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Helicobacter Pylori Infection Causes Persistent Platelet Activation In Vivo Through Enhanced Lipid Peroxidation

Giovanni Davì, Matteo Neri, Angela Falco, Davide Festi, Tea Taraborelli, Giovanni Ciabattoni, Stefania Basili, Franco Cuccurullo, Carlo Patrono

Objective—We aimed at investigating the relationship between *Helicobacter pylori* infection and in vivo lipid peroxidation and platelet activation, as reflected by urinary 8-iso-prostaglandin (PG) $F_{2\alpha}$ and 11-dehydro-thromboxane (TX) B_2 , respectively, in otherwise healthy dyspeptic subjects.

Methods and Results—We measured urinary 8-iso-PGF $_{2\alpha}$ and 11-dehydro-TXB $_2$ excretion in 40 dyspeptic subjects with a positive ^{13}C -urea breath test and 38 dyspeptic individuals with a negative test. Moreover, we investigated the effects of *H pylori* eradication on prostanoid metabolite excretion in 23 *H pylori*-positive subjects. We also measured prostanoid metabolite excretion before and after selective cyclooxygenase-2 inhibition with rofecoxib in 4 *H pylori*-positive subjects. Urinary 8-iso-PGF $_{2\alpha}$ and 11-dehydro-TXB $_2$ excretion was significantly higher in the *H pylori*-positive individuals than in controls. A significant direct correlation was found between the degree of positivity to the ^{13}C -urea breath test and urinary 8-iso-PGF $_{2\alpha}$ excretion. The latter was linearly correlated with urinary 11-dehydro-TXB $_2$. Successful eradication of *H pylori* infection led to a significant reduction in both 8-iso-PGF $_{2\alpha}$ and 11-dehydro-TXB $_2$. Furthermore, their levels were unaffected after treatment with rofecoxib.

Conclusions—Our study provides evidence of enhanced in vivo lipid peroxidation and platelet activation in association with *H pylori* infection and suggests a novel mechanism by which an infectious agent could contribute to atherothrombosis. (*Arterioscler Thromb Vasc Biol.* 2005;25:246-251.)

Key Words: risk factors ■ oxidant stress ■ platelets ■ infection ■ inflammation

Inflammatory mechanisms have been implicated in the pathogenesis of atherosclerosis, and a significant association between infectious burden and the extent and long-term prognosis of atherosclerosis has been reported.¹

Helicobacter pylori infection represents one of the most widespread human infectious diseases.² Several studies have reported an association between infection with *H pylori* and incidence of vascular disease, particularly coronary heart disease (CHD).³ However, most of these data derived from cross-sectional and retrospective studies and potential confounders, such as socioeconomic status or smoking pattern,^{3,4} may complicate their interpretation. Analysis restricted to the available prospective studies in socioeconomically homogeneous populations provides limited evidence for an association between *H pylori* and CHD.⁵ However, a case-control and sibling pairs study of early-onset myocardial infarction reported strong evidence for an association between *H pylori* infection and CHD, suggesting that the potential proinflammatory effects of *H pylori* might be of greater importance at younger ages.⁶

Experimental studies have shown that *H pylori* induces platelet aggregation in gastric mucosal microcirculation.^{7,8}

However, no previous study has examined the occurrence and mechanism(s) of platelet activation in vivo that might be associated with *H pylori* infection in humans.

Several cardiovascular risk factors are associated with low-grade inflammation, increased oxidant stress, and lipid peroxidation.⁹ F $_2$ -isoprostanes, a family of bioactive prostaglandin (PG)F $_2$ -like compounds¹⁰ produced from arachidonic acid through a nonenzymatic process of lipid peroxidation catalyzed by oxygen-free radicals on cell membranes and low-density lipoprotein (LDL) particles,⁹ represent a reliable marker of in vivo lipid peroxidation. Among F $_2$ -isoprostanes, of particular interest is 8-iso-PGF $_{2\alpha}$, which induces vasoconstriction and modulates function of human platelets.¹¹ Measurement of urinary F $_2$ -isoprostanes has been used extensively in clinical settings putatively associated with increased oxidant stress.⁹

