

Effect of *Helicobacter pylori* infection and its eradication on cell proliferation, DNA status, and oncogene expression in patients with chronic gastritis

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Abstract

Background—*Helicobacter pylori*, the main cause of chronic gastritis, is a class I gastric carcinogen. Chronic gastritis progresses to cancer through atrophy, metaplasia, and dysplasia. Precancerous phenotypic expression is generally associated with acquired genomic instability.

Aim—To evaluate the effect of *H pylori* infection and its eradication on gastric histology, cell proliferation, DNA status, and oncogene expression.

Methods/Subjects—Morphometric and immunohistochemical techniques were used to examine gastric mucosal biopsy specimens from eight controls, 10 patients with *H pylori* negative chronic gastritis, 53 with *H pylori* positive chronic gastritis, and 11 with gastric cancer.

Results—All patients with chronic gastritis were in a hyperproliferative state related to mucosal inflammation, regardless of *H pylori* infection. Atrophy was present in three of 10 patients with *H pylori* negative chronic gastritis and in 26 of 53 with *H pylori* positive chronic gastritis, associated in 18 with intestinal metaplasia. DNA content was abnormal in only 11 patients with atrophy and *H pylori* infection; eight of these also had c-Myc expression, associated in six cases with p53 expression. Fifty three patients with *H pylori* positive chronic gastritis were monitored for 12 months after antibiotic treatment: three dropped out; infection was eradicated in 45, in whom cell proliferation decreased in parallel with the reduction in gastritis activity; atrophy previously detected in 21/45 disappeared in five, regressed from moderate to mild in nine, and remained unchanged in seven; complete metaplasia disappeared in 4/14, and markers of genomic instability disappeared where previously present. In the five patients in whom *H pylori* persisted, atrophy, metaplasia, dysplasia, and markers of genomic instability remained unchanged.

Conclusions—Chronic *H pylori* infection seems to be responsible for genomic instability in a subset of cases of *H pylori* positive chronic atrophic gastritis; eradication of *H pylori* infection can reverse inflammation and the related atrophy, metaplasia, and genomic instability.

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Keywords: *H pylori* infection; atrophic gastritis; genomic instability; eradication therapy

Helicobacter pylori, the main cause of chronic gastric disorders, has been defined as a class I gastric carcinogen.¹ However, only a minority of *H pylori* positive patients with chronic gastritis develop gastric cancer, and the link between *H pylori* and gastric carcinoma is unclear.²

Gastric carcinogenesis is a multistep process progressing from chronic gastritis through glandular atrophy, metaplasia, and dysplasia.³⁻⁶ Atrophic gastritis and intestinal metaplasia may be the long term consequences of *H pylori* infection,^{7,8} but it is debatable whether or not bacterial eradication reverses these lesions.⁹⁻¹² Acquired genomic instability, which is typical of the phenotypic expression of precancerous lesions, generally precedes neoplastic clonal expansion.^{13,14}

Chronic *H pylori* infection damages gastric barrier function^{15,16} and stimulates gastric cell proliferation,^{3,17-22} which leads to mucosal repair,^{22,23} but which can also induce cellular DNA damage.^{3,17-22,24,25} The most frequent epiphenomenon of DNA alteration is activation of oncogenes and/or mutation of oncosuppressor genes.²⁶⁻²⁹ The role of these genes has been studied in colon carcinogenesis³⁰⁻³⁴ and, to a lesser extent, in gastric carcinogenesis,³⁴⁻³⁹ but their interrelation with *H pylori* infection has yet to be defined. The aim of our study was to detect the relation between *H pylori* infection and gastric carcinogenesis evaluated as the appearance of genomic instability and the associated phenotypic expression. To this aim we investigated gastric histology, cell proliferation, DNA content, and bcl-2, p53, and c-Myc expression in patients with chronic gastritis with and without *H pylori* infection and after *H pylori* eradication therapy.

Materials and methods

SUBJECTS

The study population consisted of 82 subjects (52 men and 30 women, age range 24-75 years). They were consecutively recruited from 1 January 1995 to 30 June 1995 from a population of 680 subjects referred to our department for dyspepsia according to the following

Abbreviations used in this paper: PCNA, proliferating cell nuclear antigen; AgNOR, nucleolar organiser region.

