Homocysteine serum levels are increased and correlate with disease severity in patients with lupus erythematosus

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Abstract

Objective

To determine homocysteine (Hcy) serum levels in patients with cutaneous lupus erythematosus (CLE) and a possible correlation with the disease activity.

Methods

Ninety-three patients with LE and 30 healthy controls were included in the study. For each patient, disease activity was calculated and plasma levels of Hcy was measured by enzymatic colorimetric assay.

Results

Forty-six patients had chronic cutaneous LE (CCLE), 14 had LE tumidus (LET), 17 had subacute CLE (SCLE) and 16 had SLE. Median values [25°–75° percentile] were 7[4-9] for CCLE, 3.5[2.3–4.8] for LET, and 8[7–10] for SCLE; for SLE the RCLASI score was 7.5[4.8–13] and the SELENA/SLEDAI score was 10.5[9–13.3].

HHcy was present in 73.9% of patients with CCLE, 35.7% with LET, 82.4% with SCLE, 81.2% with SLE, 20% of healthy controls. Overall, patients with LE showed a higher median serum Hcy level than the control group (15[13–18.2] vs. 11[8.8–12.2], p<0.001).

There was a significant correlation between Hcy serum levels and disease activity, both in patients with CLE and SLE.

Conclusion

We demonstrated that Hcy levels were higher in patients with different forms of CLE and correlated with disease activity calculated by CLASI. Therefore, HHcy could be related to LE pathogenesis and might be a triggering factor in predisposed individuals.

Key words

homocysteine, lupus erythematosus, disease activity, folic acid, vitamin B12

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Introduction

Homocysteine (Hcy) is a non-protein amino acid obtained from the diet, but biosynthesised from methionine by the removal of its terminal methyl group. Mild hyperhomocysteinaemia (HHcy), as defined on the basis of plasma concentration ranging between 14 and 30 μmol/l, may result from enzyme and/or vitamin (folate, vitamins B6 and B12) deficiencies. The enzyme homocysteine methyltransferase for the regeneration of methionine from homocysteine (Hcy) in the activated methyl cycle (1) requires both vitamin B12 and folic acid as cofactors.

Recent studies have demonstrated new and interesting ancillary effects for Hcy either in the enhancement of inflammatory activation or in the autoimmunity triggering mechanisms. This suggests a possible role for Hcy in the pathogenesis of autoimmune diseases (AD). The most recent data seem to suggest a strict and bi-univocal relationship between immune activation, inflammation and Hcy levels. As a consequence, an explanatory mechanism contributing to the above mentioned high prevalence of HHcy in patients with AD may be provided. Moreover, Hcy may also have a putative role in the progression of the disease-associated inflammatory damage (3). Although not completely clarified, the mechanisms involved in the development of HHcy as a consequence of a persistent immuno-inflammatory activation, are probably multiple and intriguing. Hcy is able to activate the immune system and induce the expression of proinflammatory molecules in the pathogenesis of autoimmune diseases.

In particular, many authors have demonstrated that Hcy can induce chemokine and chemokine receptor expression in human vascular cells and monocytes, including IL-1β, IL-6, IL-8, IL-12, IL-18, IL-1 receptor antagonist, CRP, adhesion molecules and metalloproteinases (4). In this context, the relationship between Hcy and IL-18, which is a novel pro-inflammatory Th1-dependent cytokine, appears of particular interest since it has also been demonstrated in a paradigmatic autoimmune disease, such as systemic lupus erythematosus (SLE) (5).

However, the role of the depletion in vitamins implicated in the Hcy metabolism, folate, B12 and B6, seems particularly relevant (3).

The aim of this study was to determine the level of Hcy in the blood of patients with lupus erythematosus (LE) and a possible correlation to the disease activity as a first step in revealing whether this level could be related to the pathogenesis and, consequently, if this alteration will have an impact on the treatment protocol.

Materials and methods

Study population

Ninety-three patients with LE (mean age: 57 years; range: 24–82 years) were selected and classified according to the ACR criteria. Eighty-one (87.1%) were women, while 12 were men (12.9%), with a female/male ratio of 7.

