Homocysteine Plasma Concentration Is Related to Severity of Lung Impairment in Scleroderma

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ABSTRACT. ***Objective.*** To investigate the correlation between plasma concentration of total homocysteine and pulmonary involvement in patients with limited or diffuse scleroderma (systemic sclerosis, SSc).

Methods. Seventy-one patients with scleroderma were divided into 3 groups based on pulmonary involvement: Group A comprised patients without lung involvement (9 cases); Group B patients with lung involvement of mild and moderate stages (44 cases); and Group C patients with lung involvement of severe stage and endstage (18 cases). At the time of evaluation of lung involvement all patients underwent determination of plasma homocysteine concentration. Homocysteine concentration was also measured in 30 healthy controls homogeneous for sex and age.

Results. In patients with scleroderma the homocysteine concentration was significantly higher than in controls (11.1 and 6.9 µmol/l, respectively; $p < 0.001$). We found a significant association between plasma homocysteine concentration and severity of lung involvement that was not modified by correction for age, time from the diagnosis, type of scleroderma pattern, and serum creatinine and folate levels. Homocysteine concentration progressively increases in scleroderma patients with more severe pulmonary involvement. Subjects with high homocysteine concentration (i.e., ≥ 75th percentile of homocysteine concentration in patients with scleroderma without lung involvement) were mostly present in the group with the greatest lung involvement.

Conclusion. High level of homocysteinemia is associated with an increased risk of pulmonary disease in patients with scleroderma. We hypothesize that hyperhomocysteinemia may worsen injury of the endothelium, a key lesion in scleroderma disease, favoring the development of lung involvement. Our data support the hypothesis that homocysteine could be involved in the pathogenetic process of scleroderma pulmonary involvement. (J Rheumatol 2003;30:298–304)

Key Indexing Terms:
SCLERODERMA HOMOCYSTEINE LUNG INVOLVEMENT

Scleroderma is a multisystemic disorder of unknown etiology. The pathogenetic mechanisms involve 3 interactive components represented by vascular dysfunction and injury, immune system activation, and increased biosynthesis of collagen by fibroblasts. It is not clear which of these abnormalities is first expressed, although it is known that Raynaud's phenomenon (RP), an overt sign of vascular dysfunction, may precede other clinical manifestations of the disease by many years[1]. Endothelium is damaged early in the progress of scleroderma as indicated by increased plasma concentration of von Willebrand factor antigen early in the course of the disease[2]. The relationship between the 3 pathogenetic components that cooperate in realizing scleroderma damage is complex. For example, mediators of endothelial or immune cell origin influence fibroblast production of collagen in different ways: transforming growth factor-ß[3], interleukin 1 (IL-1)[4], and IL-4[5] favor the fibrotic process, while interferon-γ[6,7] and tumor necrosis factor-α[8] perform a negative regulation. Although genetic factors may play a role in determining susceptibility to scleroderma[9], a twin study indicated that one or more environmental agents likely operate in causing the disease[10]; some environmental factors incriminated in the development of scleroderma have been reviewed[11].

The clinical expression, the visceral involvement, and the evolution of scleroderma disease present a great deal of variability. Pulmonary involvement that includes both lung fibrosis and pulmonary hypertension represents a frequent manifestation with significant morbidity and mortality[12]. Lung fibrosis is more frequent in the diffuse pattern of scleroderma, while pulmonary hypertension may be a complication of longstanding limited scleroderma[13]. Not all patients develop pulmonary involvement and the variability of its severity is broad; a recent study has described that in diffuse scleroderma severe organ involvement does not develop in all patients and most often occurs early in the course of the illness; for example, severe lung involvement has been reported in 16% of cases[14].

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Considering the great variability of both visceral involvement and clinical course of the disease, it is likely that some factors different from the etiological agents contribute to pathogenetic mechanisms. We hypothesized that hyperhomocysteinemia, a well recognized independent risk factor for vascular disease, might favor endothelium damage in scleroderma. We evaluated the relationship between pulmonary involvement in patients with scleroderma and plasma concentrations of total homocysteine.

