

Hyperhomocyst(e)inemia and *Chlamydia pneumoniae* IgG seropositivity in patients with coronary artery disease

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Abstract

Elevated levels of homocyst(e)ine and infection by *Chlamydia pneumoniae* have been hypothesized individually to play a role in coronary artery disease (CAD), but the mechanisms are unclear. Data on a possible association are not available. We investigated the correlation between IgG antibody titers against *C. pneumoniae* and fasting plasma homocyst(e)ine in 234 consecutive male patients with CAD. Chlamydial antibodies to a recombinant genus-specific lipopolysaccharide (LPS) were measured with ELISA. Total homocyst(e)ine (tHcy) concentrations were measured by high-performance liquid chromatography (HPLC). Thirty-seven subjects were classified hyperhomocyst(e)inemic (fasting homocyst(e)ine > 14 $\mu\text{mol/l}$, group A), and 197 subjects were below cut-off (tHcy < 14 $\mu\text{mol/l}$, group B). Prevalence of IgG seropositivity against *C. pneumoniae* was significantly higher in group A (68%) as compared to group B (39%, $P = 0.002$). Antibody titers were also significantly higher in hyperhomocyst(e)inemic subjects than in cases with low homocyst(e)ine levels ($P = 0.002$). Overall titers correlated significantly with tHcy levels ($r^2 = 0.222$, $P = 0.001$). Hyperhomocyst(e)inemia was associated with arterial hypertension ($P = 0.003$), intake of lipid lowering drugs ($P = 0.022$) and quite not with low folate concentration ($P = 0.052$). No association was seen for IgG seropositivity or homocyst(e)ine and age, body mass index, smoking, diabetes, vitamin B₆ and B₁₂, cholesterol and triglycerides. These data indicate an association between elevated plasma homocyst(e)ine concentrations and chlamydial IgG antibody titers in patients with CAD. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Hyperhomocyst(e)inemia is recognized as an independent risk factor for coronary artery disease (CAD) [1,2]. Even mild elevation of plasma homocyst(e)ine is prospectively associated with myocardial infarction (MI) [3] and a higher mortality in patients with preexisting CAD [4]. Homocyst(e)ine was found in animal and in vitro studies to have toxic effects on endothelial cells (EC) [5], enhance monocyte chemotaxis [6] and

promote proliferation of vascular smooth-muscle cells (VSMC) [7]. However, most of these investigations used unphysiological, high homocyst(e)ine concentrations and therefore the atherogenic causality of mildly elevated homocysteine in man remains unclear.

Chlamydia pneumoniae (*C. pneumoniae*) is an obligate intracellular bacterium and a common cause of respiratory tract infections [8]. Several epidemiologic studies, but not all [9], have linked higher *C. pneumoniae* antibody titers with CAD [10,11] and acute (MI) [12], supported by histopathological evidence of *C. pneumoniae* in atherosclerotic plaque examination [13]. Successful cultivations of *C. pneumoniae* from plaques suggest the endovascular presence of viable bacteria [14]. Fur-

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thermore it was recently demonstrated by Davidson et al. [15], that IgG titers, but not IgA or IgM, correlate with the ability to directly detect *C. pneumoniae* within human coronary arteries obtained at autopsy. *C. pneumoniae* has been shown in vitro to replicate in EC, VSMS and monocytes [16], as well as to stimulate the secretion of cytokines [17]. At present, however, the evidence for an association of *C. pneumoniae* and CAD does not constitute causation.

Homocysteine and *C. pneumoniae* may potentially cause synergic endothelial cell injury and initiate an immune-mediated inflammatory response, compatible with the response-to-injury model of atherosclerosis [18], but to date, no study has controlled for an association between homocyst(e)ine and *C. pneumoniae*.

We therefore conducted a study involving patients with CAD to investigate a hypothesized relationship between homocyst(e)ine and serological evidence of *C. pneumoniae* infection.