Also 30 age-matched healthy controls (mean age: 54 years; age range 21–83 years) were included in the study: 6 (20%) were male and 24 (80%) female. Patients were recruited from the attendees at the Section of Dermatology (Immunopathological Unit) of the University of Florence.

For each patient, the data on disease activity were recorded: they had been calculated by the Cutaneous Lupus Erythematosus Disease Area and Severity Index (CLASI) (6) in cutaneous LE (CLE) patients and by the Safety of Estrogens in Lupus Erythematosus National Assessment/Systemic Lupus Erythematosus Disease Activity Index (SELENA/SLEDAI) (7), in patients with SLE. It was also include a detailed medical history with data on smoking, personal history of cardiovascular disease (hypertension, diabetes), family history of ischaemic heart disease, intercurrent illnesses, body mass index and medications.

All the causes of Hcy alterations or Hcy serum variability, were considered as exclusion criteria. They were included the following: under 18 years of age, pregnancy, concurrent therapy with folic acid, vitamin B6 or B12, methotrexate or antiepileptics therapy, active nephritic syndrome, myositis, liver disease, renal failure, hypercholesterolemia on treatment at enrolment, smoking and alcohol abuse.

As controls, 30 healthy subjects, sex- and age-matched with the patients, with no acute or chronic diseases, genetic syndromes or familial history of autoimmune disease, and who were not smokers or alcohol abusers were included.

After explaining the procedure and obtaining consent from every patient and control, at the first visit a serum sample was collected and then stored at -80°C to perform the serologic testing (see below). A comparison of Hcy serum levels was performed between cutaneous LE patients, SLE patients and healthy controls.

Determination of Hcy serum level

Serum Hcy was measured using the Axis Homocysteine Enzyme Immunoassay kit (Axis-Shield Diagnostics Ltd., Dundee, U.K.) (8). This kit is an enzyme immunoassay for the determination of Hcy in blood. Protein-bound Hcy is reduced to free Hcy and enzymatically converted to S-adenosyl-L- homocysteine (SAH) in a separate procedure prior to the immunoassay. The enzyme is specific for the L-form of Hcy, which is the only form present in the blood. Hcy, mixed disulphide and protein-bound forms of Hcy in the sample are reduced to free Hcy by using dithiothreitol. Hcy in the test sample is converted to SAH by using SAH hydrolase and excess adenosine. The following solid-phase enzyme immunoassay is based on competition between SAH in the sample and immobilised SAH bound to the walls of the microtitre plate for binding sites on a monoclonal anti-SAH antibody.After removal of unbound anti-SAH antibody, a secondary rabbit anti-mouse antibody labelled with the enzyme horseradish peroxidase is added. The peroxidase activity is measured spectrophotometrically after addition of substrate, and the absorbance is inversely related to the concentration of Hcy in the sample. For preparing the calibration curve and calculation of unknown samples (9, 10), a four-parameter logistic curve fit was used.

Statistical analysis

Levels of Hcy were presented as median [25°–75° percentile] and compared using Mann-Whitney U-test. To evaluate the association between the severity of the disease calculated by CLASI in cutaneous LE patients and with SELENA/SLEDAI in SLE and Hcy levels, both parametric Pearson's correlation test and non-parametric Spearman's correlation test were used. Differences were considered as statistically significant when $p<0.05$. All the analyses were conducted using SPSS software for Windows (SPSS for Windows, Chicago, IL, USA).

Table I. Hcy serum levels in patients with lupus erythematosus and healthy controls.

Group	Hcy (Median [25°-75° percentile])	Patients *vs*. healthy controls
CCLE (n=46)	15 [12.6-18]	0.009
LET (n=14)	12 [10.2-14]	ns
SCLE (n=17)	16.8 [14.9-19.7]	<0.001
SLE (n=16)	18 [14.7-21.7]	<0.001
All patients (n=93)	15 [13-18.2]	0.002
Healthy controls (n=30)	11 [8.8-12.2]	<0.001

Hcy: homocysteine; CCLE: chronic cutaneous lupus erythematosus; LET: lupus erythematosus tumidus; SCLE: subacute cutaneous lupus erythematosus; SLE: systemic lupus erythematosus.

