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RESEARCH ARTICLE

Lingguizhugan Decoction, a Chinese herbal formula, improves insulin resistance in overweight/obese subjects with non-alcoholic fatty liver disease: a translational approach

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Abstract **Lingguizhugan Decoction (LGZG) has been investigated in basic studies, with satisfactory effects on insulin resistance in non-alcoholic fatty liver disease (NAFLD). This translational approach aimed to explore the effect and underlying mechanism of LGZG in clinical setting. A randomized, double-blinded, placebo-controlled trial was performed. A total of 243 eligible participants with NAFLD were equally allocated to receive LGZG (two groups: standard dose and low dose) or placebo for 12 weeks on the basis of lifestyle modifications. The primary efficacy variable was homeostasis model assessment of insulin resistance (HOMA-IR). Analyses were performed in two populations in accordance with body mass index (BMI; overweight/obese, BMI ⩾ 24 kg/m^2; lean, BMI < 24 kg/m^2). For overweight/obese participants, low-dose LGZG significantly decreased their HOMA-IR level compared with placebo (−0.19 (1.47) versus 0.08 (1.99), P = 0.038). For lean subjects, neither dose of LGZG showed a superior effect compared with placebo. Methylated DNA immunoprecipitation sequencing and real-time qPCR found that the DNA N6-methyladenine modification levels of protein phosphatase 1 regulatory subunit 3A (PPP1R3A) and autophagy related 3 (ATG3) significantly increased after LGZG intervention in overweight/obese population. Low-dose LGZG effectively improved insulin resistance in overweight/obese subjects with NAFLD. The underlying mechanism may be related to the regulation of DNA N6-methyladenine modification of PPP1R3A and ATG3. Lean subjects may not be a targeted population for LGZG.**

Keywords insulin resistance; non-alcoholic fatty liver disease; Chinese herbal medicine; randomized controlled trial; DNA N6-methyladenine modification

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a frequently encountered chronic disease in clinical practice, and it afflicts more than one-fourth of the global population [1]. A recent systematic review in China indicated that the prevalence of NAFLD was approximately 29.88%, and it was accompanied by a rapidly increasing trend [2,3]. Insulin resistance (IR) was demonstrated repeatedly during NAFLD development and progression [4–8], and it naturally became a therapeutic target. Thiazolidinediones are the conventional option in daily practice against IR in NAFLD at present [9]. However, their application is only limited to non-alcoholic steatohepatitis (NASH) [10,11], and they may induce unwanted adverse effects [12,13]. For early-stage NAFLD, lifestyle modification is the sole intervention recommended by guidelines, but the execution of this intervention remains a big concern [14].

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Considering these issues, many clinicians and patients in China introduce Chinese herbal medicines in NAFLD management. Lingguizhugan Decoction (LGZG) is a representative formula. It is composed of four herbs, namely, Poria (Fuling), Ramulus Cinnamomi (Guizhi), Rhizoma Atractylodis Macrocephalae (Baizhu), and Radix Glycyrrhizae (Gancao). The previous studies showed a satisfactory effect of LGZG on improving IR, regulating lipid metabolism, and alleviating hepatic steatosis in NAFLD rats [15–18]. One of the underlying mechanisms may be related to the N6-methyladenosine level of the suppressor of cytokine signaling 2 (SOCS2) [19]. However, rigorous randomized controlled trials (RCTs) to verify these perspectives in a clinical setting are lacking.

Approximately two-fifths of patients with NAFLD in China are non-obese [20]. The NAFLD models in the animal studies were generally based on a high-fat diet [15–18], thus more like overweight/obese patients with NAFLD. For lean patients with NAFLD, the data on LGZG are limited. Previous studies suggested that the clinical features and pathophysiological mechanisms were dissimilar between overweight/obese and lean subjects with NAFLD, possibly resulting in different treatment responses [21–24]. Therefore, evaluating the effects of LGZG separately in overweight/obese and lean populations is more appropriate.

On the foundation of the long-term successful application of LGZG and previous animal studies, this translational approach composed of a pilot randomized, double-blinded, controlled trial and the following methylated DNA immunoprecipitation sequencing (MeDIP-seq) was performed. As LGZG was first evaluated in clinical setting, dose optimization was introduced in the study design, in reference to the 2015 Chinese Pharmacopoeia [25]. This clinical trial aimed to assess the efficacy and safety of LGZG in overweight/obese and lean patients with NAFLD, and MeDIP-seq aimed to discover whether the regulation of DNA N6-methyladenine (6mA) modification by LGZG could be reproduced in humans.

Materials and methods

Study design

The pilot clinical trial was a multicenter, three-arm, double-blinded, placebo-controlled clinical trial performed in Zhangjiang Health Center, Beicai Health Center, and Sanlin Health Center. A 2-week wash-out period was arranged to allow participants to discontinue relevant interventions against NAFLD. Eligible participants were randomly allocated to a standard dose of LGZG (SLGZG), a low dose of LGZG (LLGZG), and a placebo at 1:1:1 ratio. A 12-week treatment period and a 4-week follow-up period were set for every participant. Six visits were arranged in total, namely, screening (Week −2), baseline (Week 0), during treatment (Weeks 4 and 8), treatment endpoint (Week 12), and follow-up endpoint (Week 16).

The study protocol was published elsewhere [26], and the reporting of this clinical trial followed the CONSORT statement and CHM Formulas extension [27,28]. The corresponding checklist could be found in Table S1.

Participants

All participants were recruited from the public via advertisements placed in community centers. The diagnostic criteria of NAFLD referred to the 2017 American Association for the Study of Liver Diseases NAFLD practice guideline [29]. In brief, the diagnosis should fulfil the following criteria: imaging or histological evidence of hepatic steatosis; no significant alcohol consumption; exclusion of other reasons inducing hepatic steatosis; and no coexistence of other chronic liver disease. LGZG was originally prescribed for patients with a traditional Chinese medicine (TCM) pattern of spleen-yang deficiency. Therefore, this standard was introduced in participant recruitment. The pattern differentiation was based on a previous study and certain guidelines [30–32]. Ten signs and symptoms were assessed using a continuous 100-point scale, including laziness to speak, easy perspiration, tastelessness, loose stool, increased sweat, gingival bleeding, unwarm limbs, insomnia, easy getting cold, and diet habit alterations. Higher scores indicated a more severe degree. Every symptom or sign possessed a certain weight. The scoring was calculated by multiplying the rating score by the weight. A spleen-yang deficiency pattern was defined as a total score of 10 dimensions equal or greater than 20. The Chinese overweight and obesity guideline was used to determine the body mass index (BMI) cutoff value for overweight/obese and lean populations, and BMI = 24 kg/m^2 was chosen [33].

The participants who met the following criteria were included: either gender; aged 18–80 years old; confirmed diagnosis of NAFLD and a spleen-yang deficiency pattern; and voluntary signed informed consent. Participants were excluded if they had other combined liver diseases or conditions that could lead to hepatic steatosis. Those with diabetes, under anti-diabetic medication treatment, and receiving other agents for NAFLD were excluded to assess the absolute effect of LGZG on IR. The detailed eligible criteria for participants were listed in the protocol [26].

