Shimokata et al. 2006), who assessed the cognitive function of elderly Japanese individuals using the Japanese Wechsler Adult Intelligence Scales—Revised Short Forms and MMSE. That study included 2234 participants aged 40–79, including a subgroup of 1116 elderly people (60–79 years). These researchers found that the A allele (GA/AA) was associated with superior cognitive function compared with the GG phenotype. However, the intelligence quotient, information, picture completion, and similarity abilities significantly differed only in the 60–79-year-old subgroup. The mean MMSE scores were 27.4 and 27.2 for the A carriers and GG phenotypes, respectively ($p=0.099$). These researchers speculated that the relationship between KLOTHO gene polymorphism and cognitive function changes with age. In the present study, people with the GG phenotype had significantly lower MMSE scores than A carriers (15.2 ± 6.3 vs. 17.0 ± 5.9 ; $p=0.001$). Our study extends the findings of Shimokata et al., and we speculate that KLOTHO G-395A polymorphism plays a more important role in cognitive function than age.

Although the KLOTHO gene was not associated with senile plaques, amyloid deposits, or other

pathological brain changes (Kuro-o et al. 1997; Shimokata et al. 2006), it plays an increasingly important role in brain function with age in both mice and humans (Deary et al. 2005; Nagai et al. 2003; Shimokata et al. 2006). A previous study found that KLOTHO mutant mice exhibited more impaired visual recognition and associative fear memory at 7 weeks than wild-type mice, whereas these animals displayed normal memory function at 6 weeks (Nagai et al. 2003). Age-dependent memory impairment in KLOTHO gene mutant mice may result from age-associated oxidative neuronal membrane and DNA damage in the hippocampus (Nagai et al. 2003). Recently, Dubal and colleagues conducted important studies of the Klotho gene and cognitive function and also found that KLOTHO KL-VS heterozygosity is associated with improved cognition and a larger right dorsolateral prefrontal cortex volume (Dubal et al. 2014, 2015; Yokoyama et al. 2015); these differences are mediated by mechanisms that involve the regulation of *N*-methyl-*D*-aspartate receptors and enriched synaptic GluN2B. Our study highlights the role of KLOTHO G-395A polymorphism in cognitive function. Further functional studies to explore the exact mechanism of KLOTHO of G-395A polymorphism in the pathogenesis of cognitive impairment would be highly desirable.

As an aging-suppressor gene, KLOTHO encodes a transmembrane protein that is predominantly secreted by the choroid plexus of the brain and kidney (Kuro-o 2009; Kuro-o et al. 1997; Wang and Sun 2009). The potential involvement of the SNP G-395A in KLOTHO gene function remains speculative. Previous studies reported that G-395A can influence the affinity of binding transcription factors and that the A allele exhibits higher promoter activity in vitro (Kawano et al. 2002; Wang et al. 2010). Furthermore, G-395A exhibits strong linkage disequilibrium, which may allow it to also act as a surrogate for other nearby functional variants (such as G110C, C1818T, and C2298T) (Zhang et al. 2007). However, previous studies did not identify significant differences in KLOTHO expression in human cell culture between the G and A alleles of G-395A (Kawano et al. 2002). Dubal and colleagues reported that a higher Klotho level associated with better cognitive function and decreased mortality (Dubal et al. 2014, 2015). Moreover, old people with Alzheimer's disease have lower cerebrospinal fluid KLOTHO protein levels than those without (Semba et al. 2014). Therefore, Klotho protein may function as a humoral factor (Wang et al. 2010). Based on these studies,

we hypothesized that the A allele can enhance the levels or activity of Klotho in humans.

Our findings must be interpreted with caution because of the following limitations. First, the data were derived from self-reported questionnaires. Subjects who refused to participate in the PLAD study might also have lower cognitive functioning, which may have resulted in nonresponse or selection bias. Second, the present study included only Han Chinese individuals. Therefore, our results might not be representative of other ethnic population, such as Caucasians. Third, a cross-sectional design was used. Therefore, causal inferences with respect to the relationship between KLOTHO G-395A polymorphism and cognitive impairment could not be made. Fourth, the confidence intervals of the multivariate analysis are wide and close to non-significant, and the sample size was insufficient in our study. Therefore, further studies are needed to confirm our findings. Finally, because of missing data, we did not adjust for some important potential confounders, such as socio-economic status, serum KLOTHO protein, and family dementia history. This omission may have induced additional bias.

Conclusions

In summary, KLOTHO G-395A polymorphism is associated with reduced cognitive impairment in a sample of Chinese community-dwelling people aged 90 years or older. Specifically, the GA and AA genotypes were associated with a lower risk of cognitive impairment than the GG genotype.

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Compliance with ethical standards Written informed consent was obtained from all subjects or their legal proxies. The Research Ethics Committee of Sichuan University approved the study protocol.

Conflict of interests The authors declare that they have no competing interests.

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