

# Total homocysteine in patients with angiographic coronary artery disease correlates with inflammation markers

Katharina Schroecksnadel<sup>1</sup>; Tanja B. Grammer<sup>2</sup>; Bernhard O. Boehm<sup>3</sup>; Winfried März<sup>2,4,5</sup>; Dietmar Fuchs<sup>1</sup>

<sup>1</sup>Division of Biological Chemistry, Biocenter, Innsbruck Medical University, Innsbruck, Austria; <sup>2</sup>Synlab Center of Laboratory Diagnostics, Heidelberg, Germany; <sup>3</sup>Division of Endocrinology, Department of Medicine, University Hospital Ulm, Ulm, Germany; <sup>4</sup>Clinical Department of Medical and Chemical Laboratory Diagnostics, Graz Medical University, Graz, Austria; <sup>5</sup>Mannheim Institute of Public Health, Chemical Faculty Mannheim, Ruprecht Karls University of Heidelberg, Heidelberg, Germany

## Summary

Moderate hyperhomocysteinaemia is considered as an independent risk marker for cardiovascular disease and stroke. Earlier, increased homocysteine production was detected in stimulated immunocompetent cells in vitro, and several markers of inflammation like neopterin or C-reactive protein (CRP) were demonstrated as significant indicators of cardiovascular risk. The relationship between coronary artery disease (CAD), homocysteine metabolism and markers of immune activation and inflammation was investigated in a population of 1717 patients undergoing coronary angiography, recruited as participants of the Ludwigshafen Risk and Cardiovascular Health (LURIC) study. 1325 patients (77.2%) suffered from coronary artery disease (CAD), which was defined as the occurrence of a visible luminal narrowing ( $\geq 20\%$  stenosis) in at least 1 of 15 coronary segments according to the classification of the American Heart Association, the remaining 392 individuals of the study population served as controls. Significant differ-

ences regarding systolic blood pressure, homocysteine, neopterin and folic acid concentrations were observed between patients and controls. Older age, decreased creatinine-clearance and higher concentrations of homocysteine and CRP were indicative for CAD. Low B-vitamin availability, therapy and the extent of immune activation strongly influenced homocysteine concentrations. Homocysteine concentrations were correlated with neopterin levels ( $r_s = 0.325$ ,  $p < 0.001$ ), and hyperhomocysteinaemic patients also presented with significantly higher CRP concentrations. Homocysteine accumulation coincided with impaired renal and heart function (as reflected by ProBNP[Brain natriuretic peptide]-concentrations). We conclude that homocysteine accumulation could result from B-vitamin deficiency which is related to chronic immune activation.

## Keywords

Homocysteine, coronary artery disease, inflammation, neopterin, CRP

## Correspondence to:

Dietmar Fuchs  
Division of Biological Chemistry, Biocenter  
Innsbruck Medical University  
Fritz Pregl Strasse 3  
A-6020 Innsbruck, Austria  
Tel.: +43 512 9003 70350, Fax: +43 512 9003 73330  
E-mail: dietmar.fuchs@i-med.ac.at

Received: July 2, 2009

Accepted after major revision: December 30, 2009

Prepublished online: March 9, 2010

doi:10.1160/TH09-07-0422

Thromb Haemost 2010; 103: 926–935

## Introduction

Elevated total homocysteine concentrations in the blood are considered as an independent risk marker for cardiovascular disease and stroke (1–3). Homocysteine accumulation is supposed to trigger endothelial dysfunction and increase oxidative stress (4, 5) and thus is often regarded to be a primary event in atherogenesis. Also inflammation and immune activation are well established to be crucially involved in the pathogenesis of cardiovascular disease (6, 7). Activated macrophages play a key role in plaque formation and the development of oxidative stress, and increased numbers of macrophages are observed in plaques of patients with acute coronary syndromes (8). They release pro-inflammatory cytokines (e.g. interleukins or tumour necrosis factor- $\alpha$  (TNF- $\alpha$ )) and generate reactive oxygen species (ROS) (9). Additionally, activated macrophages are able to release proteases and to form neopterin (9), which serves as a sensitive marker of cell-mediated immune activation (10).

In patients with unstable angina and acute myocardial infarction, higher neopterin concentrations have been observed as compared with control subjects and patients with stable angina pectoris (11). Interestingly, neopterin concentrations did not differ between patients with chronic stable angina and unstable angina/myocardial infarction at baseline, but within 72 hours after the onset of symptoms in another study (12). Also in a recent study neopterin has been associated with plaque destabilisation, where abundant neopterin-positive macrophages were found at the sites of coronary culprit lesions in patients with unstable angina (13).

Very recently, neopterin has been demonstrated to be a good marker to identify patients at long-term risk of death or recurrent acute coronary events after acute coronary syndrome (ACS) (14). Furthermore, serum neopterin concentrations were also identified as an independent predictor of major adverse coronary events in patients with chronic stable angina pectoris (15).

Neopterin as well as the combination of neopterin and C-reactive protein (CRP) have been shown to be predictive of adverse

outcome in patients with both stable coronary artery disease (CAD) and ACS, as well as non-Q-wave myocardial infarction, respectively (16, 17). Similarly, both inflammation markers have been shown to be associated with coronary artery disease progression in patients with stable angina pectoris (18). C-reactive protein has been shown to predict the outcome of patients with acute coronary syndrome and has been suggested as useful marker for cardiovascular risk (19, 20). Recently, neopterin was demonstrated as probably the strongest predictor of total and cardiovascular mortality in a large cohort of individuals undergoing angiography, the so-called Ludwigshafen Risk and Cardiovascular Health (LURIC) cohort (21).

Associations between immune activation and moderate hyperhomocysteinaemia have been demonstrated in smaller studies of patients suffering from rheumatoid arthritis, Alzheimer's disease and also coronary heart disease (22–24). These clinical data as well as results of *in vitro*-studies (25), in which immunocompetent cells were shown to release homocysteine in parallel with neopterin, have indicated that hyperhomocysteinaemia may develop secondarily – as a consequence of immune activation. In this study, the relationship between CAD, homocysteine metabolism and immune activation by means of soluble and cellular markers was investigated in a large population of patients undergoing coronary angiography.

## Methods

### Study design

We studied 1717 Caucasian German patients (1181 men, 536 women) who were recruited as participants of the LURIC study and were hospitalised for coronary angiography between June 1997 and January 2000 (26). The study was approved by the ethics committee at the “Ärztchamber Rheinland-Pfalz”. Informed written consent was obtained from all participants.

Inclusion criteria were: German ancestry, clinical stability (except for acute coronary syndromes), and the availability of a coronary angiogram. The indications for angiography in individuals in a clinically stable condition were chest pain and/or non-invasive test results consistent with myocardial ischaemia. Individuals who were suffering from acute illness other than ACS or who had chronic non-cardiac diseases (e.g. chronic renal failure) or malignancy within the five past years and those unable to understand the purpose of the study were excluded. Patients with acute myocardial infarction were generally not enrolled within the first 24 hours after the onset of the myocardial infarction, but within a few days post-infarction, after they had been transferred from the intensive care unit to the general ward and they presented in a stable clinical setting.

