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Intracellular homocysteine metabolites in SLE: plasma S-adenosylhomocysteine correlates with coronary plaque burden

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ABSTRACT

Background and aims We hypothesised that intracellular homocysteine and homocysteine metabolite levels in patients with SLE are disproportionately elevated compared with the levels seen in healthy subjects and that they are independently associated with coronary plaque in SLE.

Methods A liquid chromatography–tandem mass spectrometry absolute quantification assay was used for the determination of six analytes in both plasma and peripheral blood mononuclear cells (PBMCs): homocysteine (Hcy), S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH), methionine (Met), cystathionine (Cysta) and 5-methyltetrahydrofolate (5m-THF). We then compared intracellular (PBMC) and extracellular (plasma) Hcy and Hcy metabolite (SAM, SAH, Met, Cysta and 5m-THF) concentrations in 10 patients with SLE and in 10 age, sex and ethnicity matched controls. Subjects with a history of diabetes mellitus, cardiovascular disease, hypertension, alcohol consumption in excess of 3 units per day, anaemia, renal insufficiency (serum creatinine >1.5 mg/dL) and pregnancy were excluded. All patients with SLE had two coronary CT angiography studies as screening for occult coronary atherosclerotic disease.

Results Plasma from patients with SLE had higher levels of Hcy ($p<0.0001$), SAH ($p<0.05$), SAM ($p<0.001$) and lower levels of Met ($p<0.05$) and Cysta ($p<0.001$) compared with controls. PBMC intracellular concentrations from patients with SLE had higher levels of Cysta ($p<0.05$), SAH ($p<0.05$), SAM ($p<0.001$) and lower levels of 5m-THF ($p<0.001$). Plasma SAH showed a positive correlation with total coronary plaque, calcified plaque and non-calcified plaque ($p<0.05$).

Conclusion Intracellular concentrations of Hcy metabolites were significantly different between patients with SLE and controls, despite similar intracellular Hcy levels. Plasma SAH was positively correlated with total coronary plaque, calcified plaque and non-calcified plaque.

Key messages

What is already known about this subject?

- Epidemiological studies have identified homocysteine (Hcy) as an independent risk factor for cardiovascular disease in the general population and in SLE, but lowering Hcy levels via folate supplementation did not improve cardiovascular outcomes in randomised trials

What does this study add?

- We pursued the first study of intracellular and extracellular Hcy metabolites in SLE to assess their potential contribution to the elevated cardiovascular risk seen in this population.
- Intracellular (PBMC) concentrations of homocysteine metabolites (cystathionine (Cysta), S-adenosylhomocysteine (SAH), S-adenosylmethionine (SAM) and 5-methyltetrahydrofolate) were significantly higher in patients with SLE compared with controls, despite similar intracellular Hcy levels.
- Extracellular (plasma) concentrations of Hcy, SAH and SAM were significantly higher, while methionine and Cysta levels were significantly lower in patients with SLE compared with controls.
- Plasma SAH was positively correlated with total coronary plaque, calcified plaque and non-calcified plaque in patient with SLE.

How might this impact on clinical practice or future developments?

- This is the first study identifying SAH as a potential cardiovascular risk factor in SLE. Folate supplementation, which lowers plasma Hcy levels, does not affect SAH levels that could be a potential target for future interventions in prevention and treatment of accelerated atherosclerosis in SLE.

INTRODUCTION

Increased plasma concentrations of homocysteine (Hcy) have been linked to a variety of diseases ranging from neural tube defects to cardiovascular disease (CVD).[1–3] In SLE, Hcy has been shown to be associated with disease activity[4] and coronary calcification[5] and is an independent risk factor for stroke and thrombotic events.[5] A number of clinical trials addressed the potentially beneficial effects of folic acid supplementation on treatment of hyperhomocysteinaemia. Even though folic acid indeed effectively decreased plasma Hcy, it failed to lower cardiovascular risk.[6] One of the proposed explanations included the strong inter-relationship between Hcy and S-adenosylhomocysteine (SAH) in the one

