· Advances in Medical Sciences · Vol. 53(2) · 2008 · pp 205-213 · DOI: 10.2478/v10039-008-0050-8

Association between *Helicobacter pylori*-infection, C-reactive protein and status of B vitamins

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Received 04.07.2008
Accepted 13.10.2008
Advances in Medical Sciences
Vol. 53(2) · 2008 · pp 205-213
DOI: 10.2478/v10039-008-0050-8

ABSTRACT

Purpose: Some investigations, but not all, found that a chronic infection with *Helicobacter pylori* (*Hp*) is associated with deficiencies in B vitamins, elevated plasma total homocysteine concentrations (tHcy) and increased plasma levels of proinflammatory acute-phase proteins. It has been suggested that these factors promote atherogenesis and therefore could mechanistically explain why people infected with *Hp* might have an increased risk for cardiovascular diseases (CVD). Therefore we evaluated the association between *Hp*-infection, concentration of C-reactive protein (CRP), and status of various B vitamins in apparently healthy subjects.

Material and methods: In 69 subjects with proved *Hp*-infection and 21 healthy control subjects identified in a cross sectional study, blood samples were collected to determine serum folate, serum vitamin B_{12}, serum methylmalonic acid (MMA), serum CRP and plasma vitamin B_6 and plasma total homocysteine (tHcy).

Results: The mean concentration of CRP was significantly higher in the *Hp*-positive collective than in controls. Although mean concentrations of vitamin B_{12}, B_6, and MMA differed between the groups, statistical significance was missed. However, the mean concentrations of homocysteine and folic acid were nearly the same in both groups. In univariate analysis a significant impact of *Hp*-status was shown on cobalamin (p=0.028; eta square: 0.055), and in multivariate analysis of variance the *Hp*-status had an impact on vitamin B_{12}-values (p=0.028; eta square: 0.057).

Conclusions: In this study *Hp*-infection shows no significant impact on status of B vitamins, but has a significant influence on CRP concentration. However, this study does not support the hypothesis that *Hp*-infection is related to CVD *via* elevated levels of tHcy.

Key words: *Helicobacter pylori*, homocysteine, B vitamins, CRP, inflammation

INTRODUCTION

In Western Europe, 20-50% of the apparently healthy people are infected with *Helicobacter pylori (Hp)* [1]. It is well known that *Hp* is involved in the pathogenesis of several gastro duodenal diseases such as atrophic gastritis, gastric carcinoma, and duodenal ulcer [2,3]. At the onset of *Hp*-infection, there is reduced secretion of gastric acid [4], but after a few months, there may be either normal or increased gastric acid production in *Hp*-gastritis [5]. If *Hp*-gastritis progresses to gastric atrophy, the secretion of gastric acid decreases (gastric hypoacidity). Furthermore, the secretion of pepsinogen and the intrinsic factor can also be reduced. Together, these mechanisms might impair the bioavailability of several B vitamins, mostly of cobalamin (vitamin B_{12}), folic acid and pyridoxine (vitamin B_6) [6-8]. An inadequate supply of these B vitamins, associated with an elevated concentration of plasma total homocysteine (tHcy), has been associated with cardiovascular disease (CVD) [9], although the strength of these relation may be weaker than had previously been thought [10,11]. Therefore, the association between *Hp*-infection, impaired bioavailability of B vitamins and hyperhomocysteinemia offers a pathophysiological explanation why people with *Hp* are at higher risks for CVD [12]. However, results from more than 25 studies evaluating the possible association between *Hp*-infection, impaired B vitamin status, and elevated homocysteine concentrations are

still inconsistent [13]. Whereas some studies have not found any associations [14-17], other investigations showed that persons with *Hp*-infection and higher grades of gastric mucosa atrophy had lower serum concentrations of cobalamin, folic acid, and hyperhomocysteinemia than persons without *Hp*-infection [18-22]. These latter data are consistent with another study showing that *Hp* eradication resulted in an improvement of B vitamin status in 40 % of the study collective [23]. Beside the "homocysteine CVD connection", infection with *Hp* may lead to a chronic low-grade systemic inflammation response, characterized by increased plasma levels of proinflammatory cytokines and acute-phase proteins [24-27]. Up to now there is growing evidence that systemic inflammation is associated with CVD [28-30]. Beside this, C-reactive protein (CRP), an important marker of inflammation, may also be a causal agent promoting atherosclerotic initiation and progression [31,32]. Thus, an indirect mechanism that could link *Hp* with atherosclerosis might be through an increase of CRP levels in plasma.

