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Randomized Control Trials

Effect of green mediterranean diet on serum folate and its interaction with genetic variation in folate metabolism: The DIRECT PLUS 18-month dietary randomized controlled trial

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SUMMARY

Background & Aims: Folate metabolism can be regulated by diet and genetic variation, particularly Methylenetetrahydrofolate reductase (MTHFR) rs1801133 polymorphism. We explored the effect of plant-based green-Mediterranean (green-MED) diet on serum folate and whether this effect varies by genetic variation in folate metabolism over 18-months

Methods: In the DIRECT-PLUS trial, 294 participants were randomized to healthy dietary guidelines (HDG), MED, or plant-based green-MED diets. Both isocaloric MED groups consumed walnuts (28g/day), while green-MED group also avoided red/processed meat, consumed Wolffia-globosa Mankai green shake (500 ml/day), and green–tea (3–4 cups/day). MRI assessed visceral adipose tissue (VAT). MTHFR rs1801133 single-nucleotide-polymorphism (SNP), and folate-pathway mRNA expression were evaluated.

Results: Participants (age = 50.1years; 90.6% men; body-mass-index = 31.4 kg/m^2; serum folate = 7.6 ng/mL; rs1801133 frequencies: CC = 38.5%; CT = 49.8%; TT = 11.7%) had 89% 18-month retention rate. Folate deficiency at baseline was rare (~3%, n = 8), indicating effects within the normal range. After 18-months of intervention, the green-MED diet significantly increased serum folate (+1.2 ng/mL) compared to MED (+0.41 ng/mL) and HDG diets (+0.1 ng/mL), $p < 0.05$ between groups, with higher Mankai intake strongly associated with folate elevation ($p = 0.003$). rs1801133 TT-genotype carriers had lower folate at baseline ($p = 0.037$) and post-intervention ($p = 0.04$; recessive mode-of-

Abbreviations: ALT, Alanine aminotransferase; ANOVA, Analysis of variance; AST, Aspartate aminotransferase; BMI, Body mass index; BP, Blood pressure; CBS, Cystathionine beta-synthase; CV, Coefficient of variation; CVD, Cardiovascular disease; DHFR, Dihydrofolate reductase; DIRECT-PLUS, Dietary Intervention Randomized Controlled Trial Polyphenols Unprocessed Study; DRIs, Dietary reference intakes; ECLIA, Electrochemiluminescence immunoassay; ELISA, Enzyme-linked immunosorbent assay; FRS, Framingham risk score; GAE, Gallic acid equivalents; HDG, Healthy dietary guidelines; HOMA-IR, Homeostatic model assessment of insulin resistance; hsCRP, High-sensitivity C-reactive protein; IL-6, Interleukin 6; MED, Mediterranean diet; MET, Metabolic equivalent; MRI, Magnetic resonance imaging; MRS, Magnetic resonance spectroscopy; MTHFD1, Methylenetetrahydrofolate dehydrogenase 1; MTHFD2, Methylenetetrahydrofolate dehydrogenase 2; MTHFR, Methylenetetrahydrofolate reductase; MTR, Methionine synthase; MTRR, Methionine synthase reductase; PA, Physical activity; PUFA, Polyunsaturated fatty acids; RCT, Randomized controlled trial; SNP, Single-nucleotide polymorphism; TG, Triglycerides; THF, Tetrahydrofolate; VAT, Visceral adipose tissue; WC, Waist circumference.

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inheritance), while CC/CT-genotype carriers showed the highest folate increase following the green-MED intervention ($p = 0.03$). TT-genotype carriers showed lower baseline *MTHFD2* mRNA expression ($p = 0.005$), but an enriched transcription, compared to CC/CT-carriers post-intervention ($p = 0.001$). Mankai intake interacted with rs1801133 genotype on VAT ($p = 0.028$) and Framingham risk score ($p = 0.024$), with CC/CT-carriers showing dose–response benefits.

Conclusions: The green-MED diet, enriched with Mankai, significantly increased serum folate compared with the MED and HDG diets. The response differed by MTHFR rs1801133-genotype, with CC/CT carriers showing greater increases in folate. These findings support a gene–diet interaction with potential implications for visceral adiposity and cardiometabolic risk.

1. Introduction

Folate, naturally found in green leafy vegetables and legumes, and also consumed as folic acid through fortified foods, is essential not only for DNA synthesis but also for its involvement in complex metabolic pathways, regulatory mechanisms, and disease prevention [1–9]. Insufficient folate concentrations, influenced by dietary patterns, genetic variation (e.g., Methylenetetrahydrofolate reductase (*MTHFR*) polymorphisms), and lifestyle factors, can lead to elevated homocysteine concentrations, increasing the risk of cardiovascular disease (CVD) [10–12]. Conversely, a diet high in folate-rich foods has been associated with lower metabolic syndrome scores, reduced inflammation, enhanced neurocognitive function [5], and a lower risk of all-cause mortality and CVD mortality [13], suggesting potential mechanisms through which plant-based diets might influence health outcomes. While traditional Mediterranean (MED) diets are known for their high plant-based content and associated health benefits [14], the relationship between specific plant-based dietary patterns, serum folate concentrations, and cardiometabolic outcomes remains incompletely understood, particularly in the context of long-term dietary interventions.

Genetic variations, particularly single-nucleotide polymorphisms (SNPs) in genes encoding folate-metabolizing enzymes, can significantly influence folate metabolism and bioavailability [15]. The rs1801133 polymorphism in the *MTHFR* gene is of particular interest due to its prevalence and impact on folate metabolism [15]. Genome-wide association studies have demonstrated that this genetic variant has been associated with altered folate concentrations and potentially modified responses to dietary interventions [16], suggesting that genetic factors may influence the effectiveness of dietary approaches to optimize folate status and associated health outcomes [17].

The current study is a secondary analysis of the DIRECT PLUS trial. We investigated the effect of a green MED diet, enriched in plant-based proteins and polyphenols and low in red meat, on serum folate concentrations and their interaction with folate-related genetics, and cardiometabolic parameters over an 18-month dietary randomized controlled trial (RCT). We hypothesized that the green-MED diet would lead to increased serum folate concentrations compared to traditional MED and healthy dietary guidelines and that these changes would be influenced by rs1801133 genotype status, potentially affecting metabolic outcomes through altered folate metabolism pathways.

2. Materials and methods

2.1. Study design

The Dietary Intervention Randomized Controlled Trial Polyphenols Unprocessed Study (DIRECT-PLUS) trial (Clinical-trials-identifier:NCT03020186), initiated in May 2017, was conducted in an isolated workplace, where a monitored lunch was provided. Of 378 volunteers, 294 met inclusion criteria: 30+ years of age with abdominal obesity [waist-circumference(WC): men>102 cm, women>88 cm] or dyslipidemia [triglycerides (TG) > 150 mg/dL and high-density-lipoprotein-cholesterol(HDL-c): men≤40 mg/dL, women:≤ 50 mg/dL] [18,19]. Exclusion criteria included an inability to partake in physical activity (PA), a serum creatinine concentration≥2 mg/dL, disturbed liver function, a major illness that might require hospitalization, pregnancy or lactation, presence of active cancer or chemotherapy treatment within the past three years, participation in another trial, current treatment with warfarin (due to the interaction with vitamin K), and implants that would preclude magnetic resonance imaging.

The Soroka University Medical Centre Ethics Board and the Institutional Review Board approved the study protocol for the DIRECT PLUS trial. All participants provided written informed consent and received no financial compensation.

2.2. Randomization and intervention

Participants who completed baseline measurements were randomly assigned to one of three intervention groups (1:1:1 ratio), stratified by sex and working sites: healthy dietary guidelines (HDG); MED diet; or green-MED diet; all included PA recommendations (Supplemental Data 1), with a free gym membership and educational sessions promoting moderate-intensity PA [18] with ~80% aerobic content. Dietary and PA interventions are fully described in Supplemental Table 1. Randomization was conducted in a single phase, as the interventions were conducted simultaneously, and participants were aware of their assigned intervention (open-label protocol). The HDG group received basic health-promotion guidelines to achieve a healthy diet. The MED group was instructed to follow a calorie-restricted traditional MED diet, low in simple carbohydrates, similar to the DIRECT [14] and CENTRAL [20] trials. Both MED and green-MED diets were equally calorie-restricted (men:1500–1800 kcal/day; women:1200–1400 kcal/day); ~40% of total fat was mainly from polyunsaturated-fatty-acids (PUFA) and monounsaturated-fatty-acids (MUFA), and consisted of less than 40gr/day carbohydrates in the first two months with increased gradual intake up to 80 gr/day. In addition, both MED groups included 28g/day of walnuts (containing ~440 mg polyphenols/day; gallic-acid-equivalents (GAE), including mostly ellagitannins, ellagic acid, and its derivatives, Phenol-Explorer database). Participants from the green-MED diet were instructed to avoid red and processed meat and were guided to consume: 3–4 cups/day of green tea and 100g of frozen Wolffia-globosa (Mankai cultivated strain) [19,21–23] plant cubes. We guided the participants to prepare a green Mankai shake with additional ingredients, part of the diet regimen (fruits, walnuts, or vegetables) each evening. The green protein shake was partially substituted for dinner, replacing beef/poultry protein sources (Supplemental Data 2). In total, both MED diets had the

Table 1
Baseline characteristics of the DIRECT-PLUS study population[a] (n = 266).