CAD was assessed by angiography, with maximum luminal narrowing estimated by visual analysis. CAD was defined by angiographic criteria, angiograms were analysed by five experienced angiographers. As described elsewhere, the three major coronary ar-

teries were divided into 15 coronary arterial segments (27). Presence of a visible luminal narrowing ( $\geq 20\%$  stenosis) in at least one of 15 coronary segments was defined as CAD-positive, however, as stenoses  $>50\%$  are regarded as clinically relevant, analyses were also conducted for this subgroup and are presented separately.

Only data of patients, for whom the concentrations of neopterin, homocysteine and B-vitamins were available, were included in the analysis.

### Laboratory procedures

Blood sampling was done in fasting subjects before cardiac catheterisation. A total of 115 ml of fasting venous blood was sampled for the determination of a pre-specified wide range of laboratory parameters in serum, plasma or whole blood, as described elsewhere (26). Total homocysteine was determined by HPLC (Waters millennium chromatography with fluorescence detector 470), creatinine by the Jaffé method (Crea/Hitachi 717, Roche), folic acid by ion capture immunoassay (Folic acid/Abbott AXYM autosampler), vitamin B12 by microparticle enzyme immunoassay (vitamin B12, Abbott AXYM autosampler), TNF- $\alpha$  by chemiluminescent assay (Immulate TNF- $\alpha$ /DPC, Immulate autosampler, ), interleukin-12 (IL-12) by ELISA (Biosource Europe SA), percentage of activated T-cells and monocytes by fluorescence-activated cell-sorting (FACS) technique (26). “Sensitive” CRP was measured by immunonephelometry (N Latex CRP mono, Dade Behring) (26). Neopterin concentrations were determined by radioimmunoassay (BRAHMS Diagnostica, Henningsdorf, Germany), ProBNP (Brain natriuretic peptide) was measured by electrochemiluminescence on an Elecsys 2010 (Roche Diagnostics). The creatinine-clearance was calculated separately for men [ $\text{crcl} = (140 - \text{age}) \cdot \text{weight} / (72 \cdot \text{crea})$ ] and women [ $\text{crcl} = 0.85 \cdot (140 - \text{age}) \cdot \text{weight} / (72 \cdot \text{crea})$ ].

To account for renal function, the homocysteine-to-creatinine and neopterin-to-creatinine ratios as well as the homocysteine-to-creatinine-clearance and neopterin-to-creatinine-clearance ratios were calculated.

### Statistical analysis

SPSS 11.0 was used for the statistical analysis of data. Normal distribution of the variables was checked with the Kolmogorov-Smirnov test with Lilliefors significance correction. As data did not show normal distribution, Spearman rank correlation analysis and non-parametric tests were used (Kruskal-Wallis test for the comparison of more than two independent groups, Mann-Whitney U-test for the comparison of two independent groups). A  $p$ -value  $< 0.05$  was considered to indicate statistical significance.

For the comparison of patients with and without various medications T-tests for independent variables were used.

Binary logistic regression analysis (univariate method) was used to figure out determinants of the presence of coronary artery disease, multivariate binary logistic regression analysis was performed with all parameters, that were significant in univariate logistic regression analysis (stepwise forward-LR).

## Results

### Characteristics of the study cohort

A total of 1,182 patients underwent elective angiography, additionally 535 patients were recruited with acute coronary syndrome. Of those, 330 had unstable angina, 62 had non-Q-wave infarction and 143 had myocardial infarction.

Among the 1,717 individuals studied, 392 were defined as CAD-negative (22.8%) and 1,325 as CAD-positive (77.2%). A total of 1,137 patients had clinically relevant stenoses (i.e. stenoses >50%), 345 patients had one vessel disease (1-VD), 330 two vessel- (2-VD) and 462 three vessel-disease (3-VD).

Most of the patients were under treatment: 22 patients were taking antibiotics, 902 ACE-inhibitors, 141 oral antidiabetic agents, 84 AT-II receptor antagonists, 1222 individuals took aspirin or other antiplatelet medication, 1,086  $\beta$ -adrenergic receptor blockers, calcium antagonists were taken by 264 patients and glucocorticoids by 28 patients, respectively. One hundred fourteen patients were treated with vitamin K antagonists, 820 with statins (CSE-inhibitors), 260 with digitalis, 468 with diuretics, 420 were heparinised, 76 women had hormonal replacement therapy, six women took oral contraceptives, and 785 were treated with miscellaneous not encoded medication, 38 patients were vitamin-supplemented.

► Table 1 shows the median values of age, body mass index (BMI), systolic and diastolic blood pressure as well as concentrations of homocysteine, B-vitamins folic acid and vitamin B12, neopterin, CRP and proBNP concentrations in all subjects, and separately for men and women. Differences between men and women concerning parameters investigated are indicated by p-values.

### Differences between CAD-cases and non-cases

► Table 2 shows medians of epidemiologic and lab parameters that were different in patients with CAD (>20% stenosis) and controls. Age, systolic blood pressure, homocysteine, neopterin, CRP and folic acid concentrations differed significantly between patients and controls. No differences between CAD-positive and -negative cases were seen regarding BMI, diastolic blood pressure and vitamin B12 concentrations.

Patients with clinically relevant stenoses (stenoses >50%; number of patients n=1,137) also were older and had higher systolic blood pressure, higher homocysteine, neopterin and CRP values and lower folic acid concentrations (all p<0.001) compared with individuals without clinically relevant stenoses – results were very similar to those obtained in patients with stenoses >20%.

► Table 3 shows the power of the investigated variables to predict the presence of CAD in univariate logistic regression analysis (in the whole population): Cases were older and more likely to be male. Thus, the ability of variables to predict the presence of CAD in univariate logistic regression analysis was also calculated for men and women separately: Age and creatinine-clearance, smoking status and higher homocysteine and CRP levels were predictive