Although a large number of studies on *Hp*-infection, B vitamin status [13] and systemic inflammatory [24] have been published during the last years, there are only limited studies which examined these associations in apparently healthy subjects. We therefore investigated the influence of *Hp* on the inflammation marker CRP in such a collective. Furthermore, this cross-sectional study evaluated the relations of *Hp*-infection to serum concentrations of cobalamin, folic acid, and MMA as well as plasma tHcy and pyridoxine.

MATERIALS AND METHODS

Study Design

Subgroup Allocation

The study protocol was approved by the Ethical Committee of the Medical School Hannover, Germany. Written informed consent was obtained from all subjects in accordance with the principles of the Helsinki Declaration. The investigation was planned as a cross sectional study. Study subjects were recruited through newspaper articles and radio announcements in the area of Hannover, Germany. After a short telephone screening study volunteers received a questionnaire which included questions about disease history, medication, age, sex, education status, and other parameters needed for Winkler's socio economic index [33]. To be included in the study the subjects had to be older than 18 years and they had to give written consent to *Helicobacter pylori*-stooltest, ^{13}C-urea breath test (UBT) for *Helicobacter pylori*, and a blood analysis. Exclusion criteria were: (i) medication for gastrointestinal disorders, (ii) pregnancy, (iii) taking antibiotics in the last month, (iv) having an ulcer disease. Of the 829 volunteers originally interested and pre-screened via telephone interview, 794 were qualified to get the screening questionnaire and stool test tube per mail. Of the 794 volunteers 579 returned the screening questionnaire and stool test tubes. From 90 of them we draw up urea breath and stool tests to determine *Hp* status. After testing, volunteers were divided into two groups: group A consisted of persons being *Hp*-positive in UBT and stool test (n=69). Group B was formed by persons being *Hp*-negative in both *Hp*-tests, in total 21 persons.

Blood sampling and analytic methods

Blood samples were drawn by venipuncture after an overnight fast. For plasma vitamin B_6 and plasma tHcy we took an EDTA blood ampule, for the other parameters we used blood tubes. All tubes were send by curier to the laboratory (Gemeinschaftspraxis für Laboratoriumsmedizin, Prof. Dr. med. Hellthaler und Dr. med. Sloot, Hannover; Medizinisches Analyse Zentrum, Leisewitzstr. 28, 30175 Hannover) within four hours after venipuncture. EDTA blood tubes were centrifuged at 2665 × g for 10 min at 19°C. After completed coagulation the blood ampules likewise were centrifuged (2000 x g for 10 min) to get serum. Serum vitamin B_{12} and folic acid were measured by electron chemiluminescence immunoassay (ECLIA) on an Elecys System (Roche Diagnostics GmbH, Germany) [34-36]. Vitamin B_6 was analysed in plasma with HPLC fluorescence detection (Recipe Chemicals & Instruments GmbH & Co KG). We measured plasma tHcy by using high performance liquid chromatography (HPLC) with fluorescence detection (Chromsystems Instruments & Chemicals GmbH) [37]. Serum MMA was measured with the use of gas chromatography–mass spectrometry (GC-MS) as previously described [8]. CRP was detected with an immunoturbidimetric test (Olympus Diagnostica GmbH).