	HDG (n = 91)	MED (n = 90)	Green-MED (n = 85)	Entire (n = 266)
Men, % of study population	82 (90.1%)	81 (90.0%)	78 (91.8%)	266 (90.6%)
N of rs1801133 genotypes absolute	28/45/7	35/35/5	26/35/15	89/115/27
and % (CC/CT/TT)	35.0%/56.3%/8.7%	46.7%/46.7%/6.6%	34.2%/46.0%/19.8%	38.5%/49.8%/11.7%
Age, years	50.7 (10.3)	50.4 (9.7)	49.3 (10.0)	50.1 (10.0)
BMI, kg/m2	31.3 (3.8)	31.2 (3.9)	31.5 (4.3)	31.4 (4.0)
WC, cm				
Men	111.0 (10.2)	110.8 (9.6)	110.7 (8.2)	110.9 (9.3)
Women	103.2 (7.3)	103.1 (9.5)	101.3 (12.3)	102.6 (9.3)
Blood pressure, mm Hg				
Diastolic	80.3 (11.7)	82.0 (8.8)	81.2 (9.6)	81.2 (10.1)
Systolic	130.0 (14.6)	129.8 (12.8)	129.4 (14.7)	129.7 (14.0)
Blood biomarkers				
Serum LDL cholesterol, mg/dL	127.6 (32.6)	126.5 (31.4)	123.9 (28.9)	126.1 (31.0)
Serum HDL cholesterol, mg/dL				
Men	43.4 (9.4)	45.8 (10.0)	43.3 (10.6)	44.2 (10.0)
Women	57.3 (11.2)	48.9 (9.3)	58.5 (12.3)	54.6 (11.3)
Fasting glucose, mg/dL	101.6 (16.1)	100.4 (13.4)	102.9 (20.2)	101.6 (16.7)
Fasting insulin, μIU/mL	15.6 (9.0)	14.6 (7.2)	14.5 (7.5)	14.9 (7.9)
Anemia biomarkers				
Serum folate, ng/mL	7.5 (3.0)	7.9 (3.0)	7.3 (2.8)	7.6 (2.9)
Folate deficiency (%)	2 (2.2%)	3 (3.3%)	3 (3.5%)	8 (3.0%)
B12 vitamin, pg/mL	397.9 (157.0)	410.4 (152.4)	382.5 (124.2)	397.2 (145.6)
Ferritin, ng/mL	162.3 (112.1)	181.4 (119.9)	165.7 (110.4)	169.9 (114.2)
Transferrin, mg/dL	269.1 (35.2)	270.2 (35.5)	263.6 (29.9)	267.7 (33.7)
Hemoglobin, g/dL	14.9 (1.1)	14.9 (1.0)	15.0 (1.2)	14.9 (1.1)

[a] Out of 294 participants, 28 were excluded from the baseline analysis due to the intake of vitamin B complex, B12 vitamin, folic acid, or multivitamins. Values are presented as Mean (standard deviations) for continuous variables and as numbers (%) for categorical variables. BMI, body mass index; HDG, healthy dietary guidelines; HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol; MED, Mediterranean; WC, waist circumference. Genotype data was available for 231 samples.

same calorie restriction. Green tea and Mankai provided an additional daily intake of ~800 mg polyphenols [18] [GAE, Phenol-Explorer, and Eurofins laboratory analysis] beyond the polyphenol content in the MED diet. Participants received green tea, walnuts, and Mankai onsite, free of charge. The participants were instructed to follow their lifestyle intervention for 18 months. The lifestyle interventions included 90-min nutritional and PA sessions in the workplace with multidisciplinary guidance (physicians, clinical dietitians, and fitness instructors). These sessions were held every week during the first month, once a month over the following five months, and every other month until the 18th month. All lifestyle educational programs were provided at the same intensity to all three groups. Text messages with relevant information for each assigned intervention group were sent at fixed time intervals to keep the participants motivated. In addition, a website listing all nutritional and PA information needed for the participants was accessible to them according to their intervention group. Most clinical and medical measurements, as well as lifestyle-intervention sessions, were conducted onsite. We assessed adherence by self-reported dietary intake and lifestyle habits assessment tool, using validated food-frequency questionnaires at baseline, after 6- and 18 months, including PA, measured in metabolic equivalent (MET) units. The questionnaire includes 127 food items and 3 portion size pictures for 17 selected food items, and a PA questionnaire. In addition, the participant's closed workplace enabled monitoring the freely provided lunch and the presence of an onsite clinic. A detailed description of the provided foods is reported in Supplemental Data 2.

2.3. Wolffia globosa duckweed

A specific strain of Wolffia globosa, an aquatic plant in the duckweed family, is characterized by high protein content (more than 45% of the dry matter) and the presence of 9 essential and 6 conditional amino acids [21]. The Mankai plant is rich in insoluble fibers, vitamins (including vitamin B12 and folate), and minerals (including iron and zinc). The daily Mankai shake contributed 18% of the total protein recommended in the Dietary reference intakes (DRIs) for men, 5.7% of carbohydrates, and 2.4% of fat [24]. The vitamin contribution of Mankai (as a percentage of men's DRIs) was 61% of folate, 49% of vitamin A, 21% of vitamin B-12, and 26% of vitamin E. The mineral contribution of the Mankai shake was 168% for iron, and 35% for zinc (as percentages of men's DRIs) [25]. The Wolffia globosa supplied for this study was grown under optimized controlled conditions; thus, it was considered free of heavy metal contamination, as confirmed by laboratory analyses.

2.4. Outcome measures

2.4.1. Clinical parameters and fasting blood biomarkers

Measurements were taken at baseline, 6, and after 18 months of intervention. Height was measured to the nearest millimeter using a standard wall-mounted stadiometer. Bodyweight was measured without shoes to the nearest 0.1 kg. WC was measured halfway between the last rib and the iliac crest to the nearest millimeter by standard procedures using an anthropometric measuring tape. Two blood pressure (BP) measurements were recorded after resting using an automatic BP monitor (Accutorr-4, Datascope); the mean of the two was calculated. We used magnetic-resonance-imaging (MRI) to quantify abdominal adipose tissue and magnetic-resonance-spectroscopy (MRS) to measure intrahepatic fat percentage over 18 months [18,26].

2.5. Laboratory methodology

Blood and urine samples were taken at times 0, 6, and 18 months after a 12-h fast. Blood samples were centrifuged, and both blood and urine samples were stored at −80 °C. Serum folate was measured by the electrochemiluminescence immunoassay (ECLIA). Biochemical deficiency has been defined as a concentration of less than 3 ng/mL of serum folate [27]. Serum total cholesterol (coefficient-of-variation (CV), 1.3%), HDL-c, low-

density-lipoprotein-cholesterol (LDL-c), and TG (CV, 2.1%) were determined enzymatically with a Cobas 8000 automatic analyzer (Roche). Plasma concentrations of high-sensitivity C-reactive protein (hsCRP) were measured by a Tina-quant® hsCRP assay from Roche. Interleukin 6 (IL-6) was measured by high-sensitive Enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol. Plasma glucose concentrations were measured by Roche GLUC3 (hexokinase method). Plasma insulin concentrations were measured with a Roche Elecsis assay. The homeostatic model of insulin resistance (HOMA-IR) was calculated as follows: insulin (μIU/ml) × glucose (mg/dl)/405). All biochemical analyses were performed at the University of Leipzig, Germany.

2.6. Genotyping

The SNP variant rs1801133 (chr1:11796321; GRCh38), a well-characterized missense variant within the *MTHFR* locus, was de novo genotyped in the DIRECT-PLUS trial, including in total N = 266 participants who had available EDTA blood samples and gave written informed consent for genetic testing. To do so, we isolated genomic DNA from 200 μl EDTA blood samples using the QIAmp DNA Blood Midi Kit (Qiagen Inc., Valencia, CA, USA), according to the manufacturer's instructions. SNP genotyping was performed using the Allelic Discrimination TaqMan SNP Genotyping System (Applied Biosystems, Life Technologies, Carlsbad, CA, USA). Fluorescence signals were detected using an ABI 7500 Real-Time PCR system. To ensure data accuracy and exclude genotyping errors, 5% of all samples were randomly re-genotyped, with all results matching the initial genotypes. Additionally, water was used as a non-template control (N = 6 per run). The rs1801133 SNP was in line with Hardy–Weinberg equilibrium ($P > 0.05$). In accordance with standard genetic analysis practices, we evaluated additive, dominant, and recessive inheritance models for rs1801133. The recessive model (TT vs. CT/CC) was prioritized for presentation based on previous literature [28], reporting stronger associations between rs1801133 and essential hypertension risk under a recessive inheritance model compared with the dominant model, supporting the biological relevance of the recessive mode of inheritance.

