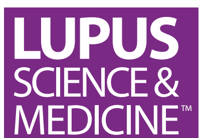

Methyl-donor supplementation in women with systemic lupus erythematosus with different nutritional status: the protocol for a randomised, double-blind, placebo-controlled trial

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ABSTRACT

Introduction DNA hypomethylation in patients with systemic lupus erythematosus (SLE) has been recently documented in the literature. Low levels of DNA methylation have been observed globally and in genes associated with immune and inflammatory pathways in SLE's CD4+T lymphocytes. Given that certain micronutrients can either donate methyl groups within one-carbon metabolism pathways or serve as cofactors for enzymes involved in the DNA methylation process, this randomised, double-blind, placebo-controlled trial aims to investigate whether a 3-month supplementation of folic acid and vitamin B_{12} will modulate the DNA methylation profile in subcutaneous adipose tissue (primary outcome) of women with SLE and normal weight or excess body weight. As secondary objectives, we will assess gene expression, telomere length and phenotypic characteristics (ie, clinical parameters, body weight and composition, abdominal circumference, food intake and disordered eating attitude, physical activity, lipid profile, serum concentrations of leptin, adiponectin, and cytokines).

Methods and analysis Patients will be classified according to their nutritional status by body mass index in normal weight or excess body weight. Subsequently, patients in each group will be randomly assigned to either a placebo or an intervention group (folic acid (400 mcg) and vitamin B_{12} (2000 mcg) supplementation). Endpoint evaluations will be conducted using both intention-to-treat and per-protocol analyses. This study has the potential to design new personalised nutritional approaches as adjunctive therapy for patients with SLE.

Ethics and dissemination This study has been reviewed and approved by the Ethical Committee from Clinical Hospital of the School of Medicine of the University of Sao Paulo, Brazil (CAAE.: 47317521.8.0000.0068).

Trial registration number NCT05097365 (first version).

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Systemic lupus erythematosus (SLE) is associated with DNA hypomethylation.

⇒ Methyl-donor nutrients supplementation can impact DNA methylation levels.

WHAT THIS STUDY ADDS

⇒ Patients with SLE and normal weight or excess body weight may have different DNA methylation patterns, telomere length and gene expression profile.

⇒ Folic acid and vitamin B_{12} supplementation can modulate DNA methylation and gene expression levels and some phenotypic characteristics in subcutaneous adipose tissue of women with SLE.

⇒ Epigenetic modulation resulting from supplementation treatment may be different depending on nutritional status.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This study could contribute to the development of new therapeutic approaches based on precision nutrition for the adjuvant treatment of SLE.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a complex autoimmune disorder that mainly affects women of reproductive age.[1] This disease is characterised by immune system dysregulation, resulting in autoimmunity against proteins and nucleic acids, as well as inflammatory processes that cause tissue damage.[2] The complex pathophysiology and wide range of clinical manifestations classify SLE as one of medicine's most challenging diseases.[3] Although the precise aetiology remains elusive, the interaction between environmental triggers and genetic predispositions is recognised as important factors in the onset and progression of this disease.[4] Immune dysregulation in SLE is primarily caused by autoantibodies that target self-antigens. This leads to a series of responses that include abnormal activation of B and T lymphocytes and the production

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of pro-inflammatory mediators like interferon-γ, tumour necrosis factor (TNF)-α and interleukin (IL)-6.[5–7]

In this regard, epigenetic changes are now recognised as major players in the aetiology of autoimmune diseases like SLE.[8–10] Epigenetics involves alterations in chromatin structure and biochemical composition without altering DNA sequence,[11] thereby regulating gene expression.[12 13] Among epigenetic mechanisms, DNA methylation stands out as a crucial process characterised by the enzymatic addition of methyl groups (CH3) onto the fifth carbon position of the cytosine, facilitated by DNA methyltransferase (DNMT) enzymes.[14] Previous research has demonstrated a state of DNA hypomethylation in patients with SLE, which is typified by a decrease in the activity and concentration of DNA methyltransferase 1 (DNMT1) in CD4+T cells.[15] Notably, specific DNA hypomethylation in several genes linked to the SLE pathophysiology have been observed, especially those connected to immunological and inflammatory processes.[16–18] This pattern of DNA hypomethylation may contribute to increased disease activity and unfavourable clinical outcomes.[16–18]

