

# Antibodies to N-homocysteinylated albumin in patients with systemic lupus erythematosus

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**Abstract: Introduction.** Hyperhomocysteinemia is known to predispose to atherosclerosis and occurs more commonly in patients with systemic lupus erythematosus (SLE) than in the general population. It has been shown that elevated plasma total homocysteine (tHcy) results in protein N-homocysteinylated and production of autoantibodies against N-homocysteinylated (N-Hcy) proteins. **Objectives.** The aim of the study was to investigate whether anti-N-Hcy-albumin antibodies occur in patients with SLE and identify factors that determine these antibodies in such a population. **Patients and methods.** In 50 subjects with SLE and 50 age- and sex-matched healthy controls, we determined serum IgG antibodies to N-Hcy-albumin using an in-house enzyme linked immunosorbent assay. **Results.** Patients had higher plasma tHcy and C-reactive protein (CRP) than controls, while serum folate and vitamin B<sub>12</sub> were lower in patients. Levels of anti-N-Hcy-albumin were higher in patients with SLE than in controls (medians: 0.31; vs. 0.19;  $p < 0.0001$ ). In SLE patients, levels of anti-N-Hcy-albumin antibodies correlated with tHcy ( $r = 0.83$ ;  $p < 0.0001$ ), CRP ( $r = 0.33$ ;  $p = 0.02$ ) and the duration of the disease ( $r = 0.3$ ;  $p = 0.04$ ). Seropositivity to anti-N-Hcy-albumin antibodies was more frequent in SLE patients than in controls (50% vs 10%;  $p < 0.001$ ). In SLE patients tHcy and CRP concentrations, along with the duration of the disease, were independent predictors of anti-N-Hcy-albumin antibodies levels. There were no associations between a type or levels of antinuclear antibodies or patient's age with anti-N-Hcy-albumin antibodies. **Conclusions.** Compared with healthy controls, in SLE patients levels of anti-N-Hcy-albumin antibodies are significantly higher and are largely determined by tHcy, CRP and the disease duration. This novel autoimmune response might contribute to an increased risk of vascular events in SLE patients.

**Key words:** antibodies to N-homocysteinylated albumin, homocysteine, systemic lupus erythematosus

## INTRODUCTION

Hyperhomocysteinemia has been reported an independent risk factor for coronary artery disease, cerebral infarction and peripheral arterial disease [1-5]. In patients with systemic lupus erythematosus (SLE), ischemic heart disease and stroke are associated with higher total plasma homocysteine (tHcy) levels compared to those without cardiovascular disease [6,7]. It has been published that in patients with SLE, hyperhomocysteinemia is associated with an increased risk of cardiac valve abnormalities [8] and the development of atherosclerotic plaques in coronary arteries [7]. Moreover, elevated tHcy may be a marker of increased vascular risk in SLE patients with low levels of antiphospholipid antibodies [9]. Despite much experimental work on Hcy role in cardiovascular diseases, mechanisms by which Hcy is involved in the pathogenesis of vascular disease remain unclear.

It has been reported that elevated Hcy levels causes: endothelial cell dysfunction, cytotoxic effects, impaired fibrinolysis, enhanced vascular oxidative stress, vascular smooth muscle hypertrophy and increase in collagen synthesis in the vascular wall [10-12]. One of mechanisms for vascular injury induced by hyperhomocysteinemia might be a metabolic conversion of Hcy to cyclic thioester, Hcy-thiolactone, catalyzed by methionyl-t-RNA synthase [13] (fig. 1). Hcy-thiolactone, like other thioesters, is a highly reactive compound which acylates nucleophil groups e.g. the NH<sub>2</sub> group. It has been documented that Hcy thiolactone reacts with proteins and forms amide bonds with amino groups of lysine residues [14]. This results in the generation of N-homocysteinylated proteins (N-Hcy proteins), which can be detected in human serum [13,15]. Protein N-homocysteinylated rates are proportional to their lysine contents [14]. Albumin constitutes the major pool of plasma N-Hcy-proteins. N-homocysteinylated of ε-amino groups of lysine residues results in the loss of positive charges and the SH group formation. In consequence, such N-Hcy proteins lose their structure and biochemical properties (e.g. catalytic functions), and also they become susceptible to further oxidation. Non-enzymatic protein modification could be observed with as little as 10 nM thiolactone and N-homocysteinylated degree increases with the Hcy thiolactone concentration. N-Hcy proteins, such as albumin, can be re-