2.7. mRNA

RNA from blood-derived samples, collected before and after the intervention, was isolated, sequenced, quality controlled, and processed as described previously [29]. For this study, we extracted normalized mRNA expression values for specific genes of the methionine and folate metabolism, including *MTHFR*, Methionine synthase reductase *(MTRR)*, Methionine synthase *(MTR)*, Cystathionine beta-synthase *(CBS)*, Dihydrofolate reductase *(DHFR)*, Methylenetetrahydrofolate dehydrogenase 1 (*MTHFD1)*, and Methylenetetrahydrofolate Dehydrogenase 2 (*MTHFD2)*.

2.8. Statistical analysis

The primary endpoint of the DIRECT-PLUS trial was changes in adiposity and ectopic fat depots, as previously reported. Serum folate concentrations and related genetic analyses were evaluated as secondary outcomes. The pre-specified primary outcome for this analysis was change in serum folate at 18 months between diet groups, excluding participants on B vitamins/folate supplements. Absolute serum folate concentrations at 18 months were also examined. Secondary analyses included genotype associations, genotype–diet interactions, and exploratory Mendelian randomization analyses. General and generalized linear regression models were used for adjustments and two-way interaction models (the specific adjustments are detailed in the Results section). For serum folate change over time and across dietary groups, generalized linear models were used to assess between-group differences, adjusted for age, sex, and weight loss or WC. To account for multiple testing, false discovery rate (FDR) correction was applied to the genotype–diet interaction analyses using the Benjamini–Hochberg method. Genotype diet interaction analyses were considered exploratory and hypothesis-generating. To explore the association between serum folate and cardiometabolic outcomes, we conducted an exploratory Mendelian randomization analysis using the MTHFR rs1801133 genotype as a single instrumental variable. This functional SNP influences folate metabolism and serum folate concentrations, and we applied a two-stage least squares approach with a single instrument. In the first stage, serum folate concentrations at 18 months were regressed on rs1801133 genotype (recessive model: TT vs. CC/CT), adjusted for age, sex, intervention group, and weight change. In the second stage, genetically predicted serum folate values were used to estimate associations with cardiometabolic outcomes. The validity of the Mendelian randomization analysis relies on three key assumptions. First, the relevance assumption is supported by the observed association between the MTHFR rs1801133 variant and serum folate concentrations ($p = 0.003$; F-statistic = 8.77). Second, the independence assumption requires that the genetic variant is not associated with potential confounders; although this cannot be directly tested, no strong evidence suggests such associations. Third, the exclusion restriction assumption requires that the genetic variant influences the outcome only through serum folate; while this cannot be formally verified, pleiotropic effects cannot be excluded. Missing data were assumed to be missing at random. Primary analyses were conducted using complete-case data. As a sensitivity analysis, multiple imputation (20 imputations) was performed for serum folate, VAT, and Framingham risk score. Continuous variables are presented as mean ± SD. Nominal variables are expressed as numbers and percentages. The Kolmogorov–Smirnov test was used to determine whether variables were normally distributed, and natural log transformations were applied when needed to achieve normal distributions. Differences in values over time were examined using a paired-sample t-test or Wilcoxon test for 18-month changes or, for the three-time points, using Analysis of variance (ANOVA) for repeated measures. Differences across groups were tested using ANOVA, the Kruskal–Wallis test, or the $\chi 2$ statistic. Multiple comparisons were adjusted using the Tukey post hoc test (for ANOVA) and Bonferroni correction (for Kruskal–Wallis). Correlations were tested using Spearman's or Pearson's correlation analysis. The Kendall Tau correlation was used to examine the trends across ordered categories. Out of 294 participants, 28 were excluded from the baseline analysis due to the intake of vitamin B complex, B12 vitamin, folic acid, or multivitamins. Similarly, 14 participants were excluded at time point 18 for the same reason. For the genetic analysis, a final number of 231 participants without supplementation and with available genotype data were included. mRNA expression analyses were performed in the subset of participants with available transcriptomic data and corresponding genotype information (n = 144). Sample sizes differed across analyses according to the availability of genotype data, phenotype measurements, follow-up data, and covariates required for the specific statistical models, after exclusion of participants using vitamin B complex, folic acid, or multivitamin supplements. SNP analysis was performed according to three inheritance models for the MTHFR rs1801133 polymorphism: additive (TT vs. CT vs. CC), dominant (TT/CT vs. CC), and recessive (TT vs. CT/CC). Phenotypic characteristics according to the different genotypes were assessed using non-parametric

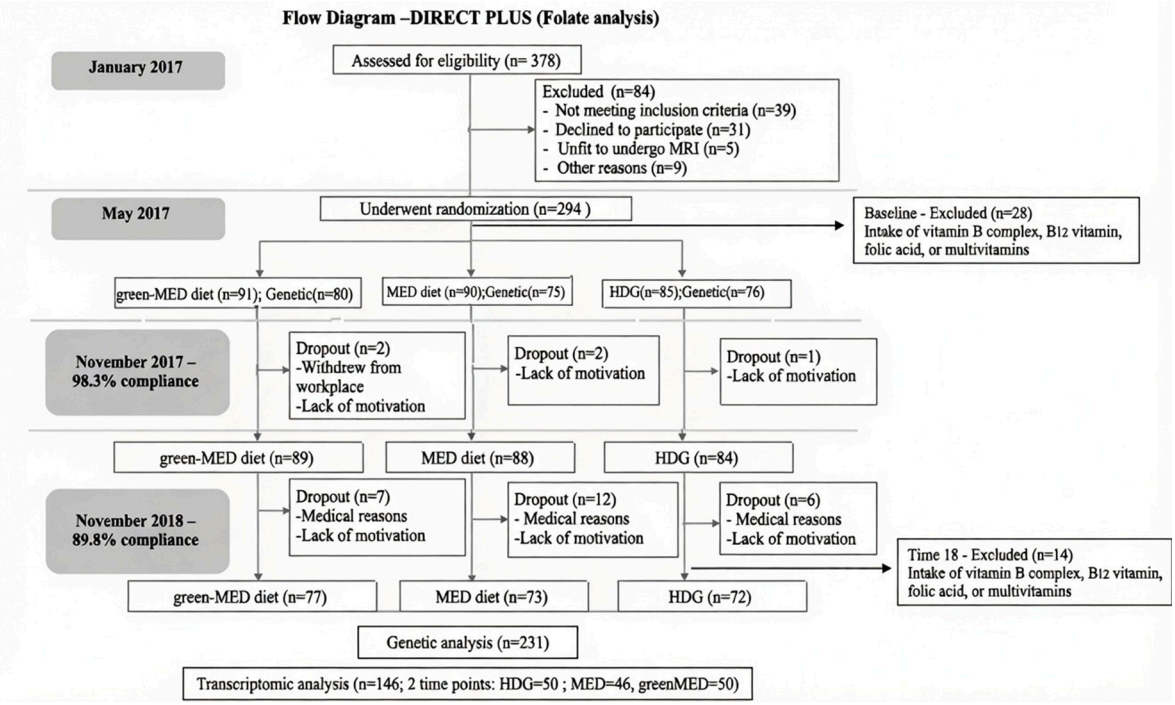

Fig. 1. DIRECT PLUS flow chart.

tests (additive: Kruskal–Wallis; dominant/recessive: Mann–Whitney U). Sample size calculations are reported in Supplemental Data 4. Statistical significance was set at a two-sided $\alpha = 0.05$. All statistical analyses were performed using R version 3.5 or SPSS Statistics software version 29 for the genetic analysis.

3. Results

3.1. Baseline characteristics

The flow diagram is shown in Fig. 1. Participants (age = 31.4–75.9 years; 90.6% men; Body mass index (BMI) = 31.4 kg/m^2; mean serum folate = 7.6 ng/mL) had an 89% 18-month retention rate (Table 1). Of the 294 randomized participants, 266 remained eligible for serum folate analyses after exclusion of participants using vitamin B complex, folic acid, or multivitamin supplements. Among these, 231 participants had available rs1801133 genotype and phenotype data and were included in genotype-based analyses. At the 18-month follow-up, 230 participants had available genotype and follow-up folate measurements, whereas adjusted complete-case multivariable analyses included 222 participants with complete covariate data. The DIRECT-PLUS participants had a baseline folate range of 2.4–20.0 ng/mL, with 8 participants (3%) having folate deficiency [27](Table 1). Serum folate, B12 vitamin, ferritin, transferrin, and hemoglobin at baseline were not different across the 3 intervention groups ($p > 0.05$; Table 1). The rs1801133 genotype frequencies were CC = 38.5%; CT = 49.8%; TT = 11.7% (minor allele frequency = 0.37). Baseline characteristics were comparable between participants included in the mRNA expression analyses, the genotyped subgroup (rs1801133), and the full cohort, with no statistically significant differences observed across the variables (Supplemental Table 2). Additional analyses examining the association between baseline folate and changes in liver enzymes are presented in Supplemental Table 3 and Supplemental Fig. 1. Phenotypic characteristics according to the different genotypes (N/%: CC = 89/38.5%; CT = 115/49.8%; TT = 27/11.7%) are presented in Supplemental Table 4. The sample sizes presented by endpoint, time point, intervention group, and genotype can be found in Supplemental Table 5.

