Patients with Systemic Lupus Erythematosus Show Increased Platelet Activation and Endothelial Dysfunction Induced by Acute Hyperhomocysteinemia

LAI-SHAN TAM, BOLI FAN, EDMUND K. LI, G. NEIL THOMAS, SO F. YIM, CHRISTOPHER J. HAINES, and BRIAN TOMLINSON

ABSTRACT. ***Objective***. Hyperhomocysteinemia adversely affects the endothelium, although the exact mechanism is unclear. Systemic lupus erythematosus (SLE) is an inflammatory disease with a high atherothrombotic tendency. We examined whether acute hyperhomocysteinemia exacerbates endothelial and platelet dysfunction in patients with SLE.

Methods. Twelve SLE patients and 15 controls were recruited. Oral methionine was used to achieve acute hyperhomocysteinemia. Endothelial function was assessed by flow-mediated dilatation (FMD) of the brachial artery; also assessed were the levels of von Willebrand factor (vWF) and plasminogen activator inhibitor type 1 (PAI-1). Platelet activation was assessed by the levels of beta-thromboglobulin (ß-TG), fibrinogen binding, and P-selectin expression using flow cytometry.

Results. After oral methionine loading, vWF levels increased significantly, whereas FMD remained unchanged in both groups. PAI-1 increased significantly only in controls. Fibrinogen binding to platelets increased significantly only in SLE patients. ß-TG remained unchanged in SLE patients but increased significantly in controls. Platelet P-selectin expression did not change in either group.

Conclusion. These results suggest that the prothrombotic tendency after acute hyperhomocysteinemia is mediated by endothelial dysfunction and platelet activation in patients with SLE and healthy controls. (J Rheumatol 2003;30:1479–84)

Key Indexing Terms:
PLATELETACTIVATION
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Systemic lupus erythematosus (SLE) is an inflammatory disease involving multiple organ systems. Among other problems, patients with SLE have higher rates of premature thrombotic and atherosclerotic disease[1-4]. In the "response to injury" hypothesis of atherosclerosis, different sources of injury to the endothelium including immune complexes, viruses, and toxins such as homocysteine can lead to endothelial cell dysfunction[5], resulting in increased permeability and adhesiveness of the endothelium, procoagulative properties, and vasoactive molecule expression[6]. All of these insults are believed to be important facilitators of atherogenesis in SLE.

Hyperhomocysteinemia occurs in 15% of SLE patients, and was found to be an independent risk factor for stroke and arterial thrombotic events[7,8]. Although the precise pathophysiological mechanisms remained unclear, experimental evidence suggests that the atherogenic propensity associated with hyperhomocysteinemia results from endothelial dysfunction and injury, followed by platelet activation and thrombus formation[9]. Methionine taken orally is converted to homocysteine by demethylation, and the acute transient rise in plasma homocysteine concentrations after an oral load of methionine can be used as a model to study the mechanisms of endothelial damage caused by homocysteine[10].

The endothelium plays an integral role in the regulation of vascular tone, platelet activity, and thrombosis and is intimately involved in the development of atherosclerosis. Estimates of different types of endothelial dysfunction may be obtained indirectly by measuring endothelial–dependent vasodilatation and high levels of endothelial-derived regulatory proteins including von Willebrand factor (vWF) and plasminogen activator inhibitor type 1 (PAI-1). Endothelial cells are highly responsive to altered shear stress. Changing shear or flow may be one of the most important physiological regulators of prostacycline and nitric oxide production, resulting in flow-dependent vasodilatation[11]. Plasma vWF is a key mediator of platelet aggregation and adhesion. Although platelets contain some vWF, virtually all plasma

From the Department of Medicine and Therapeutics and Department of Obstetrics and Gynecology, The Chinese University of Hong Kong, Prince of Wales Hospital, Hong Kong.

L.S. Tam, MRCP(UK), Medical Officer; B. Fan, PhD, Post-doctorate Fellow; E.K. Li, FRCPC, Associate Professor; G.N. Thomas, PhD, Post-doctorate Fellow, Department of Medicine and Therapeutics; S.F. Yim, MRCOG, Senior Medical Officer; C.J. Haines, FRANZCOG, Professor, Department of Obstetrics and Gynecology; B. Tomlinson, FACP, Professor, Department of Medicine and Therapeutics.