	All	Men	Women	P-value
Age (years)	63.1 (56.2–70.3)	62.1 (55.5–69.4)	64.7 (58.0–71.9)	<0.001
Body mass index (kg/m <sup>2</sup> )	27.1 (24.7–29.6)	27.4 (25.2–29.7)	26.4 (23.7–29.4)	<0.001
Systolic blood pressure (mmHg)	139 (123–155)	138 (122–155)	141 (123–156)	n.s.
Diastolic blood pressure (mmHg)	81 (73–88)	81 (73–89)	80 (72–88)	n.s.
Homocysteine ( $\mu$ M)	12.6 (10.2–15.7)	12.9 (10.5–15.8)	11.9 (9.5–15.5)	<0.001
Folic acid ( $\mu$ g/l)	8.00 (6.1–10.4)	7.90 (5.9–10.3)	8.40 (6.5–10.8)	<0.001
Vitamin B12 (ng/l)	345 (258–472)	341 (259–461)	354 (255–502)	n.s.
Neopterin (nM)	6.84 (5.60–8.42)	6.80 (5.58–8.37)	6.94 (5.65–8.68)	<0.001
C-reactive protein (mg/l)	3.9 (0.8–10.0)	3.7 (0.80–10.00)	4.3 (1.0–10.3)	n.s.
Creatinine (mg/dl)	0.9 (0.8–1.0)	1.0 (0.9–1.1)	0.8 (0.7–0.9)	<0.001
Creatinine clearance	87.4 (70.0–107.0)	93.5 (76.0–111.9)	74.2 (60.6–92.2)	<0.001
N-terminal Pro-BNP (ng/ml)	294 (103–840)	289 (97–835)	313 (132–865)	<0.05
Homocysteine/creatinine [ $\mu$ M/(mg/dl)]	13.5 (11.1–16.5)	13.1 (10.9–15.8)	14.6 (11.7–17.9)	<0.001
Neopterin/creatinine [nM/mg/dl]	7.5 (6.0–9.3)	7.0 (5.7–8.6)	8.6 (6.8–10.7)	<0.001
Homocysteine/creatinine-clearance	0.15 (0.1–0.21)	0.14 (0.1–0.20)	0.16 (0.11–0.23)	<0.001
Neopterin/creatinine-clearance	0.07 (0.05–0.11)	0.07 (0.05–0.11)	0.09 (0.06–0.14)	<0.001

**Table 1: Medians of epidemiologic and laboratory parameters determined in a population of 1,717 patients undergoing coronary angiography.** Median concentrations are shown for the whole population (all) as well as for men and women separately. P-values <0.05 indicate significant differences regarding parameters between men and women (Mann Whitney U-test; n.s. = not significant).

**Table 2: Medians of epidemiologic and laboratory parameters determined in a population of 1,717 patients undergoing coronary angiography.** Patients without coronary artery disease (no CAD) were compared with patients suffering from coronary artery disease (CAD, i.e. stenoses >20%). Interquartile ranges are shown in brackets, p-values <0.05 indicate significant differences.

	No CAD	CAD	P-value
Age (years)	60.5 (51.9–67.1)	63.8 (57.2–70.8)	<0.001
Body mass index (kg/m <sup>2</sup> )	26.6 (24.4–29.3)	27.2 (24.8–29.7)	n.s.
Mean systolic blood pressure (mmHg)	135 (121–150)	141 (123–157)	<0.001
Mean diastolic blood pressure (mmHg)	80 (73–88)	81 (73–89)	n.s.
Homocysteine (μM)	11.7 (9.5–14.6)	12.9 (10.4–16.0)	<0.001
Folic acid (μg/l)	8.2 (6.5–10.8)	8.0 (6.0–10.3)	<0.05
Vitamin B12 (ng/l)	350 (259–476)	344 (258–470)	n.s.
Creatinine (mg/dl)	0.9 (0.8–1.0)	0.9 (0.8–1.1)	<0.001
Creatinine-clearance	92.1 (73.3–113.7)	86.4 (69.5–105.0)	=0.001
N-terminal-Pro Brain natriuretic peptide (ng/ml)	179 (73–599)	327 (116–922)	<0.001
C-reactive protein (mg/l)	2.8 (0.1–7.7)	4.2 (1.1–11.0)	<0.001
Neopterin (nM)	6.4 (5.4–8.0)	6.9 (5.7–8.6)	<0.001
Homocysteine/creatinine [μM/(mg/dl)]	13.1 (10.9–16.3)	13.6 (11.2–16.5)	n.s.
Neopterin/creatinine [nM/(mg/dl)]	7.4 (5.9–9.2)	7.4 (6.0–9.4)	n.s.
Homocysteine/creatinine-clearance	0.13 (0.09–0.18)	0.15 (0.11–0.21)	<0.001
Neopterin/creatinine-clearance	0.07 (0.05–0.1)	0.08 (0.06–0.12)	<0.001

for CAD in men and in women (all  $p < 0.05$ ), furthermore also neopterin concentrations as well as systolic blood pressure were predictive of CAD in men (both  $p < 0.05$ ).

When renal function was accounted for by calculating homocysteine/creatinine, this ratio was no longer predictive for the presence of CAD (neither in the whole population, nor in the separate analyses of men and women).

Concentrations of CRP, smoking status and age were suited best to predict the presence of angiographic stenosis >20% in men and women in multivariate logistic regression analysis (Men:  $z = -2.888 + 0.069 * \text{age} + 0.16 * \text{CRP} + 0.465 * \text{smoking status}$ ; Women:  $z = -1.970 + 0.035 * \text{age} + 0.199 * \text{CRP} + 0.454 * \text{smoking status}$ ).

## Determinants of homocysteine concentrations

The median homocysteine concentration in the whole population was 12.6 μM (interquartile range: 10.2–15.7 μM). Five hundred twenty-one of 1,717 patients presented with homocysteine concentrations above 15 μM, the earlier defined 95<sup>th</sup> percentile of normal (28).

Four hundred thirty (82.5%) of those hyperhomocysteinaemic patients had stenoses >20%, whereas 91 (17.5%) were defined as CAD-negative. Homocysteine concentrations were higher in patients with progressive CAD ( $r_s = 0.161$ ;  $p < 0.001$ ), the median homocysteine concentration in patients without clinically relevant stenoses was 11.7 μM, while the median homocysteine level was 12.6 μM in patients with 1-VD, 12.9 μM in patients with 2-VD and 13.5 μM in patients with 3-VD.

Correlation analysis showed that B-vitamin concentrations (folic acid,  $r_s = -0.405$ , and vitamin B12,  $r_s = -0.230$ ; both  $p < 0.001$ ), but also age ( $r_s = 0.264$ ;  $p < 0.001$ ), creatinine ( $r_s = 0.418$ ), the creatinine-clearance ( $r_s = -0.338$ ), urea ( $r_s = 0.264$ ), uric acid

( $r_s = 0.264$ ), neopterin ( $r_s = 0.325$ ) and somewhat weaker CRP ( $r_s = 0.125$ ; all  $p < 0.001$ ) were associated with homocysteine concentrations. Also proBNP was associated significantly with homocysteine ( $r_s = 0.230$ ; all  $p < 0.001$ ), while only a very weak correlation existed between left ventricular function (estimated by angiography) and homocysteine levels ( $r_s = -0.056$ ;  $p < 0.05$ ).

**Table 3: The power of epidemiologic and laboratory parameters (determined in a population of 1,717 patients undergoing coronary angiography) to predict the presence of coronary artery disease (CAD, presence of vessel stenosis >20%) is shown (n.s. = not significant).**

	Odds ratio [95% CI]
Age (years)	1.040 [1.029–1.052]
Body mass index (kg/m <sup>2</sup> )	n.s.
Smoking status	1.946 [1.552–2.440]
Mean systolic blood pressure (mmHg)	1.010 [1.005–1.010]
Mean diastolic blood pressure (mmHg)	n.s.
Homocysteine (μM)	1.046 [1.021–1.071]
Folic acid (μg/l)	0.961 [0.926–0.998]
Vitamin B12 (ng/l)	n.s.
Creatinine (mg/dl)	3.457 [1.926–6.204]
Creatinine-clearance	0.994 [0.990–0.998]
C-reactive protein (mg/l)	1.256 [1.119–1.411]
Neopterin (nM)	1.026 [1.000–1.052]
Homocysteine/creatinine [μM/(mg/dl)]	n.s.
Neopterin/creatinine [nM/(mg/dl)]	n.s.
Homocysteine/creatinine-clearance	3.797 [1.437–10.04]
Neopterin/creatinine-clearance	n.s.