3.2. Eighteen-month dietary intervention effect on folate concentrations

After 18 months of intervention, changes in serum folate were as follows: The green-MED diet significantly increased serum folate concentrations as compared to baseline ($p < 0.05$), and the change (+1.2 ng/mL) was found significantly higher as compared to the HDG (+0.1 ng/mL) and MED (+0.41 ng/mL) diets after 18 months adjusted for age and sex (green-MED vs HDG:$p = 0.002$, green-MED vs MED $p = 0.021$; $n = 222$). These differences were significant after adjustment to age, sex, and weight or WC loss (WC: green-MED vs HDG:$p = 0.006$, green-MED vs MED $p = 0.03$; weight-loss: green-MED vs HDG:$p = 0.02$, green-MED vs MED $p = 0.04$) (Fig. 2). A greater intake of Mankai was a significant marker of higher serum folate concentrations; Participants who consumed Mankai more than three times per week experienced a significantly greater increase in folate concentrations compared to those who did not consume Mankai at all ($p = 0.003$; Fig. 3). This association remained significant after adjusting for age, sex, and weight loss, as well as after further adjustment for age, sex, weight loss, and group variables ($p < 0.05$). An 18-month increase in serum folate was associated with improved HOMA-IR ($\beta = -0.326$; $p < 0.001$), reduced TG/HDLc ratio ($\beta = -0.188$; $p = 0.017$), and decreased IL-6 concentrations ($\beta = -0.151$; $p = 0.024$; multivariate models adjusted for age, sex, intervention groups, and 18-month weight change; Table 2; Supplemental Table 6). Increased folate concentrations were associated with decreased visceral adipose tissue (VAT) ($\beta = -0.166$; $p = 0.024$), deep subcutaneous adipose tissue ($\beta = -0.184$; $p = 0.014$), and intrahepatic fat ($\beta = -0.179$; $p = 0.021$) independent of age, sex, and intervention groups (Table 2). In an exploratory Mendelian randomization analysis

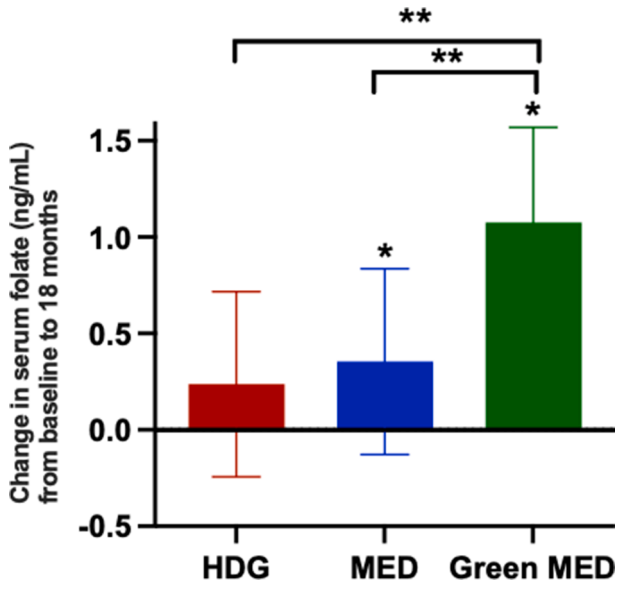

Fig. 2. The effect of green Mediterranean diet on serum folate 18-month change
Changes in Serum Folate between intervention groups after 18 months of intervention. 28 were excluded from the baseline analysis due to the intake of vitamin B complex, B12 vitamin, folic acid, or multivitamins. Similarly, 14 participants were excluded at time point 18 for the same reason. The green-MED diet significantly increased serum folate levels and was found significantly different as compared to the HDG and MED diet after 18 months, adjusted for age and sex (green-MED vs HDG:p = 0.002, green-MED vs MED p = 0.021). After adjustment to age, sex, and weight or WC loss (WC: green-MED vs HDG:p = 0.006, green-MED vs MED p = 0.03; weight-loss: green-MED vs HDG:p = 0.02, green-MED vs MED p = 0.04). HDG (N = 77); MED (N = 73); Green-MED (N = 72); Bars represent adjusted means (adjusted for age, sex, and weight change). Error bars indicate 95% confidence intervals. *denotes significant within-group change vs. baseline at the 0.05 level. ** indicates significant differences between groups at the 0.05 level. HDG, healthy dietary guidelines; MED, Mediterranean.

using a two-stage least squares with a single genetic instrument (MTHFR rs1801133), the genotype was significantly associated with serum folate concentrations at 18 months ($\beta = -1.938$; $p = 0.003$), explaining 2.5% of the variance ($R^2 = 0.025$) with an F-statistic of 8.77. In the second-stage regression, genetically predicted serum folate concentrations were significantly and inversely associated with the TG/HDL-c ratio ($\beta = -0.390$; $p = 0.016$). No significant associations were observed between genetically predicted folate and other cardiometabolic outcomes, including HOMA-IR, Intrahepatic fat%, IL-6. Data regarding adherence is presented in Supplemental Data 3.

3.3. Effects of genetic variation on folate metabolism

Carriers of the rs1801133 TT genotype showed significantly lower serum folate concentrations at baseline ($p = 0.037$) and after the intervention ($p = 0.045$, recessive mode of inheritance; Fig. 3) as compared to CC/CT carriers. In addition, we observed genotype-specific associations on ALKP baseline concentrations, HDL cholesterol concentrations, and higher WC values after the intervention (Supplemental Table 3). Carriers of the CC/CT genotype showed the highest increase in folate concentrations following the green-MED intervention ($p = 0.03$; Fig. 4). A significant interaction was observed between intervention groups and the rs1801133 TT genotype ($p = 0.02$). After 18 months of intervention, TT carriers showed a significantly greater reduction in methionine concentrations (median: −10.7) compared to CC/CT carriers (median: −4.5; $p = 0.04$; Supplemental Fig. 3).

Although we did not observe a direct effect of the genotype on *MTHFR* blood gene expression (Supplemental Fig. 4), we demonstrated that the TT-genotype carriers showed reduced *MTHFD2* mRNA expression at baseline ($p = 0.005$), but an enriched transcription compared to CC/CT carriers after the intervention ($p = 0.001$) (Fig. 5). Consistently, we found the *DHFR* to be higher expressed in the TT-genotype carriers after the intervention ($p = 0.02$; Supplemental Fig. 5).

3.4. Genotype-diet interaction

A significant rs1801133 genotype-Mankai intake interaction was observed for VAT change over 18 months (p interaction = 0.028) adjusted for age and sex. Higher Mankai intake was significantly associated with VAT reduction over 18 months ($p = 0.002$). However, this association was modified by MTHFR genotype. Among CC/CT individuals, VAT change showed a dose-dependent response to Mankai: None: −8%; 1–2/week: −20%; 3+/week: −33% (unadjusted means). Among TT individuals, VAT loss was greater at low intake (−16%), but the trend lacked consistency in this population. Furthermore, MTHFR genotype was significantly associated with the change in Framingham risk score (FRS) after 18 months ($p = 0.008$). A significant genotype-Mankai intake interaction was found for FRS change (p interaction = 0.030), adjusted for age and sex. Among CC/CT individuals, there was a dose–response relationship between Mankai intake and reduction in FRS, with the greatest decrease (−4 points) seen in those consuming Mankai ≥3 times/week. While TT carriers also showed a substantial FRS reduction at high Mankai intake (−7.74), the overall trend was less consistent. Notably, TT carriers who did not consume Mankai had a mean increase in FRS (+1.58). The genotype–diet interaction findings remained significant after false discovery rate correction (FDR-adjusted $p = 0.030$ for VAT and Framingham risk score). Additional information on the relationship between folate and methionine can be found in Supplemental Data 5. Sensitivity analyses using multiple imputation yielded results consistent with the primary analyses (Supplemental Table 7).

4. Discussion

In this 18-month dietary RCT (DIRECT-PLUS), we demonstrated that adherence to the green-MED diet significantly increased serum folate concentrations compared to the HDG and the traditional MED diet, with Mankai intake, a folate-rich green aquatic plant, being a significant predictor of increased serum folate concentrations. Genetic analysis revealed that carriers of the rs1801133 TT genotype had lower serum folate concentrations at baseline and post-intervention, but these concentrations were substantially improved among CC/CT carriers following adherence to the green-MED diet. Furthermore, TT-genotype carriers demonstrated an upregulation of *MTHFD2* and *DHFR* mRNA expressions following the dietary intervention, which may reflect a potential adaptive response in folate metabolism. Genetically predicted folate was significantly associated with the TG/HDL-c ratio using a Mendelian randomization analysis. Furthermore, Mankai intake appears particularly associated with VAT and cardiovascular risk reduction among CC/CT carriers, highlighting a potential gene–diet interaction. Notably, TT carriers who did not consume Mankai had an increase in FRS, potentially suggesting a genotype-specific vulnerability in the absence of dietary folate.