Address reprint requests to Dr. L.S. Tam, Department of Medicine and Therapeutics, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, Hong Kong. E-mail: tamls_813@yahoo.com

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vWF is derived from the endothelium[12]. Homocysteine has been found to be cytotoxic to endothelial cells *in vitro*[13], and vWF is thought to be a good marker for this injury[14]. However, acute hyperhomocysteinemia induced by oral methionine loading was associated with impaired endothelial function in most[10,15-20] but not all studies in healthy subjects[21,22].

PAI-1 is a fast-acting inhibitor of plasminogen activation. It is produced by the vascular endothelium but is also present in platelets, adipocytes, smooth muscle cells, and monocytes, and is considered to be an important regulatory element in fibrinolysis that is intimately linked to the risk of thrombosis[23-26]. The suppression of fibrinolysis due to high plasma concentrations of PAI-1 is associated with the development of myocardial infarction[27]. An *in vitro* study described that PAI-1 levels in cultured human vascular endothelial and smooth muscle cells were increased by high homocysteine concentration[28]. In human subjects, the association of hyperhomocysteinemia and plasma PAI-1 levels remains controversial[29-31].

In addition to procoagulant effects on the vessel wall, hyperhomocysteinemia may induce vascular occlusion through platelet actions[32]. Platelet activation results in changes in the level of expression of surface glycoproteins — which act as receptors for platelet agonists and for adhesive proteins involved in platelet aggregation[33] — and in secretion of preformed granules. During platelet activation, P-selectin (CD62p) translocates from the membrane of α-granules to the plasma membrane[34], and the GPIIb-IIIa complex on the plasma membrane also undergoes a conformation change that exposes a fibrinogen binding site[35]. Assays to show that platelets have been activated *in vivo* include measurement of plasma concentrations of platelet-specific proteins such as beta-thromboglobulin (ß-TG), which are released from the α-granules. However, measurement of degranulation markers alone may limit the ability to detect platelet activation. Detection of activation-dependent platelet surface changes by specific antibodies using flow cytometry has been used as a sensitive assay of platelet activation in whole blood; antibodies include anti-fibrinogen antibody, which directs against the epitopes of the fibrinogen molecule bound to activated platelet, and antibody against P-selectin[36]. The effects of homocysteine on platelet functions have been mainly studied in homocysteinuric patients, showing *in vivo* platelet activation[32]. However, platelet activation was not observed after acute hyperhomocysteinemia in healthy volunteers and patients with occlusive vascular diseases[22,37].

There are no data on the effect of acute hyperhomocysteinemia on platelet activation and endothelial function in patients with SLE, and there are limited data on the effect of acute hyperhomocysteinemia on platelet activation in healthy subjects. We hypothesized that experimental hyperhomocysteinemia, produced by methionine loading, would lead to endothelial dysfunction and platelet activation in both SLE patients and healthy subjects. Thus we examined the effects of acute hyperhomocysteinemia following an oral methionine load on endothelial function and platelet activation in SLE patients and healthy subjects.

MATERIALS AND METHODS

Patient selection. Twelve female SLE patients were recruited from the Rheumatology Clinic of the Prince of Wales Hospital. All patients fulfilled the 1997 American College of Rheumatology revised criteria for the classification of SLE[38]. Medical history review and physical examination were conducted by a rheumatologist, and laboratory tests were performed as listed below. Current corticosteroid dosage, concomitant use of hydroxychloroquine, and immunosuppressants were noted. Fifteen age-matched healthy controls (8 females and 7 males) were also recruited. The study protocol was approved by the Clinical Research Ethics Committee of the Chinese University of Hong Kong and all subjects gave written consent.

SLE related factors. SLE activity was determined with the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI)[39]. Organ damage was assessed according to the Systemic Lupus International Collaborating Clinics/American College of Rheumatology (SLICC/ACR) damage index[40].

Screening tests. Blood samples were taken after an overnight fast. Both groups had complete blood counts, renal and liver function tests, glucose, lipids (total cholesterol, low density lipoprotein- and high density lipoprotein-cholesterol, and total triglycerides), vitamin B_{12}, red cell folate, and fibrinogen levels. Complement levels, anti-dsDNAbinding, and antiphospholipid antibody status (anticardiolipin antibody and lupus anticoagulant screen using Russell viper venom test) were measured in SLE patients.

