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Methyl donor micronutrients, CD40-ligand methylation and disease activity in systemic lupus erythematosus: A cross-sectional association study

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Abstract

Objective: Hypomethylation of CD40-ligand (CD40L) in T-cells is associated with increased disease activity in systemic lupus erythematosus (SLE). We therefore investigated possible associations of dietary methyl donors and products with CD40L methylation status in SLE.

Methods: Food frequency questionnaires were employed to calculate methyl donor micronutrients in 61 female SLE patients (age 45.7 ± 12.0 years, disease duration 16.2 ± 8.4 years) and compared to methylation levels of previously identified key DNA methylation sites (CpG17 and CpG22) within CD40L promotor of T-cells using quantitative DNA methylation analysis on the EpiTYPER mass spectrometry platform. Disease activity was assessed by SLE Disease Activity Index (SLEDAI). Linear regression modelling was used. P values were adjusted according to Benjamini & Hochberg.

Results: Amongst the micronutrients assessed (g per day), methionine and cysteine were associated with methylation of CpG17 ($\beta = 5.0$ (95%CI: 0.6-9.4), $p = 0.04$; and $\beta = 2.4$ (0.6-4.1), $p = 0.02$, respectively). Methionine, choline, and cysteine were additionally associated with the mean methylation of the entire *CD40L* ($\beta = 9.5$ (1.0-18.0), $p = 0.04$; $\beta = 1.6$ (0.4-3.0), $p = 0.04$; and $\beta = 4.3$ (0.9-7.7), $p = 0.02$, respectively). Associations of the SLEDAI with hypomethylation were confirmed for CpG17 (β=-32.6 (-60.6 to -4.6), $p = 0.04$) and CpG22 (β=-38.3 (-61.2 to -15.4), $p = 0.004$), but not the mean methylation of *CD40L*. Dietary products with the highest impact on methylation included meat, ice cream, white bread, and cooked potatoes.

Conclusions: Dietary methyl donors may influence DNA methylation levels and thereby disease activity in SLE.

Keywords

Systemic lupus erythematosus, nutrition, methylation, CD40-ligand, T-cells, disease activity

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Introduction

The heterogeneous clinical course of systemic lupus erythematosus (SLE) with varying disease activity states is considered to be a consequence of a combination of distinct genetic and environmental factors in each patient.[1–4] Epigenetic modifications such as DNA methylation are controlled in part by environmental factors such as diet or vitamin D levels, and therefore constitute an important link between genetic

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and environmental factors.[3–10] DNA methylation at CpG sites within promotor and enhancer regions generally results in silencing of the methylated gene.[4,10,11] Global hypomethylation has been observed in T-cells of SLE patients.[9,12] Co-expression of a number of hypomethylated genes results in a distinct autoreactive T-cell subtype that correlates with SLE disease activity.[5] Moreover, inefficient gene silencing by X-chromosomal DNA hypomethylation is likely to be one of the key reasons for a striking preponderance of SLE in women.[4,13] In particular, X-chromosomal *CD40L* is hypomethylated in $CD4^+$-T-cells of women with SLE.[14] Consecutive overexpression of CD40L in turn [14] leads to increased stimulation of B-cells with increased autoantibody production.[15,16] In this context, distinct CpG sites within CD40L are reported to be particularly associated to increased SLE disease activity.[17] Accordingly, reduced methyl donor content of food increased *CD40L* expression and increased disease activity in experimental systemic lupus.[5]

One-carbon (1 C) metabolism consists of interlinking metabolic pathways (Figure 1) resulting in generation of S-adenosylmethionine (SAM) which is the principal substrate for DNA-methylation.[18] Methionine is a precursor of SAM which may use folate and choline as 1 C donors.[18] Cysteine is generated from homocysteine via the transsulfuration pathway to generate the antioxidant glutathione, and this pathway is stimulated by SAM,[19] thereby indicating active ongoing 1 C metabolisms. Vitamin B6 catalyzes reactions within the folate cycle and transsulfuration pathway, while vitamin B12 is a cofactor for methionine synthetase thereby linking the methionine and folate cycles.[18]

In the present study, we sought to explore if the content of methyl donor micronutrients and certain foods rich in methyl donors are associated with SLE disease activity in human patients.