Cutoff values

Because elevated MMA and tHcy concentrations have been reported in association with plasma cobalamin <258 pmol/L [37], this value was taken to indicate inadequate cobalamin status. For vitamin B_6, a plasma cutoff of 25 nmol/L has been proposed as an index of adequacy [38]. A serum folate concentration of <7 nmol/L indicates a negative folate balance [39]. Suggested normal tHcy values ranged in the literature from <14 µmol [40] to 5–13.6 µmol/L [41] and 4.9–11.7 µmol/L [42]. Since the results of many studies showed that the prevalence and mortality of CVD is increased if the concentration exceedes 10 µmol/L [6,43,44], this limit has been suggested as desirable [45]. Because many studies agree that a tHcy concentration of ≤10 µmol/L is optimal [8,44,46-48], a tHcy concentration >12 µmol/L was taken as cutoff. Such values are classified as moderate hyperhomocysteinemia [49]. The range of serum MMA values in healthy subjects (mean ± 2 standard deviations) is 73–271 nmol/L [6,7,50]. MMA concentrations rise because of an inadequate vitamin B_{12} intake or absorption [41]. Based on the position statement of the American Heart Association [51] CRP levels <1 mg/L were considered to be low risk, 1-3 mg/L as average risk, and >3 mg/L as high risk for CVD, a cutoff of >3 mg/L was taken. All defined cutoffs are shown in *Tab. 1*.

Table 1. **Defined cutoffs of concentrations of the variables.**

Blood variable and reference	Cutoff
Serum cobalamin [80]	<258 pmol/L
Serum folate [39]	<7 nmol/L
Plasma pyridoxin [38]	< 25 nmol/L
Plasma total homocysteine [46,47,48,44]	>12 µmol/L
Serum methylmalonic acid [44]	>271 nmol/L
Serum C-reactive proteine [32]	>3 mg/L

Assessment of Hp Status

Hp-infection was assessed using two non-invasive methods: an enzyme-linked immunoassay (*H. pylori* FemtoLab CnX-stooltest, Connex GmbH) against *Hp*-antigens in stool; and the urea breath test (UBT), which relies on bacterial hydrolysis of ^{13}C isotope-labeled urea (INFAI GmbH-*Helicobacter pylori* breath test, INFAI GmbH).

Hp-antigens in stool were analyzed in microtiter format with monoclonal antibodies against *Hp*-katalase by courtesy of ELISA-tests (enzyme linked immunosorbent assay), specific antigens were marked and visualized through process. To evaluate the test method there was a photometric measuring of colour intensity by 450 nm against reference between 620 to 650 nm. The cut off was 0.150 units of absorbance. Values ≥ 0,170 units of absorbance were classified as *Hp*-positive and values < 0,170 *Hp*-negative. Every analysis had run a positive and a negative sample in order to validate the analysis.

Stool samples should be collected from the study participants in the morning or during the evening before attending to the study centre [52]. They were frozen (-20°C) and send to the laboratory (Gemeinschaftspraxis für Laboratoriumsmedizin, Prof. Dr. med. Hellthaler und Dr. med. Sloot, Hannover).

The sensitivity and specifity of this test method were proven to be excellent against the gold standard gastroscopy and biopsy [52-54].

UBT-tests were made in the morning after having fasted for at least 6 hours. Breath samples were collected in plastic tubes closed with a stopper. After collecting the baseline values the test persons got a test meal (200 ml 100 % orange juice or 200 ml 1 g citric acid solved in tap water) and then the test solution (75 mg ^{13}C-urea powder solved in 30 ml tap water). After 30 minutes a second breath sample was collected. Breath samples were analyzed with an isotope ratio mass spectrometry in laboratory of the fabricator (INFAI GmbH, Institut für biomedizinische Analytik und NMR-Imaging GmbH, Bochum). In each sample the $^{13}C/^{12}C$-ratio was determined. *Hp*-infection is present if the difference in $^{13}C/^{12}C$-ratios of baseline-value and 30 minute-value exceeds 4.0 ‰ [55]. The test is a highly sensitive and specific method for diagnosis of *Hp*-infection [56,57].