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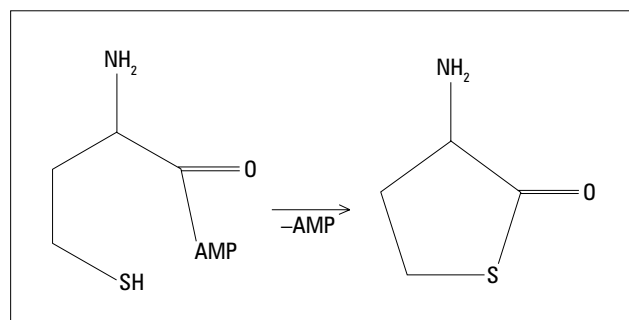


Fig. 1. Homocysteine thiolactone synthesis

cognized as antigens and might activate humoral response of the immune system. Anti-N-Hcy protein IgG antibodies have been detected in humans [17-19]. Significant differences in levels of anti-N-Hcy protein antibodies were found between young male patients (<50 years old) with ischemic heart disease and healthy subjects [17]. It is worth noting that higher levels of anti-N-Hcy-protein antibodies were observed in patients with coronary artery disease compared with age, sex-matched controls even if there was no differences in plasma tHcy concentrations between these groups. [20]. Anti-N-Hcy-protein IgG antibodies have been also detected in patients after stroke [17] and in subjects with end-stage renal disease treated with hemodialysis [19]. Immunological consequences of hyperhomocysteinemia might be of importance in our understanding of a pathological Hcy influence on the vascular system. It is not known whether there is a tendency to produce anti-N-Hcy protein antibodies in patients with SLE. If so, whether these antibodies might contribute to the development of atherosclerosis in patients with SLE. [21,22].

The aim of our study was to assess the occurrence of anti-N-Hcy-albumin antibodies in patients with SLE compared to healthy controls. We also sought to determine factors which may determine the antibody levels in subjects with SLE.

## PATIENTS AND METHODS

We enrolled 50 patients with SLE (diagnosis in accordance with the American Collage of Rheumatology revised criteria for the classification of SLE), aged between 18 and 60 years, to this case-control study. Patient were recruited in a stable phase of SLE. Exclusion criteria were symptoms of acute infection, cancer, diabetes (fasting plasma glucose >7 mmol/L, measured twice), chronic renal disease (serum creatinine  $\geq 177$   $\mu\text{mol/L}$ ), coronary artery disease, acute or previous deep vein thrombosis or pulmonary embolism, pregnancy, liver insufficiency, hypo- or hyperthyroidism, treatment with drugs or vitamins known to affect tHcy concentration (e.g metotrexate, folic acid). Patients were allowed to take chloroquine, aspirin, statins, inhibitors of angiotensin converting enzyme (ACE-inhibitors), glucocorticosteroids. Previous cyclophosphamide and

azathioprine treatment was also allowed if discontinued within at least 3 months preceding the onset of the study. Age, sex-matched apparently healthy individuals, free of any signs or symptoms, with normal results of routine laboratory blood tests, served as controls.