Dietary micronutrients (eg, vitamins B_2, B_6 and B_{12}, folic acid, betaine, choline and methionine) might affect DNA methylation patterns by acting as methyl group donors within one-carbon metabolic pathways or as cofactors for enzymes engaged in this process.[19] For instance, vitamin B_{12} plays a crucial role in one-carbon metabolism by acting as a cofactor in the conversion of homocysteine to methionine, which acts as a substrate for S-adenosylmethionine synthesis, a key methyl group donor for DNA methylation reaction. Folic acid (vitamin B_9) also significantly contributes to this cycle, particularly in its reduced forms.[19 20]

In this context, conditions such as low intake or poor absorption of methyl donor nutrients,[21] or polymorphisms in genes encoding essential enzymes involved in one-carbon metabolism[22] can lead to alterations in gene expression due to changes in DNA methylation patterns. For example, folic acid deficiency has been associated with hypomethylation of specific genes and, consequently, with an increased risk of cancer and a worse clinical prognosis.[23 24] Indeed, the effects of methyl donor supplementation on DNA methylation profiles in several genes, clinical conditions and populations have been investigated.[19] For instance, patients with colorectal adenoma supplemented with folic acid (400 µg/day for 10 weeks) increased DNA methylation in both leucocytes and colonic mucosa.[25] In addition, high doses of folic acid (5 mg/day) supplementation for 3 months resulted in an increase in global DNA methylation in patients with schizophrenia and metabolic syndrome under treatment with antipsychotics.[26] On the other hand, other authors evidenced that folic acid supplementation (600 µg/day for 6 months) resulted in a decrease in global DNA methylation level compared with placebo.[27] Although studies evaluated the effects of methyl donor micronutrient supplementation in different clinical conditions, there are few studies addressing SLE. Therefore, it becomes relevant to investigate whether supplementation with methyl-donor nutrients can modulate DNA methylation profile and consequently gene expression, telomere length and phenotypic characteristics in patients with SLE disease. In this regard, personalised treatment strategies based on precision nutrition can be defined to promote better management and quality of life.

METHODS AND ANALYSIS

Study aims and outcomes

The main goal of the proposed intervention is to determine the effect of a 12-week methyl donor supplementation protocol on epigenetic profile in patients with SLE with normal weight and overweight/obese. The primary outcome is DNA methylation level in subcutaneous adipose tissue (SAT). The secondary outcomes are anthropometric and body composition parameters (ie, body weight, body mass index (BMI), abdominal circumference, fat mass and fat-free mass], serum blood biochemical profile (glucose, lipid profile, reactive C protein, folic acid, vitamin B_{12}), serum adipokines (leptin, adiponectin) and cytokines levels (IL-2, IL-6, IL-17, TNF-a), dietary intake and presence of disordered eating attitudes, gene expression in SAT and blood telomere length. The main hypotheses are that (1) the supplementation will modify the DNA methylation profile, (2) the supplementation will alter gene expression, telomere length and inflammatory markers and (3) the supplementation will not modify the other phenotypic characteristics.

Sample size

According to recent recommendations,[28 29] sample size was determined by feasibility criteria. The following factors were taken into consideration for determining sample size: (1) the number of potentially eligible patients from our outpatient clinic, (b) the staff capacity of our research team and (c) the availability of resources to conduct the project. Namely, the high cost of the DNA methylation array technology that will be employed in the present trial was the main limiting factor for sample size determination. Altogether, the feasibility analysis of these factors allowed the a priori determination of 40 patients per intervention arm (80 patients in total).