2. Subjects and methods

We enrolled 234 male patients with angiographically documented CAD in this study. CAD was defined as $\geq 50\%$ luminal obstruction of at least one of the major epicardial coronary arteries as assessed by two independent cardiologists. All were under 60 years of age (mean 52.6 ± 5.9 years, range 32.4–60.4) at the time of investigation. Written informed consent was obtained from each subject and the study was approved by the Ethics Board of the Karl-Franzens University. All patients were residents of the Country of Styria.

Exclusion criteria were: intake of vitamins, antidepressants, antibiotics and medication known to interfere with folate, vitamin B₁₂ or B₆ metabolism. Other exclusion criteria were: liver dysfunction (abnormal aminotransferase concentrations), impaired kidney function (serum creatinine $> 110 \mu\text{mol/l}$), myocardial infarction or invasive procedures (surgery, coronary angioplasty, stenting) within the past 6 months, a history of respiratory and thyroid disease, psychiatric illness, neoplasia and clinical signs of acute or chronic infection. Information was obtained on smoking habits, diabetes, history of CAD, weight, height and medication use. Blood pressure was recorded (after 15 min of supine rest). Hypertension was defined as diastolic blood pressure > 90 mmHg and/or systolic blood pressure > 140 mmHg and/or use of antihypertensive drugs having been prescribed for the subject on the basis of a previous diagnosis of hypertension.

2.1. Measurements

Blood samples were taken from an antecubital vein between 07:00 and 08:00 h after an overnight fasting

period of at least 8 h. Samples were processed immediately, centrifuged at 4 °C ($3000 \times g$ for 10 min) within 15 min and stored at -70 °C until analysis. All serological analyses were carried out without prior knowledge of clinical data. Measurements of plasma homocyst(e)ine in EDTA plasma were performed using high-performance liquid chromatography and fluorescence detection according to the method of Araki [19] with modifications by Ubbink [20] and Vester [21]. Briefly, after reduction with tri-*N*-butylphosphine the free thiol groups were derivatized with SBD-F (7-fluorobenzofurazane-4-sulfonic acid). Separation was performed under isocratic conditions on a reversed phase column at pH 2.1 with mercaptopropionylglycine as internal standard. The intraassay variability of the method was between 1.3 (27 $\mu\text{mol/l}$) and 4.5% (10 $\mu\text{mol/l}$).

Because this procedure involved a reducing step, the method did not distinguish between homocysteine and its oxidized analogues. Therefore the measured moiety is referred to as homocyst(e)ine.

Tests for IgG antibodies to chlamydial lipopolysaccharide (LPS) were done with a commercially available, recombinant ELISA kit (MEDAC GmbH, Hamburg, Germany). This ELISA includes a chemically pure structure of a recombinant LPS which contains a genus-specific epitope of the human pathogens *Chlamydia* spp. [22]. The IgG cut-off values were calculated as prescribed by the manufacturer. Concentrations of chlamydial antibodies were expressed as index (optical density of the sample/cut-off).

Vitamin B₆ (serum) was analysed by an online derivatization method according to the procedure of Kurioka [23] using a stable bond column instead of the graphitic carbon column and a slight modification of the mobile phase.

Folate and vitamin B₁₂ (plasma) concentrations were determined by commercially available 'ion capture assay' and 'microparticle enzyme immunoassay', respectively (Abbott Diagnostics, Wiesbaden, Germany). Standard methods were used for serum lipids.

2.2. Data and statistical analysis

Statistical analysis was performed using the version 10.0 SPSS software package (SPSS, Chicago). Analysis of 2×2 contingency tables were performed with Fisher's exact test for small samples. Larger tables were analysed by Pearson's χ^2 test. Because serum parameter values did not show normal distribution, log transformation of raw data was performed which resulted in fairly normal distribution for all variables. Student's *t*-test was thus used to test for differences between groups. Influence of smoking status on *C. pneumoniae* antibody levels was tested by ANOVA after log transformation of antibody titers. All tests were two-sided

and P -values smaller than 0.05 were considered statistically significant. Post hoc estimation reveals a power of 89% for the χ^2 test (seroprevalence data) and a power of 95% for the Student t -test (antibody titers) to detect the relation between homocyst(e)ine levels and *C. pneumoniae* antibodies.

