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Citation

Rosendaal, F. R. (2002). The relation between *Helicobacter pylori* and atherosclerosis cannot be explained by a high homocysteine concentration, 549-55. Retrieved from <https://hdl.handle.net/1887/1583>

Version: Not Applicable (or Unknown)

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The relation between *Helicobacter pylori* and atherosclerosis cannot be explained by a high homocysteine concentration

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Abstract

Background Recent studies have suggested that a chronic infection with *Helicobacter pylori* might be an independent risk factor for atherosclerosis. However, a direct role in atherogenesis is not plausible, since the bacterium has not been isolated from atherosclerotic lesions. An indirect mechanism that could link *H. pylori* with atherosclerosis might be through an increase in plasma homocysteine concentration caused by deficiencies of vitamin B₁₂ and folate in plasma.

Materials and methods In 150 female patients with peripheral arterial disease (PAD) and in 412 healthy control women from a nation-wide population-based case-control study, blood samples were collected to determine the antibody titre against *H. pylori* and to measure plasma homocysteine, folate and vitamin B₁₂ levels. First, the odds ratio for PAD in women with a positive antibody titre against *H. pylori* was calculated and adjusted for homocysteine level. Secondly, mean concentrations of vitamin B₁₂, folate and homocysteine were compared in healthy controls with a positive or negative antibody titre against *H. pylori*. Thirdly, the relation between *H. pylori* and PAD in individuals with a normal or high homocysteine level was investigated.

Results A positive immunoglobulin G antibody titre against *H. pylori* was found in 42% of the PAD patients and in 27% of the controls. The age- and socio-economic-status (SES) adjusted odds ratio for PAD was 1.5 (95% CI, 1.0–2.2). Additional adjustment for homocysteine plasma concentration did not essentially change the odds ratio. Secondly, among the healthy controls, the homocysteine plasma concentration did not depend on the immunoglobulin G titre, neither did the folate plasma concentration. The concentration of vitamin B₁₂ was slightly higher in women with a positive titre. Thirdly, *H. pylori* infection was a risk factor for PAD in subjects with a normal homocysteine concentration [OR 2.0 (95% CI 1.3–3.1)].

Conclusions This study shows a relationship between a positive immunoglobulin G antibody titre against *H. pylori* and PAD in young women. Moreover, this study does not support the hypothesis that *H. pylori* infection is related to atherosclerosis via an increase in plasma homocysteine concentration.

Keywords *Helicobacter pylori*, homocysteine, peripheral arterial disease
Eur J Clin Invest 2002, 32 (8) 549–555

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Received 29 November 2001, accepted 25 March 2002

Introduction

Recent studies have suggested that chronic infections with *Chlamydia pneumoniae*, *Helicobacter pylori*, or cytomegalovirus (CMV) might be independent risk factors for atherosclerosis [1]. A direct role of these infections in the pathogenesis is supported by the detection of *C. pneumoniae* and CMV in diseased vessels [2–6]. However, for *H. pylori* a direct role is less convincing since the bacterium has not yet been isolated from atherosclerotic lesions [7]. It has been hypothesized that indirect mechanisms that could link *H. pylori* with atherosclerosis, might be through one of the conventional risk factors for atherosclerosis.

In a recent meta-analysis, involving more than 10 000 patients, the relations between *H. pylori* and the conventional risk factors have been studied [8]. Included risk factors were blood pressure, body mass index, plasma viscosity, white cell count, lipid profile, fibrinogen, blood glucose, and C reactive protein. No strong relationships between *H. pylori* seropositivity and any of these vascular risk factors were found and therefore none of the studied risk factors can be considered as a confounder or an intermediate in the relation between infection with *H. pylori* and atherosclerosis. Surprisingly, plasma homocysteine concentration was not included. Prospective and case-control studies have shown that an elevated homocysteine concentration is an independent risk factor for atherothrombotic vascular disease [9].