In the present study, we tested the hypothesis that low-grade inflammation associated with *H pylori* infection³ would induce increased in vivo lipid peroxidation with generation of 8-iso-PGF $_{2\alpha}$ and other biologically active isoeicosanoids and that these compounds would in turn contribute to platelet

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From the Center of Excellence on Aging (G.D., M.N., A.F., D.F., T.T., G.C., F.C.), Fondazione Università "G. d'Annunzio," and Departments of Medicine and Drug Sciences, University of Chieti "G. d'Annunzio" Schools of Medicine and Pharmacy, Italy; and Departments of Medical Therapy (S.B.) and Pharmacology (C.P.), University of Rome "La Sapienza," Italy.

Reprint requests to: Carlo Patrono, MD, Center of Excellence on Aging, Fondazione Università "G. d'Annunzio," Via Colle dell'Ara, 66013, Chieti, Italy. E-mail cpatrono@unich.it

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Baseline Characteristics of *H Pylori*-Positive and -Negative Subjects

Variable	<i>H Pylori</i> -Positive (n=40)	<i>H Pylori</i> -Negative (n=38)
Age, years	47 (30–70)	47 (26–61)
Fasting blood glucose, mg/dL	92 (63–112)	92 (69–110)
Systolic blood pressure, mm Hg	117 (95–140)	125 (95–140)
Diastolic blood pressure, mm Hg	80 (60–85)	80 (60–85)
Total cholesterol, mg/dL	182 (150–200)	182 (155–194)
LDL cholesterol, mg/dL	113 (55–140)	113 (61–138)
HDL cholesterol, mg/dL	55 (40–75)	51 (35–78)
Body weight, kg	71 (42–84)	75 (48–86)
BMI	23.9 (20.5–27.3)	24.0 (21.3–27.5)

HDL indicates high-density lipoprotein; BMI, body mass index. Data are presented as median (range).

activation in this setting. The latter was evaluated by measuring urinary excretion of 11-dehydro-thromboxane (TX)_{B2}, a stable enzymatic derivative of TXA₂, a labile eicosanoid that amplifies platelet activation in response to other stimuli and induces irreversible platelet aggregation.¹²

Thus, the aim of our study was to investigate the causal relationship between *H pylori* infection and the rates of in vivo lipid peroxidation and platelet activation in otherwise healthy dyspeptic subjects through biochemical measurements and pharmacological interventions.

Methods**Study Participants**

We initially studied 28 dyspeptic men aged <60 years who had a first-time positive ¹³C-urea breath test (UBT) for *H pylori* infection and had not received antibiotic treatment previously. Twenty-eight age-matched dyspeptic men who had a negative UBT for *H pylori* infection were recruited as a control group. To investigate potential gender-related differences, we performed a second cross-sectional study in 12 dyspeptic women aged <60 years with a first-time positive UBT who had not been treated previously. Ten age-matched dyspeptic women who had a negative UBT were enrolled as a control group. Because the results of the 2 studies were internally consistent, the data are presented as a whole. The clinical characteristics of study subjects are summarized in the Table.

To avoid confounding by other determinants of oxidant stress and platelet activation, subjects were excluded if they had a history or evidence of atherothrombotic diseases, diabetes mellitus, cigarette smoking, dyslipidemia, and arterial hypertension, as well as current or recent (<3 months) systemic or localized infections. Moreover, dyspeptic subjects were excluded if they were taking low-dose aspirin, nonsteroidal anti-inflammatory drugs, or vitamin supplements. Informed consent was obtained from each participating subject, and the protocol was approved by the University of Chieti ethics committee.

Study Design

A cross-sectional comparison of urinary 8-iso-PGF_{2α} and 11-dehydro-TXB₂, a major enzymatic metabolite of TXA₂,¹³ was performed between subjects positive and negative for *H pylori* infection. All participants were studied as outpatients after a 12-hour fast. Each subject performed an overnight urine collection. Urine samples were added with the antioxidant 4-hydroxy-Tempo (1 mmol/L; Sigma) and stored at -20°C until extraction.