MATERIALS AND METHODS

Patients and biochemical analysis. Seventy-one Italian patients with scleroderma (61 women, 10 men, mean age 58.5 ± 11.7 yrs, range 24–85 yrs; mean duration of disease 11.9 ± 9.9 yrs, range 1–45 yrs) classified using the criteria of LeRoy, *et al*[13] and consecutively observed at our department from January 2000 to September 2001 were enrolled for this case-control study. Patients underwent examination and laboratory evaluation for comprehensive blood count, renal and liver function indexes, immunoglobulins, cryoglobulins, C3 and C4 levels, antinuclear and anti-ENA antibody determination.

Forty-three patients (60.6%) presented the limited pattern of scleroderma (lSSc) and 28 patients (39.4%) the diffuse form (dSSc). Anticentromere antibodies, examined by indirect immunofluorescence on HEp-2 cells, were positive in 37 patients with lSSc (86.0%); anti-Scl-70 antibodies determined by ELISA were detected in 20 patients with dSSc (71.4%).

All patients were evaluated for fasting total plasma homocysteine, which refers to the sum of free and protein-bound homocysteine and homocysteine-cysteine mixed disulfide. Blood was collected into vacuum tubes containing EDTA and kept on ice in the dark; plasma was separated within 90 min; total homocysteine concentrations were determined by high performance liquid chromatography with fluorescent detection as described[15].

Fasting total homocysteine was also evaluated in a group of 30 healthy volunteers recruited from hospital staff and relatives; the control group was homogeneous for sex and age (26 women, 4 men, mean age 54.6 ± 14.3 yrs, range 26–82).

Serum folate and vitamin B12 levels were evaluated (normal 6.7–38.5 nmol/l and 162–708 pmol/l, respectively).

Patients taking folate, B6, or B12 vitamin or other drugs able to interfere with total homocysteine level were excluded.

Lung involvement evaluation. At the time of total homocysteine determination all patients underwent the following investigations: (1) pulmonary function tests with diffusing capacity for carbon monoxide (DLCO), adjusted to hemoglobin level; (2) chest radiograph; and (3) Doppler echocardiography to estimate pulmonary artery systolic pressure; if no tricuspid regurgitation could be detected, pulmonary artery systolic pressure was presumed normal. Pulmonary hypertension was defined as mild for a pulmonary artery systolic pressure between 30 and 60 mm Hg, as proposed[16]; values > 60 mm Hg were considered expression of moderate–severe pulmonary hypertension. All investigations were performed at the local hospital.

Based on the proposal of Medsger, *et al*[17], pulmonary involvement was judged absent (stage 0) or present with a severity grading scale grouped in 4 stages: stage 1 (mild): DLCO 70–80% predicted, or forced vital capacity (FVC) 70–80% predicted, or rales or fibrosis on radiograph; stage 2 (moderate): DLCO 50–69% predicted, or FVC 50–69% predicted, or mild pulmonary hypertension; stage 3 (severe): DLCO < 50% predicted, or FVC < 50% predicted, or moderate–severe pulmonary hypertension; stage 4 (end-stage): oxygen required.

Nine patients (6 with lSSc; 3 dSSc) had no pulmonary involvement (stage 0). Eighteen patients (13 lSSc; 5 dSSc) presented with stage 1 lung involvement based on isolated DLCO reduction of 70–80% predicted (15 cases), DLCO reduction of 70–80% predicted associated with interstitial fibrosis observed on chest radiograph (2 cases), and isolated FVC reduction of 70–80% predicted (1 case). Twenty-six patients (16 lSSc; 10 dSSc) presented with stage 2 lung involvement; all these cases had moderate DLCO reduction of 50–69% predicted; moreover in this subgroup we found mild pulmonary hypertension (2 cases), interstitial fibrosis observed on chest radiograph (5 cases), and FVC reduction always of mild grade (70–80% predicted, 5 cases). Seventeen patients (7 lSSc; 10 dSSc) presented with stage 3 lung involvement; all these cases had severe DLCO reduction < 50% predicted, moreover in this subgroup of cases we found moderate–severe pulmonary hypertension (3 cases), interstitial fibrosis on chest radiograph (8 cases), and FVC reduction mild grade (1 case) and moderate grade (7 cases). Only one patient required continuous oxygen therapy (stage 4); this patient had severe DLCO reduction, moderate FVC reduction, moderate–severe pulmonary hypertension, and interstitial fibrosis on chest radiograph.