Methods

Patients and clinical data

In this cross-sectional study, consecutive patients were recruited from the rheumatology outpatient department of Heinrich-Heine-University Düsseldorf, Germany, in case they fulfilled 1997 American College of Rheumatology (ACR) SLE criteria,[20] were- >18 years of age, and had no clinical signs of infections. Disease activity was assessed by the Systemic Lupus Disease Activity Index-2000 (SLEDAI). Patients were administered a food frequency questionnaire (FFQ) which has previously been validated in the German population.[21] To avoid errors during transferring answers from (paper) questionnaires to the electronic data base, answers to the items were digitalized in two consecutive and independent runs. The second round was blinded for entries of the first run. The resulting double-entries were cross-checked for agreement by EpiData (http://www.epidata.dk). In case of any disagreement, original questionnaires were consulted to find the intended answer. Micronutrient contents were calculated by multiplying the various items within the FFQ with micronutrient ingredient estimations derived from US Department of Agriculture (USDA) (https://fdc.nal.usda.gov/). These data were also used for ranking of dietary products according to their relative content of the respective micronutrient per serving.

Written informed consent was obtained from all patients. The study was approved by the ethics committee of the Medical Faculty of

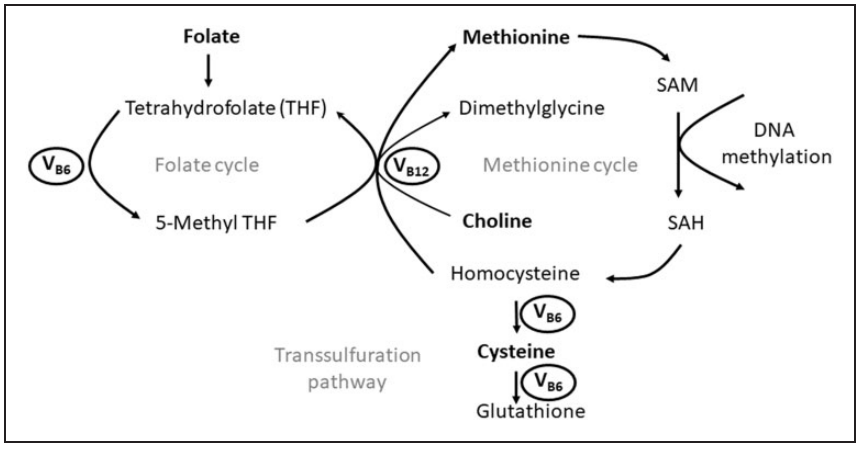

Figure 1. Simplified overview of one-carbon metabolism highlighting the nutrients addressed in the present study. V_{B6}, Vitamin B6. V_{B12}, Vitamin B12. *SAM*, S-Adenosylmethionine. *SAH*, S-Adenosylhomocysteine. Nutrients assessed in the presents study are printed in bold letters.

Heinrich-Heine-University Düsseldorf (study identification 2016034998), and adhered to the Declaration of Helsinki.

Isolation of CD4+ T-cell DNA

EDTA blood (9 ml per patient) was collected and promptly processed. Peripheral mononuclear cells were isolated by density gradient centrifugation with Leucosep (Greiner Bio-One GmbH, Frickenhausen, Germany) and Lymphoprep (AXIS-SHIELD PoC AS, Oslo, Norway) according to manufacturer's instructions. Cells were again resuspended in 4 ml autoMACS Running Buffer (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and separated according to manufacturer's instructions using CD4 MicroBeads human isolation kit (Miltenyi Biotec GmbH). Subsequently magnetic bead based automatic isolation of DNA from CD4+ cells by MagCore instrument (MagCore Genomic DNA Whole Blood Kit, RBC Bioscience, New Taipei City, Taiwan) was carried out. The isolation procedure and quality control measures have previously been described.[22]