Interventions

Health education for lifestyle modification was provided to all three groups. The participants should reduce calorie intake and increase physical exercise. In particular, the total calorie of daily diet should be approximately 1660 kcal. Moderate aerobic sports should be performed at least four times per week, and the total exercise time

should be above 150 mins [26,33]. The participants were required to complete the daily dietary and exercise records during the intervention period. The clinical investigators evaluated the execution status of lifestyle modification on the basis of the participants' records by using a continuous five-level scale (Table S2). Higher level meant better compliance. Levels "1" and "5" indicated that lifestyle modification requirements were met in less than 1 day per week and in more than 3 days per week, respectively. For drug intervention, two doses of LGZG granules were used in this trial. SLGZG was based on the original record, namely, 12 g Fuling, 9 g Guizhi, 6 g Baizhu, and 6 g Gancao per day. The dose of LLGZG was determined on the basis of consultation with pharmacy specialists and a consensus obtained from TCM practitioners [34]. Half of the routine dose was finally chosen, namely, 6 g Fuling, 4.5 g Guizhi, 3 g Baizhu, and 3 g Gancao per day. Considering that the doses of Fulin and Guizhi were already close to the upper limit of the 2015 Chinese Pharmacopoeia [25], no higher dose of LGZG was used. The placebo granules were made from soluble starch (88.19%), colorant (1.8%), bitter principle (0.01%), and SLGZG (10.0%). LGZG was added to the placebo to achieve a comparable taste and smell with the other two groups. The dose of herbs contained in the placebo were far below the recommendation of the Pharmacopoeia, and no significant therapeutic effect was shown. This preparation method is generally accepted in CHM clinical studies [35].

The specific fingerprint spectrum of LGZG on liquid chromatograph–mass spectrometry (LC–MS) was reported previously [18]. All study granules were provided by Neo-green Pharmaceutical Technology Development Limited Company (Pengzhou, Sichuan, China) and prepared strictly in accordance with the standards of Good Manufacturing Practices. The granules were also authenticated. The appearance, content of quality-control ingredients, and microbial limit conformed to the corresponding industry quality standards. The detailed authentication reports are provided in Supplementary File 1. The participants were informed to dissolve a lattice of granules in 150 mL hot water and drink the liquid once daily 30 min after breakfast on weekdays. No medication was provided on weekends. This administration mode was also based on the record of classic dosing. The use of agents clearly indicated for NAFLD was forbidden during the entire study.

Outcomes

The primary outcome was the proportions of participants with at least one-unit reduction of homeostasis model assessment of IR (HOMA-IR) after treatment [36]. HOMA-IR was calculated by multiplying fasting insulin (FINS, mU/L) by fasting plasma glucose (FPG, mmol/L) and then dividing by 22.5. Secondary outcomes included changes in BMI, lipid metabolism (total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), apolipoprotein A1 (apoA1), and apolipoprotein B (apoB)), hepatic function (alanine aminotransferase (ALT), aspartate transaminase (AST), gamma-glutamyl transpeptidase (GGT), and alkaline phosphatase (ALP)), glucose metabolism (FPG, FINS, and glycosylated hemoglobin (HbA1c)), inflammatory biomarkers (white blood cells (WBCs) and C-reactive protein (CRP)), imaging findings (assessed as the liver–kidney echo ratio on ultrasound), and questionnaire scores (36-item short form survey (SF-36), self-rating depressive scale (SDS), self-rating anxiety scale (SAS), and spleen-yang deficiency pattern scale). Four questionnaires were introduced in this trial. SF-36 was a classical questionnaire evaluating quality of life [37]. SDS and SAS were two self-rating scales assessing the emotional improvement throughout the study [38,39]. Spleen-yang deficiency pattern scale, which was described above, was used to evaluate whether LGZG could improve the TCM pattern. Safety assessments included adverse events reported throughout the entire period and by laboratory tests (hepatic and renal function).

A preliminary analysis of baseline characteristics was performed after participant recruitment was completed. The investigators noted that the baseline HOMA-IR of the entire population was lower than expected, and the original primary outcome may not be able to reflect the efficacy of LGZG. Therefore, the change in HOMA-IR was supplemented as the other primary outcome. Preliminary analysis also found that the included participants had normal ranges of liver enzymes and inflammatory biomarkers, indicating that only mild liver impairment existed. Therefore, the assessments of hepatic function and inflammatory response were converted to a safety evaluation of LGZG. Given the relatively short intervention period (12 weeks) and mild severity of NAFLD (low HOMA-IR and normal liver enzymes), changes in imaging evaluation may not appear. After consultation with specialists, the assessment of liver–kidney echo ratio was also reduced.

Sample-size estimation

Sample-size calculation was based on the original primary outcome. To the authors' knowledge, this study was the first RCT to evaluate the efficacy of a Chinese herbal formula versus placebo in the treatment of NAFLD. No available data were used for reference. Therefore, based on the consultation with specialists and the previous animal studies, the effect sizes of SLGZG, LLGZG, and placebo were presumed to be 30%, 15%, and 10%, respectively. Sixty-four participants per group were needed for an 80% power to detect an efficacy difference and a two-sided level of significance of 0.05. After a dropout rate of 20% was considered, the final sample size was determined as 243.

Randomization and blinding

Central randomization was performed on the basis of 1:1:1 ratio. An independent statistician generated the random sequence by using SPSS 19.0 for Windows software (Chicago, Illinois, USA). The pharmacists who did not participate in this clinical trial distributed the medications in accordance with the randomization numbers sealed in opaque envelopes. The granules from the three groups had comparable color, appearance, shape, smell, and taste. The opaque plastic medicine box only contained a number and administration instructions. Emergency letters were also prepared in sealed opaque envelops and maintained by the principal investigator. The treatment allocations were blinded to the participants, investigators, and statisticians and revealed when the entire trial was completed.

DNA 6mA-Seq and data analysis

Fasting whole-blood samples were obtained from voluntary participants in Weeks 0 (baseline) and 12 (post-treatment). After unblinding, DNA 6mA-Seq was performed in selected participants in accordance with the results' interpretation. DNA 6mA-Seq was provided by CloudSeq Biotech Inc. (Shanghai, China). In brief, genomic DNA was extracted with DNeasy kit (QIAGEN Inc., Germany) and sonicated to 100–300 bp fragments. Magnetic beads were added to the genomic DNA fragments and then immunoprecipitated with 6mA antibodies (Synaptic Systems Inc., Germany) overnight at 4 °C. Afterwards, DNAs were amplified and purified to obtain DNA libraries. DNA 6mA-Seq and subsequent bioinformatic analysis were performed using an Illumina Hiseq instrument. Differentially methylated sites (DMSs) were identified in accordance with fold change > 2 and P value < 0.01. The enriched peaks were visualized on UCSC Genome Browser.

DNA 6mA immunoprecipitation real-time qPCR

Differentially methylated genes (DMGs) were further validated by 6mA immunoprecipitation real-time qPCR. In brief, genomic DNA was extracted and sonicated, followed by immunoprecipitation with 6mA antibodies. The 6mA-enriched DNA was amplified and analyzed. The primer sequences in this study are listed in Table S3.

Statistical analysis

Efficacy and safety analyses were performed on the basis of intention-to-treat (ITT) principle. The last-observation-carried-forward method was used to fill in the missing values. Per-protocol (PP) analysis was also conducted to comprehensively evaluate the results. The PP analysis set contained participants who completed all treatments and follow-ups and did not violate the trial protocol. The efficacy of LGZG was further assessed in two sets on the basis of BMI level: overweight/obese population (BMI ⩾ 24 kg/m^2) and lean population (BMI < 24 kg/m^2).

SPSS 24.0 for Windows software was used for data analyses. Data are given as the mean with standard deviation (SD) or percentages. Differences within groups were evaluated using paired t-test or Wilcoxon signed rank test. For differences between groups, continuous variables were assessed using ANOVA or Kruskal–Wallis test. If statistical significance was detected among three groups, post-hoc pairwise multiple comparisons with Bonferroni adjustment were performed (least significant difference test or Dunnett method for ANOVA based on data distribution and Dunn–Bonferroni approach for Kruskal–Wallis test). Chi-square test was used for categorical variables. The significance level was set as 0.05, with two-tailed test.