Results

Forty-six (49.5%) patients had chronic CLE (CCLE), 14 (15.1%) had LE tumidus (LET), 17 (18.3%) had subacute CLE (SCLE) and 16 (17.2%) had SLE. Thirty-four out of 46 patients with CCLE (73.9%) had the localised form, while 12 (26.1%) the generalised one. Twelve out of 17 patients with SCLE (70.6%) presented the annular type, while 5 (29.4%) the psoriasi form. Of the 14 patients with SLE, 9 had butterfly rash (64.3%), while 5 the maculopapular form (35.7%).

Moreover, the severity of the disease was calculated by CLASI for all the forms of CLE and, for SLE, also by SELENA/SLEDAI. Median values [25°–75° percentile] were 7[4-9] for CCLE, 3.5[2.3–4.8] for LET, and 8[7–10] for SCLE; for SLE the RCLASI score was 7.5[4.8–13] and the SELENA/SLEDAI score was 10.5[9–13.3].

ANA were positive in 14 out of 46 patients with CCLE (30.4%), in 11 out of 17 with SCLE (64.7%), and in 14 out of 16 with SLE (87.5%), while no LET patients showed ANA positivity.

Serum levels of Hcy were calculated as μmol/l and HHcy were defined for values above 14 μmol/l. HHcy was present in 34 out of 46 patients with CCLE (73.9%), 5 out of 14 with LET (35.7%), 14 out of 17 with SCLE (82.4%), 13 out of 16 with SLE (81.2%), and 6 out of 30 of the controls (20%).

Overall, patients with LE showed a higher serum Hcy level than the control group (15[13–18.2] *vs*. 11[8.8–12.2], $p<0.001$). In particular, a significant difference was found between CCLE patients (15[12.6–18], $p=0.009$), SCLE patients (16.8[14.9–19.7], $p<0.001$) as well as SLE patients (18[14.7–21.7], $p<0.001$), and control group. By contrast, no significant differences were demonstrated between patients with LET (12[10.2–14], ns) and control group. Moreover, no differences regarding Hcy levels were found among CCLE, SCLE and SLE groups (Table I), while patients with LET showed lower Hcy levels than those with SCLE or SLE ($p=0.02$ and $p=0.007$, respectively; data not shown).

In Figure 1, the activity scores calculated by CLASI for the different variants of CLE and by both CLASI and SELENA/SLEDAI for SLE were reported. There was a significant correlation between Hcy serum levels and disease activity calculated with both parametric and non-parametric correlation tests, in patients with CCLE (Pearson's test: r=0.43, $p<0.05$; Spearman's test: r=0.35, $p<0.05$), SCLE (Pearson's test: r=0.62, $p<0.05$; Spearman's test: r=0.50, $p<0.05$) and SLE (SLE/CLASI – Pearson's test: r=0.51, $p<0.05$; Spearman's test: r=0.62, $p<0.05$; SLE/SELENA/SLEDAI – Pearson's test: r=0.64, $p<0.05$; Spearman's test: r=0.66, $p<0.05$) (Fig. 1). By contrast, patients with LET showed a significant