Methionine loading. Vitamin supplements, antiplatelet drugs, and vasoactive drugs were not allowed 2 weeks before the study. All subjects had fasted and abstained from smoking, alcohol, and caffeine for at least 12 h prior to the commencement of the study. Methionine powder was dissolved in 250 ml orange juice according to body weight (100 mg/kg), while methionine-free orange juice served as placebo. Each subject was randomized in a single blind manner to receive either methionine or placebo for the first visit, then crossed over to receive the alternate treatment after one week. Plasma total homocysteine (tHcy) concentrations were assayed with high performance liquid chromatography at baseline and 4 hours after loading[41].

Endothelial Function

Flow-mediated dilatation. Conduit vessel endothelium-dependent vasodilatation was induced by reactive hyperemia, while endothelium-independent vasodilatation was induced by administration of sublingual nitroglycerin (glyceryl trinitrate, GTN) as described[42]. Briefly, measurements were made of changes in the diameter of the brachial artery using color duplex Doppler ultrasound (Aloka S-680 with a steered 7.5 MHz peripheral vascular ultrasound probe, Aloka UST-5518-7.5). The ultrasound examination was performed in a quiet room at a temperature between 21°C and 22°C. Subjects rested in a supine position for 15 min before examination. A B-mode scan was obtained of the right brachial artery in longitudinal section. A resting measurement was taken, and a pneumatic cuff was then inflated to a pressure of 200 mm Hg for 4 min. The diameter of the artery was recorded again 45–60 s after cuff deflation. A period of 15 min was allowed for recovery before testing for endothelium-independent relaxation. A repeat baseline measurement of the diameter was made before a 400 µg dose of sublingual GTN spray was administered. The brachial artery diameter was again measured 3–4 min after the GTN was given. A single investigator performed all imaging and analysis, blinded to the subject's disease and loading status. The coefficient of variation of the measurements was determined by making 5 repeated measurements in 5 subjects over a period of 10 min. Using analysis of variance, the between-measurement of variance was found to be 0.02 mm with confidence limits of 0.24 mm[42].

Endothelial-derived regulatory proteins. vWF and PAI-1 were measured at baseline and 4 h after the administration of the methionine or placebo by ELISAusing commercial kits[43] (Diagnostica Stago, Asnieres, France).

Platelet Activation Markers

Flow cytometry. Venous blood (4.5 ml) was collected into a Diatube[44] containing citric acid, theophylline, adenosine, and dipyridamole, first diluted 1:10 in modified HEPES Tyrode's buffer within 15 min of sampling; 20 µl of either phycoerythrin (PE)-conjugated anti-CD42b monoclonal antibody (CD42b-PE, Becton Dickinson, San Diego, CA, USA) and FITC-anti-fibrinogen polyclonal antibody (anti-fibrinogen-FITC, Gene Company Limited, Glostrup, Denmark) or PE-conjugated anti-CD62p monoclonal antibody (CD62p-PE, Becton Dickinson) were added. ADPor thrombin was added to make a final concentration of 5 µmol/l or 2 U/ml, respectively, mixed, and incubated for 20 min at room temperature in the dark. Samples were then fixed with 1000 µl 1% paraformaldehyde in phosphate buffered saline, pH 7.4, prior to flow cytometric analysis[45]. For fixed samples, whole blood was fixed within 1 min after venipuncture diluted 1:10 with 1% buffered paraformaldehyde. Analysis of the fibrinogen binding to platelet membrane receptors and platelet P-selectin expression was performed in a flow cytometer (Becton Dickinson) at baseline and 4 h after methionine or placebo loading. Five thousand individual platelets were examined for FITC or PE fluorescence, indicating binding of the anti-fibrinogen or anti-P-selectin antibody. Antibody binding was expressed as mean fluorescence intensity.

ß-*Thromboglobulin.* The tube for measurement of ß-thromboglobulin (ß-TG) was prepared as described by Tanaka, *et al*[46]. Venous blood (2.5 ml) was drawn into a tube containing 0.3 ml of EDTA (3.3 mg/ml), theophylline (5.4 mg/ml), and prostaglandin E1 (1 mg/ml). Blood samples were put on ice within 15 min after sampling, and were centrifuged as soon as possible. Separated plasma was stored at –20°C until analysis. ß-TG concentration was assayed by ELISA[47,48] at baseline and 4 h after methionine loading test.

Statistical analyses. Results are expressed as mean ± standard deviation (SD) for normally distributed data. Non-normally distributed data are expressed as median (interquartile range). The changes observed before and after loading were assessed using the Wilcoxon signed rank test. A p value < 0.05 was considered statistically significant. All tests for the changes before and after loading were 1 tailed as the markers were expected to increase after intervention. Changes in brachial artery diameter were expressed as percentage change in diameter of the artery from the baseline obtained just before increasing flow (flow-mediated dilatation) or GTN. The Statistics Package for Social Sciences (SPSS for Windows, v. 10.0, 1999, SPSS Inc., Chicago, IL, USA) was used for the analyses.