One plausible pathway by which *H. pylori* infection could be linked to atherogenesis is via an increase in the homocysteine concentration. In detail, chronic gastritis caused by *H. pylori* infection will negatively influence the uptake of vitamin B₁₂, either through reduced acid output leading to malabsorption of food-bound vitamin B₁₂ or through decreased intrinsic factor production [10,11]. Moreover, folate uptake could also be decreased. Lucock *et al* [12] have studied factors which affect the stability, and thus the bioavailability, of dietary folates. Based on their *in vitro* experiments they conclude that patients with a normal gastric juice pH but a low ascorbate acid concentration or patients with a high gastric juice pH are likely to have reduced dietary folate bioavailability. A gastric juice with an increased pH which contains decreased ascorbate is precisely the situation precipitated by chronic infection. As a result of decreased bioavailability or uptake capacity, deficiencies may develop.

One important pathway by which homocysteine is metabolized, is the remethylation cycle, in this reaction homocysteine is converted into methionine by methionine synthase [9,13]. This conversion is dependent on the presence of both vitamin B₁₂ and folate. Therefore, anyone with a nutritional deficiency or with a malabsorption that has led to low blood concentrations of folate or vitamin B₁₂, is at increased risk of hyperhomocysteinaemia. Homocysteine is directly toxic to endothelial cells and impairs endothelium-dependent vasodilatation [14]. Thus, the possible role of plasma homocysteine concentration as an intermediate in the relation between *H. pylori* infection and atherosclerosis, is a field for investigation.

We hypothesize that chronic gastritis caused by *H. pylori*, results in malabsorption of vitamin B₁₂ and folate which will, eventually, lead to an increase in the homocysteine concentration. We performed a case-control study to investigate if the relation between *H. pylori* and atherosclerosis might be explained by an increased plasma homocysteine concentration.

Methods

Study design

Analyses are performed within the framework of the RATIO (Risk of Arterial Thrombosis In relation to Oral Contraceptives) study. This multicentre, population-based case-control study was conducted to investigate the relation between vascular diseases (stroke, myocardial infarction and peripheral arterial disease) and oral contraceptive use among women 18–49 years of age in the Netherlands [15]. The study protocol was approved by the ethics committees of all the participating hospitals.

Patients with peripheral arterial disease (PAD)

Female patients were eligible if (i) they had been referred to one of the five collaborating hospitals (see Acknowledgements) between January 1990 and December 1999, (ii) they had an angiographically confirmed diagnosis of PAD, (iii) they were aged 18–49 years at the time of referral and (iv) they gave informed consent. PAD was considered if a patient presented with typical symptoms of intermittent claudication (cramping pain in the lower leg(s) during exercise) or with rest pain, nonhealing ulcers, or gangrene. A stenotic lesion of more than 50% reduction (angiography) of the lumen in at least one major peripheral artery (distal abdominal aorta, common iliac artery, internal and external iliac artery, femoral artery, popliteal artery, anterior and posterior tibial artery, peroneal artery) was considered diagnostic for PAD. Patients were excluded if they had a history of cerebral or coronary heart disease, used folic acid, vitamin B dietary supplements, or were diagnosed with severe hyperhomocysteinaemia ($> 100 \mu\text{mol L}^{-1}$) [13].

Controls

The group of population-based control women was recruited by random digit dialling (RDD), random phone numbers (in a certain area) were dialled and households were ascertained for eligible individuals (female, aged 18–49 years) who were subsequently asked to participate [16,17]. This method resulted in controls who were approximately (5-year strata) the same age as the patients and who lived in the service areas of the participating hospitals. Controls were excluded if they used folic acid, vitamin B dietary supplements, or were diagnosed with a severe hyperhomocysteinaemia ($> 100 \mu\text{mol L}^{-1}$).

Data collection

Between June 1998 and May 2000, all participants had their blood pressure measured, nonfasting venous blood samples were taken and a structured questionnaire was completed. Blood pressure was measured semiautomatically by a physician (Omron M1 OMRON Healthcare GmbH, Hamburg, Germany) at one time-point. Serum or plasma were stored at -80°C until processed.

Conventional risk factors

Data obtained from the questionnaire included current medication use and classical risk factors [body mass index (BMI), smoking, history of hypercholesterolaemia, diabetes and hypertension]. We categorized smokers as current, former, or never. A positive history of hypercholesterolaemia was defined by the use of cholesterol-lowering medication or a serum total cholesterol $\geq 5.0\text{ mmol L}^{-1}$. This definition is based on the consensus text for lipid-lowering therapy (the third consensus 'Cholesterol' which was published in 1998 [18]).