Results

Baseline characteristics

A total of 284 individuals were screened from July 2018 to January 2019. Among them, 243 were randomized and 224 completed the study (Fig. 1). Nineteen participants withdrew from the trial due to various reasons. Among the participants who completed the study, 218 entered the PP analysis. The compliance rates for the SLGZG, LLGZG, and placebo groups were 88.9%, 87.7%, and 92.6%, respectively. The reasons for exclusion included concomitant medication (n = 1) and protocol violation of laboratory tests (n = 5). The baseline characteristics are shown in Table 1, and all variables were well balanced among the three groups. The execution status of lifestyle modification is given in Table S4. Approximately 80% of participants had the execution status of level 4 or 5, and no significant difference was found among the three groups. The execution status results indicated that most of the participants followed lifestyle modification as suggested in the trial protocol. In other words, around 80% of participants reduced their daily calorie intake to 1660 kcal for at least 4 days per week and took physical exercise for at least 120 min weekly.

The distributions of overweight/obese and lean populations are presented in Table S5. The BMI levels ranged from 24.02 kg/m^2 to 36.80 kg/m^2 for the overweight/obese population, and from 17.74 kg/m^2 to 23.92 kg/m^2 for the lean population (ITT set). The baseline characteristics of overweight/obese and lean participants are shown in Tables S6 and S7. No significant differences were found among the three groups in the two analysis sets.

HOMA-IR

After 12-week treatment, ITT analysis showed that the proportions of participants who experienced at least a

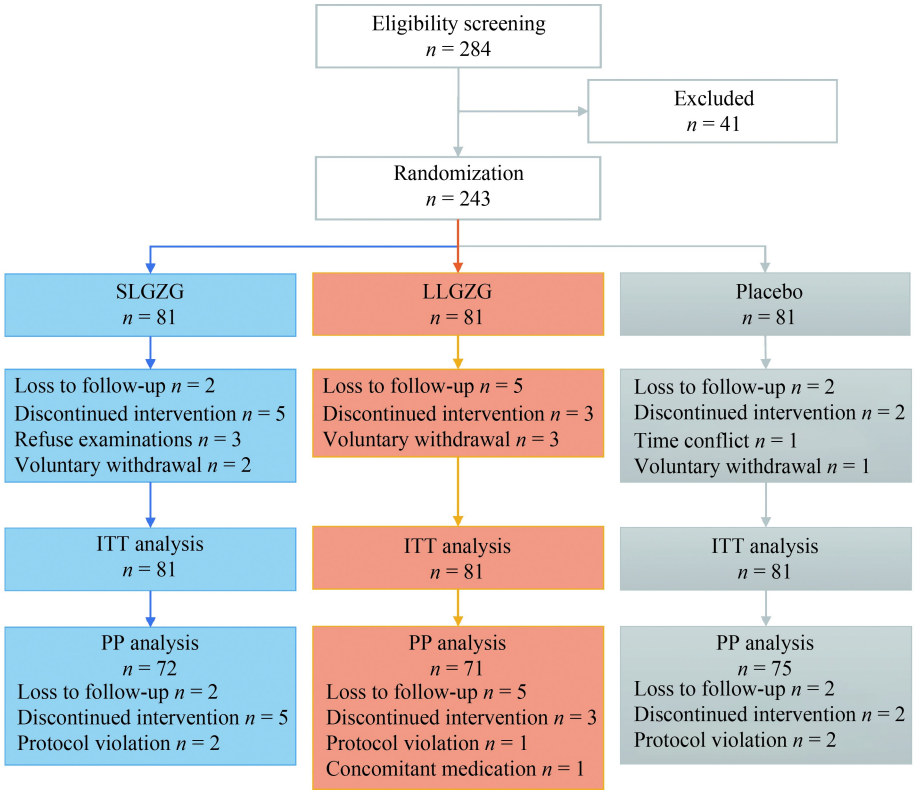

Fig. 1 Participant flowchart. ITT, intention-to-treat; LGZG, Lingguizhugan Decoction; LLGZG, low-dose Lingguizhugan Decoction; SLGZG, standard-dose Lingguizhugan Decoction; PP, per-protocol.

one-unit reduction in HOMA-IR were 12.3% (n = 10), 18.5% (n = 15), and 12.3% (n = 10) for SLGZG, LLGZG, and placebo, respectively (P = 0.434, Fig. 2A). In the overweight/obese population, 21.5% (n = 14) of participants in LLGZG achieved the prescribed HOMA-IR reduction compared with 14.8% (n = 9) in SLGZG and 8.8% (n = 5) in placebo (P = 0.147, Fig. 2B). The corresponding proportions in the lean population were 5.0% (n = 1), 6.3% (n = 1), and 20.8% (n = 5) for SLGZG, LLGZG, and placebo, respectively (P = 0.194, Fig. 2C).

For the overall population, the mean (SD) changes in HOMA-IR from baseline to treatment endpoint were −0.01 (2.44), −0.12 (1.38), and −0.02 (1.77) for SLGZG, LLGZG, and placebo on the basis of ITT analysis, respectively. The difference among the three groups was not statistically significant (P = 0.246). Analysis based on the PP set did not reveal any obvious change. ITT analysis in the overweight/obese population indicated that LLGZG significantly reduced the HOMA-IR level compared with placebo (−0.19 (1.47) versus 0.08 (1.99), P = 0.038). Comparisons of SLGZG versus placebo and SLGZG versus LLGZG did not show meaningful differences. PP analysis also found similar results. No statistical significance was noted among the three groups in either analysis in lean population. The details of HOMA-IR changes from baseline in the three groups are presented in Table 2 and Fig. 3.

Secondary outcomes

The BMI and lipid metabolism-related variables of the three groups showed a slight elevation after treatment, except TG, but the differences were not statistically significant. The changes in variables in overweight/obese and lean populations were comparable to the values in the overall population. The detailed changes in BMI and lipid profiles from baseline are presented in Tables 3 and S8.

The changes in FPG and FINS after treatment among the three groups were also not significant in the overall population. However, LLGZG markedly reduced the FINS level compared with placebo in the overweight/obese population (−0.72 (5.83) versus 0.09 (7.48) in the ITT analysis, P = 0.041, and −1.35 (4.64) versus 0.92 (5.39) in the PP analysis, P = 0.008), but no significance appeared in the SLGZG versus placebo or SLGZG versus LLGZG. The lean population did not exhibit consistent results. Some statistical significances were also found in HbA1c among the three groups in the overall and lean populations, indicating no particular clinical significance. The detailed changes in the above variables are listed in Tables 3 and S8.