Statistical Analysis

Data analysis was made with SPSS Software (Version 13.0 SPSS Inc., Chicago). Metric data are expressed as mean ± standard deviation (SD). In some cases the median is given additionally. Nominal and ordinal data are shown as absolute and relative frequencies. Normally distributed metric data were analysed with the Student t test, ordinal data with the Mann-Whitney U test, and dichotomic variables with the chi-square test or Fisher`s exact test. Analysis of variance (ANOVA; one way), in this case a one-way and multivariate analysis, were used to test for one or more independent variables on a dependent variable. P<0,05 was considered statistically significant.

RESULTS

Subject characteristics

The characteristics of the study population are shown in *Tab. 2.* No significant differences were shown between the two groups regarding to sex, age, school education, vocational status, and socio economic status.

CRP values

The mean concentration of CRP was significantly higher in the *Hp*-positive collective than in *Hp*-negative subjects (p=0.011) (*Tab. 3*).

Status of vitamins and associated parameters

Serum and plasma values and the percentage of subjects with concentrations not meeting the defined cutoffs are presented in Tab. 3. With a difference of 17.5% (58 pmol/L), the mean concentration of vitamin B_{12} was slightly lower in *Hp*-positive than in *Hp*-negative subjects. However, statistical significance was missed. Contrary to this, the mean folic acid level was nearly the same in the two groups. Surprisingly, vitamin B_6 levels were higher in *Hp*-positive volunteers. However, statistical significance was also missed here. Levels of MMA were higher in group A than in group B but again without a significant difference. Furthermore, the mean concentrations of homocysteine differ not significantly between *Hp*-positive and *Hp*-negative subjects.

Correlations

However, in group A a statistical significant correlation was observed between MMA concentrations and *Hp*-antigens in stooltest (p=0.001, r_s=0.404). Significant negative correlations were found between vitamin B_{12}, folic acid and homocysteine (*Tab. 4*). Correlations between CRP and homocysteine or body mass index (BMI) could not be found.

GLM (General Linear Model)

First, in univariate analysis the influence of the factors age, sex, and *Hp*-status on blood parameters was evaluated. *Hp*-status had a significant impact on cobalamin concentration

Table 2. **Description of the two study groups.**

	Group A (Hp-positive) [n=69]	*Group B (Hp-negative)* [n=21]	*P*
Sex			
women	42 (60.9 %)	13 (61.9 %)	0.932$
men	27 (39.1 %)	8 (38.1 %)	
Age [years]	50.8 ± 12.9	47.3 ± 10.8	0.266*
BMI# [kg/m²]	26.4 ± 4.74	25.6 ± 4.02	0.506*
School leaving certificate			
without / elementary school	21 (30.4 %)	4 (19.0 %)	0.506§
secondary school level	29 (42.0 %)	8 (38.1 %)	
advanced technical college entrance qualification	8 (11.6 %)	2 (9.52 %)	
general qualification for university entrance	11 (16.0 %)	7 (33.3 %)	
Vocational status			
without	5 (7.24 %)	1 (4.76 %)	0.386§
part-craftsman	0	1 (4.76 %)	
certificate of apprenticeship	34 (49.3 %)	7 (33.3 %)	
master craftsman´s diploma	3 (4.35 %)	1 (4.76 %)	
technician	3 (4.35 %)	0	
advanced technical college/ university degree	11 (15.9 %)	6 (28.6 %)	
commercial school	10 (14.5 %)	5 (23.8 %)	
Socio economic class			
lower class	6 (8.70 %)	3 (14.3 %)	0.113§
middle class	44 (63.8 %)	8 (11.6 %)	
upper class	19 (27.5 %)	10 (47.6 %)	

$ Fisher`s exact test, * Student-t-test, #BMI: body mass index, § chi-square test

Table 3. **Concentrations of CRP, homocysteine, methyl malonic acid, and B vitamins.**