A positive history of diabetes was defined by the use of glucose-lowering medication or a (nonfasting) serum glucose $\geq 11.0\text{ mmol L}^{-1}$. A positive history of hypertension was defined by the use of antihypertensive drugs or a systolic blood pressure $\geq 160\text{ mmHg}$ or a diastolic blood pressure $\geq 95\text{ mmHg}$. The socio-economic status (SES) was defined as low, intermediate, or high and was determined according to the highest level of education attended by the participant. Highest education level could be primary school (low SES), secondary school (intermediate SES) or higher education/university (high SES).

Serum total cholesterol, high-density lipoprotein (HDL)-cholesterol, triglyceride and glucose concentrations were measured with a colorimetric test on a clinical analyser (Roche/Hitachi[®] 747, Mannheim, Germany). Low-density lipoprotein (LDL)-cholesterol was calculated by the Friedewald equation. The plasma C reactive protein (CRP) concentration was determined by a commercial enzyme immunoassay (EIA, CRP EAI HS, Kordia, Leusden, the Netherlands). Sensitivity was $5\text{ }\mu\text{g L}^{-1}$ and intra-assay and interassay coefficients of variation were 5.2% and 7.5%, respectively.

All assays were performed by a single technician who was unaware of the origin of the samples (case or control).

Determination of homocysteine concentration

Non-fasting blood samples were drawn from the antecubital vein in 5-mL Stabilyte[®] (Monovette[®]) tubes for determination of homocysteine. Withdrawal on acidic citrate is a good alternative for blood tubes on crushed ice, when screening patients in epidemiological field studies, because total homocysteine (tHcy) concentrations stay stable for 6 h [19]. The Stabilyte[®] blood sample was centrifuged at 2000 r p m for 10–15 min, and the plasma was separated and stored at -80°C until analysis.

The determination of plasma homocysteine concentration was carried out in the Laboratory of Paediatrics and

Neurology of the University Medical Centre Nijmegen by high-performance liquid chromatography (HPLC). The HPLC sodium borohydride/monobromobimane ($\text{NaBH}_4/\text{mBrB}$) method used HaBH_4 for reduction and mBrB for derivatization essentially according to Fiskerstrand *et al* with cysteamine as external standard [20]. A programmable sample processor (Gilson 232–401 sample processor, Spectra Physics 8800 solvent delivery system and Spectra Physics LC 304 fluorometer) was used for automated homocysteine reduction, derivatization and sample injection. Values for plasma tHcy, expressed as homocysteine concentration in mmol L^{-1} , included the sum of free and bound forms of homocysteine, homocystine, and homocysteine–cysteine mixed disulphide. Hyperhomocysteinaemia was defined as a homocysteine concentration $> 16\text{ }\mu\text{mol L}^{-1}$ (90th percentile of the female control population).

Determination of immunoglobulin G antibodies to *H. pylori*

The presence of immunoglobulin G (IgG) antibodies to *H. pylori* was determined by the use of commercial EIA. Positivity was defined according to the instructions of the manufacturer (Enzygnost[®] Anti-*Helicobacter pylori* II/IgG, Dade Behring, Marburg, Germany).

Determination of vitamin B₁₂ and folate concentrations

Vitamin B₁₂ and folate concentrations were simultaneously measured in the control group using a standard radioassay (Dualcount[®] SPB, DPC, Los Angeles, CA, USA). The concentration vitamin B₁₂ was expressed in pg mL^{-1} and the concentration of folate was expressed in ng mL^{-1} .

Statistical analysis

Mean or median values or proportions of cardiovascular risk factors were calculated. To assess whether the relation between *H. pylori* and PAD was due to an increase in the homocysteine concentration, three different analyses were performed. Firstly, the age- and SES-adjusted odds ratio for PAD and *H. pylori* was calculated and compared to the homocysteine-adjusted odds ratio. The mean homocysteine, vitamin B₁₂ and folate concentrations and the percentage of individuals with hyperhomocysteinaemia were compared in women with positive and negative IgG antibody titre against *H. pylori*. Thirdly, the age- and SES-adjusted odds ratios for PAD were calculated in four subgroups according to the IgG antibody titre against *H. pylori* (positive or negative) and the homocysteine plasma concentration (hyperhomocysteinaemia or no hyperhomocysteinaemia).