Table 1 Demographic, clinical and laboratory characteristics of patients with SLE

Characteristic	Value
No. of patients	10
Age at diagnosis, mean±SD years	39.9±12.5
Age at sample collection, mean±SD years	57.9±8.8
Female, %	90
Race/ethnicity, %	
Caucasian	50
African-American	40
Asian	10
Disease duration*, mean±SD years	18.1±11.6
ACR or SLICC classification criteria, %	
Malar rash	40
Discoid rash	10
Photosensitivity	50
Oral/nasopharyngeal ulcers	90
Arthritis	80
Proteinuria	30
Pleurisy	50
Pericarditis	30
Psychosis	0
Seizures	10
ANA	90
Anti-dsDNA	70
Anti-Sm	10
Lupus anticoagulant (by dRVVT)	40
Leucopenia	40
Thrombocytopenia	20
Haemolytic anaemia	10
Coombs test	10
At time of sample collection	
SLEDAI, mean±SD	2.5±2.2
PGA, mean±SD	1.0±0.9
Antihypertensive use, %	80
Statin use, %	80
Prednisone use, %	30
Immunosuppressant use, %	30
Plaquenil use, %	70

*Up to their date of sample collection.
ACR, American College of Rheumatology; ANA, antinuclear antibody; dRVVT, Dilute Russell's viper venom time; dsDNA, double stranded DNA; PGA, Physician Global Assessment; SD, standard deviation; SLEDAI, SLE Disease Activity Index; SLICC, Systemic Lupus Erythematosus International Collaborating Clinics.

carbon metabolism and the observation that vitamin supplementation, which lowers plasma Hcy levels, does not affect SAH levels.[7 8] This led to several studies that showed that higher concentrations of plasma SAH were independently associated with an increased risk of cardiovascular events[9] and presence of subclinical atherosclerosis.[10] Another hypothesis was that cardiovascular risk might be mediated by intracellular Hcy and its metabolites since folate supplementation lowers only extracellular Hcy levels, while intracellular Hcy concentrations remain unaffected.[11]

We thus hypothesised that plasma SAH and intracellular Hcy and Hcy metabolite levels in patients with SLE are disproportionately elevated compared with the levels seen in healthy subjects and that they are independently associated with coronary plaque in SLE.

PATIENTS AND METHODS

The Hopkins Lupus Cohort is an ongoing longitudinal cohort of patients with SLE presenting from the community surrounding Johns Hopkins University School of Medicine, which was begun in 1986. Patients who met classification criteria for SLE (either the revised American College of Rheumatology (ACR) criteria[7] or Systemic Lupus Erythematosus International Collaborating Clinics (SLICC) Classification criteria[8] were enrolled. All patients furnished written, informed consent to participate in the Cohort.

At enrolment into the cohort, a detailed patient history of SLE was collected based on patient report and medical record review. Subsequently, patients were seen quarterly (or more often if medically necessary). At each visit, disease activity indices (including the Physician Global Assessment as part of the Lupus Activity Index[9] and SELENA revision of the SLE Disease Activity Index[10] were completed.

Ten patients who met the SLICC classification criteria for SLE were included in the study. All patients with SLE had two coronary CT angiography (CCTA) studies (follow-up mean=3.77 years, SD=0.94 years) as screening for occult coronary atherosclerotic disease in asymptomatic individuals. CT images were acquired using a 320×0.5 mm detector row CT system (Aquilion ONE, Canon Medical Systems, Otawara, Japan). Patient preparation included oral (75–150 mg) metoprolol. Fifty to 70 mL of iodinated contrast (iopamidol 370 or iohexol 350 mg iodine/mL) was injected intravenously at 4.0–6.0 mL/s for prospectively ECG-triggered acquisitions (typically 75% of the R–R interval). If coronary calcification was evident on angiographic images, a non-contrast CT acquisition was added to quantify calcium for the Agatston score. CT angiographic data were reconstructed to generate 0.5 mm slice thickness images with a 0.25 mm increment using both a standard (FC43) and a sharp (FC05) convolution kernel. Reconstructed images were transferred to a dedicated workstation for further analysis (Vitrea FX version 3.0 workstation, Vital Images, Minnetonka, Minnesota, USA). All images were interpreted by a physician, blinded to any clinical information, with extensive experience (level III certified) in the interpretation of CCTA. The left anterior descending coronary artery, left