RESULTS

Clinical characteristics. All 12 SLE patients and 15 controls completed the crossover protocol. No adverse events were reported. Patients with SLE had a median disease duration of 10.9 (5.4–14.3) years. Most patients had mild disease activity with a median disease activity score (SLEDAI) of 6.0 (0.5-11.5), and the damage score (SLICC/ACR) was 1 (0–2). Seven patients had nephritis. Three patients were positive for antiphospholipid antibodies. One patient had a history of stroke. No other patient had clinical cardiovascular disease or diabetes. Five (41.7%) SLE patients were taking hydroxychloroquine and 5 (41.7%) immunosuppressants. Nine (75%) were taking prednisolone with a median dosage of 5.0 (0.5–5.0) mg/day. The demographic and metabolic profiles of the SLE patients and controls are summarized in Table 1.

Methionine loading. Following methionine loading, tHcy increased from 8.2 (6.0–9.3) µmol/l to 21.0 (18.7–23.2) µmol/l ($p < 0.005$) in SLE patients, and increased from 6.6 (5.3–8.8) µmol/l to 18.6 (14.5–20.8) µmol/l ($p < 0.001$) in controls. Homocysteine concentrations did not change significantly after placebo administration in either SLE [7.4 (6.8–9.3) µmol/l to 7.7 (6.0–8.5) µmol/l] or controls [7.2 (6.5–9.3) µmol/l to 7.3 (5.7–8.0) µmol/l].

Effects on Endothelial Function

Flow-mediated endothelium-dependent vasodilation and endothelium-independent vasodilation. Under basal conditions, the brachial artery diameter of SLE patients was similar to that found in controls (Table 2). After methionine loading, no significant change in endothelium-dependent vasodilatation was observed in either SLE patients or controls. There were no differences in the percentage change in endothelium-dependent vasodilatation before and after placebo loading in either controls or SLE patients. Neither placebo loading nor methionine loading affected endothelium-independent vasodilation in either SLE patients or controls.

Endothelial-derived regulatory proteins. After methionine loading, the levels of vWF increased significantly in both SLE patients and controls (Table 2, Figure 1). There was no significant change after placebo loading in either group.

The level of PAI-1 increased significantly only in controls after methionine loading. There was no significant change after placebo loading in either group (Table 2).

Effect on Platelet Activation

Fibrinogen binding to platelets and platelet p-selectin expression. The *in vivo* assay for fibrinogen binding to platelets increased significantly after methionine loading in SLE patients, but decreased slightly in controls (Table 3).

Table 1. Baseline demographic and metabolic variables. Data are expressed as median (interquartile range).

	Controls, n = 15	SLE Patients, n = 12
Age, yrs	37 (32–40)	38 (29–41)
BMI, kg/m^2	24 (21–25)	21 (21–24)
Systolic blood pressure, mm Hg	105 (101-115)	119 (106-126)
Diastolic blood pressure, mm Hg	56 (54–62)	70 (57–78)
Urea, mmol/l	4.8 (3.8–5.8)	4.8 (3.8–5.5)
Creatinine, µmol/l	71.0 (63.0–89.0)	63.5 (59.5–78.5)
Platelet, 10^9/l	226 (199–264)	235 (148–245)
Glucose, mmol/l	4.7 (4.5–5.0)	4.5 (4.2–4.8)
Total cholesterol, mmol/l	4.7 (3.8–5.0)	4.7 (3.9–5.3)
Total triglycerides, mmol/l	0.8 (0.5–1.4)	1.1 (0.9–1.9)
HDLcholesterol, mmol/l	1.6 (1.2–1.9)	1.3 (1.1–1.8)
LDLcholesterol, mmol/l	2.4 (2.0–3.2)	2.5 (2.1–3.4)
Folate, nmol/l	695 (612–965)	803 (611–905)
Vitamin B_{12}, pmol/l	324 (266–411)	301 (262–349)
Fibrinogen, g/l	3.1 (2.6–3.9)	4.2 (3.7–6.1)

BMI: body mass index.

Table 2. Effects of oral methionine loading on endothelial function and markers of impaired fibrinolysis. Values are expressed as median (interquartile range).