Results

Initially 212 PAD patients and 464 control women were included. However, eight patients and five control women were excluded because of a severe hyperhomocysteinaemia.

Table 1 Characteristics of PAD patients and control women

	PAD patients (<i>n</i> = 150)	Control women (<i>n</i> = 412)
Age in years (mean ± SD)	48.7 ± 6.9	45.5 ± 7.9
BMI in kg m ⁻² (mean ± SD)	26.4 ± 5.8	24.9 ± 4.3
Smoking in percentage (<i>n</i>)		
current	59 (88)	33 (135)
former	36 (54)	33 (137)
never	5 (7)	34 (140)
Education in percentage		
primary school or less	22 (32)	9 (36)
secondary school	68 (100)	67 (276)
higher education or university	10 (14)	24 (98)
History of hypercholesterolaemia* in percentage (<i>n</i>)	87 (129)	61 (252)
Cholesterol in mmol L ⁻¹ (mean ± SD)	5.61 ± 1.21	5.38 ± 1.10
HDL-cholesterol in mmol L ⁻¹ (mean ± SD)	1.31 ± 0.37	1.42 ± 0.33
Triglyceride in mmol L ⁻¹ (mean ± SD)	2.07 ± 1.12	1.49 ± 0.90
LDL-cholesterol in mmol L ⁻¹ (mean ± SD)	3.36 ± 1.13	3.29 ± 0.97
Ratio of cholesterol/HDL-cholesterol (mean ± SD)	4.80 ± 2.91	4.00 ± 1.31
History of diabetes mellitus† in percentage (<i>n</i>)	14 (20)	1 (4)
Glucose in mmol L ⁻¹ (mean ± SD)	6.16 ± 3.48	4.01 ± 1.38
History of hypertension‡ in percentage (<i>n</i>)	61 (91)	25 (103)
Mean systolic blood pressure in mmHg (mean ± SD)	142 ± 24	130 ± 20
Mean diastolic blood pressure in mmHg (mean ± SD)	86 ± 11	83 ± 11
Hyperhomocysteinaemia§ in percentage (<i>n</i>)	23 (35)	10 (43)
Homocysteine in µmol L ⁻¹ (mean ± SD)	13.3 ± 5.3	12.4 ± 3.3
CRP in mg L ⁻¹ (median, quartiles)	3.8 (1.6, > 10.0)	1.3 (0.5, 5.2)
Positive IgG antibody titre against <i>H. pylori</i> in percentage (<i>n</i>)	42 (63)	27 (110)

*Positive history of hypercholesterolaemia was defined as use of lipid-lowering medication and/or cholesterol plasma concentration ≥ 5.0 mmol L⁻¹

†Positive history of diabetes was defined as use of blood-glucose-lowering medication and/or (nonfasting) glucose plasma concentration ≥ 11.0 mmol L⁻¹

‡Positive history of hypertension was defined as the use of antihypertensives and/or systolic blood pressure ≥ 160 mmHg and/or diastolic BP ≥ 90 mmHg

§Hyperhomocysteinaemia was defined as a homocysteine concentration > 16 µmol L⁻¹ (90th percentile of the control population)

(> 100 µmol L⁻¹) Of the remaining participants, 54 patients and 47 controls were excluded because they used folic acid or vitamin B dietary supplements. Thus, in the present analysis 150 PAD patients and 412 control women were included. All cardiovascular risk factors were more prevalent in the group of PAD patients compared to the control group (Table 1). The percentage of individuals classified as suffering from hyperhomocysteinaemia, was statistically significantly higher in PAD patients [OR 2.6 (95%CI 1.6–4.3)]. The mean homocysteine concentration was higher in PAD patients [mean difference 0.9 (95%CI 0.2–1.7)].