Assessment of *CD40L* promotor and enhancer methylation status in CD4+ T-cells

Mass spectrometry analysis of bisulfite-converted DNA was employed as described previously.[23] For quantitative analyses of aberrantly methylated CG-rich regions in a larger patient cohort, we selected 22 CpG sites (7 of the promotor, and 10 and 5 of the two enhancer regions, respectively), one of which could not be quantified satisfactorily due to technical issues, resulting in 21 CpG sites (listed in Supplementary Table 1). Analysis was by Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) of bisulfite converted DNA.[24] Genomic sequences of selected regions were extracted from the UCSC genome browser (www.genome.ucsc.edu/). PCR primers were designed using Methprimer (www.urogene.org/methprimer/) and sequences are provided in Supplementary Table 1. Sodium bisulfite conversion was performed using EZ DNA methylation kit (Zymo Research, California, USA) using 300 ng of genomic DNA and an alternative conversion protocol. The amplification of target regions was followed by Shrimp alkaline phosphatase (SAP) treatment, reverse transcription and subsequent RNA base-specific cleavage (MassCLEAVE, Agena, San Diego, CA) as previously described.[24] Cleavage products were loaded onto silicon chips (spectroCHIP, Agena, San Diego, CA) and analyzed by MALDI-TOF mass spectrometry (MassARRAY Compact MALDI-TOF, Agena, San Diego, CA). Methylation was quantified from mass spectra using the Epityper software v1.0 (Agena, San Diego, CA). For CpG dinucleotides that were covered by more than one amplicon we calculated the mean methylation ratio. For the present study, mean methylation rate of the total CD40L along with previously identified key methylation sites at CpG17 (nucleotide position 135730265 to 13,57,30,266 of chromosome X (GRCh37/hg19) reference assembly) and CpG22 (135730445 – 135730446 of chromosome X (GRCh37/hg19) reference assembly) were further considered.[17]

Statistical analysis

Descriptive for continuous variables are reported as mean ± empirical standard deviation. Associations of the fraction of methylated DNA at the specific sites to micronutrient intake and disease activity were assessed by linear regression adjusted for age and body mass index. Associations are reported in terms of the regression coefficients (β). For instance, when assessing the association of a food product (measured in g intake per day) with the methylation rate (micronutrient intake (g per day) $\sim \beta$ * CpG methylation rate), a β of 5 would indicate a 0.1 (10%) increase in methylation with each 0.5 g (500 mg) higher intake per day. P-values were adjusted according to Benjamini and Hochberg.[25] Associations of methylation rates and dietary products were assessed by univariable linear regression followed by multivariable linear regression of those products with a significant or borderline significant association ($p < 0.1$) in the univariable analysis. The statistical software R version 3.5.2 was used for all analyses.

Results

Patients' characteristics

Sixty-one female SLE patients, aged 45.7 ± 12 years, disease duration 16.2 ± 8.4 years participated in the study. Treatment consisted mainly of antimalarials (68.9%), and glucocorticoids (63.9% at a mean daily dose of 4.8 ± 2.4 mg). Details are outlined in Table 1. Weekly micronutrient intake was calculated by adding the products of daily food intake from the FFQ with respective micronutrient ingredients as determined by the USDA. Details are depicted in Table 1.

CD40L methylation status and disease activity

Previously identified distinct *CD40L* promotor methylation sites CpG17 (β=-32.6 (-60.6 to – 4.6), $p = 0.035$) and CpG22 (β=-38.3 (-61.2 to -15.4), $p = 0.004$), but not the mean methylation of *CD40L* (β=-21.9 (-77.7

Table 1. Patients' characteristics (n = 61).