Table 2 Average concentration of PBMC and plasma analytes in patients with SLE patients and controls

	PBMC (pmol/million cells)			Plasma (nM)		
	Control	Patient	P value	Control	Patient	P value
Homocysteine	1.57 (SD=0.6)	1.7 (SD=0.92)	0.68	10807.5 (SD=3033.5)	16453.4 (SD=1764.4)	0.00008
Methionine	286.8 (SD=197.9)	210 (SD=103.1)	0.29	24930.56 (SD=5099.2)	17877.6 (SD=9216.53)	0.048
Cystathionine	0.13 (SD=0.07)	0.65 (SD=0.774)	0.049	97.4 (SD=32)	180.2 (SD=84)	0.009
SAH	2.46 (SD=0.86)	3.53 (SD=1.36)	0.049	30.31 (SD=10.74)	47.78 (SD=16.4)	0.01
SAM	5.04 (SD=1.74)	8.51 (SD=2.94)	0.005	119.67 (SD=20.1)	229.62 (SD=91.27)	0.001
5m-THF	0.88 (SD=0.48)	3.07 (SD=1.96)	0.005	N/A	N/A	

5m-THF, 5-methyltetrahydrofolate; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine.

circumflex coronary artery and right coronary artery were divided into proximal, mid and distal segments, respectively, according to standard segmentation guidelines.[12] The left main coronary artery was evaluated separately, resulting in 10 segments evaluated in total. Each segment was assessed for the presence and severity of atherosclerotic plaques on a semiquantitative scale. Plaque characteristics were noted for each segment.

Coronary plaque area was measured by manual tracing for the difference between the area within the external elastic membrane and the area of the vessel lumen at the site of maximal luminal narrowing as observed on a cross-sectional CCTA image. A non-calcified plaque was defined as a low-density mass >1 mm^2 in size with a density <150 Hounsfield units, located within the vessel wall, and clearly distinguishable from the contrast-enhanced coronary lumen and the surrounding pericardial tissue. The threshold for a calcific lesion was set at a computed tomographic density of >150 Hounsfield units having an area >1 mm^2. All images were interpreted by a physician, blinded to any clinical information.

Subjects with a history of diabetes mellitus, CVD, hypertension, alcohol consumption in excess of 3 units per day, anaemia, renal insufficiency (serum creatinine >1.5 mg/dL) and pregnancy were excluded. The standard blood sample volume used to isolate an adequate number of PBMC's for mass spectrometry studies is 10 mL. Due to an inherently lower number of PBMC's present in patients with SLE, 20 mL of blood was collected from each patient to assure an adequate number of isolated cells.

Ten healthy volunteers matched by age, sex and ethnicity to the enrolled patients with SLE were enrolled in the study. A creatinine was measured for each control; all controls with a creatinine over 1.5 mg/dL were excluded from the study. Ten millilitres of whole blood was drawn for baseline Hcy measurements.

A liquid chromatography–tandem mass spectrometry absolute quantification assay was used for the determination of six analytes in both plasma and PBMCs: Hcy, S-adenosylmethionine (SAM), SAH, methionine (Met), cystathionine (Cysta) and 5-methyltetrahydrofolate (5m-THF).