Several limitations should be acknowledged. The higher proportion of men limits the generalizability of findings to women. In addition, we cannot identify the exact components responsible for the dietary effects as we compared dietary regimens and not

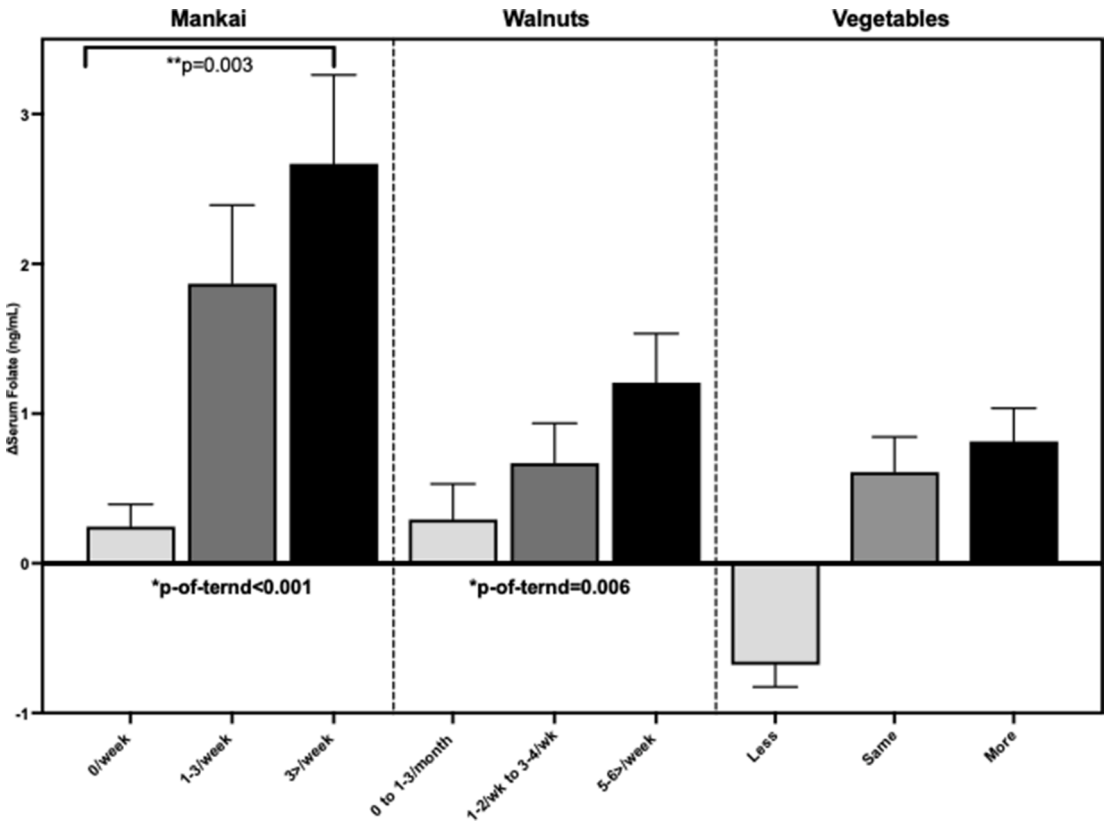

Fig. 3. Nutritional components and changes in serum Folate after 18 months of intervention
Multivariate models for assessment of associations between nutritional components with changes in serum folate adjusted for age, sex, and weight loss. Mankai consumption categories (18 months): non:0/week, Medium: 1–3/week, High: 3≥/week; walnut consumption categories (18 months): Low/non: 0 to 1–3 times/month; Medium: 1–2/week to 3–4/week; High: more than 5–6/week. Values represent raw mean changes; p-values are derived from multivariable-adjusted models adjusted for age, sex, and weight loss. Excluded vitamin B complex, folic acid, and multivitamin consumers. *P trend<0.05, **indicated significant differences between groups at the 0.05 level.

Table 2
Associations between 18-m serum folate change and 18-month cardiometabolic outcomes.

	Model 1		Model 2		Model 3	
	Beta coefficient	p value	Beta coefficient	p value	Beta coefficient	p value
Anthropometric						
ΔWeight	**−0.219**	**0.001**	**−0.173**	**0.012**	**-**	
ΔWaist circumference	**−0.172**	**0.011**	**−0.139**	**0.041**	**-**	
ΔSBP	**−0.140**	**0.039**	**−0.134**	**0.043**	−0.105	0.119
ΔDBP	**−0.172**	**0.012**	**−0.177**	**0.008**	**−0.136**	**0.050**
Abdominal adipose tissues and IHF (MRI)						
ΔVAT	**−0.224**	**0.002**	**−0.166**	**0.024**	–	
ΔDeep-SAT	**−0.246**	**0.001**	**−0.184**	**0.014**	–	
ΔSuperficial-SAT	−0.140	0.071	−0.094	0.218	–	
ΔIHF	**−0.245**	**0.001**	**−0.179**	**0.021**	–	
Glycemic biomarkers						
ΔGlucose	−0.070	0.314	−0.077	0.259	−0.040	0.568
Δhoma IR	**−0.348**	**<0.001**	**−0.332**	**<0.001**	**−0.326**	**<0.001**
ΔInsulin	**−0.345**	**<0.001**	**−0.329**	**<0.001**	**−0.324**	**<0.001**
ΔHbA1C	**−0.145**	**0.034**	**−0.134**	**0.046**	−0.096	0.165
Lipid biomarkers						
ΔTriglycerides	**−0.272**	**<0.001**	**−0.250**	**<0.001**	**−0.221**	**0.004**
ΔHDLc	0.097	0.159	0.104	0.122	0.044	0.550
ΔLDLc	−0.003	0.963	0.011	0.874	0.025	0.710
ΔTG/HDLc ratio	**−0.239**	**<0.001**	**−0.223**	**0.001**	**−0.188**	**0.017**
ΔFFA	**0.142**	**0.035**	**0.147**	**0.025**	**0.170**	**0.009**

Model 1: Adjusted for age and sex.
Model 2: Adjusted for age, sex, and intervention groups.
Model 3: Adjusted for age, sex, intervention groups, and 18-m weight change.
BP, blood pressure; HDLc, high density lipoprotein cholesterol; LDLc, low density lipoprotein cholesterol; HOMA-IR, homeostatic model assessment of insulin resistance.

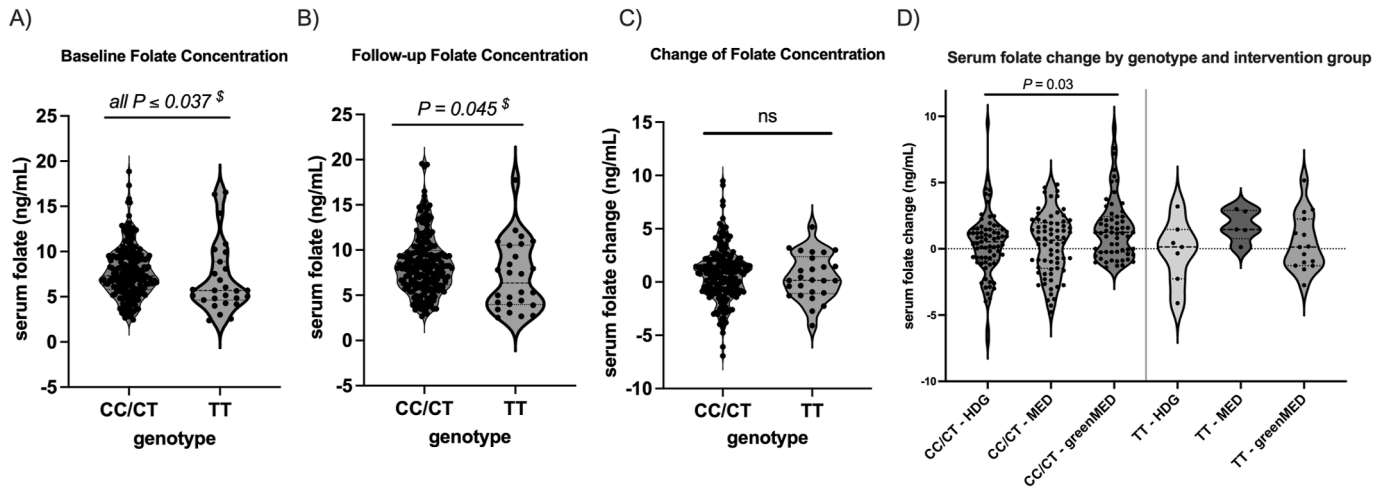

Fig. 4. Serum folate concentrations and changes across genetic variations.
Shows average serum folate concentrations and changes as violin plots. For A) baseline, B) follow-up (18 months) and C) the change (T18-T0) as a recessive mode of inheritance. Genotype distribution (N) for A: CC/CT vs TT/204 vs 27 and for B and C: CC/CT vs TT/204 vs 26. D) Shows changes per intervention group in the recessive mode of inheritance. The raw differences between the groups were assessed with non-parametric Mann–Whitney U test.