To examine the effects of *H pylori* eradication on indexes of lipid peroxidation and platelet activation, an open pilot intervention study

was performed in *H pylori*-positive subjects. Thus, we investigated the effects of a standard antibiotic therapy on urinary 8-iso-PGF_{2α} and 11-dehydro-TXB₂ excretion in 15 of the 28 *H pylori*-positive men and in 8 of the 12 *H pylori*-positive women. Entry criteria were a clear indication of antibiotic therapy, lack of contraindications, and willingness to participate in this additional study. These subjects were given 1000 mg amoxicillin twice daily plus 500 mg clarithromycin twice daily for 7 days. A standard regimen of a proton pump inhibitor (20 mg omeprazole twice daily) was also given.¹⁴ Before and after treatment, participants were instructed to perform an overnight urine collection. At least 4 weeks after completing the triple therapy, the UBT was repeated to assess eradication. In case of treatment failure (11 subjects), a new treatment regimen was prescribed. Five subjects who were found still infected after the first course of therapy were treated with 500 mg clarithromycin twice daily and 500 mg tinidazole twice daily for 7 days. A dosage of 400 mg ranitidine bismuth citrate twice daily was also given. Six subjects who failed to respond to the first treatment regimen were lost to follow-up.

Because *H pylori* infection is associated with cyclooxygenase-2 (COX-2) expression in the gastric mucosa^{15–17} and 8-iso-PGF_{2α} and TXA₂ can be formed by human monocytes through a COX-2-dependent mechanism,¹⁸ a third study was performed to evaluate whether selective inhibition of COX-2 activity had any influence on 8-iso-PGF_{2α} in subjects positive for *H pylori* infection. For this purpose, 4 of the *H pylori*-positive subjects were given 12.5 mg rofecoxib, a highly selective COX-2 inhibitor,¹⁹ once daily for 7 days. Participants collected overnight urine samples at the beginning and the end of the rofecoxib treatment for measurement of 8-iso-PGF_{2α} and 11-dehydro-TXB₂ excretion.

Analytical Measurements

The presence of *H pylori* was determined by UBT. UBT consisted of a baseline breath sample and a second sample 30 minutes after administration of 75 mg of ¹³C-labeled urea (Isotec) dissolved in orange juice.²⁰ Subjects were fasted overnight. The ¹³C-enrichment in breath was analyzed using an isotope ratio mass spectrometer (ABCA; Europa Scientific) The UBT was considered positive when the difference with baseline at 30' (DOB30; ie, the difference between δ-value at 30' and δ-value at baseline) was ≥5%. This noninvasive test is as accurate in predicting *H pylori* status as invasive tests and is the recommended test for diagnosis of *H pylori* infection.²¹ Urinary 8-iso-PGF_{2α} and 11-dehydro-TXB₂ were measured by previously described and validated radioimmunoassay methods.^{22,23}

Statistical Analysis

Data were analyzed by nonparametric methods to avoid assumptions about the distribution of the measured variables. An ANOVA was performed with the Kruskal-Wallis method. Subsequent pairwise comparisons were made with the Mann-Whitney *U* test. Differences between baseline and post-treatment values were analyzed with the Wilcoxon signed-rank test. Moreover, the association of eicosanoid measurements with other biochemical parameters was assessed by the Spearman rank correlation test. A multiple linear regression analysis was performed to further quantify the relationship between 11-dehydro-TXB₂ excretion and the other variables in the cross-sectional studies.

The cross-sectional and intervention studies had a >80% power to detect an *H pylori*-related difference in urinary 11-dehydro-TXB₂ excretion of 1 SD between groups with a 2-tailed α of 0.05.

All values are reported as median (range). *P* values <0.05 were regarded as statistically significant. All tests were 2-tailed, and analyses were performed using a computer software package (Statistica 1999 edition; StatSoft; or Statistical Package for the Social Sciences, version 12.0; SPSS).