Chemicals

Hcy, Met, Cysta, SAH and SAM were purchased from Sigma Aldrich (St. Louis, Missouri, USA). 5m-THF was

Table 3 Correlations between analyte concentrations (PBMC and plasma) and coronary plaque volumes

		Total plaque		Total calcified plaque		Total non-calcified plaque	
		Correlation coefficient	P value	Correlation coefficient	P value	Correlation coefficient	P value
PBMC	Hcy	0.02	0.97	−0.31	0.45	0.060	0.89
	Methionine	0.34	0.41	0.11	0.80	0.37	0.37
	Cystathionine	−0.58	0.13	−0.31	0.46	−0.60	0.11
	SAH	0.06	0.88	−0.03	0.95	0.07	0.86
	SAM	−0.26	0.54	−0.04	0.91	−0.28	0.5
	5m-THF	−0.17	0.69	−0.05	0.90	−0.18	0.67
Plasma	Hcy	0.41	0.31	0.46	0.25	0.39	0.33
	Methionine	0.26	0.52	0.03	0.93	0.29	0.48
	Cystathionine	0.48	0.22	0.38	0.35	0.49	0.21
	SAH	0.81	0.01	0.81	0.01	0.8	0.02
	SAM	−0.15	0.71	0.12	0.77	−0.19	0.66

Hcy, homocysteine; 5m-THF, 5-methyltetrahydrofolate; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine.

Table 4 Correlation between analyte concentrations (PBMC and plasma) and progression of non-calcified and calcified plaque

		Non-calcified plaque			Calcified plaque		
		Progressor (n=6)	Regressor (n=2)	P value	Progressor (n=4)	Regressor (n=4)	P value
PBMC (pmol/ million cells)	Hcy	389 (226)	282.9 (139.1)	0.56	269.8 (140)	455.2 (233.8)	0.22
	Methionine	43 883 (21178.1)	29 713.5 (9066.9)	0.41	41 378.2 (17555.8)	39 303 (23753.7)	0.89
	Cystathionine	79.1 (53.4)	102.8 (79.3)	0.64	73.8 (51)	96.3 (64.8)	0.60
	SAH	725.8 (299.4)	667.8 (328.1)	0.82	704.4 (236.3)	718.3 (362)	0.95
	SAM	1620.5 (389.9)	2397.7 (958.4)	0.12	1804.4 (390.6)	1825.2 (841.8)	0.96
	5m-THF	548.5 (447.9)	599.2 (153.2)	0.88	590.2 (418)	532.2 (408)	0.83
Plasma (nM)	Hcy	16 657.3 (623.5)	15 871.7 (2427.9)	0.73	16 271.3 (1519)	16 650.4 (720)	0.67
	Methionine	19 272.2 (10522.1)	19 303 (7588.4)	0.99	21 381 (3332.1)	17 178.7 (13447.8)	0.58
	Cystathionine	177.2 (62.9)	101.2 (10.6)	0.16	154.4 (86.3)	161.9 (45.1)	0.88
	SAH	53.1 (18.5)	41.9 (12.7)	0.46	53.1 (23.4)	47.5 (10.8)	0.68
	SAM	216.4 (99.1)	276.7 (69.7)	0.46	288.6 (93.3)	174.3 (48.8)	0.07

Hcy, homocysteine; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine.

purchased from Caymen Chemical (Ann Arbor, Michigan, USA). SAM-d_3, Hcy-d_4, Met-d_4 and SAH-d_4 were purchased from Toronto Research Chemicals (Toronto, Canada). Cysta-d_4 and 5m-THF-d_3 were purchased from 13C Molecular, Inc (Fayetteville, North Carolina, USA). Optima liquid chromatography–mass spectrometry grade water (H_2O), acetonitrile and formic acid were purchased from Fisher Scientific (Pittsburg, Pennsylvania, USA).

Sample preparation

Stock solutions were prepared in water with 0.1% formic acid. One hundred millimolar DL-dithiothreitol (DTT) was prepared in water (freshly prepared every day). The internal standard solution was prepared in water, containing 5 µM of each internal standard (IS). To either a lysate of 2×10^8 PBMC or 50 µL plasma, 10 µL of the IS solution and 10 µL of the 100 mM DTT solution were added. The solution was vortex mixed at 2000 rpm for 1 min and then reacted at 4°C for 30 min. Proteins were precipitated by the addition of 170 µL acetonitrile with 0.1% formic acid followed by vortex mixing at 2000 rpm for 1 min and then centrifugation at 15 000 rpm for 10 min at 4°C. The supernatant was then used for liquid chromatography–tandem mass spectrometry analyses.