Parameter	n (%) or mean ± SD
Female	61 (100%)
Age (years)	45.7 ± 12
Disease duration	16.2 ± 8.4
SLEDAI	4.7 ± 5.2
C3c (mg/dl)	91.9 ± 16.7
C4 (mg/dl)	17.1 ± 8.5
Anti-dsDNA (U/l)	205.7 ± 345.0
Antimalarials	42 (68.9%)
Glucocorticoids	39 (63.9%)
Azathioprine	9 (14.8%)
Mycophenolate	5 (8.2%)
Rituximab	4 (2.4%)
Belimumab	3 (4.9%)
Methotrexate	2 (1.2%)
Ciclosporine	3 (1.8%)
Choline (mg/d)	302.9 ± 137.9
Cysteine (mg/d)	871.4 ± 351.6
Methionine (g/d)	1.72 ± 0.85
Vitamin B6 (mg/d)	1.97 ± 1.15
Folate (μg/d)	269.6 ± 129.1
Vitamin B12 (μg/d)	4.38 ± 2.98

SLEDAI, Systemic lupus erythematosus disease activity index 2000; micronutrient intake is quantified based on food frequency questionnaire items including food and supplements.

to 33.9), p = 0.4) were associated to disease activity measured by the SLEDAI, as was expected based on previous observations.[17] In other words, each 10% decrease of methylation of CpG17 was associated to a 3.3 point (95%CI 6.1 – 4.6) increase of the SLEDAI, while each 10% decrease of methylation of CpG22 was associated to a 3.8 point (95%CI 6.1-1.5) increase of the SLEDAI (Figure 2).

CD40L methylation status and micronutrients

Next, we were interested to determine, if methyl donor micronutrient intake was associated to the methylation status of the above assessed methylation sites within *CD40L* of SLE patients' T-cells. As outlined in the introduction, methionine, folate, choline, and cysteine intake were assessed due to their involvement in 1 C metabolism along with catalytic vitamins B12 and B6. An increase of 500 mg methionine intake per day was associated to a 10% higher methylation rate of CpG17. Similarly, an increase of 950 mg methionine intake per day was associated to a 10% higher mean methylation rate of the entire CD40L promotor. Concerning cysteine, an increase of 240 mg per day was associated to a 10% higher methylation rate of CpG17, and an increase of 430 mg per day was associated to a 10% higher mean methylation rate of the entire CD40L promotor. Additionally, an increase of 168 mg choline per day was associated to a 10% higher mean methylation rate of the entire CD40L promotor.

Neither vitamins nor the other micronutrients assessed displayed a significant association after adjustment for multiple testing. Details of the analysis are summarized in Table 2 and depicted in Figure 2.

Association of distinct dietary products with methylation rate

We were interested to analyze which distinct dietary products had the largest impact on *CD40L* methylation rates. Therefore, dietary products with the highest content of methyl donors methionine (>20 mg/serving), choline (>15 mg/serving), and cysteine (> 40 mg/serving) were selected from the FFQ for further analyses. Of note, foods rich in methionine, choline and cysteine were largely overlapping and there was considerable correlation between the daily intake of all three methyl donors (correlation coefficient according to Spearman: choline ~ cysteine r = 0.85, p < 2e-16; choline ~ methionine r = 0.89, p < 2e-16; cysteine ~ methionine 0.93, p < 2e-16). Details on the respective methyl donor content of the foods assessed are provided in Supplementary Table 2. The most robust associations were noted for meat (2.5% increase in mean *CD40L* methylation with each serving per day), ice cream (6.3% increase in mean *CD40L* methylation with each serving per day), white bread (3% increase in CpG22 methylation with each serving per day) and cooked potatoes (6% increase in CpG17 methylation with each serving per day). Details are given in Table 3.