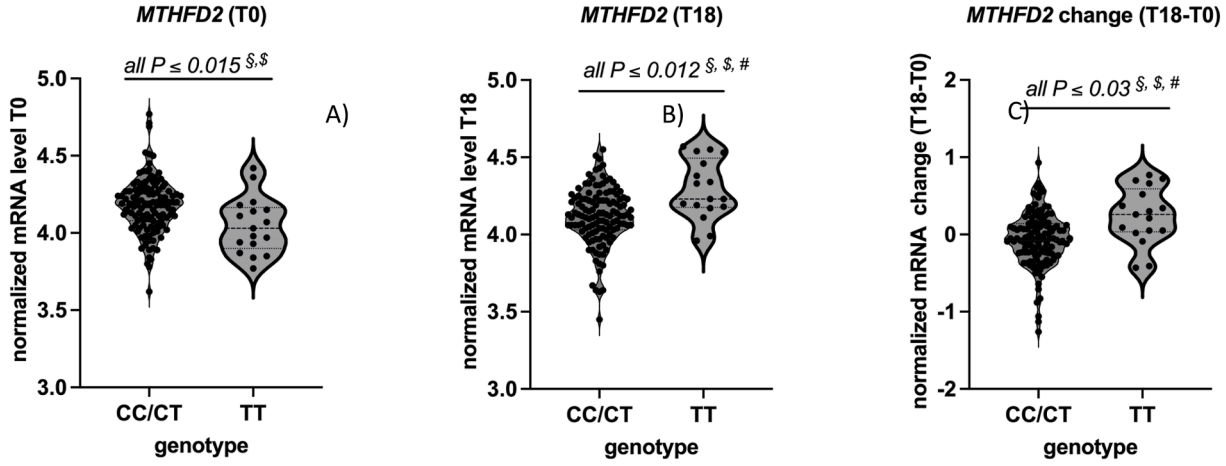

Fig. 5. MTHFD2 mRNA expression during 18-month intervention.
Shows average, normalized MTHFD2 mRNA expression detected in blood as violin plots. For A) baseline, B) follow-up (18 months) and C) the change (T18-T0) as a recessive mode of inheritance. § = additive; $ = dominant and # = recessive mode of inheritance. Differences between the groups were assessed with non-parametric test statistics. Available mRNA expression data stratified by genotype distribution (N) for A–C: CC/CT vs TT - 127 vs 17.

specific nutrients. Although the sample size is relatively small to study genetic associations, we observed significant genotype-specific effects, suggesting that the tested variant is functionally effective also in our study. We assessed adherence by the self-reported dietary intake assessment tool, which is subject to error, although the instrument has been validated [30]. Of note, the rs1801133 SNP in the MTHFR gene influences enzyme activity, affecting folate metabolism and homocysteine concentrations, which may have additional implications beyond those assessed in our study. While our findings based on blood transcriptomics suggest a potential adaptive response to the genetic effect in folate metabolism, further mechanistic studies specifically involving MTHFD2 enzyme activity are required to elucidate the precise biological pathways involved. The Mendelian randomization analysis should be interpreted with caution, as it is based on a single functional variant and a relatively modest sample size, which may limit statistical power and the strength of causal inference. Genotype-diet interaction analyses were conducted for multiple outcomes and were considered exploratory and hypothesis-generating. The strengths of our study include the long-term randomized controlled design, relatively large sample size, high retention rate, the use of a well-defined dietary intervention, and comprehensive genetic and biochemical analyses. Furthermore, the closed workplace enabled monitoring of the freely provided lunch, the presence of an onsite clinic, intense dietary guidance, group meetings with multidisciplinary guidance, and access to polyphenol-rich foods provided at no charge.

Serum folate concentrations reflect recent dietary intake but do not accurately represent long-term folate tissue stores [27]. Based on data from the U.S. National Health and Nutrition Examination Survey (NHANES) conducted between 1988 and 1994, the reference interval for serum folate was reported as 2.6–12.2 ng/ml. Since then, approximately 60 countries worldwide have implemented mandatory folic acid fortification of staple foods such as cereals and bread to address folate deficiency and reduce the risk of neural tube defects [31–33]. In the United States, the FDA mandated folic acid fortification of enriched grain products in 1998 (140 μg/100 g), which has since resulted in a doubling of average

folate concentrations in the population. Currently, folate deficiency is typically defined as serum concentrations below 3 ng/mL [25]. In our study, only 3% of participants were classified as folate-deficient (<3 ng/mL), which aligns with recent US data showing a prevalence of around 0.7% in the general population [33]. Our results highlight the importance of re-evaluating what constitutes a "normal" folate range in diverse populations and suggest a clinical advantage in optimizing folate status even within currently accepted ranges.

The long-term effects of plant-based dietary patterns or MED diets on serum folate concentrations remain unclear. Observational studies suggest that vegan and vegetarian populations typically do not exhibit folate deficiency [34,35]. However, findings from clinical studies assessing the effect of plant-based diets on folate concentrations are limited and inconsistent. For example, a previous RCT [36], evaluated the effects of varying dietary protein sources, 70% animal/30% plant, 50% animal/50% plant, and 30% animal/70% plant, over a 12-week intervention. The study found no significant differences in serum folate concentrations between the groups, suggesting that short-term dietary shifts in protein sources may not substantially impact folate status, and highlights the need for further investigations with a long-term dietary effect on folate concentrations.

The green-MED diet, rich in plant-based proteins, offers a unique nutritional composition that supports increased serum folate concentrations. A key component of this diet is Mankai, a novel folate-rich aquatic plant, which contributes approximately 61% of the recommended daily intake of folate for men [25]. In our study, Mankai intake emerged as a significant dietary marker for elevated serum folate status. Participants consuming Mankai more than three times per week exhibited significantly greater increases in serum folate concentrations, independent of weight loss, suggesting that the green-MED diet can meaningfully enhance folate status through targeted plant-based components.

Elevated serum folate concentrations were associated with multiple cardiometabolic improvements in our study, including enhanced insulin sensitivity, improved lipid profiles, and reduced inflammatory markers. Mechanistically, folate functions as a critical methyl donor in one-carbon metabolism, facilitating the remethylation of homocysteine to methionine, thereby reducing homocysteine accumulation, a known contributor to endothelial dysfunction and cardiovascular risk [12,37,38]. Folate-dependent enzymatic processes also support lipid metabolism and glucose regulation by modulating hepatic lipid synthesis and clearance, as well as mitochondrial energy metabolism and insulin signaling pathways. Notably, increases in folate concentrations were associated with reductions in intrahepatic fat, VAT, and deep subcutaneous adipose tissue. While these associations suggest a role for folate in hepatic and metabolic regulation, the Mendelian randomization analysis indicates a possible association with the TG/HDL-c ratio, but should be interpreted with caution given the modest sample size and single, relatively weak instrument. This finding highlights a possible specific role for folate in lipid metabolism, whereas other observed benefits may reflect the broader effects of the green-MED dietary pattern.

Genetic variability in folate metabolism significantly influences serum folate concentrations and response to dietary intake [39]. Within the folate and homocysteine metabolism, the enzyme MTHFR plays a critical role by catalyzing the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the methyl donor of homocysteine to methionine [40](Fig. 6). The most studied common genetic variant associated with serum folate concentrations is the c.677 C > T MTHFR polymorphism (rs1801133; missense-mutation), reducing MTHFR activity by 50–60% in homozygous individuals [41]. It replaces an alanine with valine in the catalytic domain, resulting in a thermolabile enzyme [42]. This SNP has been consistently associated with increased cardiovascular risk, particularly in individuals with low folate intake [43]. The homozygous TT genotype has been reported in approximately 12% of Asian and White populations, which is consistent with our population (11.7%). Similarly, the heterozygous CT genotype can be prevalent in up to 50% of individuals, aligning with our observed prevalence of 49.8% [27,44]. A previous study demonstrated that individuals carrying the TT genotype exhibited a significantly higher risk of CVD, with a 71% increased risk compared to CC carriers, reinforcing the critical role of folate metabolism in cardiovascular health [15]. Meta-analysis further supports these findings, indicating a 16% increased risk of coronary heart disease among TT carriers, particularly in populations with low folate status [45]. Moreover, studies identified significant associations between the rs1801133 polymorphism and