A positive IgG antibody titre against *H. pylori* was found in 42% of the PAD patients compared to 27% of the controls [crude OR 2.0 (95%CI 1.3–2.0)]. After adjustment for age and SES the odds ratio decreased to 1.5 (95%CI 1.0–2.2) (Table 2). Additional adjustment for homocysteine plasma concentration, or for hyperhomocysteinaemia, did not change the odds ratio essentially, both adjusted odds ratios were 1.6 (95%CI 1.0–2.4).

Table 3 shows that among controls the homocysteine plasma concentration did not differ between subjects with a positive or a negative IgG titre against *H. pylori* [mean difference -0.1 (95%CI -0.8 to 0.6)], neither did the folate

Table 2 Crude and adjusted odds ratios (95%CI) for PAD in women with a positive IgG antibody compared to women with a negative titre against *H. pylori*

Adjustment	<i>H. pylori</i> OR (95%CI)
None	2.0 (1.3–2.9)
Age (years)	1.7 (1.1–2.6)
Age (years) and SES (low, middle, high)	1.5 (1.0–2.2)
Age (years) and SES SES (low, middle, high) and homocysteine concentration (in µmol L ⁻¹)	1.6 (1.0–2.4)
Age (years) and SES (low, middle, high) and hyperhomocysteinaemia* (yes/no)	1.6 (1.0–2.4)

*Hyperhomocysteinaemia was defined as a homocysteine concentration > 16 µmol L⁻¹ (90th percentile of the control population)

Table 3 Prevalence of hyperhomocysteinaemia, plasma total homocysteine, vitamin B₁₂ and folate concentration in a population based sample of young women according to their antibody titre against *H. pylori*

	Antibody titre against <i>H. pylori</i>		Difference (95%CI)
	Negative (n = 302)	Positive (n = 110)	
Hyperhomocysteinaemia* in percentage (n)	11 (32)	10 (11)	0.9 (0.5–1.9) [†]
Homocysteine in µmol L ⁻¹ (mean ± SD)	12.4 (3.4)	12.3 (3.0)	-0.1 (-0.8–0.6)
Vitamin B ₁₂ in pg mL ⁻¹ (mean ± SD)	368 (181)	412 (193)	44.5 (4.0–85.1)
Folate in ng mL ⁻¹ (mean ± SD)	8.2 (4.0)	8.0 (4.1)	-0.2 (-1.1–0.7)

*Hyperhomocysteinaemia was defined as a homocysteine concentration > 16 µmol L⁻¹ (90th percentile of the control population)

[†]Odds ratio instead of difference of the mean values

Table 4 Hyperhomocysteinaemia and *H. pylori* separate and combined effects on PAD

IgG antibody titre <i>H. pylori</i>	Hyperhomocysteinaemia*	PAD (n)	Controls (n)	OR (95%CI) [†]
Negative	No	62	270	1
Positive	No	53	99	1.7 (1.1–2.7)
Negative	Yes	25	32	2.7 (1.5–5.1)
Positive	Yes	10	11	2.6 (1.0–6.8)
Total		150	412	

*Hyperhomocysteinaemia was defined as a homocysteine concentration > 16 µmol L⁻¹ (90th percentile of the control population)

[†]Adjusted for age and socio-economic status (SES)

plasma concentration [mean difference -0.2 (95%CI -1.1 to 0.7)]. However, the concentration of vitamin B₁₂ in plasma was higher in women with a positive titre compared to those with a negative titre [mean difference 44.5 (95%CI 4.0–85.1)].

The age- and SES-adjusted odds ratios for PAD according to the presence of hyperhomocysteinaemia and to the IgG antibody titre against *H. pylori* are shown in Table 4. Individuals classified as 'no hyperhomocysteinaemia' but who were seropositive against *H. pylori* had a 1.7-fold risk of PAD compared to subjects without hyperhomocysteinaemia and a negative IgG antibody titre [OR 1.7 (95%CI 1.1–2.7)]. Seronegative individuals with hyperhomocysteinaemia had a 2.7-fold risk of PAD compared to the reference group [OR 2.7 (95%CI 1.1–5.1)]. When subjects were exposed to both risk factors their risk of PAD was increased by a factor of 2.6 [OR 2.6 (95%CI 1.0–6.8)].