Liquid chromatography–tandem mass spectrometry quantitation

Liquid chromatography–tandem mass spectrometry was performed using a Dionex Ultimate 3000 RS liquid chromatography system coupled to a TSQ Altis tandem quadrupole mass spectrometer equipped with a heated electrospray ionisation source operated in positive ion mode. Separation was effected on a Phenomenex Kinetex HILIC column (2.6 µm, 100 mm × 4.6 mm) using eluent A as water with 0.01% formic acid and eluent B as acetonitrile with 0.01% formic acid. The following linear gradient separation was used: (time, % B) – min, 90% B; 0 min, 90% B; 3 min, 20% B; 5.5 min, 20% B. The flow rate was 0.4 mL/min, the column temperature was 30°C and the injection volume was 2 µL. Mass spectrometry parameters were as follows: spray voltage: 3500 V; sheath gas: 50 arbitrary units (a.u.); auxillary gas: 10 a.u.; sweep gas: 1 a.u.; ion transfer tube temperature; 350°C; vaporiser temperature; 300°C; cycle time; 0.4 s; Q1 resolution; 0.7 full width at half-maximum (FWHM); Q3 resolution; 0.7 FWHM; and CID gas; 2 mTorr. Analytes and IS were detected according to a unique selected reaction monitoring mass transition. The *m/z* transition, retention time, collision energy and RF lens values for each analyte were as follows: Hcy, 136→90, 1.7 min, 10 V, 56 V; Hcy-d_4, 140→94, 1.7 min, 10 V, 56 V; Met, 150→133, 1.7 min, 10 V, 31 V; Met-d4, 154→137, 1.7 min, 10 V, 31 V; Cysta, 223→134, 2.3 min, 14 V, 41 V; Cysta-d_4, 227→138, 2.3 min, 14 V, 41 V; SAH, 385→136, 2.3 min, 20 V, 60 V; SAH-d_4, 389→136, 2.3 min, 20 V, 60 V; SAM, 399→250, 2.8 min, 17 V, 55 V; SAM-d_3, 402→250, 2.8 min, 17 V, 55 V; 5m-THF, 460→313, 2.2 min, 19 V, 63 V; and 5m-THF-d_3, 463→316, 2.2 min, 19 V, 63 V. Lower limits of quantitation in neat

solution were as follows: 1 nM for SAM, SAH and Cysta; 2 nM for Hcy and Met; and 10 nM for 5m-THF. Calibration standards for PBMC and plasma analyses of Hcy and Met ranged from 50 nM to 500 µM, while others (SAM, SAH, Cysta and 5m-THF) ranged from 25 nM to 50 µM. Intracellular (PBMC) and extracellular (plasma) Hcy and Hcy metabolite (SAM, SAH, Met, Cysta and 5m-THF) concentrations were compared in patients with SLE and controls.

Fisher's t-test was used to analyse the differences between Hcy and Hcy metabolite levels in patients with SLE and controls. The correlation between metabolite levels and coronary plaque volumes was analysed using Pearson correlation.

Patients or the public were not involved in the design, or conduct, or reporting, or dissemination plans of our research.

RESULTS

Table 1 details the demographic, clinical and laboratory characteristics of the included patients with SLE. The average age at sample collection was 57.9±8.8 years. Ninety per cent of patients were female and 40% were of African-American ethnicity. Disease activity expressed as SLEDAI at time of sample collection was 2.5±2.2. Proteinuria was present in 30% of patients, while 80% had hypertension.

Table 2 includes the comparison of intracellular (PBMC) and extracellular (plasma) Hcy and Hcy metabolite concentrations between SLE patients and controls. Plasma from patients with SLE had higher levels of Hcy ($p<0.0001$), SAH ($p<0.05$) and SAM ($p<0.001$) and lower levels of Met ($p<0.05$) and Cysta ($p<0.001$) compared with controls (table 1). PBMC from patients with SLE had higher levels of Cysta ($p<0.05$), SAH ($p<0.05$), SAM ($p<0.001$) and 5m-THF ($p<0.001$) (table 1).