The results of various questionnaires are shown in Table S9. For SF-36, only the vitality dimension showed a significant difference among the three groups. For SAS and SDS, the changes among the three groups were not statistically significant. Improvement in the spleen-yang

Table 1 Baseline characteristics of participants (ITT and PP analyses)

Characteristic	ITT				PP			
	SLGZG (n = 81)	LLGZG (n = 81)	Placebo (n = 81)	P value	SLGZG (n = 72)	LLGZG (n = 71)	Placebo (n = 75)	P value
Gender, male, n (%)	34 (41.98%)	28 (34.57%)	34 (41.98%)	0.538	31 (43.06%)	22 (30.99%)	31 (41.33%)	0.276
Age (year)	57.00 (12.12)	59.63 (11.69)	56.67 (13.83)	0.352	58.04 (11.86)	59.61 (12.10)	56.81 (13.89)	0.574
BMI (kg/m^2)	26.56 (3.54)	26.62 (3.29)	26.09 (3.16)	0.539	26.47 (3.33)	26.78 (3.27)	26.08 (3.15)	0.433
HOMA-IR	2.94 (2.45)	2.67 (1.38)	3.06 (1.95)	0.519	2.86 (2.49)	2.76 (1.41)	2.94 (1.74)	0.402
Lipid metabolism								
TC (mmol/L)	4.76 (0.82)	4.72 (0.82)	4.76 (0.75)	0.938	4.75 (0.84)	4.73 (0.79)	4.77 (0.75)	0.951
TG (mmol/L)	1.98 (1.04)	1.94 (0.93)	2.21 (1.39)	0.308	1.99 (1.06)	1.99 (0.95)	2.20 (1.44)	0.590
LDL-C (mmol/L)	3.04 (0.76)	3.01 (0.77)	3.02 (0.72)	0.951	3.02 (0.78)	3.00 (0.75)	3.03 (0.71)	0.963
HDL-C (mmol/L)	1.20 (0.33)	1.19 (0.28)	1.16 (0.28)	0.718	1.20 (0.32)	1.19 (0.29)	1.16 (0.27)	0.900
ApoA1 (g/L)	1.31 (0.21)	1.29 (0.19)	1.27 (0.20)	0.349	1.31 (0.20)	1.30 (0.19)	1.28 (0.20)	0.499
ApoB (g/L)	1.02 (0.21)	1.02 (0.22)	1.01 (0.20)	0.990	1.01 (0.22)	1.02 (0.21)	1.02 (0.20)	0.994
Hepatic function								
ALT (U/L)	28.37 (18.45)	24.40 (18.47)	27.44 (22.50)	0.099	26.97 (16.00)	24.75 (17.88)	27.56 (23.25)	0.313
AST (U/L)	22.57 (7.97)	21.05 (9.32)	22.32 (11.74)	0.173	22.29 (7.49)	21.34 (9.01)	22.39 (12.15)	0.411
GGT (U/L)	40.04 (44.41)	40.79 (51.73)	37.05 (40.00)	0.640	40.24 (46.73)	42.92 (54.44)	36.76 (41.43)	0.737
ALP (U/L)	76.27 (21.07)	81.86 (23.63)	76.12 (20.29)	0.162	78.36 (20.12)	83.66 (23.99)	78.19 (20.23)	0.199
Glucose metabolism								
FPG (mmol/L)	5.23 (0.62)	5.28 (0.58)	5.24 (0.64)	0.730	5.20 (0.58)	5.31 (0.60)	5.21 (0.60)	0.521
FINS (μU/L)	12.29 (9.11)	11.26 (5.52)	12.98 (7.54)	0.471	12.05 (9.31)	11.62 (5.64)	12.55 (7.01)	0.488
HbA1c (%)	5.72 (0.41)	5.76 (0.41)	5.76 (0.46)	0.816	5.73 (0.41)	5.77 (0.42)	5.75 (0.46)	0.745
Inflammatory biomarkers								
CRP (mg/L)	1.38 (1.22)	3.49 (14.60)	1.95 (2.45)	0.291	1.42 (1.27)	2.00 (3.12)	1.90 (2.45)	0.365
WBC counts (× 10^9/L)	6.08 (1.35)	6.38 (1.86)	6.45 (1.52)	0.328	6.06 (1.37)	6.38 (1.84)	6.44 (1.54)	0.346

Data are presented as mean (SD). P values were obtained using ANOVA, Kruskal–Wallis test, or Chi-square test among the three groups. ALP, alkaline phospha-tase; ALT, alanine aminotransferase; ApoA1, apolipoprotein A1; ApoB, apolipoprotein B; AST, aspartate transaminase; BMI, body mass index; CRP, C-reactive protein; FINS, fasting insulin; FPG, fasting plasma glucose; GGT, gamma-glutamyl transpeptidase; HbA1c, glycosylated hemoglobin; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment of insulin resistance; ITT, intention-to-treat; LDL-C, low-density lipoprotein cholesterol; LLGZG, low-dose Lingguizhugan Decoction; PP, per-protocol; SLGZG, standard-dose Lingguizhugan Decoction; TC, total cholesterol; TG, triglycerides.

deficiency pattern scale was found in all groups. Post-hoc analysis indicated that SLGZG and LLGZG produced a larger reduction than placebo ($P < 0.001$ and $P = 0.003$, respectively).

Safety assessment

No adverse events were reported during the entire study. Both doses of LGZG were well tolerated. The changes in the four hepatic function indices and two inflammatory biomarkers, namely, ALT, AST, GGT, ALP, WBC counts, and CRP, are presented in Table S10. SLGZG, LLGZG, and placebo did not obviously affect these variables. No meaningful change was observed in renal function within or among the three groups.

Analysis of DNA 6mA modification

Based on clinical data, the effect of LLGZG varied between overweight/obese and lean NAFLD subjects. LLGZG significantly improved IR in overweight/obese NAFLD but showed no obvious effect on lean NAFLD. Therefore, 10 baseline samples were selected from the lean NAFLD population (lean group), 10 post-treatment samples were selected from the LGZG well-responded population (effective group), and 10 post-treatment samples were selected from the LGZG no-response population (ineffective group) for DNA 6mA-Seq. The levels of DNA 6mA modification on chromosomes 21 and Y were much higher among the three groups, and they were mainly distributed among intergenic, intron, and upstream (Fig. S1A and S1B). DNA 6mA modification was highly enriched near the transcription start site (TSS), and it had a similar canonical motif among the three groups (Fig. S1C and S1D). In accordance with fold change > 2 and P value < 0.01, 122 downregulated DMSs and 153 upregulated DMSs were identified in the effective group compared