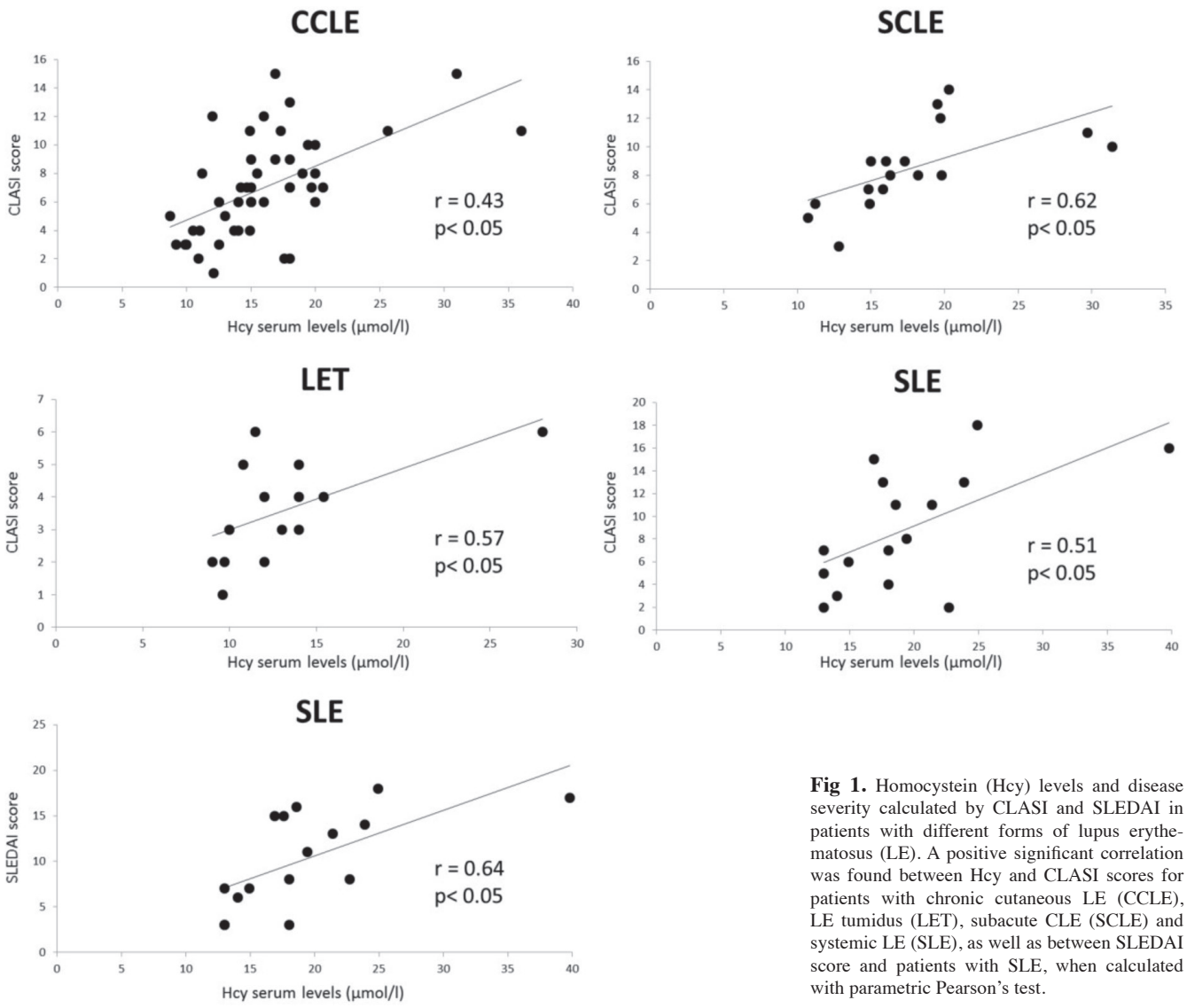

Fig 1. Homocystein (Hcy) levels and disease severity calculated by CLASI and SLEDAI in patients with different forms of lupus erythematosus (LE). A positive significant correlation was found between Hcy and CLASI scores for patients with chronic cutaneous LE (CCLE), LE tumidus (LET), subacute CLE (SCLE) and systemic LE (SLE), as well as between SLEDAI score and patients with SLE, when calculated with parametric Pearson's test.

positive correlation with Hcy levels only when calculated with parametric Pearson's test (r=0.57, p<0.05), but not with non-parametric Spearman's test (r=0.35, p=not significant).

Discussion

In the last decade, many studies have investigated the relationship among SLE, Hcy and cardiovascular diseases. The first and largest study was performed by Petri *et al*. in 337 SLE patients, followed up for a mean of 4.8 years, in order to detect thrombotic events. The authors reported HHcy in 15% of the patients with a significant association between raised Hcy concentrations and stroke and arterial thrombosis (also after adjustment for established risk factors). Later, these data were confirmed by several studies, some of which also emphasised the link between Hcy and CVD in SLE patients (11-24). There are still lacking data regarding the possible correlation and pathogenetic implication between diseases activity of CLE, SLE and HHcy. Our study showed for the first time a significant correlation between Hcy levels and the disease activity both in patients with CLE and SLE; this finding suggests a potential role of HHcy in LE, at least as a disease marker.