Results

Urinary 8-iso-PGF_{2α} excretion was significantly higher in the 40 *H pylori*-positive than in the 38 *H pylori*-negative

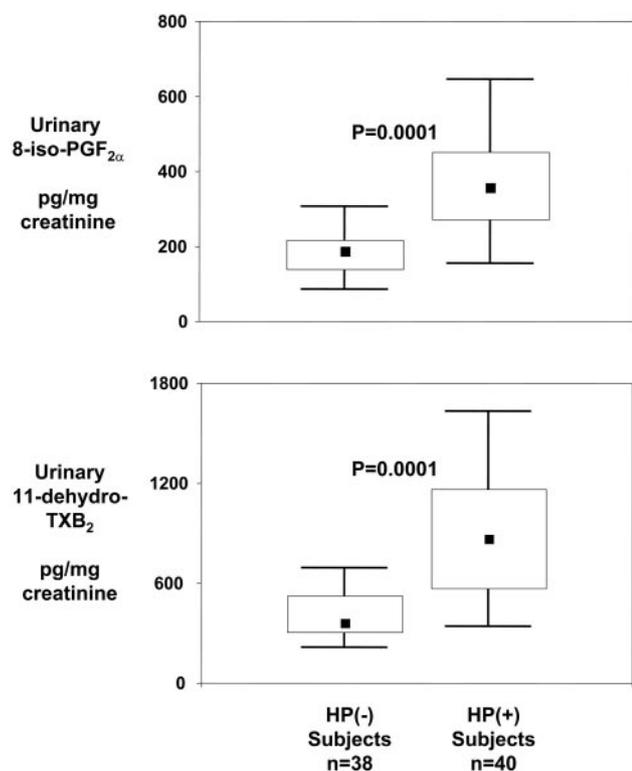


Figure 1. Urinary excretion of 8-iso-PGF_{2α} (top) and 11-dehydro-TXB₂ (bottom) in *H pylori*-negative vs *H pylori*-positive subjects. Error bars represent minimum and maximum values; ■, median values; boxes, interquartile range.

subjects (357 [154 to 645] pg/mg versus 189 [85 to 306] pg/mg creatinine; median (range) $P=0.0001$; Figure 1). Urinary 11-dehydro-TXB₂ excretion rate was also significantly increased in *H pylori*-positive compared with *H pylori*-negative subjects (868 [339 to 1630] pg/mg versus 378 [212 to 690] pg/mg creatinine; $P=0.0001$; Figure 1). In the 40 *H pylori*-positive individuals, a statistically significant direct correlation was found between DOB30 in breath samples and urinary 8-iso-PGF_{2α} excretion rates ($\rho=0.505$; $P=0.0016$). The latter index of lipid peroxidation was in turn linearly correlated with the rate of TX biosynthesis, as reflected by urinary 11-dehydro-TXB₂ excretion ($\rho=0.52$; $P=0.0012$).

A multiple regression analysis performed in 78 subjects showed that DOB30 in breath samples (regression coefficient 0.44; SE 0.11; $P=0.000128$), male sex (regression coefficient 0.39; SE 0.08; $P=0.0150$), and urinary 8-iso-PGF_{2α} excretion rates (regression coefficient 0.39; SE 0.11; $P=0.000456$) independently correlated with 11-dehydro-TXB₂ excretion.

Effects of Eradication Therapy

We also investigated the effects of *H pylori* eradication on urinary 8-iso-PGF_{2α} and 11-dehydro-TXB₂ excretion to test the hypothesis of a cause-effect relationship between *H pylori* infection and enhanced lipid peroxidation and platelet activation in this setting. Thus, we evaluated the effects of eradication therapy in 15 *H pylori*-positive men and 8 *H pylori*-positive women.