Blood samples were collected after a 14-hour overnight fast. Complete blood counts, creatinine and glucose concentrations, lipid profile, anti-nuclear antibody titers, anticardiolipin antibodies, lupus antycoagulant (LA), folic acid and vitamin B<sub>12</sub>, methylenetetrahydrofolate reductase (MTHFR) C677T polymorphism and anti-N-Hcy albumin IgG antibody levels were determined. Plasma tHcy levels were determined by high-performance liquid chromatography (HPLC). High sensitivity CRP was measured by using latex nephelometry (Dade Behring). Titers and types of anti-nuclear antibody were assessed with indirect immunofluorescence by using the Hep-2010 cells. Anticardiolipin antibody levels were measured by immunofluorescence according to a modified method by Harris et al. [23]. The LA presence was assessed in citrated plasma according to the three stage method recommended by Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis [24]. Serum folic acid and vitamin B<sub>12</sub> levels were measured using a Immunolite analyzer (Diagnostic Products Corp.) Serum levels of IgG antibodies against N-Hcy-albumin were determined using an in-house enzyme-linked immunosorbent assay; this method was developed in the Department of Molecular Genetics in New Jersey Medical School in Newark and modified in the Department of Medicine Jagiellonian University School of Medicine in Cracow [17-19]. The MTHFR C677T polymorphism was determined by the polymerase chain reaction [25].

## Statistical analysis

Normally distributed data are given as mean ( $\bar{x}$ )  $\pm$  standard deviation (SD). Variables, which were not normally distributed are presented as median and interquartile range. Inter-group differences were assessed using t-test for continuous variables with normal distribution and Mann-Whitney test for continuous not normally distributed data. Categorical variables were analyzed by chi2 test. Correlation between continuous variables were assessed using Pearson's test for normally distributed data or Spearman test for remaining variables, respectively. Multiple linear regression analysis was used to determine independent factors which influence anti-N-Hcy-albumin antibody levels. A p-value below 0.05 was considered to be statistically significant.

## RESULTS

The characteristics of patients and controls group are shown in the table 1. In the patient group a mean disease duration was 10 years ( $9.9 \pm 5.5$  years). Proteinuria (>0.5 g/24 h) was observed in 19 (38%) patients, neurological symptoms,

**Table 1.**

	Patients (n = 50)	Controls (n = 50)	p
Age (years)	42 (34–46)	42.2 (35–48)	NS
Women/Men, n/n	45/5	45/5	NS
Haemoglobin (g/dl)	13.4 (12.6–13.7)	13.6 ±1.5	NS
RBC (x 10 <sup>6</sup> /ml)	4.4 (4.13–4.72)	4.5 ±0.4	NS
WBC (x 10 <sup>3</sup> /ml)	4.85 (3.7–5.7)	5.2 (4.5–6)	NS
Platelets (x 10 <sup>3</sup> /ml)	218.5 ±81.9	235.1 ±52.9	NS
Creatinine (μmol/l)	73.1 ±11.8	72.1 ±12.3	NS
C <sub>4</sub> (g/l)	0.128 (0.082–0.173)	0.227 (0.175–0.26)	<0.0001
Total cholesterol (mmol/l)	4.55 (4–5.2)	5 (4.4–5.8)	0.05
LDL cholesterol (mmol/l)	2.7 ±1.1	2.9 ±1	NS
HDL cholesterol (mmol/l)	1.4 ±0.3	1.6 ±0.4	0.01
Triglycerides (mmol/l)	1.23 (0.87–1.48)	0.91 (0.73–1.52)	NS
Glucose (mmol/l)	4.67 ±0.6	4.95 ±0.5	0.01
C-reactive protein (mg/l)	2.29 (0.8–4.81)	0.79 (0.26–1.44)	0.0002
Folic acid (nmol/l)	5.13 (3.99–6.35)	9.18 (6.99–11.1)	<0.0001
Vitamin B <sub>12</sub> (pg/ml)	241 (203–290)	313.5 (248–402.5)	0.001
C677T MTHFR CC n (%)	26 (52%)	26 (52%)	NS
C677T MTHFR CT n (%)	20 (40%)	19 (38%)	NS
C677T MTHFR TT n (%)	4 (8%)	5 (10%)	NS
Homocysteine (μmol/l)	11.45 (9.2–13.6)	8.93 (8.1–10.4)	0.0002
Homocysteine > 15 μmol/l n (%)	10 (20)	1 (2)	p <0.0001
Anti-N-Hcy-albumin IgG antibodies (A <sub>490</sub> )	0.31 (0.23–0.39)	0.19 (0.16–0.23)	<0.0001
Seropositivity status n (%)	25 (50)	5 (10)	p <0.001