	Group A (Hp-positive) [n=69]	*Group B (Hp-negative)* [n=21]	*P*
Serum C-reactive protein [mg/L]			
Mean ± SD	3.72 ± 2.53	2.76 ± 0.97	**0.011***
Median	2.8	2.3	
% >3 mg/L	49.3	42.9	0.628$
Plasma homocysteine [µmol/L]			
Mean ± SD	13.3 ± 5.00	13.5 ± 3.03	0.321^
Median	12.4	13.4	
% >12 µmol/L	53.6%	71.4%	0.208$
Serum methyl malonic acid [µmol/L]			
Mean ± SD	179 ± 81.5	164 ± 54.2	0.633^
Median	161	152	
% >271 µmol/L	8.70%	4.76%	>0.999$
Serum folic acid [nmol/L]			
Mean ± SD	23.7 ± 7.20	24.0 ± 8.15	0.851*
Median	22.9	26.7	
% <7 nmol/L	0%	0%	/
Serum vitamin B_{12} [pmol/L]			
Mean ± SD	274 ± 95.3	333 ± 164	0.130*
Median	269	300	
% <258 pmol/L	46.4%	38.1%	0.618$
Plasma vitamin B_6 [nmol/L]			
Mean ± SD	162 ± 196	107 ± 45.9	0.970^
Median	89.3	111	
% <25 nmol/L	0%	0%	/

* Student-t-test, $ Fisher`s exact test, ^ Mann-Whitney U test

Table 4. **Correlations between status of B vitamins, MMA, tHcys, CRP and BMI.**

Correlations	r_s	p
Vitamin B_{12} [pmol/L] and Methyl malonic acid [µmol/L]	-0.118	0.267
Vitamin B_{12} [pmol/L] and Homocysteine [µmol/L]	-0.223	**0.035**
Vitamin B_6 [nmol/L] and Homocysteine [µmol/L]	-0.057	0.595
Folic acid [nmol/L] and Homocysteine [µmol/L]	-0.440	**<0.001**
C-reactive protein [mg/L] and Homocysteine [µmol/L]	-0.003	0.979
C-reactive protein [mg/L] and BMI [kg/m^2]	0.186	0.083

(p=0.028; eta square: 0.055). Sex showed a significant impact on homocysteine (p=0.008; eta square: 0.079). Serum concentrations of folic acid, methyl malonic acid, CRP, and plasma concentration of vitamin B_6 were not associated to age, sex and *Hp*-status.

Further multivariate analysis showed that the *Hp*-status had an impact on vitamin B_{12} values (p=0.028; eta square: 0.057) only.