Successful eradication, achieved in 12 of the 23 treated patients, was associated with a statistically significant reduc-

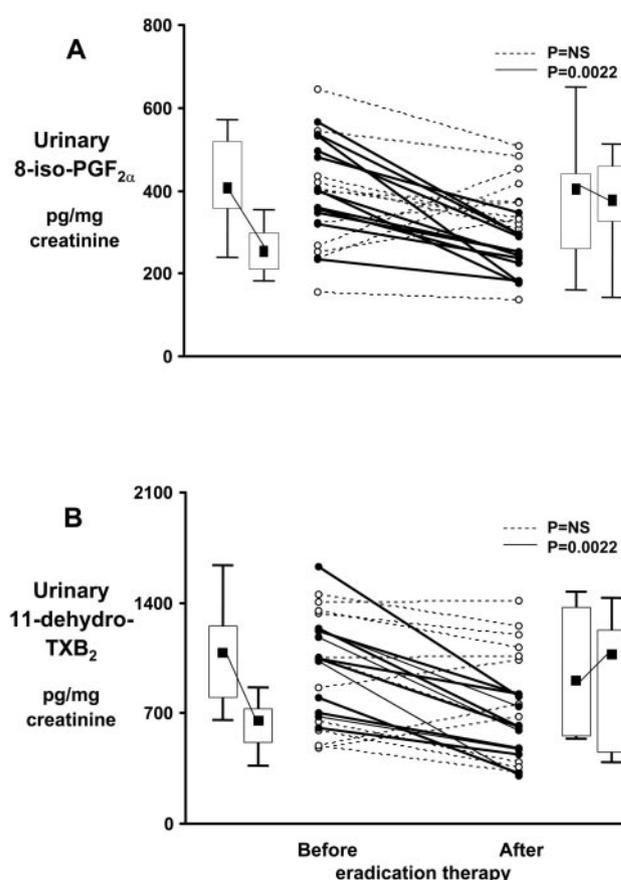


Figure 2. Effects of antibiotic treatment on urinary 8-iso-PGF_{2α} (top) and 11-dehydro-TXB₂ (bottom) in *H pylori*-positive subjects. Dots and lines connecting dots represent individual measurements; solid symbols and continuous lines, values of subjects with successful eradication; open symbols and broken lines, values of subjects with unsuccessful eradication; error bars, minimum and maximum values; ■, median values; boxes, interquartile range, represented on the left and on the right side of each panel.

tion in 8-iso-PGF_{2α} (from 400 [233 to 566] pg/mg to 247 [176 to 348] pg/mg creatinine; $P=0.0022$) and 11-dehydro-TXB₂ urinary excretion (from 1049 [606 to 1630] pg/mg to 600 [303 to 822] pg/mg creatinine; $P=0.0022$). Excretion rates of the 2 metabolites remained substantially unchanged in association with unsuccessful eradication in the other 11 subjects (Figure 2). The coefficient of variation for 3 repeated measurements of urinary 8-iso-PGF_{2α} and 11-dehydro-TXB₂, obtained from 7 subjects who failed eradication therapy, averaged $21.3 \pm 10.3\%$ and $18.2 \pm 10.5\%$, respectively.

In 5 of the 11 subjects in whom *H pylori* failed to be eradicated, a different treatment regimen was prescribed after a period of 10 ± 2 weeks. This second cycle of therapy led to successful eradication in 3 subjects, with a consistent reduction in 8-iso-PGF_{2α} (from 371 [331 to 483] pg/mg to 285 [132 to 288] pg/mg creatinine) and 11-dehydro-TXB₂ excretion (from 1040 [680 to 1200] pg/mg to 327 [254–375] pg/mg creatinine), whereas urinary levels of both metabolites remained unchanged in the 2 subjects who failed to be eradicated (data not shown).