Participants

Patients with SLE diagnosis according to the European League Against Rheumatism and American College of Rheumatology classification criteria of 2019[30] will be recruited from the SLE Outpatient Clinic of the Rheumatology Division, Clinical Hospital of the School of Medicine of the University of Sao Paulo (HC-FMUSP). Patients will be considered eligible according to the following inclusion criteria: (1) premenopausal women, (2) aged between 18 and 45 years, (3) BMI>18.5 kg/m², (4) inactive disease according to Systemic Lupus Erythematosus Disease Activity Index 2000 (SLEDAI-2K) score ≤4,[31] (5) prednisone use ≤10 mg/day and (6) chloroquine use at a stable dose. Exclusion criteria will include: (1) chronic

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disease (diabetes mellitus, arterial hypertension, cancer), (2) diseases associated with reduced absorption of vitamin B_{12} and folic acid (ie, atrophic gastritis, pernicious anaemia); (3) current smokers, (4) use of anticoagulants, (5) current methotrexate use, (6) current infection, (7) pregnancy, (8) current use of any supplementation with vitamin B_{12}, folic acid or similar and (9) cognitive impairments that impede the understanding of the intervention recommendations. Patients engaged in physical training programmes and/or prescriptive diets will be excluded.

Trial design and randomisation

This is a randomised, double-blind, placebo-controlled trial. Pre-screening of patients will be determined by telephone calls or messages and personal interviews, concomitant with analysis of medical records, will be used to confirm eligibility. Included patients will be evaluated twice: before and 12 weeks after the intervention. At baseline visit, patients will be stratified according to nutritional status according to BMI: Group 1 will enrol women with normal weight (BMI between 18.5 and 24.9 kg/m²) and Group 2 will enrol patients with overweight or obesity (BMI>25.0 kg/m²). After baseline assessments, women from each group will be randomly allocated to either treatment (supplementation) or placebo groups using stratified permuted block randomisation (block size=2, allocation ratio=1:1). The Random platform (random.org) will be used for generation of random allocation sequences. The randomisation list will be provided to a single researcher, who will store the coded randomisation list and will conceal allocation by only displaying the woman's identification number. Investigators, patients and data analysers will be blinded to supplementation assignments until final analyses are conducted. The schedule for enrolment, interventions and assessments was determined according to the Standard Protocol Items: Recommendations for Interventional Trials flow diagram and is presented in figure 1.[32]

	STUDY PERIOD				
	Enrolment	Allocation	Pos-allocation		Close-out
TIMEPOINT	-4 to 1 w	0 w	0 w	12 w	12 w
Enrolment					
Eligibility screen	•				
Informed consent	•				
Run-in period	•				
Allocation		•			
Intervention					
Methyl donor supplementation			•——	——•	
Placebo			•——	——•	
Assessment					
Clinical characteristics					
Anthropometric measures		•			•
Body composition					
Food intake		•			•
Determinants of food choices		•			•
Physical activity level		•			•
SAT collection		•			•
Biochemical assays		•			•
DNA methylation profile		•			•
Gene expression		•			•
Medication's monitoring			•	•	
Participant compliance			•	•	•

Figure 1 SPIRIT figure for the schedule of enrolment, interventions and assessments in patients with SLE. SAT, subcutaneous adipose tissue; SLE, systemic lupus erythematosus; SPIRIT, Standard Protocol Items: Recommendations for Interventional Trials; w, week.

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Supplementation and safety consideration

Patients will be randomly assigned to receive a daily oral dose of vitamin B_{12}+folic acid or placebo. The placebo and vitamin B_{12}+folic acid supplement will be indistinguishable in terms of taste, smell and appearance. Each capsule will contain 400 mcg of folic acid and 2000 mcg of vitamin B_{12} or the same amount of flour. Patients will receive one bottle (with 35 capsules) every 4 weeks and will be advised to ingest one capsule per day, in the morning, for 12 weeks. Patients will be contacted by telephone on a weekly basis and will be encouraged to keep a log file registering every time they ingest the capsules. They will be instructed not to change their dietary pattern or their level of physical activity and not to include or exclude any activity or behaviour that could culminate in weight loss or gain.

Considering the Tolerable Upper Intake Levels established by Dietary Reference Intake,[33] no considerable adverse effects are expected in response to the consumption of methyl-donor supplements or placebo capsules at the mentioned doses.