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Table 1 Frequency of clinical, histological and immunohistochemical variables in the population studied

	No of (subjects)	Age (y)	Sex (% men)	Erosion/ulcer	PMN	Lymphocytes/monocytes	Atrophy	IM	Dysplasia	DNA aneuploidy	p53	bcl-2	c-Myc
Controls	8	35 (10)	37	-	-	-	-	-	-	-	-	100	-
nCG	10	44 (11)	50	30	-	++	30	10	-	-	-	100	-
pCG	53	50 (11)	69	55	+++	+	49	34*	6	21	15	100	15
GC	11	63 (7)	90	-	-	-	-	-	-	100	64	27	36

Values are percentages except for age which is mean (SD). nCG, *H pylori* negative chronic gastritis; pCG, *H pylori* positive chronic gastritis; GC, gastric cancer; PMN, polymorphonuclear leucocytes; IM, intestinal metaplasia (*total number 18: 16 complete and two incomplete).

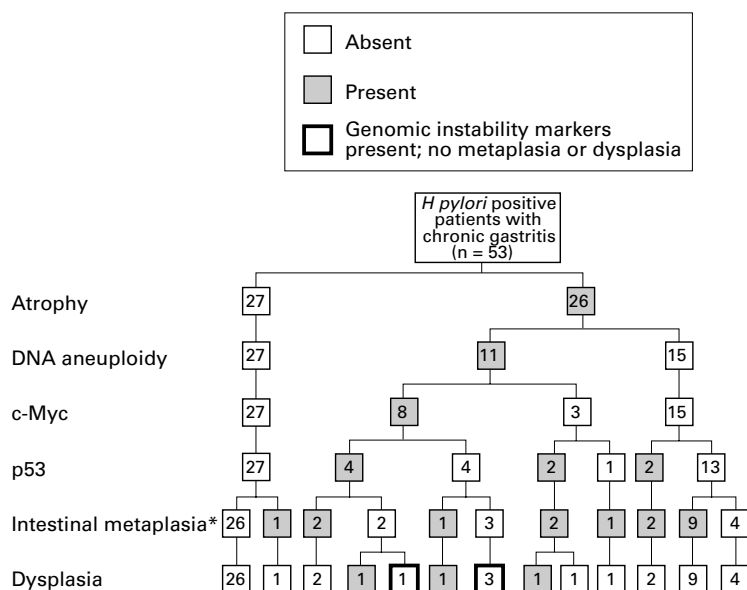


Figure 1 Distribution of histological and immunohistochemical features and DNA content in 53 *H pylori* positive patients with chronic gastritis. *16 complete and two incomplete.