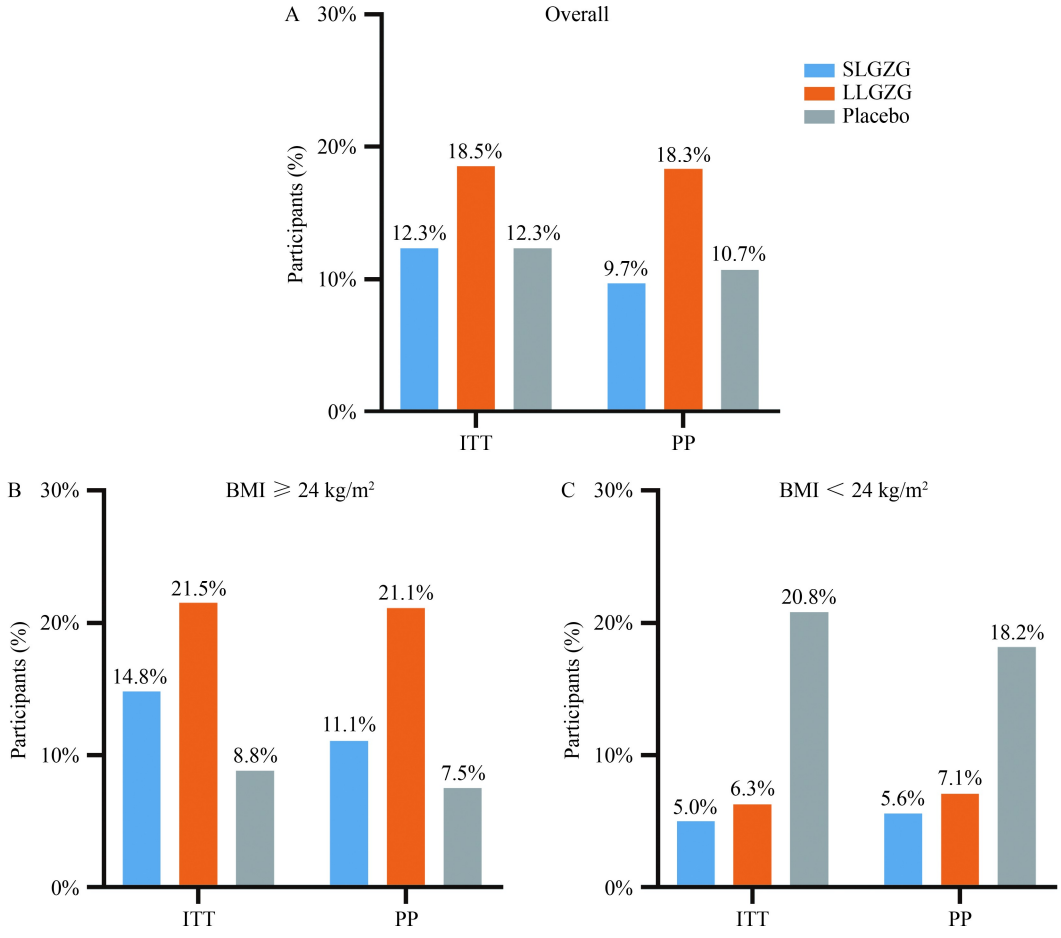

Fig. 2 Proportion of participants who achieved at least a one-unit reduction in homeostasis model assessment of insulin resistance after 12 weeks of treatment. BMI, body mass index; ITT, intention-to-treat; LGZG, Lingguizhugan Decoction; LLGZG, low-dose Lingguizhugan Decoction; SLGZG, standard-dose Lingguizhugan Decoction; PP, per-protocol.

with the lean group, and 501 downregulated DMSs and 246 upregulated DMSs were identified in the effective group compared with the ineffective group (Fig. 4A). The overlapped DMGs (124) are shown in Fig. 4B, and they focused on insulin resistance, vibrio cholerae infection, regulation of autophagy, and butanoate metabolism (Fig. 4C and 4D). The DNA 6mA levels of protein phosphatase 1 regulatory subunit 3A (PPP1R3A), autophagy related 3 (ATG3), potassium voltage-gated channel subfamily Q member 1 (KCNQ1), and inturned planar cell polarity protein (INTU) were significantly increased in the effective group based on UCSC Genome Browser.

Afterwards, immunoprecipitation-qPCR was employed to verify the above results in the overweight/obese and lean NAFLD populations that received LGZG. Due to sample limitation, 33 overweight/obese (fat group) and 16 lean (lean group) participants were included for qPCR detection. The DNA 6mA levels of PPP1R3A and ATG3 significantly increased after LGZG intervention in the overweight/obese patients with NAFLD, but no significant differences were noted in the lean patients with NAFLD. The DNA 6mA level of KCNQ1 and INTU showed no significant changes after LGZG intervention in both groups (Fig. 5).

Discussion

To the authors' knowledge, this study was the first randomized clinical trial to evaluate a Chinese herbal formula for the treatment of NAFLD by using a placebo as a comparator and an objective variable (HOMA-IR) as the primary outcomes. Moreover, following the translational medical research's concept, MeDIP-seq was used to explore the potential therapeutic mechanism of LGZG. The results indicated that LLGZG was superior to placebo, and it benefited the improvement of IR in overweight/obese patients with NAFLD. The therapeutic mechanism may be related to the upregulated DNA 6mA levels of PPP1R3A and ATG3. Some statistical differences in HbA1c were observed, but the effect sizes were likely not clinically significant. This trial was performed in three regional health centers instead of comprehensive large-scale hospitals. Therefore, it was close to real clinical practice.

This trial involved the concept of dose optimization in

Table 2 Comparison of treatment effect of change in HOMA-IR from baseline to 12 weeks

	Population	Groups			Three groups *P* value
		SLGZG	LLGZG	Placebo	
ITT	Overall	−0.01 (2.44)	−0.12 (1.38)	−0.02 (1.77)	0.246
	BMI ⩾ 24 kg/m^2	−0.19 (2.58)	−0.19 (1.47)	0.08 (1.99)	0.044
	BMI < 24 kg/m^2	0.53 (1.88)	0.20 (0.92)	−0.26 (1.08)	0.316
PP	Overall	−0.11 (2.21)	−0.23 (1.18)	0.18 (1.24)	0.085
	BMI ⩾ 24 kg/m^2	−0.34 (2.25)	−0.34 (1.21)	0.31 (1.33)	0.009
	BMI < 24 kg/m^2	0.59 (0.15)	0.23 (0.98)	−0.15 (0.94)	0.397

	Population	Pairwise multiple comparisons		
		SLGZG versus Placebo *P* value	LLGZG versus Placebo *P* value	SLGZG versus LLGZG *P* value
ITT	Overall	/	/	/
	BMI ⩾ 24 kg/m^2	0.630	0.038	0.638
	BMI < 24 kg/m^2	/	/	/
PP	Overall	/	/	/
	BMI ⩾ 24 kg/m^2	0.462	0.007	0.318
	BMI < 24 kg/m^2	/	/	/

Data are presented as the mean (SD). *P* values within the three groups were obtained using Kruskal–Wallis test. *P* values between different groups were obtained from post-hoc pairwise multiple comparisons with Dunn−Bonferroni approach. BMI, body mass index; HOMA-IR, homeostasis model assessment of insulin resistance; ITT, intention-to-treat; LLGZG, low-dose Lingguizhugan Decoction; PP, per-protocol; SLGZG, standard-dose Lingguizhugan Decoction.

the design. Therefore, a low-dose group was set. Only this group notably exhibited meaningful IR improvement, and the routine dose did not show any difference compared with placebo. This result partially conformed to the previous basic research, in which a low dose of LGZG was superior to the standard dose in improving the liver index in ob/ob mouse model with leptin deficiency [40]. However, the underlying mechanism is not unclear. These findings suggested that future research sets ladder doses. This "lower dose indicated better effect" phenomenon is not new in native products [41]. Active compounds analysis may be another potential starting point to explain this issue.

The PIVENS trial, which provided high-quality clinical evidence supporting pioglitazone usage in non-alcoholic steatohepatitis, reported that HOMA-IR was reduced 14% after 96 weeks of pioglitazone intervention [9]. The HOMA-IR of overweight/obese subjects decreased by approximately 11.6% after 12 weeks of LLGZG administration in the present study. The participants had milder case than those in the PIVENS trial for NAFLD and obesity. The effect of placebo was also consistent with that in the PIVENS trial [9], indicating the reliability of the assessment. The safety assessment indicated good LGZG tolerance. Therefore, the effects of LLGZG in overweight/obese subjects were acceptable.

Four questionnaires were included in this trial to evaluate the changes in the participants' quality of life, emotional status, and TCM pattern. Based on the results, improvements on quality of life and emotional status were limited from LGZG treatment. The potential reasons may include short intervention period and relatively complicated administration methods. For spleen-yang deficiency scale, SLGZG and LLGZG showed greater improvement than placebo, demonstrating that LGZG was a reasonable option for NAFLD with spleen-yang deficiency scale.