Six of our 71 patients had pulmonary hypertension, 2 of mild grade and 4 of moderate–severe grade; in 3 patients (1 lSSc; 2 dSSc) pulmonary hypertension was associated with lung fibrosis; in 3 patients with lSSc, pulmonary hypertension was isolated.

Because the number of cases in each stage was low, particularly in stage 4, we combined the patients with pulmonary involvement stages 1 and 2 (Group B) and the patients with pulmonary involvement stages 3 and 4 (Group C) for statistical analysis. Patients without lung involvement represented Group A.

Statistical analysis. All calculations were performed with SSPS 10.0 statistical package (SPSS Inc., Chicago, IL, USA). Distributions of continuous variables in groups are expressed as means ± standard deviation. Logarithmic transformation was performed on all skewed variables, including total homocysteine, folate, and creatinine levels; therefore these variables are expressed as geometric means with 95% confidence interval (CI). Quantitative data were assessed using Student's t test or by ANOVA with Tukey's post-hoc comparison of means, whereas qualitative data were analyzed with the chi-square test. Correlation between continuous variables was evaluated using Spearman's test. A value of $p < 0.05$ was considered significant. For evaluation of possible association between high levels of total homocysteine and lung involvement, an arbitrary threshold of hyperhomocysteinemia was defined as ≥ 75th percentile of total homocysteine concentration in patients with scleroderma without lung involvement.

To assess the association of "hyperhomocysteinemia" with different degrees of pulmonary involvement, odds ratio (OR) with 95% CI was first obtained using univariate logistic regression analysis. Then adjustment for other factors such as age, time from diagnosis, scleroderma pattern, and serum creatinine and folate levels was performed by including these covariates in a multivariate logistic regression analysis.

RESULTS

Homocysteine concentrations were significantly higher in patients than in controls (11.1 and 6.9 µmol/l, respectively; $p < 0.001$). However, if we considered the 3 scleroderma subgroups on the basis of lung involvement, we observed that the levels of homocysteine increased progressively from Group A (9.2 µmol/l) to Group B (10.6 µmol/l) and to Group C (13.5 µmol/l), as shown in Figure 1; this pattern was highly statistically significant by ANOVA ($p < 0.001$). Notably, there was no significant difference between the control group and Group A ($p = 0.132$ by Tukey's post-hoc comparison), whereas there was a highly significant difference between the control group and Group B and between the control group and Group C (for both, $p < 0.001$ by Tukey's post-hoc comparison); further, homocysteine level in Group C was significantly higher than in Group A ($p = 0.048$ by Tukey's post-hoc comparison).

We performed specific analysis on subjects with scleroderma. General characteristics of the study population are summarized in Table 1. Table 2 shows characteristics of the 3 subgroups with different lung involvement. Only homocysteine