DISCUSSION

Observational studies have suggested that a chronic infection with *Hp* might be an independent, although weak, risk factor for CVD [24,58]. It has been hypothesized that there are indirect mechanisms that could link *Hp* with atherosclerosis. One hypothesis postulates that the gastric damage induced by *Hp*-infection may affect atherogenesis by lower vitamin bioavailability and increased serum homocysteine levels [12]. As a sulfur-containing intermediate product in the metabolism of methionine, homocysteine is a highly reactive molecule. It has been demonstrated that hyperhomocysteinemia is associated with endothelial dysfunction, alterations in vascular morphology and other atherogenetic mechanisms [6,7,45]. Because folic acid, vitamin B_{12}, and vitamin B_6 are involved in the breakdown of homocysteine [6,7,45], it seems to be plausible that decreasing intestinal absorption of these B vitamins by *Hp* induced atrophic gastritis should elevate homocysteine levels, hence increasing the risk of CVD. However, biochemical plausibility does not implicit proof. Actually the results of our study do not support the assumption that *Hp*-infection is associated with significantly lower serum vitamin B_{12} levels, compared to *Hp*-negative subjects [18,21,59]. In adults, the limit between sufficient and insufficient vitamin B_{12} status is probably between serum concentrations of 220-258 pmol/L [6]. In comparison with the defined cutoff of <258 nmol/L, the mean vitamin B_{12} level (273 ± 94.8 nmol/L) of the *Hp*-infected people in this study is well above it. Only 46.4 % have values below <258 pmol/L. In addition, the mean serum concentration of MMA (179 ± 81.5) suggest that *Hp*-infection by itself is not associated with subclinical cobalamin deficiency. This interpretation is in consistence to the findings of other investigators. For example, neither in elderly individuals [60,61], nor in patients with diabetes mellitus type 2 [17] and women with peripheral artery disease [59] there was an association between *Hp*-infection and subclinical cobalamin deficiency. However, it should be reminded that manifestation of vitamin B_{12} deficiency occurs slowly over years due to the low requirement and the liver storages [6,7,62]. Taken this and the results of a study by Carmel et al. [63] showing that *Hp*-infection is associated with lower food cobalamin absorption, chronic *Hp*-infection could affect cobalamin status in the long run. This might be especially the case if *Hp*-infection induces more severe gastric damage [16]. Beside of this, the results of our study suggest that it is unlikely that *Hp*-induces folate deficiency and thus leads to hyperhomocysteinemia. We were not able to find any differences in the folate and homocysteine levels between *Hp*-positive and *Hp*-negative volunteers. This results are in accordance with the findings of several other observational studies [15,16,19,21,59,64-66]. For example Bloemenkamp et al. [59] investigated 150 women with peripheral artery disease and 412 control women. In this study, neither folate nore homocysteine levels were different between *Hp*-positive and *Hp*-negative subjects. Accordingly the authors conclude that the relation between *Hp* and atherosclerosis cannot be explained by a high homocysteine concentration [59]. In contrast to our results, other studies reported significantly higher homocysteine concentrations in *Hp*-infected patients [17,18,67]. However, it should be noted that the association between *Hp*-infection and homocysteine levels in this studies was not very strong [18,67] and differences in folate status could not be detected [17]. Overall, atrophic gastritis, rather than *Hp*-infection by itself, seems to induce hyperhomocysteinemia [16,20].

Beside the "homocysteine CVD connection", infection with *Hp* may lead to a chronic low-grade systemic inflammation response, characterised by increased plasma levels of proinflammatory cytokines and acute-phase proteins such as CRP [24-27]. CRP is well recognized as an independent predictor of CVD [28-30]. Furthermore, CRP may directly promote atherosclerosis [31,32]. However, the role of *Hp* in the pathogenesis of atherosclerosis remains controversial. While several seroepidemiological studies have shown an association between *Hp* and atherosclerosis, a meta-analysis revealed only limited evidence of a positive relationship [58]. In the present study *Hp*-infection was significantly associated with elevated CRP levels. These finding is supported by the results of other observational studies [68-71]. In contrary,

other investigators were not able to detect such a finding [72-74]. As the results of some studies suggest, not a single infection, but mostly an infection with multiple pathogens could cause a chronic low-grade inflammation [75,76]. As shown in the method part, we have not carried out tests to detect other proinflammatory pathogens such as *Chlamydia pneumoniea* and cytomegalovirus. Therefore we could not rule out the possibility that the differences in CRP levels between *Hp*-positive and *Hp*-negative volunteers shown in this study can be traced back to multiple pathogens, rather than to *Hp* itself. Mechanistically, increasing levels of CRP may be the earliest event in vascular inflammatory process, inducing endothelial dysfunction, the first step in atherosclerosis [68,77]. There are several mechanisms CRP could induce a proatherogenic environment in endothelial cells including the following [32]: (i) decreasing prostacyclin and nitric oxide synthesis, (ii) increasing endothelin-1 concentration and cell adhesion molecules such as monocyte chemoattractant protein-1. Furthermore, in vascular smooth muscle cells CRP has been shown to (i) increase NF kappa B and (ii) upregulate angiotensin type-1 receptor resulting in increased reactive oxygen species and vascular smooth muscle cell proliferation. Taken this findings together with the results of observational studies that CRP levels >3mg/L predict future risk for CVD in apparently healthy subjects [78], our study supports the hypothesis that infection with *Hp* might be a causal component in the development of CVD through CRP [76,79]. However, based on our results it seems unlikely that *Hp*-infection is related to CVD by elevated levels of tHcy.

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