In the 15 subjects who eventually achieved successful eradication (12 after the first regimen plus 3 after the second

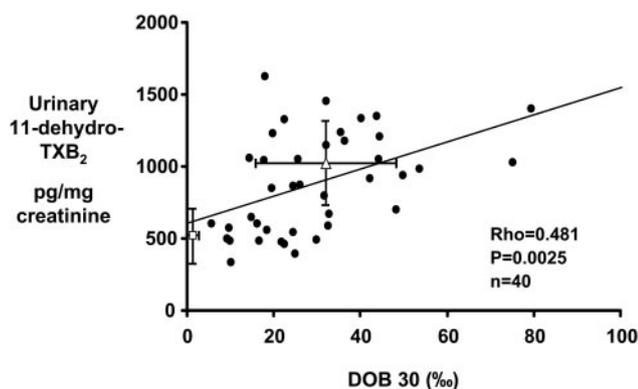


Figure 3. Correlation between DOB30 and urinary 11-dehydro-TXB₂ excretion in *H pylori*-positive subjects. The 40 data points represent the baseline measurements for the 28 and 12 dyspeptic men and women, respectively. Of these, 15 and 8 were subjected to treatment. Twelve subjects responded to the first treatment regimen, and the average results for these 12 before treatment are shown by the Δ . The average results for these 12 responding subjects after treatment are shown by the \square .

regimen), the reduction in DOB30 was associated with a fall in TX biosynthesis, the average extent of which showed a remarkably good fitting with the linear relationship between DOB30 values and 11-dehydro-TXB₂ excretion rates, as established in the whole group of *H pylori*-positive subjects at baseline (Figure 3).

Effects of Selective COX-2 Inhibition

Urinary 8-iso-PGF_{2 α} and 11-dehydro-TXB₂ excretion rates were not affected after 1 week of selective COX-2 inhibition achieved with rofecoxib (from 280 [247 to 338] pg/mg to 303 [259 to 320] pg/mg, and from 553 [463 to 578] pg/mg to 579 [363 to 606] pg/mg creatinine, respectively). This finding is consistent with a non-COX-dependent mechanism of F₂-isoprostane formation in association with *H pylori* infection. Moreover, these results demonstrate that enhanced TX biosynthesis is not a byproduct of COX-2 expression in response to *H pylori* infection.

Discussion

Atherothrombotic events do occur among individuals without readily apparent cardiovascular risk factors.²⁴ In recent years, inflammation has been suggested to play a key role in the initiation and progression of the atherosclerotic process.²⁵ Circulating markers of persistent low-grade inflammation, such as C-reactive protein, can predict recurrence of major vascular events in patients with established ischemic heart disease,^{26–28} as well as the risk of a first myocardial infarction in apparently healthy subjects.²⁹ Persistent infections may represent a potential trigger of systemic inflammation, and evidence for a link between total infectious burden and atherosclerotic severity has been provided.^{1,30,31} A weak and controversial association between *H pylori* infection and CHD has been described.³² *H pylori*, a primary pathogen for peptic ulcer disease, gastric cancer, and lymphoma² is a potential source of inflammatory cytokines possibly contributing to the atherosclerotic process.³³ It has been hypothesized that *H pylori* infection might modify serum lipid concentrations, thus increasing the risk for cardiovascular

disease.³⁴ Moreover, *H pylori* is independently associated with increased fibrinogen levels in healthy subjects,³⁵ and an association between an IgG antibody response to multiple pathogens, including *H pylori*, and endothelial dysfunction has been reported.³⁶ Finally, it has been suggested that chronic atrophic gastritis induced by *H pylori*, causing malabsorption of vitamin B₁₂ and folate, may lead to increased plasma levels of homocysteine, a known risk factor for vascular disease.³⁷ *H pylori*-specific DNA has been detected recently in atheromatous plaques of patients with severe coronary artery disease, supporting the hypothesis of direct involvement of the bacterium in the progression and instability of atherosclerotic lesions.³⁸

An important link has been shown between coronary artery disease and infection with *H pylori*, and its eradication significantly attenuated the reduction in coronary lumen after coronary angioplasty.³⁹ Moreover, *H pylori* eradication increased high-density lipoprotein cholesterol and decreased C-reactive protein, thrombin-antithrombin complexes, and lipoprotein(a) levels in type 1 diabetic patients.⁴⁰