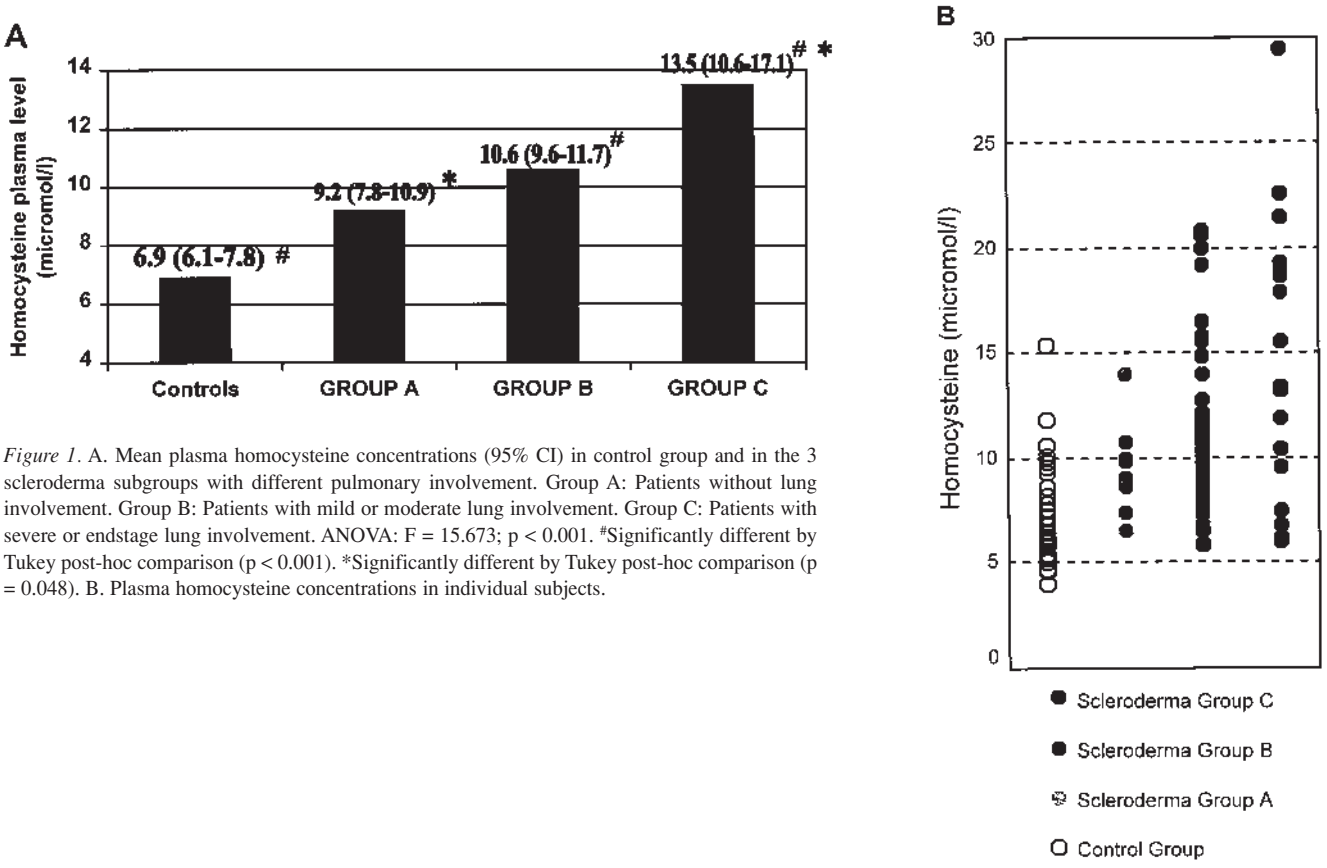

Figure 1. A. Mean plasma homocysteine concentrations (95% CI) in control group and in the 3 scleroderma subgroups with different pulmonary involvement. Group A: Patients without lung involvement. Group B: Patients with mild or moderate lung involvement. Group C: Patients with severe or endstage lung involvement. ANOVA: $F = 15.673$; $p < 0.001$. #Significantly different by Tukey post-hoc comparison ($p < 0.001$). *Significantly different by Tukey post-hoc comparison ($p = 0.048$). B. Plasma homocysteine concentrations in individual subjects.

Table 1. General characteristics of the study population (data are expressed as mean value ± standard deviation for age and time from diagnosis, and as geometric mean with 95% confidence intervals for homocysteine, folate, and creatinine concentrations).

	Total Population, n = 71
Sex, M/F (%)	10/61 (14.1/85.9)
Age, yrs	58.5 ± 11.7
Time from diagnosis, yrs	11.9 ± 9.9
Pattern of scleroderma, limited/diffused (%)	43/28 (60.6/39.4)
Lung disease, n (%)	
Absent	9 (12.7)
Present, Degree	
1	18 (25.4)
2	26 (36.6)
3	17 (23.9)
4	1 (1.4)
Homocysteine, µmol/l, mean (CI)	11.1 (10.1–12.1)
Folate, nmol/l, mean (CI)	11.1 (9.4–13)
Creatinine, mg/dl, mean (CI)	0.83 (0.79–0.87)

concentration presented a significant pattern, increasing progressively from the lowest to the highest degree of the pulmonary involvement. No other variable (age, sex, time from diagnosis, type of scleroderma pattern, serum creatinine and serum folate levels) showed a statistically significant difference between these groups. However, age and serum folate level tended to present a proportional pattern, the first directly, the latter inversely, according to the severity of the lung disease; moreover, subjects with dSSc tended to be more represented in Group C. Vitamin B12 level was normal in all the patients, with no difference between the 3 groups.