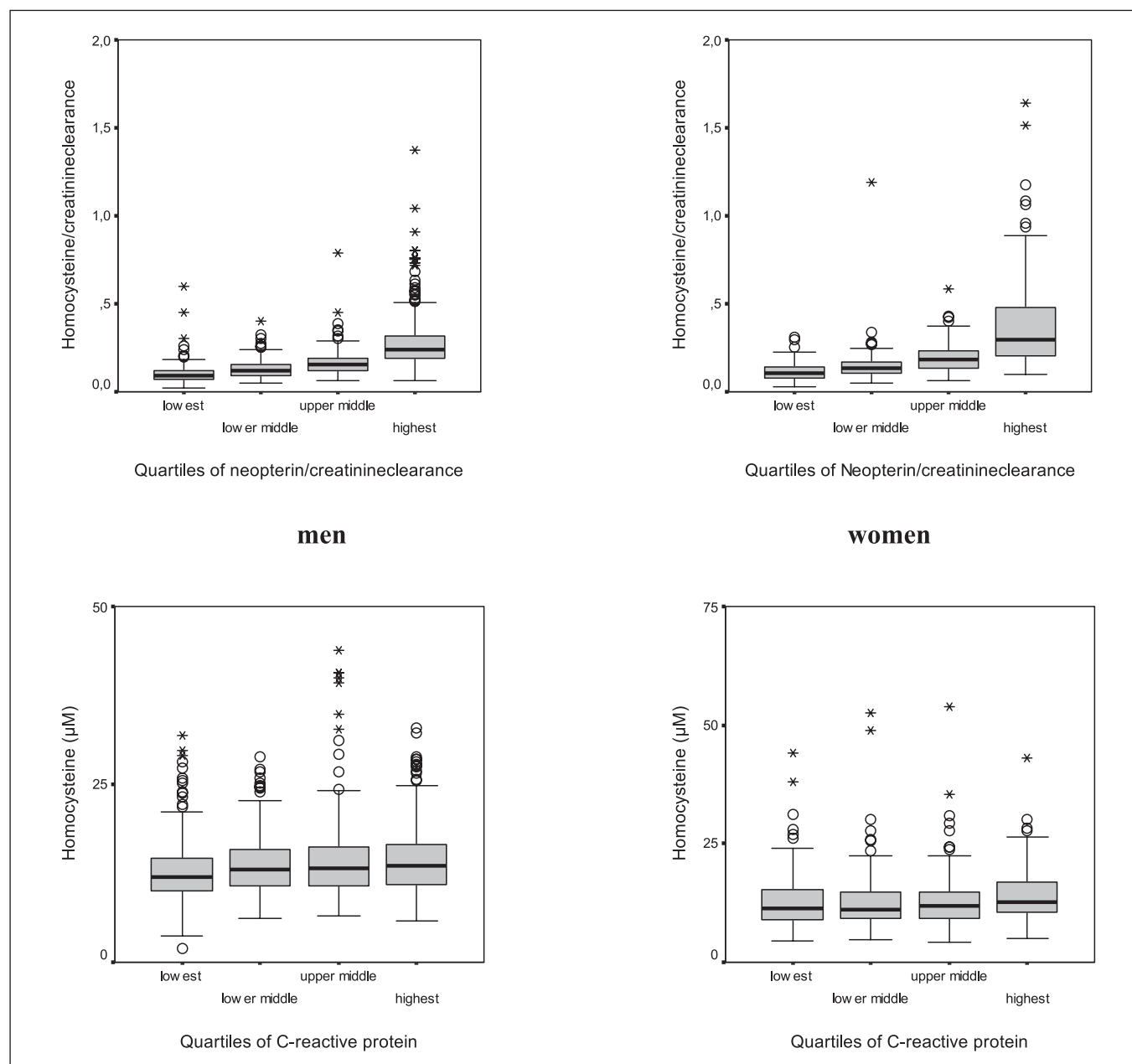
### Relationship between inflammation, immune activation, homocysteine accumulation and B-vitamins

Patients with moderate hyperhomocysteinaemia (i.e. homocysteine levels >15 µM) had higher concentrations of neopterin (median: 7.86 vs. 6.45 nM) and CRP (3.84 vs. 2.95 mg/l; both  $p < 0.001$ ) than normohomocysteinaemic patients.

Only 15 patients presented with low folate status according to the definition of the World Health Organization (WHO) (<7 nM

Folate), 12 of them were CAD-positive. Patients with low folate status presented with higher homocysteine concentrations (median 21.1 µM vs. 12.6 µM,  $p < 0.001$ ), and lower B12 concentrations (245 vs. 365 pg/ml  $p < 0.05$ ), concentrations of inflammation markers did not differ.

Significant associations existed between homocysteine concentrations and markers of inflammation and immune activation: Neopterin concentrations were associated strongest with homocysteine concentrations ( $r_s = 0.325$ ,  $p < 0.001$ ). The correlation remained significant after adjustment for renal function by calculat-