Normally distributed data are presented as mean ± standard deviation (x ±SD), remaining continuous data are presented as median and interquartile interval. Abbreviations: C<sub>4</sub> – complement, HDL – high density lipoprotein, LDL – low density lipoprotein, MTHFR – methylenetetrahydrofolate reductase, NS – non-significant, RBC – red blood count, WBC – white blood count. Seropositivity status denotes anti-N-Hcy-albumin IgG antibodies level over 90th percentile in controls

which fulfilled the criteria of SLE, were found in 11 (22 %) patients and hematologic abnormalities (leucopenia, thrombocytopenia) were observed in 19 patients (38%). The SLE-like syndrome, which is characterized mainly by skin changes and joints involvement, was observed in 8 patients (16%). Significant titers of anti-nuclear antibodies were detected in all patients. Anti-double-stranded DNA antibodies were detected in 17 patients (34%), anti-Ro antibodies in 20 (40%) and anti-La in 15 patients (30%). Moreover, 11 patients had anti-RNP antibodies and 2 patients had anti-Sm antibodies. Antiphospholipid syndrome was diagnosed in 3 (6%) patients (diagnosis according to the current criteria [27,28]). In 3 other patients elevated levels of anticardiolipin or anti-β<sub>2</sub> glycoprotein I were found, while LA was detected in 10 (20%) patients. Treatment profile was as follows: 20% of patients (10 from 50) did not take any treatment at the time of enrollment in the study, 74 % of patients (37 out of 50) were treated with oral glucocorticosteroids, 24% (12 out of 50) took chloroquine, 22% (11 out

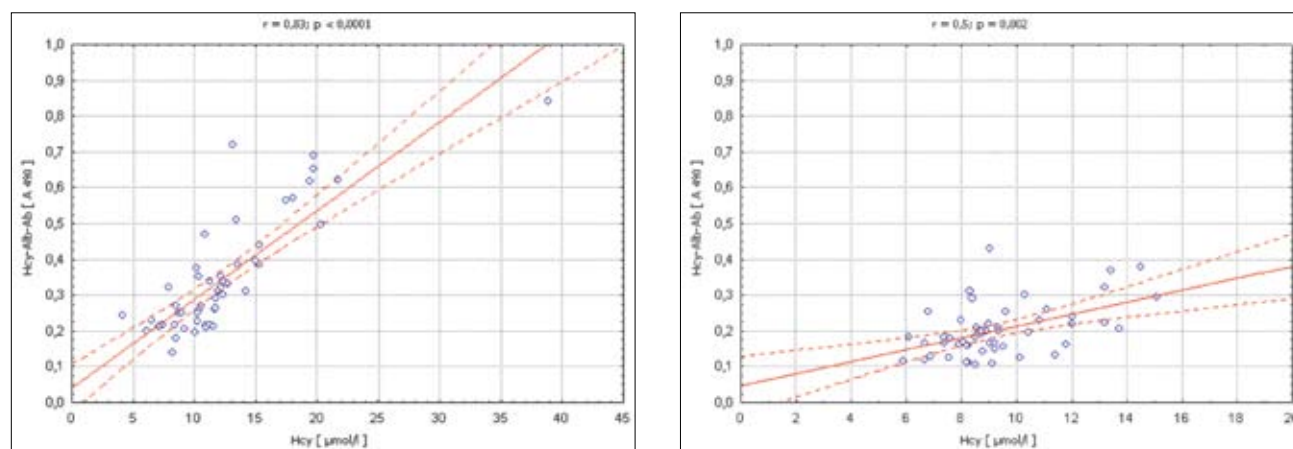
of 50 ) ACE inhibitor, 4% (2 out of 50) statins, 50% (25 out of 50) cyclophosphamide or azathioprine in the past.