	SLE		Control	
	Methionine	Placebo	Methionine	Placebo
Basal brachial artery diameter, mm	3.43 (3.24–3.69)	3.43 (3.23–3.65)	3.17 (2.93–3.46)	3.20 (2.77–3.55)
FMD, %				
0 h	7.23 (2.35–16.53)	8.21 (3.23–17.44)	3.99 (-0.99–10.23)	2.00 (-6.25–13.64)
4 h	6.86 (-0.72–11.24)	10.36 (4.49–22.63)	4.07 (0.67–10.00)	4.69 (-4.94–6.13)
GMD, %				
0 h	12.5 (7.1–14.3)	13.2 (1.8–18.1)	10.1 (-1.0–16.7)	6.2 (-1.0–14.5)
4 h	5.6 (-1.0–15.6)	7.9 (2.7–13.9)	1.8 (-2.0–12.6)	2.9 (-4.1–15.6)
vWF, %				
0 h	82.6 (72.6–104.5)	94.4 (76.3–105.9)	80.4 (32.7–94.7)	62.8 (39.7–79.8)
4 h	102.9 (80.9–123.6)*	104.4 (73.8–117.6)	86.6 (73.2–90.8)*	64.1 (30.1–95.7)
PAI-1, ng/ml				
0 h	56.1 (45.0–62.1)	52.5 (39.8–87.8)	32.3 (23.9–44.3)	40.0 (28.2–45.8)
4 h	72.1 (39.4–130.9)	54.4 (31.9–77.0)	53.5 (40.5–98.4)*	35.9 (24.9–50.1)

FMD: flow-mediated dilation; GMD: glyceryl trinitrate-mediated dilation; vWF: von Willebrand factor; PAI-1: plasminogen activator inhibitor–1. * $p < 0.05$, before vs after methionine loading.

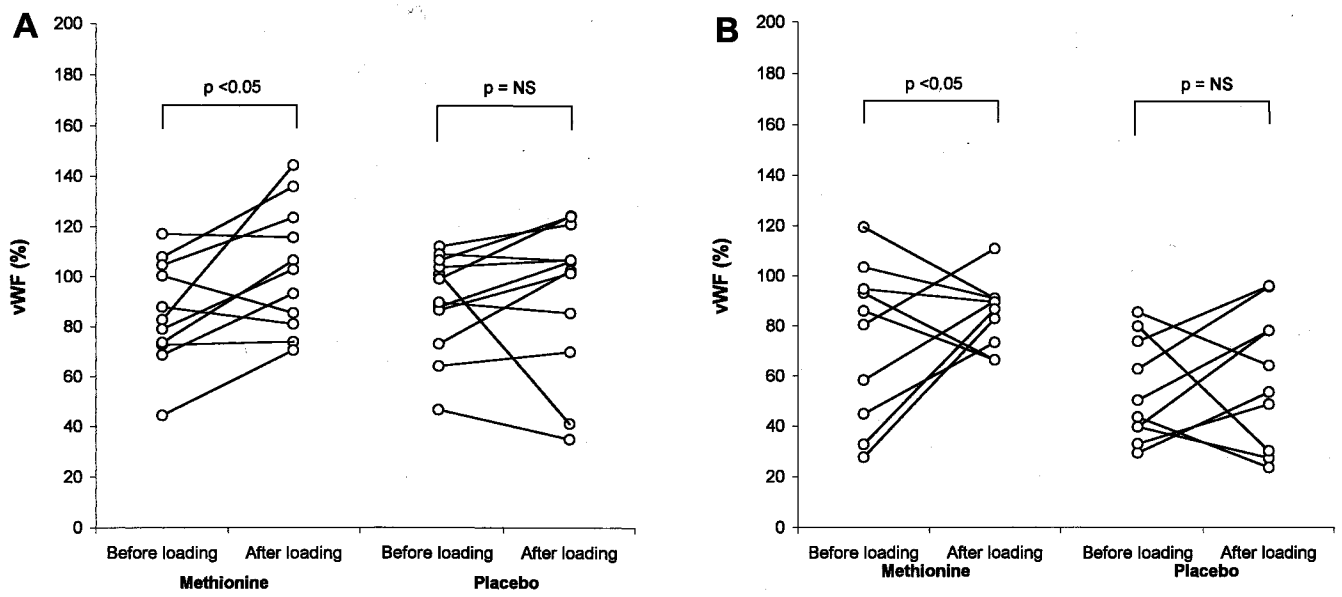

Figure 1. Changes in vWF level in patients with SLE (A) and in controls (B).