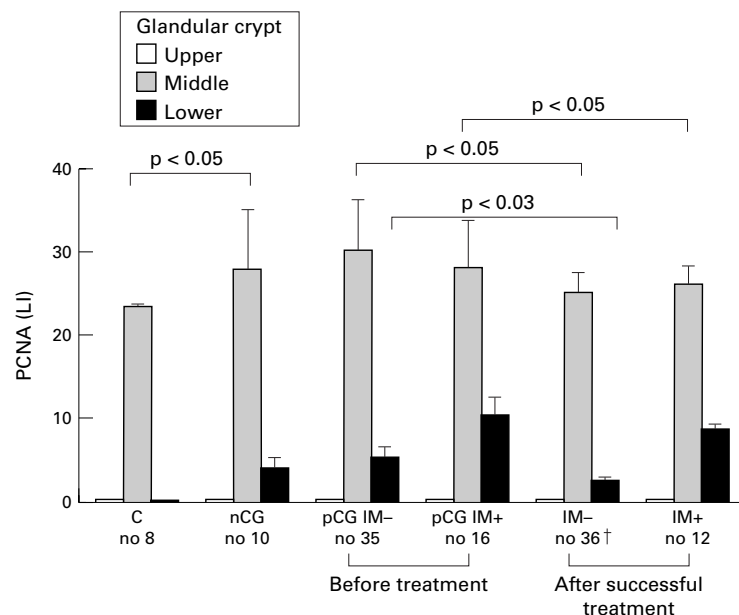


Figure 2 Immunohistochemical expression of proliferating cell nuclear antigen (PCNA) in gastric crypt sections (as indicated on the figure; a mean of five well oriented crypts for each patient) of patients with functional dyspepsia, *H pylori* negative chronic gastritis, or *H pylori* positive chronic gastritis, with or without complete intestinal metaplasia (IM), before and after *H pylori* eradication. LI, labelling index, expressed as percentage of PCNA positive nuclei; C, controls with functional dyspepsia; nCG, *H pylori* negative chronic gastritis; pCG IM-, *H pylori* positive chronic gastritis without intestinal metaplasia; pCG IM+, *H pylori* positive chronic gastritis with complete intestinal metaplasia; IM-, chronic gastritis without intestinal metaplasia after *H pylori* eradication; IM+, chronic gastritis with intestinal metaplasia after *H pylori* eradication. †Three patients dropped out; intestinal metaplasia disappeared in four subjects.

entry criteria: no antisecretory or antibiotic drugs in the previous two months; no anticoagulant drugs in the last week; no severe associated diseases (liver, kidney, cardiovascular, etc). They were subdivided into four groups according to histological features and *H pylori* status: eight *H pylori* negative controls affected by functional dyspepsia with normal gastric histology; 10 patients with *H pylori* negative chronic gastritis (nCG); 53 patients with *H pylori* positive chronic gastritis (pCG); and 11 patients with gastric cancer (three early and eight invasive, of which five were intestinal and three diffuse types). The pCG patients were also examined 12 months after eradication therapy (15 days of treatment with omeprazole 20 mg twice a day, clarithromycin 500 mg twice a day, and tinidazole 500 mg twice a day). *H pylori* status was evaluated by histology and the ¹³C-urea breath test as described in the Maastricht consensus report.⁴⁰ The presence of CagA protein, a marker of *H pylori* virulence,⁴¹ was investigated only in 25/53 of pCG patients. Informed consent was obtained from all subjects.

At study entry and at one year after eradication therapy, at least nine gastric biopsy specimens were taken during upper endoscopy in the body, angulus, and antrum of each subject (three from each site). For cancer patients, gastric samples were obtained from the surgical specimen. The specimens were immediately fixed in 10% neutral buffered formalin, embedded in paraffin wax, and routinely processed. Histological, immunohistochemical, morphometric, and DNA ploidy evaluations were performed on all biopsy samples independently by two pathologists. After the independent reviews, each case was evaluated jointly and disagreements were resolved by consensus.

HISTOPATHOLOGICAL DIAGNOSIS

Haematoxylin and eosin stains were used for the histopathological diagnosis; the modified Giemsa stain was used for *H pylori* identification. Presence of *H pylori*, degree of inflammatory reaction and glandular atrophy, intestinal metaplasia, and cellular dysplasia were diagnosed and classified according to the updated Sydney system⁴² in each biopsy sample. The diagnosis of atrophy, defined as loss of glandular tissue, was based on agreement between the pathologists on at least two samples for each site investigated.

IMMUNOHISTOCHEMISTRY

For each case, 4 µm thick serial sections were cut from paraffin wax blocks, mounted on acid-cleaned glass slides, and heated at 55°C for 60 minutes. Slides were dewaxed and rehydrated,

then the endogenous peroxidase activity was inhibited by incubation with 3% H₂O₂ in methanol (20 minutes at room temperature). To reduce non-specific background staining, slides were incubated with 5% goat serum (15 minutes

at room temperature). To enhance immunostaining, sections were treated with an antigen retrieval solution (10 mM citric acid monohydrate, pH 6.0, adjusted with 2 M NaOH) and heated three times in a microwave oven at high