3. Results

Between April 1997 and May 2000, 234 male CAD patients under 60 years of age met the inclusion criteria and were enrolled in the study. Overall mean plasma homocyst(e)ine was 10.8 ± 3.9 (range 4.59–22.99). Based on data of the multi-center European Concerted Action Project [24], a cut-off value of $14 \mu\text{mol/l}$ was used to define groups of hyperhomocyst(e)inemic patients (group A, $n = 37$) and patients with normal homocyst(e)ine levels (group B, $n = 197$). Mean fasting homocyst(e)ine concentrations were 17.3 ± 3.1 (range 14.1–29.0) $\mu\text{mol/l}$ and 9.2 ± 2.2 (range 4.6–13.9) $\mu\text{mol/l}$ in groups A and B, respectively.

Clinical characteristics and risk factors for both groups are presented in Table 1. Age and body mass index were virtually identical between groups and clinical characteristics were similar except for arterial hypertension ($P = 0.003$). Overall prevalence of Chlamydia IgG antibodies was 44%. Importantly, 68% of subjects with homocyst(e)ine levels $> 14 \mu\text{mol/l}$ were IgG seropositive as compared to 39% of subjects with homocyst(e)ine levels $< 14 \mu\text{mol/l}$ ($P = 0.002$). Table 2

displays risk factors and medication according to IgG serostatus. Anti chlamydial IgG seropositivity was strongly associated only with hyperhomocyst(e)inemia ($P = 0.007$). We found no evidence of an additional association between IgG antibody titers and other risk factors as listed in Table 2. Importantly, smoking status had no effect on *C. pneumoniae* antibody titers and seroprevalence.

Further quantitative analysis revealed that apart from seropositivity, chlamydial IgG antibody titers were also significantly higher in group A (geometric mean 1.50) as compared to group B (geometric mean 0.82, $P = 0.002$). Importantly, overall homocyst(e)ine concentrations correlated with chlamydial IgG antibody titers ($r^2 = 0.222$, $P = 0.001$). In the present study a power of 95% is achieved at a significance level of 0.05 to exclude a type 2 error. The cumulative distribution showed a marked right-shift for the hyperhomocyst(e)inemic group (Fig. 1).

4. Discussion

The major finding of our study was a significant association between elevated plasma homocyst(e)ine concentrations and *C. pneumoniae* IgG seropositivity in patients with CAD. Furthermore, homocyst(e)ine concentrations corresponded significantly with chlamydial IgG antibody titers.

Earlier studies reported increasing prevalences of *C. pneumoniae* seropositivity with lower socioeconomic

Table 1
Clinical characteristics and risk factors in patients with elevated (group A) and normal (group B) total plasma homocyst(e)ine concentrations ($n = 234$)

Parameter	Group A ($n = 37$)	Group B ($n = 197$)	P
Age, years (mean \pm S.D.)	52.5 ± 6.8	52.6 ± 5.8	0.895
Body mass index, kg/m^2 (mean \pm S.D.)	27.3 ± 3.7	27.0 ± 3.0	0.619
Smoking			0.621
Never	27%	35%	
Former	60%	54%	
Current	13%	11%	
Arterial hypertension	68%	40%	0.003
Diabetes mellitus	19%	10%	0.157
Medication			
Aspirin	73%	75%	0.839
β -Blocker	73%	83%	0.176
Lipid lowering	60%	78%	0.022
Nitrates	32%	35%	0.852
Plasma folate, ng/ml	6.5 (5.5;7.6)	7.7 (7.3;8.1)	0.052
Anti- <i>Chlamydia</i> IgG seropositivity	68%	39%	0.002
IgG antibody titer	1.50 (1.03;2.19)	0.82 (0.71;0.95)	0.002
Plasma vitamin B_{12} , ng/l	350 (297;413)	381 (359;405)	0.278
Serum vitamin B_6 , ng/l	10.1 (7.5;13.5)	10.9 (9.6;12.4)	0.597
Plasma cholesterol, mmol/l	5.17 (4.75;5.62)	5.18 (5.00;5.37)	0.957
Plasma triglycerides, mmol/l	1.93 (1.60;2.34)	1.97 (1.81;2.13)	0.858

Data are expressed as geometric means and 95% confidence intervals.