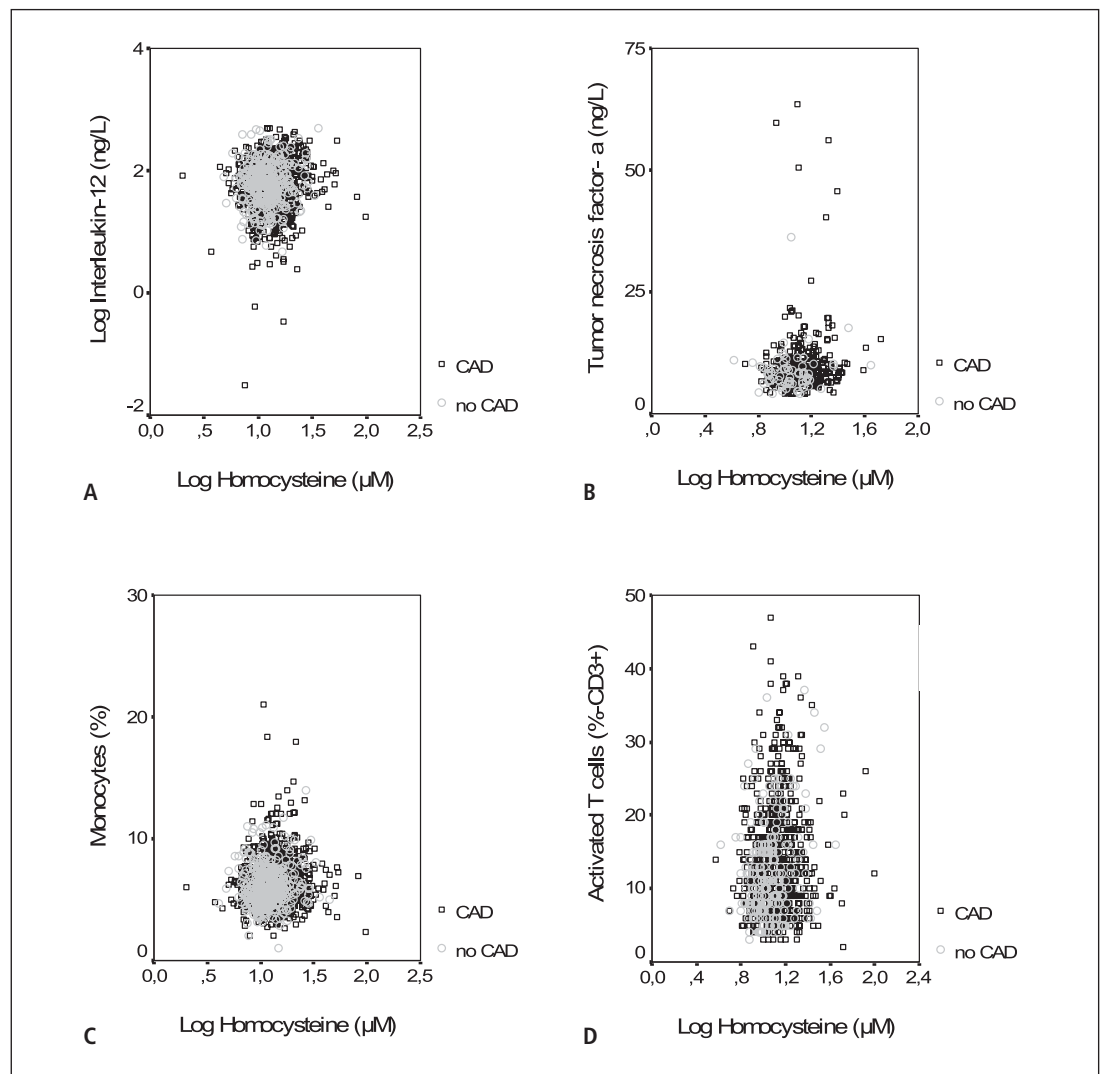
**Figure 1: Homocysteine/creatinine clearance as well as homocysteine concentrations are increasing in patients with increasing markers of immune activation and inflammation.** Patients were divided into quartiles of neopterin/creatinine clearance (upper part of the figure) and quartiles of CRP-concentrations (lower part of the figure): results are depicted separately for men (left side) and women (right side).

ing ratios of parameters with creatinine and creatinine-clearance (all  $p < 0.001$ ). Homocysteine/creatinine-clearance of men and women in dependence of neopterin/creatinine-clearance quartiles are depicted in ► Figure 1. Quartiles of neopterin/creatinine differed significantly regarding homocysteine/creatinine and folate concentrations ( $p < 0.001$ ).

Similarly, also the inflammation marker CRP showed an association with homocysteine concentrations ( $r_s = 0.131$ ;  $p < 0.001$ ; Fig. 1). Concentrations of other markers of cellular immune activation, namely TNF- $\alpha$  concentrations ( $r_s = 0.117$ ;  $p < 0.05$ , measured in  $n = 380$  patients), IL-12 concentrations ( $r_s = 0.081$ ;  $p < 0.01$ ,  $n = 1,513$ ), and the number of activated T-cells ( $r_s = 0.114$ ;  $p < 0.001$ ,  $n = 1,025$ ) and monocytes ( $r_s = 0.069$ ;  $p < 0.01$ ,  $n = 1,717$ ) were also associated weakly, but still significantly, with homocysteine concentrations (► Fig. 2). Folate concentrations were inversely associated with neopterin concentrations ( $r_s = -0.140$ ;  $p < 0.001$ ) as well as CRP concentrations ( $r_s = -0.097$ ;  $p < 0.001$ ) in all patients.

## Results for patients without vitamin supplementation or antibiotic medication

As we were interested in the relationship between inflammation and homocysteine metabolism, we conducted further analyses excluding patients with antibiotic treatment ( $n = 22$ ) as antibiotics might interfere with immune activation and inflammation cascades and those with vitamin supplementation ( $n = 38$ ). In these analyses homocysteine concentrations were still associated significantly with B-vitamin levels (folic acid,  $r_s = -0.396$ , and vitamin B12,  $r_s = -0.228$ ; both  $p < 0.001$ ), and age ( $r_s = 0.264$ ;  $p < 0.001$ ). Furthermore, also inflammation markers neopterin ( $r_s = 0.320$ ,  $p < 0.001$ ) and CRP ( $r_s = 0.126$ ;  $p < 0.001$ ) were associated with homocysteine concentrations. Very weak correlations existed between homocysteine and interleukin-12 ( $r_s = 0.079$ ;  $p < 0.01$ ;  $n = 1461$ ) and the percentage of activated T-cells ( $r_s = 0.113$ ;  $p < 0.001$ ;  $n = 992$ ) as well as the percentage of monocytes ( $r_s = 0.071$ ;  $p < 0.01$ ;  $n = 1657$ ).



**Figure 2:** Association of homocysteine concentrations (presented in log-scale!) with concentrations of IL-12 (A;  $r_s = 0.081$ ;  $p < 0.01$ ) and TNF- $\alpha$  (B;  $r_s = 0.117$ ;  $p < 0.05$ ), as well as the percentage of monocytes (C;  $r_s = 0.069$ ;  $p < 0.01$ ) and activated T-cells (D;  $r_s = 0.114$ ;  $p < 0.001$ ). Patients with CAD (>20% stenoses) are depicted as black squares, controls as grey circles.