DNA C5-methylcytosine (5mC) and 6mA are two representative directed DNA methylation forms. 5mC is considered as the most abundant DNA modification in eukaryotes [42], and it has been extensively studied in NAFLD research. Previous studies have demonstrated that DNA 5mC participates in various pathological mechanisms of NAFLD development, such as epigenetic organ remodelling, one-carbon metabolite levels, and mitochondria's function [43–45]. The function of DNA 6mA is still not fully understood. Available studies found that it was closely related to the regulation of gene's transcription and function [46,47]. Unlike DNA 5mC modification, DNA 6mA is seldom reported in NAFLD studies. This translational approach, for the first time, illustrated that DNA 6mA was associated with NAFLD, and LGZG could increase the DNA 6mA level of PPP1R3A and ATG3 in overweight/obese patients with NAFLD, but not lean patients with NAFLD. PPP1R3A is a subunit of protein phosphatase 1, and the association with IR has been reported recently [48,49]. Therefore, the benefit of LGZG in overweight/obese NAFLD population may be generated by regulating the DNA 6mA level of PPP1R3A. The other identified gene, ATG3, encodes an autophagy-related protein [50,51]. Its effect on IR needs

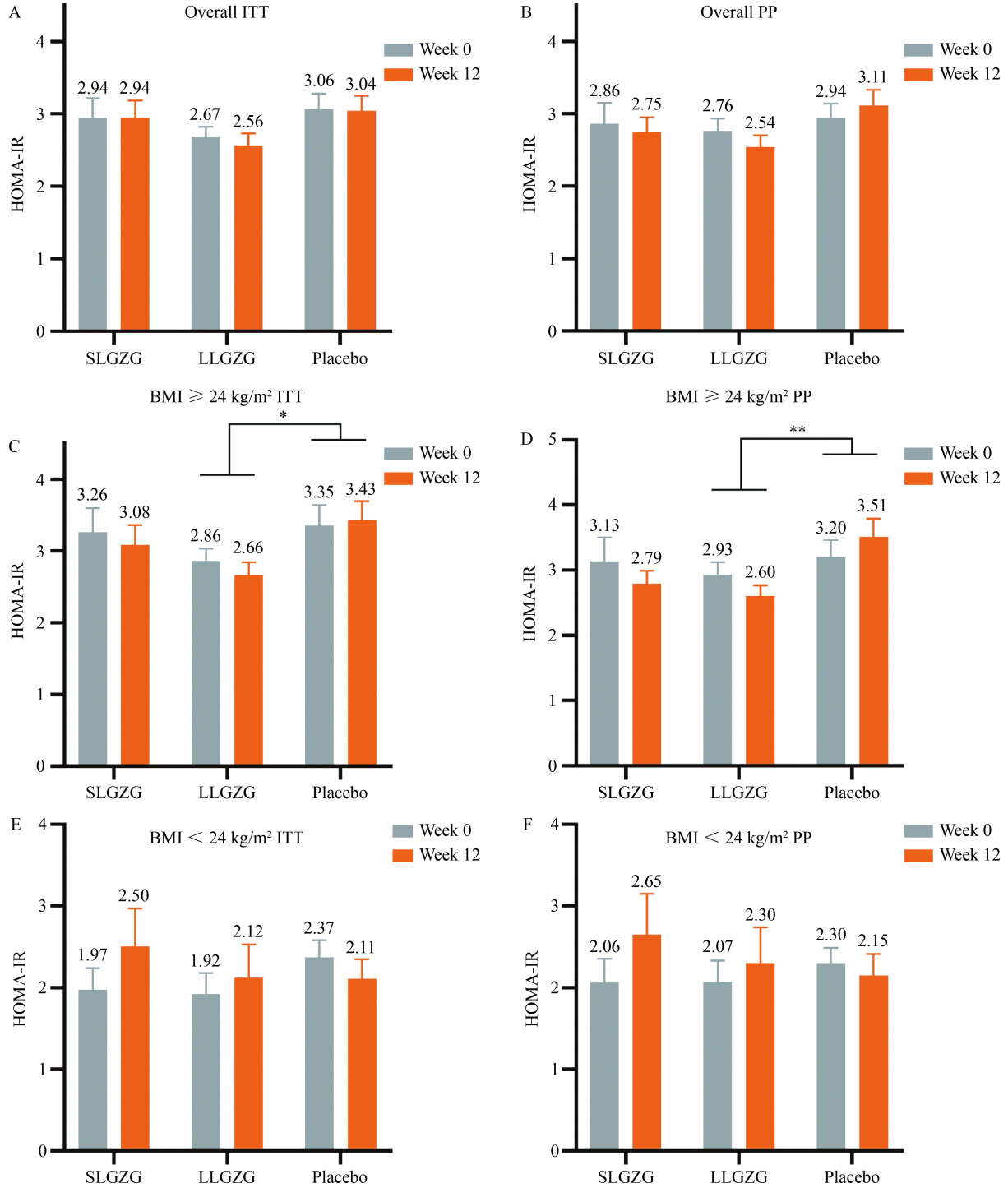

Fig. 3 Change in HOMA-IR after treatment. (A) Overall population, ITT analysis; (B) Overall population, PP analysis; (C) BMI ⩾ 24 kg/m^2 population, ITT analysis; (D) BMI ⩾ 24 kg/m^2 population, PP analysis; (E) BMI < 24 kg/m^2 population, ITT analysis; (F) BMI < 24 kg/m^2 population, PP analysis. LLGZG versus placebo, $^*P < 0.05$, $^{**}P < 0.01$. BMI, body mass index; HOMA-IR, homeostasis model assessment of insulin resistance; ITT, intention-to-treat; LLGZG, low-dose Lingguizhugan Decoction; SLGZG, standard-dose Lingguizhugan Decoction; PP, per-protocol.

further investigation. In addition, according to the fingerprint spectrum of LGZG in a previous study [18], the active ingredients of LGZG mainly included liquiritin, cinnamic acid, cinnamaldehyde, glycyrrhizic acid, and atractylenolide III. Although studies have reported that cinnamic acid, cinnamaldehyde, and glycyrrhizic acid could regulate IR [52–55], the association between active ingredients and DNA 6mA level, especially 6mA levels

Table 3 Comparison of treatment effect of changes in BMI, lipid metabolism, and glucose metabolism from baseline to 12 weeks (ITT analysis)