Table 2
Clinical characteristics and risk factors in seronegative and seropositive (anti-LPS IgG antibodies) patients with CAD ($n = 234$)

Parameter	Seropositive ($n = 102$)	Seronegative ($n = 132$)	<i>P</i>
Age, years (mean \pm S.D.)	52.4 \pm 5.9	52.8 \pm 6.0	0.612
Body mass index, kg/m ² (mean \pm SD)	26.8 \pm 3.1	27.3 \pm 3.2	0.292
Smoking			0.813
Never	32%	35%	
Former	57%	53%	
Current	11%	12%	
Arterial hypertension	49%	41%	0.146
Diabetes mellitus	12%	11%	1.000
Medication			
Aspirin	78%	71%	0.127
β -Blocker	78%	83%	0.214
Lipid lowering	75%	76%	0.485
Nitrates	36%	34%	0.386
Plasma homocyst(e)ine, μ mol/l	10.6 (9.8;11.3)	9.4 (9.0;9.9)	0.007
Plasma folate, ng/ml	7.2 (6.6;7.9)	7.7 (7.2;8.2)	0.374
Plasma vitamin B ₁₂ , ng/l	396 (365;431)	360 (333;389)	0.156
Serum vitamin B ₆ , ng/l	11.3 (9.5;13.6)	10.2 (8.6;12.0)	0.243
Plasma cholesterol, mmol/l	5.02 (4.78;5.27)	5.32 (5.10;5.55)	0.075
Plasma triglycerides, mmol/l	1.81 (1.61;2.03)	2.08 (1.89;2.30)	0.141

CAD, coronary artery disease. Data are expressed as geometric means and 95% confidence intervals.

status [25] and increasing age [26]. Furthermore, seroprevalence is considerably higher in men than in women [26] and age-adjusted homocyst(e)ine levels differ significantly between sexes and even menopausal status [27]. In our study all subjects were residents of the Province of Styria and middle-class males with an upper age limit of 60 years. We found no association between smoking status and *C. pneumoniae* IgG seropositivity, in agreement with several cross-sectional studies [12,29]. Other authors have shown that even after controlling for cigarette smoking (and other traditional risk factors), *C. pneumoniae* remains an independent risk factor for CAD [30]. In contrast, Hahn et al. reported smoking to be a potential confounder in *C. pneumoniae* seroprevalence [31]. It is important to note that the population investigated by Hahn had consisted exclusively of outpatients with respiratory illnesses regardless of CAD status. The differing results may be explained by the different study populations.

The overall prevalence of IgG antibodies in our cohort of CAD patients was 44%, which is consistent with earlier reports [22,32,33]. It must be considered, however, that because of the relatively short half-time and different kinetics of LPS antibodies, the LPS-ELISA test usually generates lower prevalence rates than the microimmuno-fluorescence (MIF) technique [34]. Though the MIF test is considered to be the gold standard for determination of *Chlamydia* serostatus, it is known to be subjective, difficult to interpret and requires considerable experience on the part of the investigator [35]. We used a commercially available LPS-ELISA which is a standardized and objective method [22]. Furthermore, we have shown previously

that this ELISA has an excellent concordance with the MIF in classifying CAD patients as seropositive or seronegative [32]. This LPS ELISA is not able to differentiate between antibodies against various *Chlamydia* species, as the LPS used as antigen is also part of *C. trachomatis* and *C. psittaci*. However, it is very unlikely that these species contributed substantially to seropositivity in our study. Infections with *C. trachomatis* are rare in the age group of our patients, who furthermore do not belong to a high-risk population for *C. trachomatis*. In recent reports on the serological data of patients with CAD, IgG seroprevalence for *C. trachomatis* was less than 1%, and no cases of *C. psittaci* were identified [36].