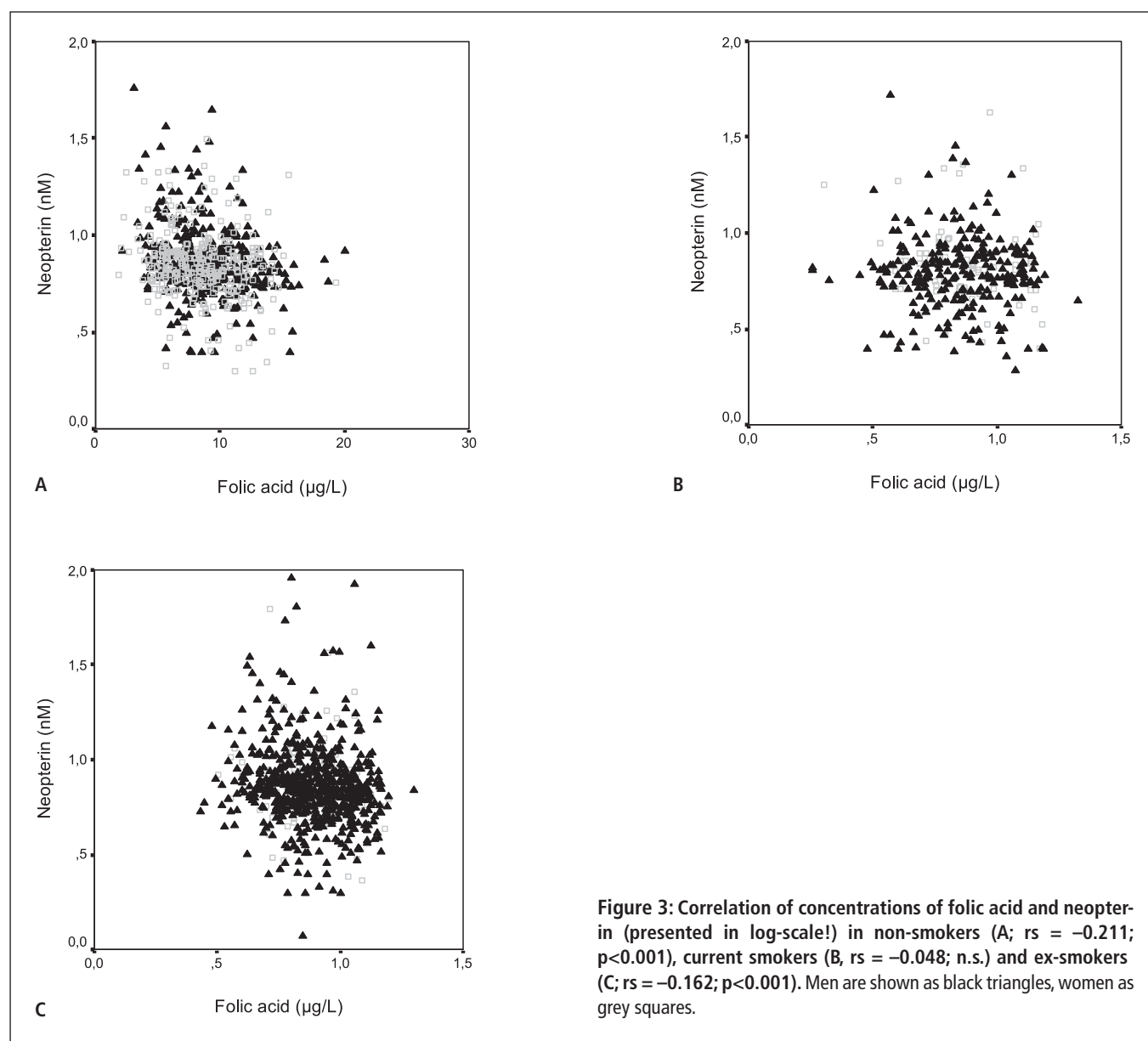
### Relationship between smoking, inflammation and immune activation

When patients were divided into subgroups according to smoking status (current smokers, ex-smokers and non-smokers), significant differences of folate, CRP and neopterin concentrations were found between the groups. Smoking status did not influence homocysteine concentrations, but current smokers presented with higher CRP (median: 5.41 mg/l) and lower folate concentrations (median: 7.2 µg/l) than ex-smokers (CRP: 3.03 mg/l; folate: 8.0 µg/l) and non-smokers (CRP: 2.65 mg/l; folate: 8.5 µg/l). On the contrary, neopterin concentrations were significantly lower in smokers than in non-smokers (6.9 nM vs. 7.8 nM). In non-smokers as well as ex-smokers inverse correlations existed between folate and neopterin (► Fig. 3), furthermore, folate and CRP con-

centrations ( $r_s = -0.143$ ;  $p < 0.001$ ) were significantly associated in ex-smokers.

### Influence of medication on homocysteine concentrations

Different medication regimens were associated with moderately elevated homocysteine concentrations in patients. Patients treated with ACE-inhibitors, diuretics and digitalis had higher homocysteine concentrations (all  $p < 0.001$ , t-test for independent variables), while patients treated with statins or hormonal oestrogen replacement had lower homocysteine concentrations (all  $p < 0.05$ , t-test for independent variables).



**Figure 3:** Correlation of concentrations of folic acid and neopterin (presented in log-scale!) in non-smokers (A;  $r_s = -0.211$ ;  $p < 0.001$ ), current smokers (B,  $r_s = -0.048$ ; n.s.) and ex-smokers (C;  $r_s = -0.162$ ;  $p < 0.001$ ). Men are shown as black triangles, women as grey squares.

## Discussion

Our study shows that homocysteine concentrations are not only influenced by B-vitamin availability (28), but also by other important covariates like immune activation and renal function. In fact, inflammation and immune activation may be responsible for both, disease progression in cardiovascular disease (finally also leading to impaired heart function by myocardial ischaemia) as well as the development of hyperhomocysteinaemia by folate depletion. Patients in this study had different disease progression status according to angiography and differed also regarding other clinical parameters. However, the association between enhanced cellular immune activation and homocysteine concentrations found earlier in other patients with chronic diseases could be confirmed: moderate hyperhomocysteinaemia was associated with lower concentrations of folate and vitamin B12, but also with higher concentrations of immune activation and inflammation markers, like neopterin, CRP, TNF- $\alpha$  or IL-12. Thus, these data also confirm and extend earlier data from a small population of patients with coronary artery disease (24), in which an association between moderate hyperhomocysteinaemia and cellular immune activation was observed.

Homocysteine accumulation in the blood is supposed to actively trigger atherogenesis. By auto-oxidation, homocysteine may further enhance oxidative stress, but whether it is really a primary cause of atherogenesis has still to be questioned (29). Recent longitudinal studies in patients with vascular disease showed that B-vitamin supplementation was very effective to lower homocysteine levels, but did not reduce cardiovascular risk (30, 31). Thus, these data rather indicate that increased homocysteine levels do not play a crucial role in the development of atherosclerotic lesions. On the other hand, it has to be kept in mind that patients investigated in these longitudinal studies had severe manifest CAD and that the time of observation was only a few years. Possibly, vitamin supplementation over a longer period might be beneficial for patients with an earlier stage of cardiovascular disease – how vitamin supplementation influences patients' cardiovascular risk has to be examined in further large prospective studies. Recently, also intervention trials counteracting immune activation cascades have been proposed to slow down atherogenesis and the progression of cardiovascular disease (32). Such studies would certainly enable a deeper insight into the pathogenesis of cardiovascular disease, and would possibly answer the question, whether the beneficial effects of statins are rather due to their anti-inflammatory and anti-oxidative or their lipid-lowering capacity. Such studies would also shed more light on the relationship between immune activation/inflammation and other risk factors like homocysteine.

Also *in vitro*-studies of our group indicate that the accumulation of homocysteine might itself derive from immune activation, as stimulated peripheral blood mononuclear cells release homocysteine (25). In parallel they also produce neopterin, and the interaction between activated T-cells and monocytes within immune activation also leads to enhanced production of ROS. The fact that lower folate concentrations were associated with higher neopterin and CRP concentrations, may indicate that immune

activation is partly responsible for decreased availability of B-vitamins, e.g. by increased production of ROS, which can oxidise oxidation-sensitive B-vitamins, which are necessary for the re-methylation of homocysteine to methionine (33). Furthermore an increased demand of B-vitamins by proliferating immunocompetent cells may result in B-vitamin deficiency.