The SLE patients had higher tHcy and CRP concentrations and lower folate, vitamin B<sub>12</sub> and serum complement (C<sub>4</sub>) levels (tab. 1). Hyperhomocysteinemia, defined as tHcy concentration >15 μM/L, was found in 20% in SLE patients and in 2% in the control group

(p <0.0001). The prevalence of the particular types of MTHFR C677T polymorphism did not significantly differ between patients and controls group (tab. 1).

In the patient group levels of anti-N-Hcy-albumin antibody were significantly higher as compared to the control group (median, interquartile range: 0.31; 0.23–0.39 vs. 0.19; 0.16–0.23; p <0.0001).

Sex, age and creatinine concentration showed no association with levels of anti-N-Hcy-albumin antibodies in either group. As expected, in SLE patients there was a significant positive correlation between tHcy and anti-N-Hcy-albumin



**Fig. 2A.** Association between plasma total homocysteine (tHcy) concentrations and anti-N-homocysteinylation-albumin antibody levels in patients group. **B.** Association between plasma total homocysteine (tHcy) concentrations and anti-N-homocysteinylation-albumin antibody levels in controls group

antibodies ( $r = 0.83$ ;  $p < 0.0001$ ; fig. 2A). However, there was no association between folic acid or vitamin B<sub>12</sub> and these antibodies. Interestingly, in SLE patients we observed weak positive correlations between anti-N-Hcy-albumin antibodies and CRP concentration ( $r = 0.33$ ;  $p = 0.02$ ) and disease duration ( $r = 0.3$ ;  $p = 0.04$ ). There were no associations between anti-N-Hcy-albumin antibodies and others autoantibodies (anti-cardiolipin, anti- $\beta 2$  glycoprotein I and antinuclear antibodies). We only found significantly higher levels of anti-N-Hcy-albumin antibody in patients with LA compared to the remaining SLE patients (median, interquartile range: 0.41; 0.34–0.51 vs. 0.26; 0.22–0.36;  $p = 0.02$ ). There was no significant association between MTHFR C677T genotype and anti-N-Hcy-albumin antibodies levels.

In the control group anti-N-Hcy-albumin antibodies correlated only with tHcy concentration ( $r = 0.5$ ;  $p = 0.002$ ; fig. 2B).

Seropositivity, which was defined as the ELISA reading above the value corresponding to the 90<sup>th</sup> percentile of the control group, i.e. 0.3055 (tab. 1), was 5-fold more frequent in patients than in controls ( $p < 0.001$ ). Multiple linear regression analysis showed that tHcy concentration, SLE presence and CRP concentration were independent predictors of anti-N-Hcy-albumin antibody levels in both groups (tab. 2). Similarly, in SLE patients tHcy concentration, disease duration and CRP concentration independently predicted levels of these antibodies (data not shown).

## DISCUSSION

The present study is the first to demonstrate that in SLE patients there is a tendency to form anti-N-Hcy-albumin IgG antibodies and levels of these antibodies are significantly higher than in healthy individuals matched for age and sex. In line with previous observations, we found markedly higher