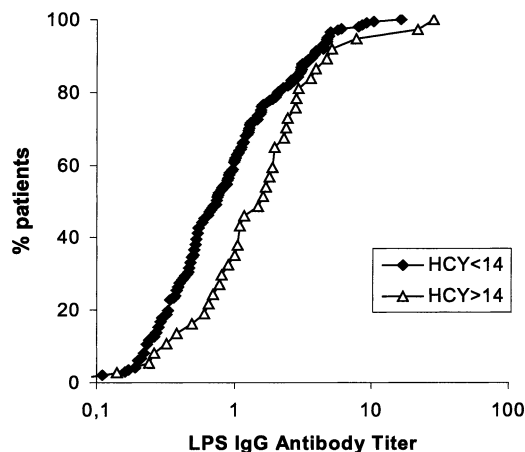


Fig. 1. Cumulative distribution of LPS IgG antibody titers in patients with CAD ($n = 234$), according to homocyst(e)ine levels (μ mol/l). Antibody titers are log transformed.

Folate status is a strong factor determining homocyst(e)ine levels [28], as we also found. Actually, folate supplementation effectively lowers elevated homocyst(e)ine levels [2]. Homocyst(e)ine may therefore be seen as a sensitive marker for the folate status. Low folate status will hence lead to impaired methionine metabolism, hypomethylation and impaired cell membrane function, which could make EC more susceptible to infection by *C. pneumoniae*.

Folate metabolism is also closely linked with one-carbon metabolism and DNA biosynthesis, which is crucial to cell growth, reproduction and differentiation. Consequently, cell-mediated immunity and phagocytic activity [37] in particular are affected by folate deficiency with decreased resistance to infections [38], which may also be relevant for infection by *C. pneumoniae*.

However, overall information about tissue folate concentrations and infectiousness of intracellular pathogens is sparse. A significantly higher infection rate in folate depleted cervix epithelium was shown for the human papilloma virus (HPV)-16 [39]. Chlamydiae, though bacteria, were also thought to have an absolute nutritional dependency on the host cell. Interestingly, Fan et al. [40] demonstrated in vitro that three Chlamydia strains, *C. trachomatis*, *C. psittaci* and *C. psittaci francis*, were all capable of (a) successful replication in folate-depleted host cells and of (b) de-novo folate synthesis if required. *C. pneumoniae* was not investigated, but it may be speculated that all species share this metabolic pathway. Certainly more studies are required to further investigate susceptibility of folate-deficient EC and VSMC to infection by intracellular pathogens, such as *C. pneumoniae*.

4.1. Limitations

Serological studies have principal limitations: a positive test is only indicative of prior infection and provides limited information on the time of infection, chronic or repeated infection. Additionally, after primary infection, antibody titers to *C. pneumoniae* may fall and become undetectably low despite persistence of the bacterium. Moreover, *C. pneumoniae* in the wall of blood vessels may only be a fraction of the total *C. pneumoniae* body load.

All subjects in our study were previously diagnosed with CAD through angiography.

Thus, most of them were being treated for their disease. Subjects on medication with known influence on homocyst(e)ine levels were excluded. We found no influence between aspirin, β -blocker intake and homocyst(e)ine levels in our study, but the population size may have been too small. No other data have been published, so we cannot completely exclude a possible interference.

Cholesterol was reported to be associated with chronic *C. pneumoniae* infection as determined by IgG antibody titers [41]. In our study, 60% resp. 78% of the subjects in both groups were using lipid lowering agents (statins), including only 3 subjects using fibrates (1 in group A and 2 in group B). An increase of homocyst(e)ine by intake of fibrates was recently reported [42], but no such association was demonstrated for statins. Due to the small number and even distribution we consider the influence of fibrates in our study as negligible. However, the intake of lipid-lowering drugs (statins) does not allow us a conclusion on the reported relationship between lipid profiles and *C. pneumoniae* serology, nor can we completely exclude an influence of statins on homocyst(e)ine levels.

In conclusion, this study provides first evidence for an association between elevated plasma homocyst(e)ine concentrations and IgG antibody titers against *C. pneumoniae* in patients with CAD. At present, accurate determination of causality requires large numbers of observations, and our findings should be regarded as preliminary. However, if confirmed they have important implications for future investigations and a better understanding in the etiology of atherosclerosis.

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