The levels of platelet P-selectin expression did not change after methionine loading in both groups (Table 3). There were no significant changes in markers of platelet activation after placebo loading in either group.

ß-Thromboglobulin. ß-TG remained unchanged after methionine loading in SLE patients but increased significantly in controls (Table 3). ß-TG level did not change after placebo loading in either group.

DISCUSSION

This is the first study showing that experimental elevation of plasma homocysteine concentrations after oral methionine load increased markers of endothelial damage and platelet activation in patients with SLE. These findings provide evidence to support the hypothesis that homocysteine is a risk factor for vascular disease in SLE by endothelial injury and platelet activation.

In contrast to previous studies[10,15-20], we have not found a significant impairment of endothelium-dependent vasodilation in acute hyperhomocysteinemia in SLE patients or healthy controls, despite the use of comparable regimen of oral methionine loading. This may be related to the relatively smaller increase in plasma homocysteine concentration achieved. Two previous studies using the same dose of methionine also did not detect any change in the endothelial function, although the designs of the studies were different[21,22]. In our study, the changes in vWF levels appeared to be a more sensitive marker of endothelial

Table 3. Effects of oral methionine loading on platelet activation. Values are expressed as median (interquartile range).

	SLE		Control	
	Methionine	Placebo	Methionine	Placebo
Fibrinogen binding, MFI				
0 h	45.0 (26.6–49.3)	31.0 (13.3–51.2)	65.1 (30.7–108.6)	46.9 (32.4–105.2)
4 h	49.1 (30.5–62.6)*	43.0 (28.2–52.6)	47.8 (29.9–74.8)	55.7 (40.0–81.6)
P-selectin expression, MFI				
0 h	56.1 (38.3–65.1)	45.5 (35.8–67.8)	57.4 (44.5–62.5)	44.7 (36.7–52.6)
4 h	57.6 (33.4–67.0)	47.4 (29.8–60.8)	64.7 (50.3–68.7)	43.1 (34.2–52.1)
ß-thromboglobulin, U/ml				
0 h	90.8 (55.7–109.6)	87.5 (54.2–132.6)	75.2 (40.1–114.7)	83.2 (44.2–121.1)
4 h	88.2 (60.4–114.6)	89.8 (54.5–118.5)	133.5 (77.7–164.5)*	74.8 (47.3–102.9)

* $p < 0.05$ before vs after methionine loading.

dysfunction caused by hyperhomocysteinemia than flow-mediated dilatation in both SLE patients as well as healthy controls. Constans, *et al* also showed that vWF was a sensitive marker of endothelial dysfunction after acute methionine load compared with other soluble markers in patients with arterial or venous occlusive disease[37]. Our results support the hypothesis that homocysteine is a risk factor for vascular disease, which may be mediated through injury to the endothelium, causing a prothrombotic tendency.

In our study, PAI-1 levels increased significantly after the oral methionine load in healthy controls, similar to the findings in young ischemic stroke patients[49]. However, this observation was not found in our SLE patients or another study on healthy controls[20]. These discrepancies may be due to gene transcription and *de novo* protein synthesis, which is required for PAI-1 release, unlike vWF, which can be readily released.

The elevated fibrinogen binding to platelets seen in SLE patients after methionine loading suggests platelet activation occurred with elevated plasma homocysteine concentration. However, CD62Pexpression was unaffected in SLE patients after methionine loading. This may suggest that fibrinogen binding to platelets is a more sensitive assay that requires lower concentrations of activation stimuli than platelet secretion[50]. Plasma ß-TG was the only marker indicating platelet activation had occurred in controls. Although ß-TG is particularly vulnerable to artifactual *in vitro* platelet activation[36], this problem was taken into account, as careful venipuncture and blood-handling techniques were employed. Nonetheless, these findings are noteworthy, as human platelet activation after acute hyperhomocysteinemia has never been reported previously to our knowledge.

A possible limitation of our study would be the small sample size. However, the post-hoc analysis of the SLE patients showed that a sample size of 12 achieves 79% power to detect a 20% difference between the pretreatment vWF level of 85.6% and the post-treatment level of 102.7%, with a standard deviation of 21.4% and with a significance level (alpha) of 0.05.

In summary, we have demonstrated that homocysteine may increase the prothrombotic tendency through both platelet activation and endothelial damage. Further studies are required to elucidate the underlying mechanisms.

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