Discussion

A complex interplay of genetic and environmental factors contributes to SLE disease activity.[1–4] The impact of food on epigenetic modification, and consequently on gene activity is increasingly recognized.[26] This connection seems especially important, since insufficient nutritional status and suboptimal composition of the regular diet are a concern in SLE patients.[27] Strikingly, lupus prone mice on a methyl donor rich diet have increased methylation rates of the important costimulatory molecule *CD40L* within T-cells and show significantly decreased disease activity.[5] We therefore explored the association of *CD40L* methylation rates and food as well as methyl donor micronutrient intake in SLE patients. Previous research has shown that hypomethylation of key CpG sites (CpG17 and 22) within T-cell's *CD40L* promotor are linked to increased disease activity in women with SLE.[17] Presently, intake of candidate methyl donor micronutrients as assessed

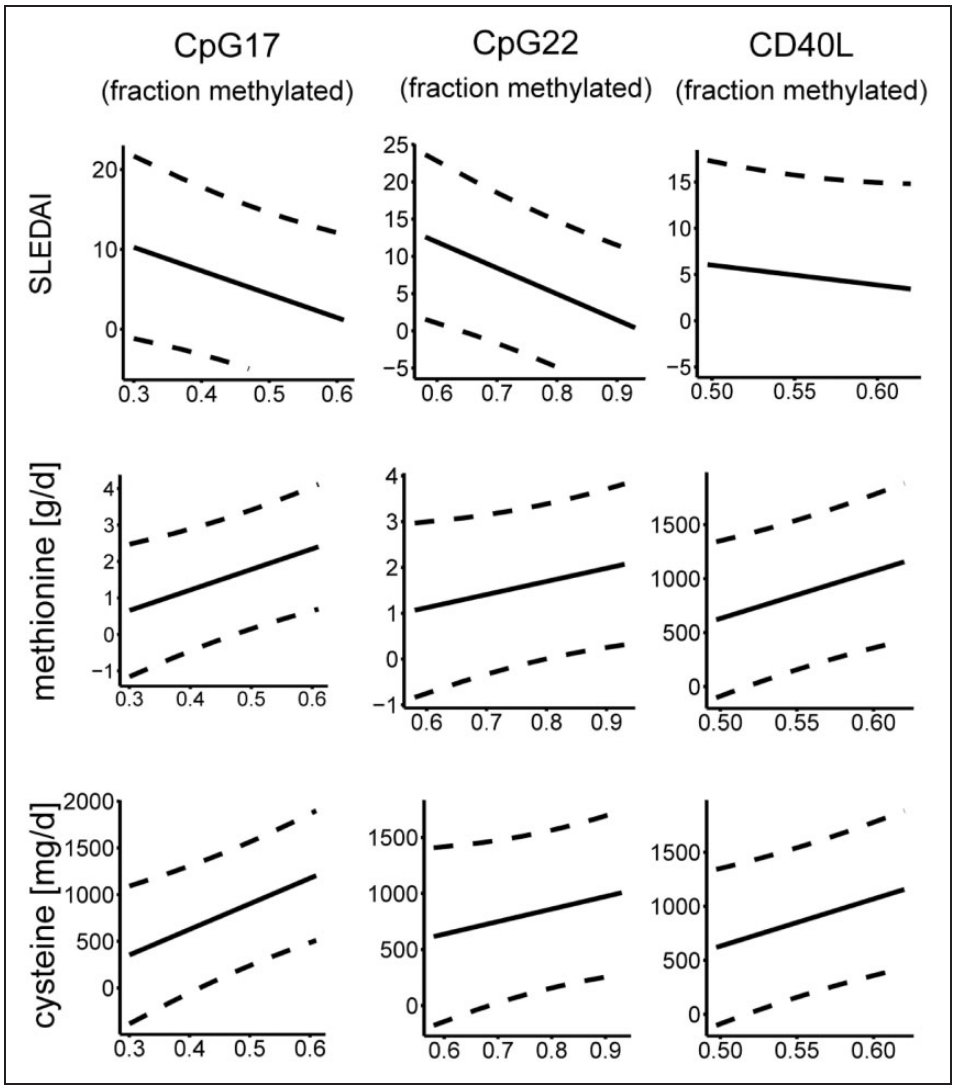

Figure 2. Associations of methylation ratio at various CD40L sites compared to disease activity and nutritional methyl donor intake. Fraction of methylated CpG sites of CD4 T-cell CD40L at two distinct sites (CpG17, CpG22) and of the whole CD40L were compared to Systemic Lupus Erythematosus Disease Activity Index (SLEDAI), and to the nutritional methionine and cysteine intake assessed by a food frequency questionnaire. Lines represent slope of linear regression analysis adjusted for age and body mass index, dashed lines represent prediction intervals.