Discussion

The results of the present study show that it is unlikely that the homocysteine plasma concentration is an intermediate between *H. pylori* infection and PAD. Firstly, Table 2 shows that the age- and SES-adjusted odds ratio for PAD did not change after additional adjustment for the homocysteine plasma concentration or for hyperhomocysteinaemia [OR 1.5 (95%CI 1.0–2.2) vs OR 1.6 (95%CI 1.0–2.4)]. If, infection with *H. pylori* had been linked with PAD via an

increase in the homocysteine concentration, one would have expected the odds ratio to decrease after adjustment for the intermediate variable. The age- and SES-adjusted odds ratio, however, is lower than the crude odds ratio. This indicates that the relation between *H. pylori* and PAD can partly be explained by the age difference and the discrepancy in SES between the PAD patients and the control women.

The odds ratios were adjusted for age and SES since these variables are related to both *H. pylori* infection and PAD and therefore they are considered as potential confounders. Other cardiovascular risk factors are not proven to be related to *H. pylori* infection and therefore were not considered potential confounders.

Secondly, Table 3 shows that there was no significant difference in plasma homocysteine concentration between *H. pylori*-infected and noninfected controls. These results contradict the hypothesis that *H. pylori* infection increases the plasma homocysteine concentration. According to our data, it is unlikely that infection with *H. pylori* negatively influences the uptake of folate and vitamin B₁₂, since the plasma concentrations in infected subjects are equally high (folate) or even higher (vitamin B₁₂) than the concentrations in noninfected subjects. Thirdly, Table 4 shows that *H. pylori* infection is a risk factor for PAD in subjects with a normal homocysteine concentration [OR 1.7 (95%CI 1.1–2.7)].

The hypothesis that *H. pylori* and atherosclerosis might be linked by the homocysteine concentration has been proposed previously [21–24]. However, original data on this hypothesis are very sparse. In 1997, two letters were published presenting data on the relation between *H. pylori* infection and homocysteine [25,26]. The first letter describes an analysis within the framework of an earlier prospective case-control study of *H. pylori* infection, in incident cases of myocardial infarction and controls within the British Regional Heart Study [27]. Within the control group (n = 118), the mean total homocysteine concentrations were similar in subjects seropositive (n = 63) or seronegative (n = 55) for *H. pylori* (P = 0.98). The second letter describes the mean homocysteine concentrations in a healthy population of 220 individuals, no significant difference was found between the group of 122 *H. pylori*-seropositive and the group of 98 seronegative subjects. More recently, Leung *et al.* showed that homocysteine concentration was not related to the presence of antibodies against *H. pylori* in 49 dyspeptic patients [28]. Moreover,

after successful eradication of the bacterium there was no significant reduction in the homocysteine concentration measured after a follow-up period of 24 weeks [28]. If a link between *H. pylori* infection and atherosclerosis exists, other mechanisms should be responsible. A possible mechanism could be mediated through the circulating cytokines induced by the infection, e.g. interleukin-1, interleukin-6 and tumour necrosis factor. These cytokines may activate or exacerbate the inflammatory reactions in the vascular endothelium [29]. Another possible mechanism is by an infection-induced autoimmune response. This mechanism requires that the infecting pathogen contains peptides homologous to those present in the host proteins. The immune response, although stimulated by and targeted to pathogen antigens, also attacks host tissues containing the cross-reacting proteins [30].

The present study has two clear advantages. Firstly, the size of the study population (150 PAD patients and 412 controls) is larger than those of earlier reported studies which made it possible to provide more precise estimates of the differences in concentrations between seropositive and seronegative individuals. Secondly, this study not only describes the homocysteine concentration but also the concentration of vitamin B₁₂ and folate, two substrates that are considered intermediates in the hypothetical relation between *H. pylori* and homocysteine. The study population consists of young women and it might be invalid to extrapolate the results to individuals who do not belong to the same domain. For example, the relation between *H. pylori* and homocysteine in older individuals might be different due to the possibility that chronic gastritis might be more advanced in the elderly.

Since in the present study nonfasting blood samples were collected, the scatter of the concentrations that were measured is larger compared to a situation where fasting blood samples were collected. Therefore, a limitation of nonfasting blood samples could be that minor differences in homocysteine, vitamin B₁₂, or folate concentration are not detected. On the contrary, the rather large size of the study population is in favour of detecting small differences.