tHcy concentrations in patients with SLE than in the control group. Levels of vitamins (folic acid and vitamin B12), which are involved in Hcy metabolism, were also lower in patients with SLE. A positive correlation between tHcy concentration and anti-N-Hcy-albumin antibodies level was confirmed in SLE patients and controls, however in patients with SLE the correlation was stronger than in the healthy group, which corroborates previous findings in patients with coronary artery disease [20]. However, in the latter study by Undas A et al. [20], subjects with coronary disease had higher tHcy plasma concentrations than SLE patients presented in this study (mean concentration 20.0 vs. 12.5  $\mu\text{mol/l}$ , respectively). Therefore, it might be speculated that a correlation value, which we found in our patient group, does not only result from higher tHcy levels than in controls. Interestingly, in accordance with the previous observations in patients with coronary disease aged 50 years or less [18], we found a weak, but significant, correlation between anti-N-Hcy-albumin antibodies and CRP levels in SLE patients. Although the association of plasma tHcy concentration and CRP levels has not been reported so far, links between hyperhomocysteinemia and inflammation might be of importance. This hypothesis might be supported by the phenomenon of Hcy participation in recruitment of leucocytes to the site of vascular injury described by Poddar et al. [29] and activation of the immune system, associated with increased production of reactive oxygen species and enhanced requirements of vitamins, which deficiencies result in an increase in tHcy concentration [30,31]. Our findings, presented in the current study, suggest that pathological activation of the immune system in patients with SLE and enhanced pro-inflammatory cytokine production, leading to increased levels of inflammatory markers (e.g. CRP), might have been also associated with undue response to N-Hcy-proteins. One might put forward a hypothesis that increased production of anti-N-Hcy-protein antibodies, like it occurs against other antigens, is associated with inflammation at the site of injured vascular



**Table 2. Multiple linear regression analysis for independent predictors of anti-N-homocysteinylated albumin antibodies levels in the whole group (patients + controls), n = 100**

Zmienna	Coefficient	95 % Confidence Interval	p
Homocysteine	0.023	0.018–0.027	<0.0001
Diagnosis of Systemic Lupus Erythematosus	0.075	0.033–0.116	0.001
C-reactive protein	0.001	0.0004–0.008	0.032

wall and it may explain one of the pathological mechanisms of premature atherosclerosis in patients with SLE.

In our study we also observed that levels of anti-N-Hcy-albumin antibodies positively correlate with the duration of clinically overt SLE. Moreover, using multiple linear regression analyses, we found the disease duration as an independent predictor of levels of the antibodies studied. It is worth emphasizing that disease duration is one of independent risk factors of coronary artery calcification [7] and atherosclerotic plaque in extracranial carotid arteries [33]. The correlation, which we observed, might be another argument to support the concept of involvement of anti-N-Hcy-albumin antibodies in the pathogenesis of atherosclerosis.

Interestingly, we found no association between anti-N-Hcy-albumin antibodies and antinuclear antibody titers nor types, anti-double stranded DNA antibodies also did not correlate with these antibodies against homocysteinylated proteins. However, the presence of SLE was an independent predictor of anti-N-Hcy-albumin antibody levels in regression analyses. This observation might be explained by lack of direct clinical association between anti-nuclear antibodies titers and SLE activity or progression. We also did not observe the association between antiphospholipid or anti- $\beta_2$ -glycoprotein I antibodies and anti-N-Hcy-albumin antibodies. We should also remember that patients with an acute or past venous or arterial thrombosis were excluded from our study because these conditions are associated with hyperhomocysteinaemia. According to this assumption we found only 3 (6%) cases of antiphospholipid syndrome, diagnosed according to the current clinical and laboratory criteria, while in other 3 patients we determined elevated levels of antiphospholipid or anti- $\beta_2$ -glycoprotein I IgG antibodies and this class of antibodies seems to be associated with anti-N-Hcy-albumin antibodies (unpublished data). However, we observed a significantly elevated level of anti-N-Hcy-albumin antibodies in LA positive patients.

In conclusion SLE patients in a stable phase of disease have significantly higher anti-N-Hcy-albumin IgG antibodies levels than healthy individuals. Total Hcy concentration, CRP level and disease duration are independent predictors of levels of these antibodies. These preliminary observations expand the current knowledge about autoantibodies which are produced in SLE and may affect the clinical presentation of this disease. It is not known how treatment with folic acid, which reduces tHcy levels, may influence the disease course. Further studies on role of autoimmunologic reactions associated with hyperhomocysteinaemia in SLE are necessary.

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