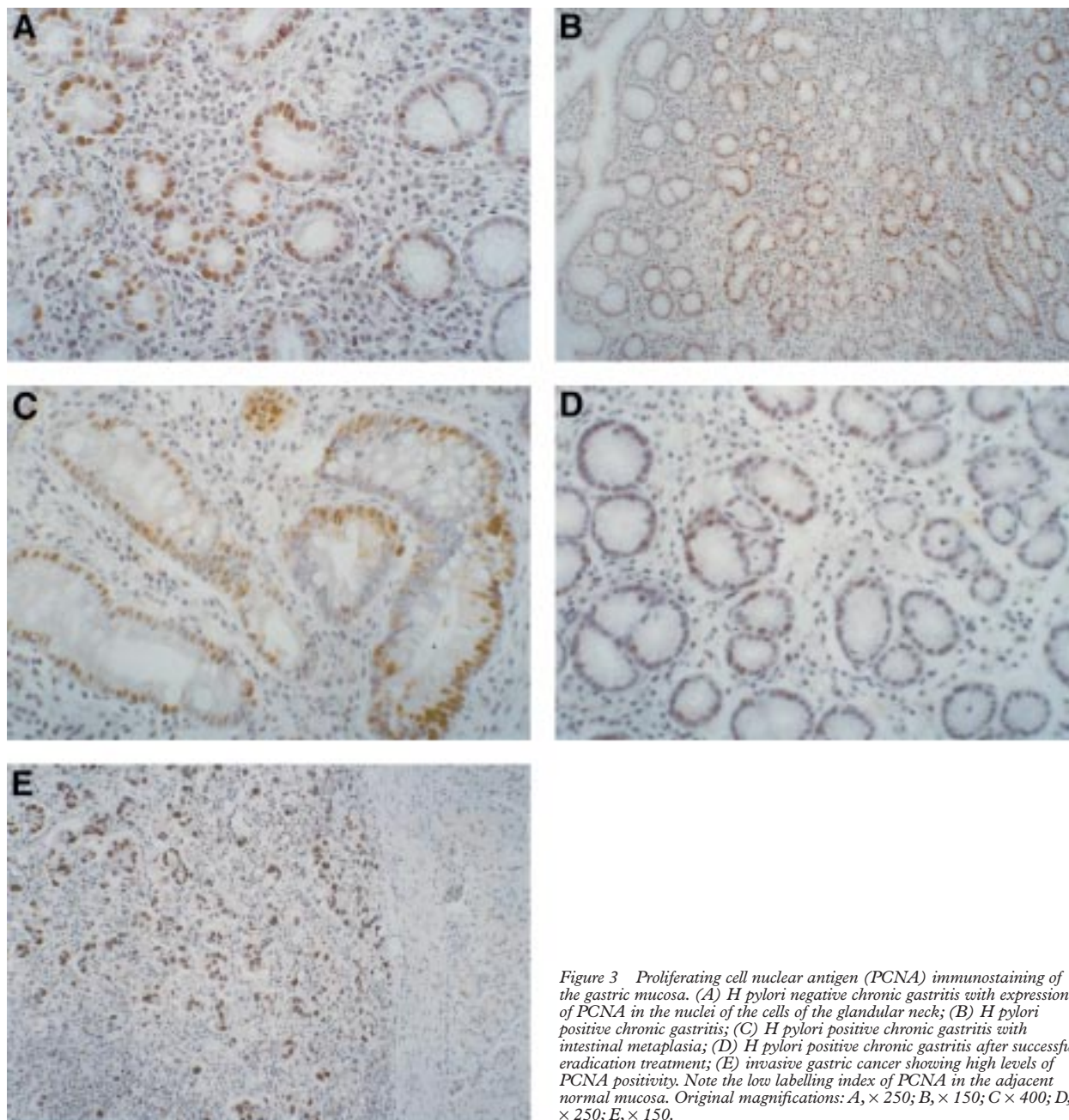


Figure 3 Proliferating cell nuclear antigen (PCNA) immunostaining of the gastric mucosa. (A) *H pylori* negative chronic gastritis with expression of PCNA in the nuclei of the cells of the glandular neck; (B) *H pylori* positive chronic gastritis; (C) *H pylori* positive chronic gastritis with intestinal metaplasia; (D) *H pylori* positive chronic gastritis after successful eradication treatment; (E) invasive gastric cancer showing high levels of PCNA positivity. Note the low labelling index of PCNA in the adjacent normal mucosa. Original magnifications: A, $\times 250$; B, $\times 150$; C $\times 400$; D, $\times 250$; E, $\times 150$.