Accordingly, several studies have demonstrated that in the course of autoimmune diseases, the degree of inflammation correlates with plasma concentration of Hcy. The most convincing data deal with rheumatoid arthritis patients, in which various authors have reported a significant correlation between the level of Hcy and the expression of immuno-inflammatory markers (2).

A role of Hcy has also been suggested in other autoimmune diseases (*e.g*. in patients with Behçet's disease, inflammatory bowel diseases and SLE). In particular, although some studies did not find HHcy in lupus patients (26), several reports have demonstrated higher Hyc levels in both children and adults with SLE than in controls, at least in a subgroup of patients (27). According to the literature, our findings confirmed the presence of HHcy in a high percentage of SLE patients, as well as in CCLE and SCLE patients.

The role of Hcy in the pathogenesis of autoimmune diseases could be related to the ability of Hcy to activate the im-

mune system and to induce the expression of proinflammatory molecules. Many authors have demonstrated that Hcy was able to induce chemokine (IL-8 and/or MCP-1) and chemokine receptor expression by human vascular cells and monocytes (28, 29). Other cytokines and pro-inflammatory molecules with a Hcy-dependent stimulatory effect include: IL-1β (30), IL-6 (30-32), IL-12 (30), IL-18 (19), IL-1 receptor antagonist (IL-1ra) (32), CRP (33), adhesion molecules (P- selectin, E-selectin, ICAM-1) (34), and metalloproteinases (MMP-9) (33). In this context, the relationship between Hcy and IL-18, a novel pro-inflammatory Th1-dependent cytokine, appears of particular interest since it has also been demonstrated in a paradigmatic AD, such as SLE (19).

The above mentioned data strongly recognised the ability of Hcy to activate the immune system and enhance the inflammatory process.

Hcy may also be a trigger of autoimmune reactions through its capability to bind and structurally modify specific proteins, then resulting in neoantigens formation. This is potentially relevant in the onset of specific autoimmune diseases, as suggested for HLA-B27 modification and the development of ankylosing spondylitis (35).

The reasons for the presence of HHcy in autoimmune diseases are not completely clarified. However, the role of the depletion in folate and vitamin B12, due to an increased consumption related to the increased oxidative stress and to the active proliferation of immune-competent cells, seems particularly relevant.

As a result, a vicious circle is established, where the reduction in vitamin levels leads to the increase of Hcy which is able to promote inflammation with consequent further consumption of folate and vitamin B12. The replacement of vitamin depletion can be hypothesised in order to counteract this self-maintaining vicious circle. *In vitro* and *in vivo* studies have recently been demonstrated that folate treatment was accompanied by a marked reduction in the release of pro-inflammatory cytokines such as IL-8 and MCP-1 (36). However, other studies reported that vitamin replacement, although producing a lowering in plasma level of Hcy, did not exert any significant change in circulating markers of inflammation (37).

In the last years, few studies have focused on Hcy levels in autoimmune skin diseases. In particular, some papers have investigated Hcy in psoriasis, where higher Hcy levels were found in patients than in controls. Instead, contrasting results are present in the literature about a correlation between Hcy serum concentrations and the severity of the disease (38-40). Furthermore, Hcy was also investigated in patients with vitiligo, where Hcy serum levels were shown to correlate with the severity of the disease. Vitamin B12 and folic acid supplementation can benefit vitiligo patients (41-43).

On the other hand, there are no studies present in the literature investigating Hcy levels in CLE. In this study we have investigated all subtypes of LE and we have demonstrated that there is an increase of Hcy not only in SLE, but also in CLE, LET and SCLE

Moreover, we showed a significant correlation between Hcy serum level and disease activity. From this point of view, HHcy could be related to LE pathogenesis and it might be a triggering factor of the disease in predisposed individuals. The limitation of our study is the small number of patients, but this was only a preliminary study to investigate the relationship between Hcy and lupus. We suggest further studies to confirm these preliminary results with emphasis on therapeutic trials in order to determine the effect of lowering elevated Hcy levels on the prognosis of both SLE and CLE. Meanwhile we recommend the routine determination of Hcy level in patients with lupus and the inclusion of Hcy lowering agents such as vitamin B6, B12 and folic acid to the treatment protocol.

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