Neopterin concentrations have been described earlier to reflect the extent of oxidative stress (34), and lower concentrations of antioxidants like vitamin C or E are observed in parallel with elevated neopterin concentrations in aged people and in patients suffering from CAD (34–36). However, neopterin might not only reflect immune activation and oxidative stress, but may also enforce oxidative stress itself: The pteridine was shown recently to induce a pro-atherogenic phenotype in human coronary artery endothelial cells by mediation of oxygen free radicals (37).

While high neopterin levels were found to be predictive for an increased risk of cardiovascular mortality (21), a reduced risk of cardiovascular events has been demonstrated in patients with high folate concentrations – independently of homocysteine levels (38). Thus, the question arises whether homocysteine really is an independent risk factor per se or rather an epiphenomenon of decreased B-vitamin availability. Recent data investigating the effects of B-vitamin supplementation on inflammatory markers including neopterin and CRP in patients with CHD (39) or various forms of dementias (40) strongly support the idea, that failure to reverse inflammatory processes may partly explain the negative results of B-vitamin intervention trials.

Well in accordance with earlier studies, homocysteine concentrations increased with older age. However, in contrast to earlier data we could not observe significantly higher homocysteine concentrations in active smokers (41, 42). Interestingly, ex-smokers differed significantly from active smokers and non-smokers regarding their CRP-levels and also folate concentrations, smokers having the highest CRP and lowest folate concentrations. Data are well in line with recent data, in which CRP concentrations were found to be higher in smokers and decreasing in ex-smokers (43). Smokers tend to have lower folate concentrations (44), thus, folate supplementation may also be useful in smokers. Interestingly, neopterin concentrations were lowest in active smokers and did not differ between ex-smokers and non-smokers, results being in accordance with earlier findings (45, 46). Higher neopterin concentrations are usually associated with oxidative stress (34), but smoking is also considered to induce oxidative stress. An “anti-inflammatory” effect of carbon monoxide (CO), which is one of the main “products” of smoking, may provide an explanation for this – at first sight – rather astonishing finding. Both *in vivo* and *in vitro*, CO at low concentrations was recently shown to inhibit the expression of LPS-induced inflammatory cytokines TNF- $\alpha$ , IL-1 and MIP1, while the expression of anti-inflammatory cytokine IL-10 was increased (47). Unfortunately – and this is certainly a limitation of our study – CO was not measured in our patients, but it would certainly be interesting to investigate effects of CO on inflammatory pathways *in vivo*.

Higher homocysteine concentrations were predictive of the presence of CAD, but the adjustment for renal function showed,

that homocysteine/creatinine was not predictive anymore. Renal function of patients, which is often impaired in patients suffering from cardiovascular disease, thus, should be taken into account. The correlation of homocysteine concentrations with creatinine levels was even stronger than the one with folate concentrations. Renal function has been described to influence homocysteine levels (41), still homocysteine concentrations have so far been regarded as independent risk factor for cardiovascular disease (1–3) and the effect of impaired renal function was – at least to our knowledge – not integrated in most analyses. To account for this problem and also for the influence of impaired renal function on neopterin concentrations, homocysteine/creatinine and neopterin/creatinine, respectively, were calculated.

As drugs influencing the renal function of patients (diuretics, ACE-inhibitors) were associated with higher homocysteine concentrations, renal impairment may partly be responsible for homocysteine accumulation, however associations between homocysteine concentrations and treatment regimes should certainly not be regarded to reflect a cause-effect-relationship. Patients with higher homocysteine concentrations mostly had to take more drugs than normohomocysteinaemic patients due to stronger disease progression and possibly also due to impaired heart function. Associations of homocysteine with N-terminal proBNP (NT-proBNP) confirm results of an earlier study (48), and additionally show that NT-proBNP is significantly associated with inflammation markers neopterin, CRP and interleukin-12 (data not shown). Thus, our data indicate that immune activation cascades may also play a role in the development of impaired heart function.

In conclusion, this study shows that the established role of moderate hyperhomocysteinaemia as risk marker for cardiovascular disease needs to be analysed more critically in future studies. Our findings suggest that there are several important determinants of homocysteine concentrations, which have not been considered consistently in analyses so far. Homocysteine accumulation relates to B-vitamin deficiency which may develop secondarily due to an increased demand for oxidation-sensitive vitamins in CAD and other clinical conditions that are associated with chronic immune activation and oxidative stress.

#### Acknowledgements

The authors thank Miss Astrid Haara for excellent technical assistance.