Study procedures

Clinical assessment

Clinical parameters, namely time of diagnosis, disease duration, current dose of prednisone and hydroxychloroquine will be obtained by reviewing medical records. Clinical disease activity will be assessed using the SLEDAI-2K questionnaire.[31]

Anthropometric and body composition evaluation

Height will be measured by a graded (0.5 cm) stadiometer. Patients will be asked to stand barefoot, with their heels touching together, back straight and arms extended along the body. Weight will be measured by a digital scale, with a capacity of 300 kg and a sensitivity of 100 g. At the moment of measurement, patients will be barefoot and wearing light clothes. BMI will be calculated using the following equation: weight (kg)/height $(m)^2$. Waist circumference will be assessed using a graded (0.1 mm) plastic measuring tape around the smallest circumference between the lowest margin of the ribs and the upper margin of the iliac crest, while subjects stand. All measurements will be conducted by the same researcher.

Body composition (ie, fat mass and fat-free mass) will be estimated by measuring skinfolds (ie, triceps, bicipital, subscapular and suprailiac) and calculated by predictive equations.[34 35]

Food intake and disordered eating attitude assessment

Food intake will be evaluated using three 24-hour food recalls undertaken on separate days (ie, 2 weekdays and 1 weekend day). The first R24h will be held on the day of data collection and the other two will be done by phone. Total energy intake (TEI, kcal), macronutrients (proteins, carbohydrates and lipids) intake (grams and percentage of TEI), folic acid (mcg) and vitamin B_{12} (mcg) intake will be assessed. Also, a qualitative analysis of food intake will be carried out regarding the level of food processing. The foods and preparations consumed will be divided and dismembered according to the NOVA recommendation for food processing level in[36]: (1) fresh or minimally processed foods; (2) processed foods; (3) ultra-processed foods and (4) culinary ingredients. The absolute amount (g/day) and energy contribution (%TEI) will be calculated for each food-processing level.

The Disordered Eating Attitude Scale questionnaire will be administered to assess the factors influencing the eating choices and behaviours of the patients enrolled in the study. This questionnaire comprises 17 objective questions that cover various psychological and emotional aspects capable of impacting food choices and consumption patterns.[37]

Physical activity level

Physical activity level will be assessed by the International Physical Activity Questionnaire (IPAQ) (Short Form; V.2.0. April 2004). IPAQ will be applied personally on each visit. For analysis, the total minutes spent engaging in physical activities throughout the week will be tallied following IPAQ guidelines. These guidelines specify evaluation criteria based on the participation in (1) moderate activities lasting at least 10 consecutive minutes (such as light cycling, swimming, dancing, playing recreational volleyball, performing light weightlifting, engaging in domestic chores like sweeping, vacuuming, gardening or any activity that moderately elevated breathing or heart rate), and (2) vigorous activities lasting at least 10 consecutive minutes (including running, aerobics, playing football, fast cycling, basketball, heavy household chores, yard work or gardening, lifting heavy weights, or any activity that significantly increases breathing or heart rate).[38 39]

Blood collection and biochemical analysis

Peripheral venous blood samples will be collected after a 12 hours overnight fast for further analysis. Serum concentrations of fasting glucose, total cholesterol and fractions, triglycerides, protein-C reactive (PCR), folic acid, vitamin B_{12}, inflammatory cytokines (TNF-α, IL-6, IL-10, IL-1ra, IL-1B e IL4) and adipokines (leptin and adiponectin) will be measured. Also, peripheral blood mononucleocytes (PBMCs) will be isolated from whole blood by density gradient using Lymphoprep (StemCell Technologies, Canada) following standard laboratory procedures.