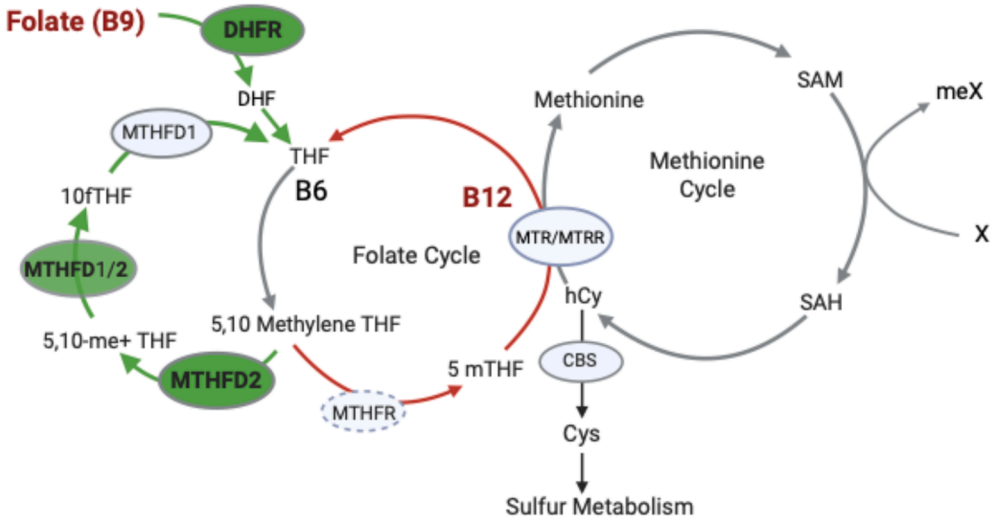

Fig. 6. Schematic representation of the folate and methionine cycle, illustrating key enzymes and metabolites involved in one-carbon metabolism and genotype-related differences in folate metabolism (created with BioRender.com). DHFR, dihydrofolate reductase; DHF, dihydrofolate; THF, tetrahydrofolate; MTHFR, methylenetetrahydrofolate reductase; MTHFD1, methylenetetrahydrofolate dehydrogenase 1; MTHFD2, methylenetetrahydrofolate dehydrogenase 2; MTR, methionine synthase; MTRR, methionine synthase reductase; hCys, homocysteine; CBS, cystathionine-β-synthase; Cys, cysteine; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine.

hyperhomocysteinemia, hypertension, and coronary artery disease, reinforcing the gene–diet interaction's role in cardiovascular risk modulation [46–49]. Our study suggests that adherence to a folate-rich diet, such as the green-MED diet, may mitigate the adverse effects of genetic predisposition by increasing serum folate concentrations and improving cardiometabolic health. More specifically, Mankai intake appears associated with VAT and cardiovascular risk reduction among CC/CT carriers, highlighting a potential gene–diet interaction. Conversely, TT carriers who did not consume Mankai experienced an increase in FRS, suggesting they may be particularly vulnerable when dietary folate is lacking. These findings suggest that genetic variation may influence individual responses to dietary interventions, with potential implications for optimizing folate metabolism and cardiometabolic risk.

In the DIRECT PLUS study, individuals with the TT-genotype carriers exhibited significantly lower baseline serum folate concentrations, which persisted post-intervention despite dietary improvements. These individuals also showed increased *MTHFD2* and *DHFR* mRNA expression following the intervention. However, these findings are based on whole-blood transcriptomics and may not reflect tissue-specific activity in key metabolic organs such as the liver or vascular tissues. Thus, while these changes may suggest a potential adaptive response in folate metabolism, this interpretation remains speculative (Fig. 6). MTHFD2 catalyzes the conversion of 5,10-methylenetetrahydrofolate (THF) to 10-formyl THF (Fig. 6) in a NADPH-dependent manner [4]. An upregulation of *MTHFD2* was previously shown to enhance one-carbon metabolic flux to purines and non-homologous end joining repair ability, which supports cell proliferation and survival [50]. In line, the cytosolic DHFR also regenerates THF from DHF. Despite the observed transcriptional changes, TT carriers exhibited a greater reduction in methionine concentrations, suggesting a limited capacity for folate-mediated remethylation of homocysteine. An inverse correlation between methionine change and folate change among TT carriers may indicate a shift in one-carbon metabolism toward nucleotide synthesis rather than methylation. While these findings are consistent with a biological interpretation, alternative explanations, including regression to the mean, differential responses to weight loss, and unmeasured confounding, may also contribute to the observed genotype-specific differences in folate-related and transcriptional responses. These findings highlight how dietary interventions can partially offset genetic limitations in folate metabolism, though the metabolic benefits may vary depending on genotype.

5. Conclusion

In conclusion, adherence to the green-MED diet significantly increased serum folate concentrations, with a strong association with Mankai intake. Elevated folate concentrations were associated with improved cardiometabolic markers, reinforcing the health benefits of plant-based dietary patterns. Genotype-specific effects were observed, with CC/CT carriers showing greater benefits, while TT carriers displayed changes in folate-related pathways that may reflect adaptive responses and appeared more vulnerable when dietary folate was lacking. These findings suggest that Mankai may contribute to increased dietary folate intake and highlight the potential role of genetic variation in modulating responses to dietary interventions, with possible implications for cardiometabolic risk.

Author contributions

I.S., F.B.H., and M.J.S. contributed to the conceptualization and design of the study. H.Z. and M.K. performed data curation and formal analysis. A.H., M.B., U.C., B.I., M.S., and I.Shelef. contributed to the methodology and investigation. I.S. secured funding. I.S., H.Z., A.Y.M., E.R., G.T., and A.K. were responsible for project administration. H.Z., M.K., I.S., and P.K. drafted the original manuscript. All authors contributed to reviewing and editing the manuscript, approved the final version for publication, and agree to be accountable for all aspects of the work. All individuals who meet the criteria for authorship have been included as authors.

Data sharing

The majority of results corresponding to the current study are included in the article or uploaded as supplementary material. No further data are available.

Ethics approval and consent to participate

The Soroka University Medical Centre Ethics Board and the Institutional Review Board approved the study protocol for the DIRECT PLUS trial. All participants provided written informed consent and received no financial compensation.

Trial registration: ClinicalTrials.gov, NCT03020186; https://clinicaltrials.gov/study/NCT03020186.

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None of the funding providers was involved in any stage of the design, conduct, or analysis of the study, and they had no access to the study results before publication.

Conflict of interest

M. Blüher received honoraria as a consultant and speaker from Abbott, Amgen, AstraZeneca, Bayer, Boehringer Ingelheim, Daiichi-Sankyo, Lilly, MSD, Novo Nordisk, Novartis, and Sanofi. All other authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clnu.2026.106701.

References

[1] Ebara S. Nutritional role of folate. Congenital Anom 2017;57(5):138–41.