Another issue might be that in this study plasma folate concentration was used instead of whole blood or erythrocyte folate concentration. Although erythrocyte folate concentration is considered a better indicator of folate status than serum folate, the latter is the variable generally measured. Moreover, a detailed analysis of 1259 consecutive requests for folate assays from a single representative laboratory showed a significant correlation between serum and erythrocyte folate levels ($r = 0.49$, $P < 0.001$) [31]. These results were moderated at a meeting of haematologists where a consensus was reached, the plasma folate assay which can be combined easily with the vitamin B₁₂ assay, was considered the most appropriate screening test for folate deficiency.

Initially patients with kidney disease were not excluded. Since there is a link between renal failure and homocysteine level it is possible that the effect of *H. pylori* infection on homocysteine is masked because it is diluted by the effect of kidney disease. However, if we performed the analysis

again, excluding all individuals (14 patients and one control) with diabetes (which in time will favour kidney disease), the results of our analyses did not change essentially.

Although the year in which the patients were diagnosed varied between 1990 and 1999, all blood samples and questionnaires were collected during the last 2 years of that period. It is possible that those patients that were diagnosed earlier have adapted a more healthy dietary pattern and life style after diagnosis. To reduce the bias which is inherent in this study design, we excluded all individuals who used folic acid or vitamin B supplements. Patients might also have received medication to control risk factors for PAD. To achieve a proper assessment of the risk factors in all individuals at the moment of diagnosis, we used definitions that included serum levels as well as the use of medication.

Some participants with a positive IgG antibody titre against *H. pylori*, might not have suffered from a chronic gastritis at the moment of blood withdrawal, it is possible that the infection and the inflammatory reactions in the gastric wall had already been resolved. To evaluate whether the results of our study are distorted by this phenomenon, we have repeated the same analyses in the subgroup of individuals with a CRP concentration above the median value (1.3 mg L^{-1}), as it is known that chronic *H. pylori* infection is associated with an increased CRP concentration. Again, the odds ratio for PAD in individuals with a positive IgG antibody titre against *H. pylori* compared to those with a negative titre, did not change after adjustment for homocysteine plasma concentration. Also, no difference in the mean homocysteine concentration between *H. pylori* seropositive and seronegative control subjects, was found (data not shown).

In this study, young (< 50 years) women were diagnosed with a first manifestation of atherosclerosis in the peripheral arterial circulation. The reasons why these women develop PAD at such an early age is not clear. Apart from *H. pylori* infection, other factors presumably also contribute to development of PAD. The high percentage of young female PAD patients who smoked or had been smoking (95%) is remarkable. Obviously, a single cause of premature PAD does not exist and the condition should be considered as a complex and multifactorial process. As has recently been stated for venous thrombosis [32], it is likely that certain environmental factors interact with one another or with one or more genetic variations, and thereby constitute the risk of PAD.

In conclusion, the results of this study do not confirm the hypothesis that *H. pylori* infection increases the homocysteine concentration and therefore, do not support the putative link between *H. pylori* infection and atherosclerosis via an increase of the homocysteine concentration.

Acknowledgements

We thank Martin van der Maas for excellent technical assistance, Tineke Kromhout and Mireille Velthuis for their tremendous efforts in data collection, D van Oppenraaij and H. J. Blom for the ability to perform the analyses of the

homocysteine concentrations in the Laboratory of Paediatrics and Neurology of the University Medical Centre Nijmegen, and K. Prinsen for conducting the vitamin determinations. We are grateful to the five participating medical centres: University Medical Centre Utrecht; W. P. Th. M. Mali, B. C. Eikelboom, Academic Medical Centre Amsterdam; M. J. H. M. Jacobs, J. A. Reekers, Leiden University Medical Centre; J. H. van Bockel, E. van der Lunden, Academic Hospital Nijmegen; J. A. van der Vliet, F. M. J. Heijstraten, Slingeland Ziekenhuis Doetinchem; J. G. J. M. van Iersel, J. Seegers, J. H. Spithoven. This project was supported by the Dutch Organization for Scientific Research (NWO). We thank all the women who participated in this study.

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