Total plasma homocysteine concentration was significantly correlated with age ($r = 0.31$, $p = 0.01$), serum creatinine level ($r = 0.3$, $p = 0.012$), and serum folate level ($r = –0.29$, $p = 0.014$). There was no statistical difference in total plasma homocysteine concentration between limited or diffuse scleroderma (11.1 and 10.9 µmol/l, respectively; $p = 0.818$ by t test).

An arbitrary threshold value of high total homocysteine (10.4 µmol/l) was determined at the level of the 75th percentile on the basis of total homocysteine concentration in scleroderma patients without lung involvement. Then we analyzed the association between this defined hyperhomocysteinemia and degree of lung disease. The hyperhomocysteinemic subjects were more represented in the groups with signs of pulmonary involvement (Table 3). Specifically, considering Group A as the reference group, the crude odds ratio was 3.5 (nonsignificant, 95% CI 0.7–18.8) in relation to Group B, and 9.1 (significant, 95% CI 1.4–59.6) in relation to Group C (Figure 2). After a logistic regression adjusted for age, time from diagnosis, type of scleroderma pattern, and creatinine and folate levels, the latter association remained statistically significant (OR 10.6, 95% CI 1.03–109.4).

Table 2. Characteristics of the study population divided into 3 groups on the basis of the pulmonary involvement.

	Group A, No Pulmonary Involvement, n = 9	Group B, Pulmonary Involvement of Degree I and II, n = 44	Group C, Pulmonary Involvement of Degree III and IV, n = 18	p
Sex, M/F (%)	1/8 (11.1, 88.9)	5/39 (11.4, 88.6)	4/14 (22.2, 77.8)	0.517*
Age, yrs	53 ± 12	58 ± 10	62 ± 15	0.143†
Time from diagnosis, yrs	10 ± 7	12 ± 10	12 ± 11	0.883†
Pattern of scleroderma, limited/diffused (%)	6/3 (66.7, 33.3)	29/15 (65.9, 34.1)	8/10 (44.4, 55.6)	0.269*
Homocysteine, µmol/l (range)	9.2 (7.8 –10.9)	10.6 (9.6–11.7)	13.5 (10.6–17.1)	0.021†
Folate, nmol/l (range)	11.9 (7.5–19.2)	12 (9.8–14.7)	8.7 (6.1–12.5)	0.232†
Creatinine, mg/dl (range)	0.81 (0.75–0.87)	0.82 (0.78–0.87)	0.86 (0.77–0.96)	0.597†

*Chi-square test; †ANOVA.

Table 3. Hyperhomocysteinemia and degree of pulmonary involvement.

	No Hyperhomocysteinemia	Hyperhomocysteinemia
Group A, n (%)	7 (77.8)	2 (22.2)
Group B, n (%)	22 (50)	22 (50)
Group C, n (%)	5 (27.8)	13 (72.2)

By chi-square test: p = 0.045.

Finally, we performed a further selection, excluding the patients with pulmonary hypertension (6 subjects): in this subanalysis as well, subjects with total plasma homocysteine > 10.4 µmol/l were significantly associated with lung involvement (comparison between Group A and Group C: p = 0.021 by chi-square test, OR 8.8, 95% CI 1.2–61.7). Homocysteine concentration for the 3 patients with isolated pulmonary hypertension was 6.5, 9.7, and 10.7 µmol/l.