[2] Bailey Lynn B, Stover Patrick J, McNulty Helene, Fenech Michael F, Gregory 3rd Jesse F, Mills James L, et al. Biomarkers of nutrition for development-folate review. J Nutr 2015;145(7):1636s–80s.
[3] Moll R, Davis B. Iron, vitamin B12 and folate. Medicine 2017;45(4):198–203.
[4] Ducker GS, Rabinowitz JD. One-carbon metabolism in health and disease. Cell Metab 2017;25(1):27–42.
[5] Navarrete-Muñoz EM, Vioque J, Toledo E, Oncina-Canovas A, Martínez-González MÁ, Salas-Salvadó J, et al. Dietary folate intake and metabolic syndrome in participants of PREDIMED-Plus study: a cross-sectional study. Eur J Nutr 2021;60(2):1125–36.
[6] Brouwer-Brolsma EM, Brandl B, Buso MEC, Skurk T, Manach C. Food intake biomarkers for green leafy vegetables, bulb vegetables, and stem vegetables: a review. Gene Nutr 2020;15(1):7.
[7] Mönch S, Netzel M, Netzel G, Ott U, Frank T, Rychlik M. Folate bioavailability from foods rich in folates assessed in a short term human study using stable isotope dilution assays. Food Funct 2015;6(1):241–7.
[8] Brevik A, Vollset SE, Tell GS, Refsum H, Ueland PM, Loeken EB, et al. Plasma concentration of folate as a biomarker for the intake of fruit and vegetables: the hordaland homocysteine study. Am J Clin Nutr 2005;81(2):434–9.
[9] Maruvada P, Stover PJ, Mason JB, Bailey RL, Davis CD, Field MS, et al. Knowledge gaps in understanding the metabolic and clinical effects of excess folates/folic acid: a summary, and perspectives, from an NIH workshop. Am J Clin Nutr 2020;112(5):1390–403.
[10] Hiraoka M, Kagawa Y. Genetic polymorphisms and folate status. Congenit Anom (Kyoto) 2017;57(5):142–9.
[11] Bird JK, Ronnenberg AG, Choi SW, Du F, Mason JB, Liu Z. Obesity is associated with increased red blood cell folate despite lower dietary intakes and serum concentrations. J Nutr 2015;145(1):79–86.
[12] Shai I, Stampfer MJ, Ma J, Manson JE, Hankinson SE, Cannuscio C, et al. Homocysteine as a risk factor for coronary heart diseases and its association with inflammatory biomarkers, lipids and dietary factors. Atherosclerosis 2004;177(2):375–81.
[13] Fallah M, Karim Dehnavi M, Lotfi K, Aminianfar A, Azadbakht L, Esmaillzadeh A. Folate biomarkers, folate intake, and risk of death from all causes, cardiovascular disease, and cancer: a systematic review and dose-response meta-analysis of prospective cohort studies. Nutr Rev 2024;83(3):e801–13.
[14] Shai I, Schwarzfuchs D, Henkin Y, Shahar DR, Witkow S, Greenberg I, et al. Weight loss with a low-carbohydrate, mediterranean, or low-fat diet. N Engl J Med 2008;359(3):229–41.
[15] Wernimont SM, Clark AG, Stover PJ, Wells MT, Litonjua AA, Weiss ST, et al. Folate network genetic variation predicts cardiovascular disease risk in non-hispanic white males. J Nutr 2012;142(7):1272–9.
[16] Grarup N, Sulem P, Sandholt CH, Thorleifsson G, Ahluwalia TS, Steinthorsdottir V, et al. Genetic architecture of vitamin B12 and folate levels uncovered applying deeply sequenced large datasets. PLoS Genet 2013;9(6):e1003530.
[17] Niforou A, Konstantinidou V, Naska A. Genetic variants shaping inter-individual differences in response to dietary Intakes—A narrative review of the case of vitamins. Front Nutr 2020;7.
[18] Zelicha H, Kloting N, Kaplan A, Yaskolka Meir A, Rinott E, Tsaban G, et al. The effect of high-polyphenol mediterranean diet on visceral adiposity: the DIRECT PLUS randomized controlled trial. BMC Med 2022;20(1):327.
[19] Yaskolka Meir A, Tsaban G, Zelicha H, Rinott E, Kaplan A, Youngster I, et al. A green-mediterranean diet, supplemented with mankai duckweed, preserves iron-homeostasis in humans and is efficient in reversal of anemia in rats. J Nutr 2019;149(6):1004–11.
[20] Gepner Y, Shelef I, Schwarzfuchs D, Zelicha H, Tene L, Yaskolka Meir A, et al. Effect of distinct lifestyle interventions on mobilization of fat storage pools: CENTRAL magnetic resonance imaging randomized controlled trial. Circulation 2018;137(11):1143–57.
[21] Kaplan A, Zelicha H, Tsaban G, Yaskolka Meir A, Rinott E, Kovsan J, et al. Protein bioavailability of Wolffia globosa duckweed, a novel aquatic plant - a randomized controlled trial. Clin Nutr 2019;38(6):2576–82.
[22] Sela I, Yaskolka Meir A, Brandis A, Krajmalnik-Brown R, Zeibich L, Chang D, et al. Wolffia globosa-Mankai plant-based protein contains bioactive vitamin B(12) and is well absorbed in humans. Nutrients 2020;12(10).
[23] Zelicha H, Kaplan A, Yaskolka Meir A, Tsaban G, Rinott E, Shelef I, et al. The effect of Wolffia globosa mankai, a green aquatic plant, on postprandial glycemic response: a randomized crossover controlled trial. Diabetes Care 2019;42(7):1162–9.
[24] OttenJJ H, Meyers LD. Dietary reference intakes: the essential guide to nutrient requirements. Washington (DC): The National Academies Press; 2006.
[25] Institute of Medicine (US) Panel on Micronutrients. Dietary reference intakes for vitamin A, v.K., arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium, and zinc. Washington (DC): The National Academies Press; 2001.
[26] Yaskolka Meir A, Rinott E, Tsaban G, Zelicha H, Kaplan A, Rosen P, et al. Effect of green-Mediterranean diet on intrahepatic fat: the DIRECT PLUS randomised controlled trial. Gut 2021;70(11):2085–95.
[27] Burtis CA AE, Bruns DE, editors. Tietz textbook of clinical chemistry and molecular diagnostics. 5th ed. Philadelphia, Pa: Saunders; 2012.
[28] Fan Y, Wu L, Zhuang W. Methylenetetrahydrofolate reductase gene rs1801133 and rs1801131 polymorphisms and essential hypertension risk: a comprehensive analysis. Cardiovasc Ther 2022;2022:2144443.
[29] Hoffmann A, Meir AY, Hagemann T, Czechowski P, Müller L, Engelmann B, et al. A polyphenol-rich green mediterranean diet enhances epigenetic regulatory potential: the DIRECT PLUS randomized controlled trial. Metabolism 2023;145:155594.
[30] Shai I, Rosner BA, Shahar DR, Vardi H, Azrad AB, Kanfi A, et al. Dietary evaluation and attenuation of relative risk: multiple comparisons between blood and urinary biomarkers, food frequency, and 24-hour recall questionnaires: the DEARR study. J Nutr 2005;135(3):573–9.
[31] Kancherla V, Botto LD, Rowe LA, Shlobin NA, Caceres A, Arynchyna-Smith A, et al. Preventing birth defects, saving lives, and promoting health equity: an urgent call to action for universal mandatory food fortification with folic acid. Lancet Global Health 2022;10(7):e1053–e1057.
[32] O'Connor DL. Establishing dietary reference intakes and laboratory reference ranges for folate. Am J Clin Nutr 2023;117(3):445–6.
[33] Zhou Y, Wang A, Yeung LF, Qi YP, Pfeiffer CM, Crider KS. Folate and vitamin B12 usual intake and biomarker status by intake source in United States adults aged ≥19 y: NHANES 2007-2018. Am J Clin Nutr 2023;118(1):241–54.
[34] Henjum S, Groufh-Jacobsen S, Lindsay A, Raael E, Israelsson AM, Shahab-Ferdows S, et al. Adequate vitamin B(12) and folate status of Norwegian vegans and vegetarians. Br J Nutr 2023;129(12):2076–83.
[35] Bakaloudi DR, Halloran A, Rippin HL, Oikonomidou AC, Dardavesis TI, Williams J, et al. Intake and adequacy of the vegan diet. A systematic review of the evidence. Clin Nutr 2021;40(5):3503–21.
[36] Pellinen T, Päivärinta E, Isotalo J, Lehtovirta M, Itkonen ST, Korkalo L, et al. Replacing dietary animal-source proteins with plant-source proteins changes dietary intake and status of vitamins and minerals in healthy adults: a 12-week randomized controlled trial. Eur J Nutr 2022;61(3):1391–404.
[37] Blom HJ, Smulders Y. Overview of homocysteine and folate metabolism. With special references to cardiovascular disease and neural tube defects. J Inherit Metab Dis 2011;34(1):75–81.
[38] Levy J, Rodriguez-Guéant RM, Oussalah A, Jeannesson E, Wahl D, Ziuly S, et al. Cardiovascular manifestations of intermediate and major hyperhomocysteinemia due to vitamin B12 and folate deficiency and/or inherited disorders of one-carbon metabolism: a 3.5-year retrospective cross-sectional study of consecutive patients. Am J Clin Nutr 2021;113(5):1157–67.
[39] Wang L, Li X, Montazeri A, MacFarlane AJ, Momoli F, Duthie S, et al. Phenome-wide association study of genetically predicted B vitamins and homocysteine biomarkers with multiple health and disease outcomes: analysis of the UK biobank. Am J Clin Nutr 2023;117(3):564–75.
[40] Watkins D, Rosenblatt DS. Update and new concepts in vitamin responsive disorders of folate transport and metabolism. J Inherit Metab Dis 2012;35(4):665–70.
[41] Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet 1995;10(1):111–3.
[42] Goyette P, Christensen B, Rosenblatt DS, Rozen R. Severe and mild mutations in cis for the methylenetetrahydrofolate reductase (MTHFR) gene, and description of five novel mutations in MTHFR. Am J Hum Genet 1996;59(6):1268–75.
[43] Jacques PF, Bostom AG, Williams RR, Ellison RC, Eckfeldt JH, Rosenberg IH, et al. Relation between folate status, a common mutation in methylenetetrahydrofolate reductase, and plasma homocysteine concentrations. Circulation 1996;93(1):7–9.
[44] Brattström L, Wilcken DE, Ohrvik J, Brudin L. Common methylenetetrahydrofolate reductase gene mutation leads to hyperhomocysteinemia but not to vascular disease. Circulation 1998;98(23):2520–6.
[45] Klerk M, Verhoef P, Clarke R, Blom HJ, Kok FJ, Schouten EG. MTHFR 677C→T polymorphism and risk of coronary heart disease: a meta-analysis. JAMA 2002;288(16):2023–31.
[46] Shivkar RR, Gawade GC, Padwal MK, Diwan AG, Mahajan SA, Kadam CY. Association of MTHFR C677T (rs1801133) and A1298C (rs1801131) polymorphisms with serum homocysteine, folate and vitamin B12 in patients with young coronary artery disease. Indian J Clin Biochem 2022;37(2):224–31.
[47] Ihan N, Kucuksu M, Kaman D, Ilhan N, Ozbay Y. The 677 C/T MTHFR polymorphism is associated with essential hypertension, coronary artery disease, and higher homocysteine levels. Arch Med Res 2008;39(1):125–30.
[48] Shane B, Pangilinan F, Mills JL, Fan R, Gong T, Cropp CD, et al. The 677C→T variant of MTHFR is the major genetic modifier of biomarkers of folate status in a young, healthy Irish population. Am J Clin Nutr 2018;108(6):1334–41.
[49] Du B, Tian H, Tian D, Zhang C, Wang W, Wang L, et al. Genetic polymorphisms of key enzymes in folate metabolism affect the efficacy of folate therapy in patients with hyperhomocysteinaemia. Br J Nutr 2018;119(8):887–95.
[50] Li G, Wu J, Li L, Jiang P. p53 deficiency induces MTHFD2 transcription to promote cell proliferation and restrain DNA damage. Proc Natl Acad Sci 2021;118(28):e2019822118.