SAT collection

Abdominal subcutaneous adipose tissue will be collected by a biopsy procedure in the patient's upper right umbilical scar. After cleaning and asepsis, approximately 5 mL of lidocaine hydrochloride will be applied to anaesthetise the area where the incision will be made. With the patient free of pain and properly anaesthetised, an incision of approximately 1.5 cm will be made with the aid of a scalpel. SAT tissue will be removed and cut using tweezers and a scalpel. The incision will be closed non-invasively using an adhesive skin suture. All biopsies will

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be performed by a qualified surgeon team. Immediately after the biopsy procedure, the tissue sample will be frozen in liquid nitrogen and stored at −80°C for posterior DNA and RNA extraction.

DNA methylation analysis

DNA from SAT will be extracted using commercial kits according to the manufacturer's instructions. Subsequently, DNA will be treated with sodium bisulfite and purified using commercial kits according to the manufacturer's instructions. Sodium bisulfite-treated DNA will be used for hybridisation on the Infinium Human Methylation EPIC Beadchip platform (Illumina, San Diego, California, USA) following the manufacturer's protocol (Infinium HD Assay Methylation Protocol Guide, Illumina). Accordingly, the Beadchip will be scanned using the Illumina iScanSQ system, which employs a two-colour fluorescent laser scanner (532 nm/660 nm) with a resolution of 0.375 µm, capable of exciting the incorporated fluorophores throughout the protocol.

Gene expression

RNA will be extracted from PBMCs and SAT using commercial kits according to the manufacturer's instructions. The reverse transcription reaction will be conducted using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) following the manufacturer's recommendations, in a thermocycler (MJ Research PTC-100). Gene expression analysis will be carried out through real-time quantitative PCR (RT-qPCR) on the Step One Plus Real-Time PCR System (Applied Biosystems) equipment, using fluorogenic TaqMan MGB 6-FAM probes (Applied Biosystems) and Gene Expression Master Mix (Applied Biosystems). The data obtained from RT-qPCR reactions will be expressed as a cycling threshold (Ct), which represents a fluorescence detection baseline. Relative gene quantification will be calculated using the $2^{-\Delta\Delta Ct}$ comparative method. Glyceraldehyde-3-phosphate dehydrogenase and β-actin genes will be employed as endogenous controls.

Telomere length

DNA from total blood will be extracted using commercial kits according to the manufacturer's instructions. The quantification of telomere length will be performed using quantitative PCR.[40] Primers will be designed based on the target sequence. PCR reactions will be carried out in 96-well plates using the 7500 Fast Real-Time PCR System (Applied Biosystems) equipment and SYBR Green PCR Mastermix kit (Applied Biosystems). The 36B4 gene will be used as the single-copy gene. All analyses will be performed in triplicate, and the agreement of values will be verified (values higher than 10% will be reanalysed). Telomere length will be obtained through the relative ratio (T/S ratio) between the number of copies of the telomeric region (T) and the number of copies of a single-copy gene (S), using the relative standard curve. The T/S ratio for each sample will be calculated using the following formula: 2−ΔCt (telomere) − ΔCt (single-copy gene) = $2^{-\Delta\Delta Ct}$.[41]

Statistical analysis

The Shapiro-Wilk test will be applied to investigate data distribution. Between-group differences at baseline will be tested accordingly (eg, Independent T or Mann-Whitney U test (quantitative variables) and Pearson χ^2 test or Fisher's exact test (qualitative variables)). To assess the impact of the intervention, the Generalised Estimating Equation will be employed, and in cases where a significant *F* value, the Bonferroni post hoc test will be conducted for multiple comparisons. Group (ie, intervention and placebo) and time (ie, pre and post) will be considered fixed factors for all dependent variables, while subjects will be defined as random factors. Evaluation of endpoints will be carried out by both intention-to-treat and per-protocol analyses. Per protocol, the evaluation will be performed on patients who provided data from both the pre-intervention and post-intervention periods with high adherence (75%) to the intervention. Quantitative data will be reported as mean±SD, and qualitative data will be presented as frequency and absolute values. SPSS Statistics software V.22 (SPSS, Chicago, Illinois, USA) will be used for statistical data analysis. All tests will be two-tailed with a significance level of 0.05.

Ethics and dissemination

This study was preregistered in ClinicalTrials.gov. Patients will be required to fill out and sign an informed consent form before being included in the study. All procedures will follow the Declaration of Helsinki in its currently applicable version.