Variable	Population	Groups			Three groups *P* value
		SLGZG	LLGZG	Placebo	
BMI (kg/m^2)	Overall	0.26 (0.57)	0.14 (0.93)	0.15 (0.85)	0.912
	BMI ⩾ 24 kg/m^2	0.32 (0.60)	0.07 (0.92)	0.17 (0.92)	0.453
	BMI < 24 kg/m^2	0.07 (0.40)	0.43 (0.95)	0.08 (0.65)	0.345
Lipid metabolism					
TC (mmol/L)	Overall	0.20 (0.65)	0.14 (0.68)	0.16 (0.61)	0.735
	BMI ⩾ 24 kg/m^2	0.31 (0.59)	0.18 (0.62)	0.14 (0.66)	0.445
	BMI < 24 kg/m^2	−0.16 (0.70)	−0.02 (0.89)	0.21 (0.48)	0.197
TG (mmol/L)	Overall	−0.01 (1.08)	−0.18 (0.58)	−0.30 (1.07)	0.204
	BMI ⩾ 24 kg/m^2	0.01 (1.22)	−0.17 (0.61)	0.08 (0.16)	0.145
	BMI < 24 kg/m^2	−0.08 (0.47)	−0.26 (0.47)	−0.18 (0.65)	0.915
LDL-C (mmol/L)	Overall	0.18 (0.66)	0.19 (0.68)	0.20 (0.54)	0.940
	BMI ⩾ 24 kg/m^2	0.26 (0.62)	0.22 (0.60)	0.21 (0.58)	0.979
	BMI < 24 kg/m^2	−0.08 (0.73)	0.06 (0.97)	0.15 (0.43)	0.924
HDL-C (mmol/L)	Overall	0.09 (0.16)	0.05 (0.15)	0.09 (0.17)	0.251
	BMI ⩾ 24 kg/m^2	0.10 (0.16)	0.06 (0.14)	−0.36 (1.21)	0.419
	BMI < 24 kg/m^2	0.05 (0.13)	0.01 (0.20)	0.10 (0.17)	0.238
ApoA1 (g/L)	Overall	0.11 (0.11)	0.08 (0.11)	0.09 (0.13)	0.405
	BMI ⩾ 24 kg/m^2	0.11 (0.12)	0.09 (0.11)	0.09 (0.13)	0.419
	BMI < 24 kg/m^2	0.08 (0.09)	0.01 (0.12)	0.11 (0.15)	0.080
ApoB (g/L)	Overall	0.04 (0.19)	0.04 (0.20)	0.05 (0.16)	0.938
	BMI ⩾ 24 kg/m^2	0.07 (0.18)	0.04 (0.18)	0.05 (0.17)	0.626
	BMI < 24 kg/m^2	−0.04 (0.19)	0.02 (0.27)	0.03 (0.14)	0.505
Glucose metabolism					
FPG (mmol/L)	Overall	0.04 (0.61)	−0.03 (0.54)	0.09 (0.49)	0.397
	BMI ⩾ 24 kg/m^2	0.04 (0.65)	−0.04 (0.54)	0.10 (0.48)	0.418
	BMI < 24 kg/m^2	0.07 (0.47)	0.05 (0.59)	0.07 (0.52)	0.997
FINS (μU/L)	Overall	−0.14 (7.60)	−0.48 (5.35)	−0.33 (6.62)	0.322
	BMI ⩾ 24 kg/m^2	−0.74 (7.95)	−0.72 (5.83)	0.09 (7.48)	0.048
	BMI < 24 kg/m^2	1.68 (6.25)	0.51 (2.52)	−1.34 (3.85)	0.177
HbA1c (%)	Overall	0.02 (0.18)	0.00 (0.22)	0.08 (0.22)	0.008
	BMI ⩾ 24 kg/m^2	0.03 (0.17)	−0.01 (0.22)	0.06 (0.23)	0.055
	BMI < 24 kg/m^2	−0.02 (0.20)	0.06 (0.24)	0.13 (0.19)	0.027

Data are presented as the mean (SD). *P* values were obtained using ANOVA or Kruskal–Wallis test. ApoA1, apolipoprotein A1; ApoB, apolipoprotein B; AST, aspartate transaminase; BMI, body mass index; FINS, fasting insulin; FPG, fasting plasma glucose; HbA1c, glycosylated hemoglobin; HDL-C, high-density lipoprotein cholesterol; ITT, intention-to-treat; LDL-C, low-density lipoprotein cholesterol; LLGZG, low-dose Lingguizhugan Decoction; SLGZG, standard-dose Lingguizhugan Decoction; TC, total cholesterol; TG, triglyceride.

of PPP1R3A and ATG3, was not reported. In the future studies, the relationship between active ingredients and the 6mA levels of PPP1R3A and ATG3 could be further explored.

Interestingly, the beneficial effect of LGZG was not repeated in lean subjects with NAFLD. Based on DNA 6mA analysis, the modification levels of PPP1R3A and ATG3 were similar between overweight/obese and lean subjects with NAFLD. However, 12 weeks of LGZG treatment could only significantly regulate the modification in overweight/obese population, without obvious effect on lean subjects. That is to say, some unknown mechanisms may interfere the regulation of LGZG in lean NAFLD, thus deserving further exploration in future studies. This finding also partially suggested that lean patients with NAFLD may possess another pathogenic trait that is different from IR.

The results from this study were inspiring. However, some confounders should be noticed in the result interpretation. Dietary intervention and exercise are the

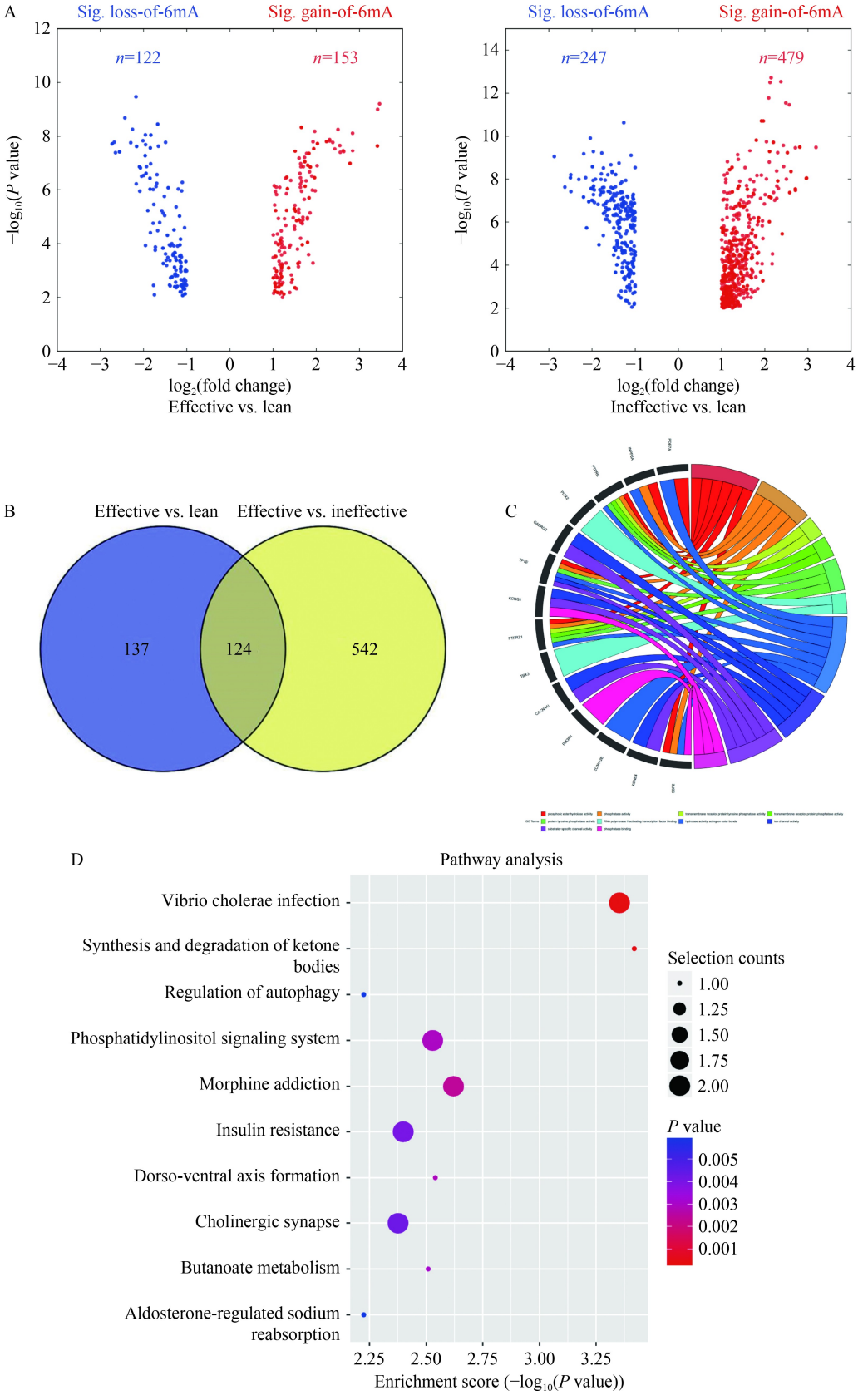

Fig. 4 Functional analysis of differentially methylated genes. (A) Numbers of differentially methylated sites in pairwise comparisons. (B) Overlapped differentially methylated genes. (C, D) GO and KEGG pathway analysis of differentially methylated genes.