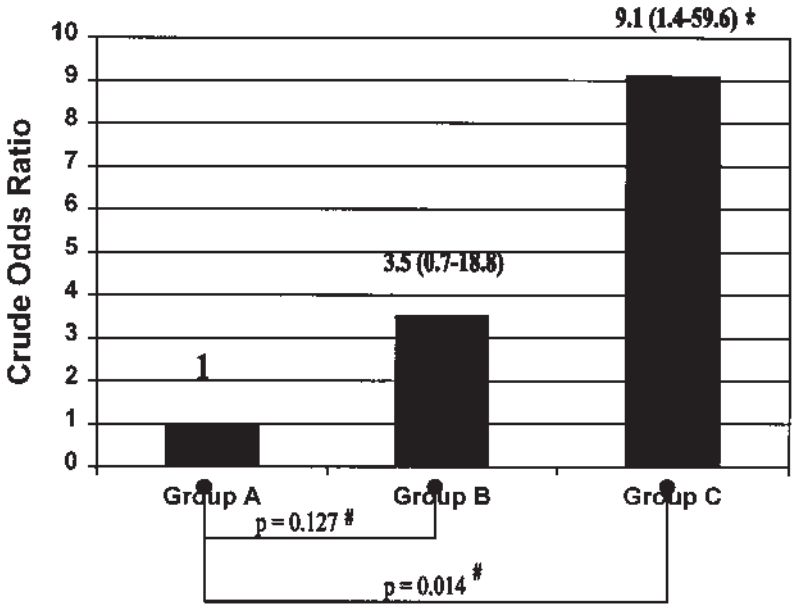

Figure 2. Hyperhomocysteinemia and degree of pulmonary involvement (OR considering Group A as the reference group). Group A: Scleroderma patients without lung involvement. Group B: Patients with mild or moderate stage lung involvement. Group C: Patients with severe or end-stage lung involvement. #Chi-square test. *The association remained statistically significant (OR 10.6, 95% CI 1.03–109.4) after logistic regression adjusted for age, time from diagnosis, type of scleroderma, and serum creatinine and folate levels.

DISCUSSION

Recently rheumatologists have paid attention to plasma homocysteine concentrations in different rheumatic diseases. In systemic lupus erythematosus it was observed that high level of plasma homocysteine represents a risk factor for coronary artery disease[18], stroke[19], and arterial thrombosis[19,20]. In rheumatoid arthritis (RA), hyperhomocysteinemia was more frequent in patients with a history of thrombosis in comparison with patients without previous thrombotic events, particularly if antiphospholipid antibody was positive[21]. In RA a persistent elevation of plasma homocysteine was found after treatment with methotrexate alone, and was more pronounced after combination therapy of methotrexate and sulfasalazine[22].

We found 2 recent reports of plasma homocysteine concentration in patients with primary RP and scleroderma associated RP in comparison with healthy subjects; the first described high plasma homocysteine in both groups of patients, particularly in those with primary RP[23]. The second study confirmed the moderate increase of plasma homocysteine concentration in scleroderma patients, but found normal concentrations in patients with primary RP, lower than controls[24]. These data are summarized in Table 4. In the latter study, determination of folic acid and vitamin B12 plasma levels and evaluation of MTHFR mutations in scleroderma patients allowed a conclusion that the hyperhomocysteinemia was due to nutritional deficiency rather than inherited factors. Neither study correlated plasma homocysteine concentrations with patients' clinical features or severity of visceral involvement.

McCully first reported that homocysteine, a nonessential sulfur-containing amino acid, was implicated in the development of arterial occlusive lesions[25]; its influence increases with age[26,27]. High concentrations of plasma homocysteine are due either to genetic defects of enzymes involved in homocysteine metabolism or to severe vitamin deficiency. The most common inherited cause of severe hyperhomocysteinemia is cystathionine ß-synthase deficiency[28]. Moderate hyperhomocysteinemia may be provoked by the frequent mutation (C677T) in gene encoding for MTHFR when folate status is inadequate[29]. Recently, mild hyperhomocysteinemia has been recognized as an independent modifiable risk factor for arterial vascular disease[28]; the association between mild hyperhomocysteinemia and occlusive disease of coronary, cerebral, and peripheral arteries has been reported[30]. Homocysteine can cause endothelium injury. Several mechanisms have been proposed to clarify the connection between homocysteine and vascular disease. Homocysteine is directly toxic to endothelial cells[31] and modifies the thromboresistant properties of normal endothelium[32], inactivating the anticoagulant substances protein C and thrombomodulin[33] and blocking the tissue plasminogen activator binding domain of annexin II[34]. Moreover, it may facilitate atherogenic processes by oxidative stress pathways; indeed, homocysteine can favor oxidation of low density lipoprotein by arterial smooth muscle cells[35]. As well, hyperhomocysteinemia alters the production of nitric oxide by cultured endothelial cells[36], generates superoxide radicals[37], and inhibits antioxidant enzymes[38]. Finally, homocysteine increases cell growth and collagen production in cultured rabbit smooth muscle cells[39].