The risk of any adverse events occurring as a consequence of the supplementation is minimal, however, any adverse events that occur as a consequence of the biopsy procedure (ie, pain or local swelling) will be recorded and patients will receive medical advice.

All collected data will be included in a data set (in Excel and SPSS version). At the beginning of data collection, all patients will be identified by a numerical code (ie, P001) and all its biological samples will be identified with the same number. Thus, the final data set will be stripped of identifiers prior to release for sharing. Epigenetic data resulting from omics technologies will be created in specific software in a computer from the University of Sao Paulo which is appropriately password-protected. Data will be retained with the main and associated research for at least 4 years after the study completion.

DISCUSSION

The literature concerning the DNA methylation profile of patients with SLE is extensive, with evidence demonstrating DNA hypomethylation both globally throughout the genome[42 43] and also in specific genes, particularly those associated with the inflammatory and autoimmune pathways.[16 17 44] For example, an experimental study involving women with SLE, and healthy controls indicated

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lower levels of DNA methylation in the promoter regions of the IL-10 and IL-13 genes in CD4+T cells, suggesting that DNA hypomethylation leads to overexpression of these ILs in SLE.[16] Notably, increased expression of IL-10 in T cells promotes B-cell activity in SLE, contributing to autoimmunity, pro-inflammatory cytokines secretion and subsequent tissue damage.[45–48] The causes of DNA hypomethylation associated with SLE have not been fully elucidated; however, decreased expression of DNMTs, particularly DNMT1, has been linked to reduced global DNA methylation of immune cells,[49] thus rendering alterations in DNA methylation profiles as a potentially interesting target to benefit patients with SLE.

In this regard, dietary methyl donor nutrients emerge as potential modulators of DNA methylation levels and, consequently, gene expression.[50] Precision nutrition is an area of research focusing on individual metabolic variability in response to factors such as lifestyle and diet, which are primarily determined by variations in the genome, epigenome and microbiome.[51 52] Precision nutrition may play a pivotal role in managing SLE, with supplementation of folic acid and vitamin B_{12} emerging as significant strategies in adjunctive treatment through modulation of the DNA methylation profile. Thus, gaining a deeper understanding of the interactions between epigenetics and nutrition is crucial for designing personalised nutritional approaches in order to promote a better quality of life for patients.

The randomised, double-blind, placebo-controlled design of this study is a notable strength, as it minimises biases and enhances the validity and robustness of the results. Additional strengths include the inclusion of patients with low disease activity, as high disease activity could potentially influence DNA methylation profiles. Furthermore, the use of advanced technology for DNA methylation analysis, specifically the Infinium Human Methylation EPIC BeadChip, ensures high sensitivity and precision in the results; the control of the patient's levels of physical activity and diet, as these factors can interfere with epigenetics patterns; the verification and control of adherence to the administered supplementation. Also, it should be noted that numerous factors can interfere with the epigenetic machinery.[53–55] Although modifications in diet and physical activity will be evaluated, other environmental and behavioural factors (eg, stress stimuli, drug use and exposure to toxins) could not be controlled in the study design.

From this perspective, the development of clinical trials becomes very important to expand the knowledge about the potential therapeutic effects of precision nutrition on DNA methylation in humans. Thus, this study has great potential to generate clinically relevant results for the management of SLE.

Contributors CFN contributed to the trial planning and drafting of the manuscript. HR, BG, JCNLdM, LMC, AAR, LLS and EFB will contribute to the data collection and statistical analysis of the manuscript. All authors carefully revised the manuscript and agreed on the final version. CFN is the guarantor and accepts full responsibility for the finished work and/or the conduct of the study, had access to the data and controlled the decision to publish.

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Competing interests None declared.

Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Not applicable.

Ethics approval The protocol of this trial has been approved by the Ethical Committee (CAAE.: 47317521.8.0000.0068) of the Clinical Hospital of the School of Medicine of the University of Sao Paulo (HCFMUSP), in Brazil.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available upon reasonable request.

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