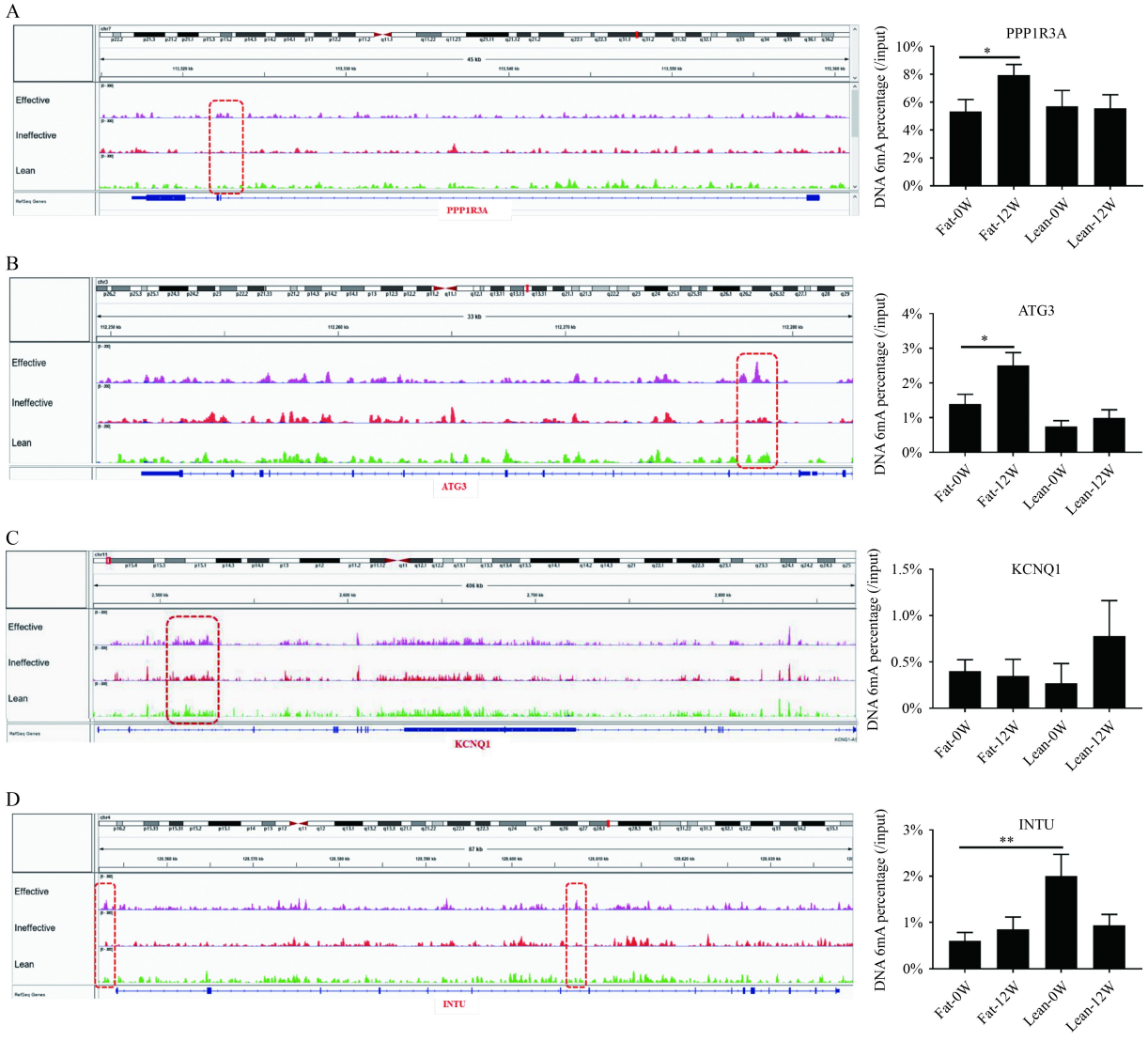

Fig. 5 DNA 6mA levels in (A) protein phosphatase 1 regulatory subunit 3A, (B) autophagy related 3, (C) potassium voltage-gated channel subfamily Q member 1, and (D) inturned planar cell polarity protein.

foundation of NAFLD management [10,11]. Throughout the present study, participants were required to follow the suggestions for lifestyle modification. Based on the execution status, most of the participants obeyed the rules. Although the effect of lifestyle modification seemed insignificant, the positive results should be determined as a combination of LGZG intervention and lifestyle modification. Besides, to achieve comparable taste and smell of placebo, 10.0% SLGZG was added in the placebo. Though the doses of herbs were not supposed to generate any therapeutic effect [25], it was actually not a "real" placebo. In future, a waitlist-control design may be considered. The introduction of waitlist control could enable the estimation of the confounding effect of doped herbs and be beneficial to the accuracy of efficacy evaluation.

This clinical trial had several inherent limitations. First, the severity of NAFLD in the study population was relatively mild. The effects of LGZG may not be fully revealed. Further trials could revise the eligibility criteria and include participants with NAFLD with abnormal transaminases or patients with confirmed NASH. Second, the sample size calculation was based on SLGZG and placebo. Based on the results, the effect estimation of placebo was reasonable, but the effect size of LGZG had a discrepancy. This trial indicated that low-dose LGZG showed enhanced efficacy in NAFLD treatment. Future studies should calculate the sample size on the basis of the effect of LLGZG. Besides, the lifestyle modification was based on health education. No fixed recipe or exercise styles was prescribed, and the execution mostly relied on the participants' self-consciousness, thus may have induced potential bias. In addition, the mean age of the included patients was close to 60 years, and only Chinese were recruited. The treatment response may not

be comparable to younger patients. The concept of TCM pattern was introduced in the screening of participants. Although TCM pattern was beneficial in the selection of specific patients, clinicians without a TCM background may be confused about the indications for LGZG.

In conclusion, LLGZG effectively improved IR in obese subjects with NAFLD, and it had a satisfactory safety profile. The DNA 6mA levels of PPP1R3A and ATG3 may serve as the effector of LLGZG in improving IR. LLGZG may be an alternative choice in NAFLD management. Further studies should extend the intervention period and use rigid outcomes to establish higher-level evidence of the efficacy of LGZG.

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Compliance with ethics guidelines

Liang Dai, Jingjuan Xu, Baocheng Liu, Yanqi Dang, Ruirui Wang, Lijie Zhuang, Dong Li, Lulu Jiao, Jianying Wang, Lei Zhang, Linda L.D. Zhong, Wenjun Zhou, and Guang Ji declare that they have no conflict of interest. The study protocol was approved by the Medical Ethics Committee of Longhua Hospital Affiliated to Shanghai University of Traditional Chinese Medicine (No. 2017LCSY069) and registered at the Chinese Clinical Trial Registry (No. ChiCTR1800014364) on January 8, 2018. All three health centers acknowledged the ethical approval from the Medical Ethics Committee of Longhua Hospital. This trial was conducted strictly following the *Helsinki Declaration*. All participants provided a written inform consent before entering the study.

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