Table 3 details the correlation between the intracellular and extracellular analytes with coronary plaque volume (total plaque, non-calcified and calcified plaque). A total of eight patients with SLE were included in the analysis. Two patients with SLE were excluded from this analysis due to poor quality of CCTA imaging during their first and second study, respectively. Only plasma SAH showed a strong positive correlation with the total coronary plaque, calcified plaque and non-calcified plaque measured by CCTA ($p<0.05$).

Table 4 includes a comparison of the intracellular Hcy analytes between patients with SLE whose total or non-calcified plaque progressed between two CCTA studies versus those who had no plaque progression. Similarly to the analysis completed for table 3, two patients with SLE were excluded from this analysis. Progressors had higher mean Hcy, methionine, SAH, and 5m-THF levels and lower mean Cystathionine and SAM levels, but none of the findings were statistically significant likely due to the small number of patients.

DISCUSSION

The role of C1 metabolism in CVD has been a matter of controversy in recent years. On the one hand, epidemiological studies have identified Hcy as an independent risk factor for CVD in the general population[13] and in SLE.[5] On the other hand, lowering Hcy levels via folate supplementation did not improve cardiovascular outcomes in randomised trials.[14] The hypotheses for the lack of improved cardiovascular outcomes with lowering Hcy levels included the potential role of plasma SAH and intracellular Hcy and Hcy metabolites in mediating the cardiovascular risk. We thus pursued the first study of intracellular and extracellular Hcy metabolites in SLE to assess their potential contribution to the elevated cardiovascular risk seen in this population.[15]

Compared with healthy controls, plasma from patients with SLE had higher levels of Hcy ($p<0.0001$), SAH ($p<0.05$) and SAM ($p<0.001$) and lower levels of Met ($p<0.05$) and Cysta ($p<0.001$). PBMC from patients with SLE had higher levels of Cysta ($p<0.05$), SAH ($p<0.05$), SAM ($p<0.001$) and 5m-THF ($p<0.001$). Interestingly, intracellular Hcy levels were similar, potentially indicating a homeostatic process that maintains the intracellular Hcy within a range regardless of its extracellular values.

Despite the significant differences compared with controls, the only Hcy metabolite in patients with SLE that significantly correlated with total ($p<0.05$), calcified ($p<0.05$) and non-calcified coronary plaque ($p<0.05$) was plasma SAH. This is an important finding, since SAH has not been previously described as a cardiovascular risk factor in SLE, although there are experimental and clinical data supporting this finding in the general population. Plasma SAH was found to be a better biomarker of atherosclerosis than Hcy and to accelerate the development of atherosclerotic lesions in apoE-deficient mice that were fed a high methionine diet.[16] In a murine model, SAH induced vascular smooth muscle proliferation and migration through an oxidative stress-dependent activation of the ERK1/2 pathway to promote atherogenesis.[17] SAH also induces phosphatidylserine exposure and apoptosis in endothelial cells independently of Hcy.[18] These findings on the pathophysiological role of SAH in the context of atherosclerosis have been supplemented by pioneering clinical studies: Kerins *et al*[19] reported a more prominent increase in plasma SAH than in plasma Hcy among 30 patients with coronary artery disease compared with 29 matched controls. Xiao *et al*[9] recently reported plasma SAH to be independently associated with increased risk of cardiovascular events among 1003 patients undergoing coronary angiography.

The limitation of our study was the small number of patients and thus lack of any cardiovascular events in the included patients. The time between blood sample collection and last CCTA varied.

In summary, we showed that intracellular and extracellular concentrations of Hcy metabolites were significantly different between patients with SLE and controls, despite similar intracellular Hcy levels. Plasma SAH was positively

correlated with total coronary plaque, calcified plaque and non-calcified plaque. Larger studies are needed to clarify the importance of Hcy metabolites as cardiovascular risk factors in SLE.

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Contributors All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. GS had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study conception and design: GS. Acquisition of data: all authors. Analysis and interpretation of data: all authors.

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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.

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