#### References

- Boushey CJ, Beresford SA, Omenn GS, et al. A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. Probable benefits of increasing folic acid intakes. *J Am Med Assoc* 1995; 274: 1049–1057.
- McCully KS. Homocysteine and vascular disease. *Nat Med* 1996; 2: 386–389.
- Hankey GJ, Eikelboom JW. Homocysteine and vascular disease. *Lancet* 1999; 354: 407–413.
- Harker LA, Slichter SJ, Scott CR, et al. Homocystinemia. Vascular injury and arterial thrombosis. *N Engl J Med* 1974; 291: 537–543.
- Lentz SR. Mechanisms of homocysteine-induced atherothrombosis. *J Thromb Haemost* 2005; 3:1646–1654.
- Libby P. Inflammation in atherosclerosis. *Nature* 2002; 420: 868–874.
- Frangogiannis NG, Smith CW, Entman ML. The inflammatory response in myocardial infarction. *Cardiovasc Res* 2002; 53: 31–47.
- Falk E. Morphological features of unstable atherothrombotic plaques underlying acute coronary syndromes. *Am J Cardiol* 1989; 63: 114E–120E.
- Nathan CF, Murray HW, Wiebe ME, et al. Identification of interferon-gamma as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. *J Exp Med* 1983; 158: 670–689.
- Fuchs D, Weiss G, Reibnegger G, et al. The role of neopterin as a monitor of cellular immune activation in transplantation, inflammatory, infectious and malignant diseases. *Crit Rev Clin Lab Sci* 1992; 29: 307–341.
- Garcia-Moll X, Coccolo F, Cole D, et al. Serum neopterin and complex stenosis morphology in patients with unstable angina. *J Am Coll Cardiol* 2000; 35: 956–962.
- Auer J, Berent R, Labetanig E, et al. Serum neopterin and activity of coronary artery disease. *Heart Dis* 2001; 3: 297–301.
- Adachi T, Naruko T, Itoh A, et al. Neopterin is associated with plaque inflammation and destabilisation in human coronary atherosclerotic lesions. *Heart* 2007; 93: 1537–1541.
- Ray KK, Morrow DA, Sabatine MS, et al. Long-term prognostic value of neopterin: a novel marker of monocyte activation in patients with acute coronary syndrome. *Circulation* 2007; 115: 3071–3078.
- Avanzas P, Arroyo-Espiguero R, Quiles J, et al. Elevated serum neopterin predicts future adverse cardiac events in patients with chronic stable angina pectoris. *Eur Heart J* 2005; 26: 457–463.
- Konstantino Y, Wolk R, Terra SG, et al. Non-traditional biomarkers of atherosclerosis in stable and unstable coronary artery disease, do they differ? *Acute Card Care* 2007; 9: 197–206.
- van Haelst PL, Liem A, van Boven AJ, et al. Usefulness of elevated neopterin and C-reactive protein levels in predicting cardiovascular events in patients with non-Q-wave myocardial infarction. *Am J Cardiol* 2003; 92: 1201–1203.
- Zouridakis E, Avanzas P, Arroyo-Espiguero R, et al. Markers of inflammation and rapid coronary artery disease progression in patients with stable angina pectoris. *Circulation* 2004; 110: 1747–1753.
- Shah SH, Newby LK. C-reactive protein: a novel marker of cardiovascular risk. *Cardiol Rev* 2003; 11: 169–179.
- Zakai NA, Katz R, Jenny NS, et al. Inflammation and hemostasis biomarkers and cardiovascular risk in the elderly: the Cardiovascular Health Study. *J Thromb Haemost* 2007; 5: 1128–1135.
- Grammer TB, Fuchs D, Böhm BO, et al. Neopterin as a predictor of total and cardiovascular mortality in individuals undergoing angiography (The Ludwigshafen Risk and Cardiovascular Health Study). *Clin Chem* 2009; 55: 115–146.
- Schroecksnadel K, Frick B, Kaser S, et al. Moderate hyperhomocysteinaemia and immune activation in patients with rheumatoid arthritis. *Clin Chim Acta* 2003; 338: 157–164.
- Schroecksnadel K, Leblhuber F, Frick B, et al. Association of hyperhomocysteinaemia in Alzheimer disease with elevated neopterin levels. *Alzheimer Dis Assoc Disord* 2004; 18: 129–133.
- Frick B, Rudzite V, Schroecksnadel K, et al. Homocysteine, B vitamins and immune activation in coronary heart disease. *Pteridines* 2003; 14: 82–87.
- Schroecksnadel K, Frick B, Wirleitner B, et al. Homocysteine accumulates in supernatants of stimulated human peripheral blood mononuclear cells. *Clin Exp Immunol* 2003; 134: 53–56.
- Winkelmann BR, Maerz W, Boehm BO, et al. Rationale and design of the LURIC study – a resource for functional genomics, pharmacogenomics and long-term prognosis of cardiovascular disease. *Pharmacogenomics* 2001; 2: S1–73.
- Austen WG, Edwards JE, Frye RL, et al. A reporting system on patients evaluated for coronary artery disease. Report of the Ad Hoc Committee for Grading of Coronary Artery Disease, Council on Cardiovascular Surgery, American Heart Association. *Circulation* 1975; 51: 5–40.
- Selhub J, Jacques PF, Rosenberg IH, et al. Serum total homocysteine concentrations in the third National Health and Nutrition Examination Survey (1991–1994): population reference ranges and contribution of vitamin status to high serum concentrations. *Ann Intern Med* 1999; 131: 331–339.
- Brattstrom L, Wilcken DE. Homocysteine and cardiovascular disease: cause or effect? *Am J Clin Nutr* 2000; 72: 315–323.
- Lonn E, Yusuf S, Arnold MJ, et al. Homocysteine lowering with folic acid and B vitamins in vascular disease. *N Engl J Med* 2006; 354: 1567–1577.

31. Bona KH, Njolstad I, Ueland PM, et al. Homocysteine lowering and cardiovascular events after acute myocardial infarction. *N Engl J Med* 2006; 354: 1578–1588.
32. Ridker PM. Testing the inflammatory hypothesis of atherothrombosis: scientific rationale for the cardiovascular inflammation reduction trial (CIRT). *J Thromb Haemost* 2009; 7 (Suppl 1): 332–339.
33. Fuchs D, Jaeger M, Widner B, et al. Is hyperhomocysteinemia due to the oxidative depletion of folate rather than to insufficient dietary intake? *Clin Chem Lab Med* 2001; 39: 691–694.
34. Murr C, Fuith LC, Widner B, et al. Increased neopterin concentrations in patients with cancer: indicator of oxidative stress? *Anticancer Res* 1999; 19: 1721–1728.
35. Solichova D, Melichar B, Svobodova I, et al. Fluorescence analysis of antioxidant vitamins and neopterin in nonagenarians. *Biomed Chromatogr* 1999; 13: 117–118.
36. Murr C, Schroeksnadel K, Winklhofer-Roob BM, et al. Inverse association between serum concentrations of neopterin and antioxidants in patients with and without angiographic coronary artery disease. *Atherosclerosis* 2009; 202: 543–549.
37. Cirillo P, Pacileo M, De Rosa S, et al. Neopterin induces pro-atherothrombotic phenotype in human coronary endothelial cells. *J Thromb Haemost* 2006; 4: 2248–2255.
38. Voutilainen S, Virtanen JK, Rissanen TH, et al. Serum folate and homocysteine and the incidence of acute coronary events: the Kuopio Ischaemic Heart Disease Risk Factor Study. *Am J Clin Nutr* 2004; 80: 317–323.
39. Bleie O, Semb AG, Grundt H, et al. Homocysteine-lowering therapy does not affect inflammatory markers of atherosclerosis in patients with stable coronary artery disease. *J Intern Med* 2007; 262: 244–253.
40. Frick B, Gruber B, Schroeksnadel K, et al. Homocysteine but not neopterin declines in demented patients on B vitamins. *J Neural Transm* 2006; 113: 1815–1819.
41. Nygard O, Vollset SE, Refsum H, et al. Total plasma homocysteine and cardiovascular risk profile. The Hordaland Homocysteine Study. *J Am Med Assoc* 1995; 274: 1526–1533.
42. de Bree A, Verschuren WM, Kromhout D, et al. Homocysteine determinants and the evidence to what extent homocysteine determines the risk of coronary heart disease. *Pharmacol Rev* 2002; 54: 599–618.
43. Ohsawa M, Okayama A, Nakamura M, et al. CRP levels are elevated in smokers but unrelated to the number of cigarettes and are decreased by long-term smoking cessation in male smokers. *Prev Med* 2005; 41: 651–656.
44. O'Callaghan P, Meleady R, Fitzgerald T, et al. Smoking and plasma homocysteine. *Eur Heart J* 2002; 23: 1580–1586.
45. Diamondstone LS, Tollerud DJ, Fuchs D, et al. Factors influencing serum neopterin and beta 2-microglobulin levels in a healthy diverse population. *J Clin Immunol* 1994; 14: 368–374.
46. Schennach H, Murr C, Larcher C, et al. Neopterin concentrations in cord blood: a single-cohort study of paired samples from 541 pregnant women and their newborns. *Clin Chem* 2002; 48: 2059–2061.
47. Otterbein LE, Bach FH, Alam J, et al. Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. *Nat Med* 2000; 6: 422–428.
48. Gueant-Rodriguez RM, Juilliere Y, Nippert M, et al. Left ventricular systolic dysfunction is an independent predictor of homocysteine in angiographically documented patients with or without coronary artery lesions. *J Thromb Haemost* 2007; 5: 1209–1216.