Table 4. Plasma homocysteine concentration in control subjects, in patients with primary Raynaud's phenomenon (RP), and in patients with scleroderma.

	Healthy Controls	Primary RP	Scleroderma
Levi[23]	5.9 ± 2.0, n = 20	15.5 ± 4.1, n = 10	11.6 ± 6.2, n = 10
Marasini[24]	9.3 ± 2.5, n = 29	7.8 ± 1.4, n = 12	13.0 ± 6.5, n = 30

Our data confirm observations that the plasma concentration of total homocysteine in patients with scleroderma is higher than in healthy subjects. Considering our 3 subgroups of patients, the plasma concentration of homocysteine in Groups B and C was significantly different from controls; in contrast the plasma homocysteine values of Group A and the controls were not significantly different; this could likely be due to the small number of subjects in the former group. In our study hyperhomocysteinemia was associated with advanced degree of pulmonary involvement. Homocysteine concentration was directly linked to the lung impairment, and the correlation was not modified by a logistic regression for age, disease duration, scleroderma pattern, renal function, or folate level. Thus our data suggest that hyperhomocysteinemia may worsen the scleroderma disease process at the pulmonary level. We are aware of the small numbers of cases in our scleroderma subgroups; this might explain the large distribution of homocysteine levels.

Several mechanisms of homocysteine activity provide biological support to the hypothesis of homocysteine-scleroderma interaction. Based on the multiple observations noted above, high homocysteine concentration seems to induce vascular dysfunction through the generation of reactive oxygen species that may inactivate nitric oxide, a powerful regulator of endothelium-dependent vasodilatation[40]. In scleroderma, vascular tone control is abnormal, with an imbalance of vasodilatation and vasoconstriction[1]; the evidence that hyperhomocysteinemia reduces nitric oxide production by endothelial cells *in vitro* may contribute to the diminished endothelial-dependent vasodilatation observed in scleroderma.

Oxygen free radicals that may be generated by Raynaud's phenomenon characterized by tissue ischemia and subsequent reperfusion may mediate endothelial injury[41]; moreover, it has been observed that reactive oxygen species may fragment autoantigens, supporting the generation of the typical autoantibodies found in patients with scleroderma[42]. The ability of hyperhomocysteinemia to produce reactive oxygen species[37] and to interfere with antioxidant enzymes[38] may exaggerate these pathogenetic mechanisms.

Moreover, the observation that hyperhomocysteinemia

increases collagen production by smooth muscle cells *in vitro* and interferes with the action of natural anticoagulants like protein C, thrombomodulin, and annexin II may contribute to worsen the scleroderma process. Finally, in scleroderma an increase in oxidized lipoproteins[43] and an increased susceptibility to oxidation of low density lipoproteins[44] have been reported; homocysteine may foster atherogenic processes by supporting oxidation of low density lipoprotein[35]. It should be noted that there is increasing evidence of macrovasculature as well as microvasculature involvement in scleroderma[45].

Thus hyperhomocysteinemia in scleroderma could act as an aggravating factor in that it amplifies pathogenetic mechanisms of the disease, contributing to the development of the vascular damage. In regard to scleroderma lung involvement, oxidative stress and the supposed increase of collagen production by high concentrations of homocysteine are of great interest. In our study, the subgroup with isolated pulmonary hypertension, in which vascular damage is considered pathogenically prominent, was too small (3 subjects) to allow any conclusion. We analyzed only a single clinical expression of scleroderma disease; other studies are needed to evaluate if other clinical features of this multifaceted disease, particularly peripheral vascular involvement, as well as pulmonary disease, are correlated to homocysteine concentration.

Two studies investigated homocysteine concentrations in lung cancer, without observing any correlation[46,47]. We found no data in the literature about homocysteine levels in other lung diseases.

Our findings support the possible role of homocysteine, a known biochemical agent of vascular system damage, in scleroderma. Moreover, our results are in keeping with the well-recognized vascular alterations in scleroderma. Larger case-control studies and above all prospective studies are needed to evaluate if lowering homocysteine concentrations could represent a new therapeutic target.

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