Table 2. Micronutrients and CD40L methylation status.

	CpG17		CpG22		Mean CD40L	
Nutrient	β (95%-CI)	p	β (95%-CI)	p	β (95%-CI)	p
Methionine (g/d)	5.0 (0.6–9.4)	**0.04**	2.6 (−1.4–6.5)	0.2	9.5 (1.0–18.0)	**0.04**
Folate (μg/d)	12.7 (−676.6–702.1)	1.0	18.9 (−576.9–614.8)	1.0	718.1 (−571.0–2007.1)	0.8
Choline (g/d)	0.63 (−0.08–1.3)	0.1	0.18 (−0.5–0.8)	0.6	1.68 (0.4–3.0)	**0.04**
Cysteine (g/d)	2.4 (0.6–4.1)	**0.02**	1.0 (−0.6–2.6)	0.2	4.3 (0.9–7.7)	**0.02**
Vitamine B6 (mg/d)	0.25 (−5.9–6.4)	0.9	0.88 (−4.5–6.2)	0.9	6.9 (−4.7–18.6)	0.7
Vitamine B12 (μg/d)	12.9 (−2.9–28.6)	0.2	5.0 (−8.8–18.9)	0.4	24.8 (−5.6–55.2)	0.2

P-values <0.05 were considered significant and printed in bold.

Association of micronutrient intake calculated based on food frequency questionnaire items with methylation rates of distinct *CD40L* promotor sites (CpG17 and CpG22) or the mean methylation of *CD40L* in T-cells of women with SLE by linear regression modelling.

Table 3. Dietary products and CD40L methylation status.

Dietary product (servings per day)	CpG17		CpG22		Mean CD40L	
	β (95%-CI)	p_{univ} (p_{mult})	β (95%-CI)	p_{univ} (p_{mult})	β (95%-CI)	p_{univ} (p_{mult})
Pizza	0.19 (0.01–0.36)	**0.04** (0.2)	−0.003 (−0.24–0.18)	0.8 (NA)	0.031 (−0.07–0.13)	0.5 (NA)
Dairy products	0.03 (0.001–0.05)	**0.047** (0.054)	0.012 (−0.02–0.04)	0.4 (NA)	0.01 (−0.001–0.02)	0.1 (NA)
Chips/french fries	0.25 (0.01–0.48)	**0.04** (0.19)	0.1 (−0.17–0.39)	0.4 (NA)	0.1 (−0.04–0.23)	0.1 (NA)
Cooked potatoes	0.06 (0.02–0.1)	**0.004 (0.005)**	0.03 (−0.02–0.08)	0.2 (NA)	0.02 (0.001–0.05)	0.046 (0.12)
White bread	0.02 (−0.01–0.05)	0.2 (NA)	0.03 (−0.001–0.06)	0.09 (**0.04**)	−0.003 (−0.02–0.01)	0.8 (NA)
Beer	0.18 (−0.19–0.55)	0.3 (NA)	0.39 (−0.03–0.81)	0.07 (0.1)	0.04 (−0.17–0.24)	0.7 (NA)
Fruit or herbal tea	0.003 (−0.01–0.01)	0.6 (NA)	0.012 (−0.001–0.02)	0.06 (0.06)	0.005 (−0.001–0.01)	0.1 (NA)
Meat	0.025 (−0.01–0.07)	0.2 (NA)	0.002 (−0.04–0.05)	0.9 (NA)	0.025 (0.001–0.05)	**0.02 (0.04)**
Ice cream	0.06 (−0.04–0.2)	0.2 (NA)	0.008 (−0.1–0.12)	0.9 (NA)	0.063 (0.01–0.11)	**0.01 (0.009)**

P-values <0.05 are printed in bold.