In the present study, we have identified a novel mechanism through which *H pylori* infection may enhance cardiovascular risk (ie, persistent platelet activation). Biochemical evidence of enhanced platelet activation in vivo in association with *H pylori* infection was obtained through noninvasive measurements of TX metabolite excretion that avoid artifactual platelet activation during and after blood sampling.⁴¹ The exclusion criteria used to recruit dyspeptic otherwise healthy subjects avoided confounding by traditional cardiovascular risk factors that can affect the rate of platelet activation. It should be emphasized that TXA₂ biosynthesis measured in *H pylori*-positive subjects in the present study is comparable to that reported previously in association with traditional cardiovascular risk factors such as hypercholesterolemia,⁴² diabetes mellitus,⁴³ obesity,⁴⁴ and hypertension.⁴⁵

Furthermore, we characterized a putative biochemical link between *H pylori* infection and platelet activation by investigating the in vivo formation of F₂-isoprostanes, as reflected by the urinary excretion of the PGF_{2 α} isomer 8-iso-PGF_{2 α} . This family of bioactive isoecosanoids is produced through free radical-catalyzed peroxidation of arachidonic acid that can occur on cell membranes and LDL particles.¹⁰ Measurement of unmetabolized F₂-isoprostanes in plasma and urine has proved to be a valuable approach to assess the actual rate of lipid peroxidation in vivo.^{9,10} Repeated measurements of 8-iso-PGF_{2 α} in those subjects in whom *H pylori* eradication failed demonstrated a persistent abnormality with limited intrasubject variability over time. Evidence for a cause-and-effect relationship between *H pylori* infection and enhanced lipid peroxidation was provided by the linear correlation between the ¹³C-UBT and urinary of 8-iso-PGF_{2 α} excretion rates as well as by the statistically significant reduction in F₂-isoprostane formation after successful eradication (Figure 2). The nonenzymatic nature of 8-iso-PGF_{2 α} production in this setting was confirmed by the failure of rofecoxib, a highly selective COX-2 inhibitor,¹⁹ to reduce its urinary excretion to any detectable extent.

Enhanced formation of 8-iso-PGF_{2 α} in *H pylori*-positive subjects correlated with increased TXA₂ biosynthesis, as

reflected by 11-dehydro-TXB₂ excretion. Although the systemic concentrations of 8-iso-PGF_{2α} may be too low to trigger platelet activation, this autacoid can synergize with sub-threshold concentrations of other agonists in inducing platelet adhesion and aggregation.^{46,47} Moreover, it is likely that enhanced formation of 8-iso-PGF_{2α} as a consequence of *H pylori* infection is associated with the release of other bioactive isoeicosanoids formed through the same mechanism of oxygen radical-catalyzed peroxidation of arachidonic acid. To assess the potential contribution to TXA₂ biosynthesis of COX-2 expressed by inflammatory and epithelial cells in response to *H pylori* infection, we investigated the short-term effects of rofecoxib on 11-dehydro-TXB₂ excretion in *H pylori*-positive subjects. The results of this intervention study are consistent with COX-1 being the main COX isoform involved in TXA₂ biosynthesis in this setting and make it unlikely that the reduction in 11-dehydro-TXB₂ excretion associated with successful *H pylori* eradication is a reflection of reduced COX-2 expression in the gastric mucosa.¹⁶

The findings in *H pylori*-positive patients extend similar observations made in other clinical settings such as hypercholesterolemia,⁴² diabetes mellitus,⁴³ cigarette smoking,^{48,49} homozygous homocystinuria,⁵⁰ visceral obesity,⁴⁴ and renovascular hypertension.⁵¹ Thus, regardless of the mechanism(s) responsible for enhanced lipid peroxidation, there is quite convincing evidence from studies in such diverse conditions that enhanced generation of bioactive isoeicosanoids may transduce the oxidant signal associated with a variety of cardiovascular risk factors into a functional platelet response, possibly contributing to enhanced thrombotic risk.

In conclusion, the present study provides biochemical evidence of enhanced *in vivo* lipid peroxidation and platelet activation in dyspeptic individuals with *H pylori* infection and identifies a novel mechanism through which an infectious agent could contribute to development of atherothrombosis. Reversibility of the hemostatic abnormality after successful eradication of *H pylori* may have clinical implications for cardiovascular risk management.

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