Association of dietary product intake calculated based on food frequency questionnaire items with methylation rates of distinct *CD40L* promotor sites (CpG17 and CpG22) or the mean methylation of *CD40L* in T-cells of women with SLE by linear regression modelling (p_{univ} p value of univariable analysis; p_{mult}, p value of multivariable analysis of selected parameters; *NA*, not applicable as parameter was not included in multivariable analysis).

by FFQ were studied. Significant associations between increased methylation of *CD40L* were noted for methionine and cysteine at CpG17 and with the mean methylation percentage of *CD40L*, and choline with the mean methylation percentage of *CD40L*. Although methyl donor levels or consumption based on FFQs may not represent the availability of methyl donors at the individual cellular level,[26] our results suggest some association of methyl donor micronutrient intake and methylation. This is in line with observations in experimental lupus, where mice fed increased amounts of methyl donors show increased methylated DNA, including the *CD40L* site.[5] Of note, there was no homogenous association of methyl donor micronutrients to all methylation sites assessed in the present study. We speculate that this may reflect the complex interplay of nutrients and epigenetic modifications.[26] For instance, besides methyl donor micronutrients, polyunsaturated fatty acids may display effects on methylation,[28] which is likely dependent on the availability of cofactors and methyl donors in the vicinity. Due to this complex interplay of dietary products, different micronutrients may be critical for DNA methylation depending on dietary backgrounds: while vitamins B6 and B12 did not appear to be associated with methylation rates in the present study, this may well be the case in populations with generally lower levels of these vitamins. Although unhealthy and healthy diets appear to contain similar products across countries,[29] there is certainly variability in food patterns across the world.[30] Moreover, these food patterns may change over time.[30] Although we did not observe such an association in the present study population, certain prescribed drugs and food supplements may further intervene in DNA methylation.[31] From the perspective of individual SLE patients, disease activity and general health are a great concern,[32] and these are influenced by other nutrients besides methyl donators, as has been shown for the composition of fatty acids within the diet for instance.[33] Moreover, methylation has been shown to be dependent upon diet in numerous studies in humans with conditions other than SLE.[34] More recently, dietary interventions and methyl donor (e.g. methionine) restriction has been assessed as a means to reduce age-dependent DNA methylation in healthy humans.[35,36] The current findings along with previously reported hypomethylation in SLE as compared to healthy controls and potential dietary effects on methylation [4,5,9,11–14] thus suggest that DNA methylation is dependent upon diet irrespective of SLE disease status. The impact of dietary interventions on methylation status may differentially impact underlying diseases. For instance, increased methylation may be desirable in

SLE to reduce disease activity, while in healthy individuals, it may increase aging effects.[35,36]

Thus, dietary counselling on an individual basis requires careful consideration of all these factors, as is best possible. Indeed, the majority of SLE patients actively seek self-empowerment and request counselling on dietary measures.[32] We were therefore also interested to identify foods with the potential to increase methylation. By screening the dietary products with the highest concentration of the methyl donor micronutrients methionine, cysteine and choline which showed the highest association with *CD40L* methylation in the present population, distinct dietary products were identified. Of note, some of these such as ice cream or chips/french fries are often included in a rather unhealthy diet.[29,30] Interestingly, polyunsaturated fatty acids, generally regarded as a feature of rather healthy foods, may be involved in DNA methylation procedures as well.[28,37] Their role in this context is hitherto incompletely understood, however, as different effects have been noted depending on sex [37] or even cell type.[28] Hence, caution should be used when applying the present data to dietary counselling as of yet. The present results concern methylation and clearly need to be balanced against health effects on other domains such as cardiovascular risk and obesity issues. Of note, the present study was focused on women with SLE. This is due to *CD40L* being located on the X-chromosome and the arguably established association of decreased X-inactivation to SLE preponderance in women.[14] Since multiple DNA sites may be subject to gene inactivation via methylation, a similar effect of dietary methyl donors may be present for the occasional male SLE patient. As this association cannot be directly concluded from our data, more research on the subject is warranted.

The present data may thus inform future studies on dietary interventions and counselling of SLE patients.

Declaration of conflicting interests

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Supplemental material

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