Table 2 Nucleolar organiser region (AgNOR) counts (number and area) in gastric crypt sections of patients with gastritis with and without *Helicobacter pylori* infection and gastric cancer

Diagnostic groups	Patients (n)	Crypt sections								
		Upper			Middle			Lower		
		Number	Area	p Value	Number	Area	p Value	Number	Area	p Value
Controls	8	1.1 (0.01)	4.0 (0.07)	} <0.05	1.1 (0.02)	4.0 (0.08)	} <0.01	1.1 (0.01)	3.8 (0.01)	} <0.05
<i>H pylori</i> negative gastritis	11	1.1 (0.70)	4.3 (0.10)		1.3 (0.75)	4.8 (0.12)		1.2 (0.63)	4.1 (0.13)	
<i>H pylori</i> positive gastritis	53	1.1 (0.02)	4.4 (0.09)	} <0.05	1.4 (0.09)	4.9 (0.13)	} <0.01	1.3 (0.09)	4.2 (0.10)	} <0.01
<i>H pylori</i> positive intestinal metaplasia	16	1.1 (0.01)	4.8 (0.08)		1.4 (0.01)	4.8 (0.09)		1.3 (0.09)	4.8 (0.11)	
Gastric cancer	11	3 (7.8)			15.8 (2.1)					

A mean number of five well oriented crypts was examined for each patient. Results are expressed as mean (SD) of positive cells. p Value was calculated using the Mann-Whitney U test. Gastric cancer values (total rate) are reported but they were not considered in the statistical evaluation.

power for five minutes. Finally, slides were incubated with the appropriate primary antisera in a moist chamber overnight at 4°C. The monoclonal primary antibodies used were: anti-proliferating cell nuclear antigen (anti-PCNA, PC10; DBA, Milan, Italy; dilution 1:500); anti-bcl-2 protein (Dako, Milan, Italy; dilution 1:400); anti-c-Myc protein (Oncogene Science, San Diego, California, USA; c-Myc p62, dilution 1:50); anti-p53 protein (NCL-CM1, YLEM, Rome, Italy; dilution 1:150).

Immunohistochemical staining reactions for each of the antibodies tested were compared within corresponding areas of the tissue sections from the consecutive slides. The avidin-biotin-peroxidase complex procedure (ABC standard; Vector Laboratories, Burlingame, California, USA) was then performed as described by Hsu *et al.*⁴³ Peroxidase activity was detected with diaminobenzidine as substrate. Finally, sections were weakly counterstained with Harris's haematoxylin and coverslipped with a synthetic mounting medium.

Negative controls with normal human serum replacing specific primary antibodies were included in each run. Positive controls were a case of colon adenocarcinoma for p53 protein, a normal lymph node for c-Myc and PCNA, and a case of a low grade follicular lymphoma for bcl-2 protein. Sections were considered positively stained only in cases of unequivocal

nuclear staining for p53, c-Myc, and PCNA, and cytoplasmic staining for bcl-2.

The degree of immunopositivity was evaluated semiquantitatively. A total of 300 cells was counted in random fields from representative areas of the lesions, and the immunoreactive cells were roughly assessed and expressed as percentages. The scoring system for all the antibodies tested was: 0–5% (negative); 5–25% (low positivity); 25–50% (moderate positivity); >50% (high positivity).

STAINING AND COUNTING OF NUCLEOLAR ORGANISER REGIONS (AgNORS)

For each case, a 4 µm section was dewaxed in xylene and rehydrated through graded ethanol to deionised distilled water. AgNOR staining was performed as described by Egan *et al.*⁴⁴ The final working solution was obtained by dissolving gelatin in 1% aqueous formic acid at a concentration of 2%, and mixing it with a 60% aqueous silver nitrate solution (1:2, v/v). This silver colloid solution was applied to the sections (60 minutes at room temperature, shielded from daylight). The sections were then washed with deionised distilled water, and counterstained with Mayer's haematoxylin, dehydrated, and mounted with a synthetic medium. The usual controls were performed as described by Crocker and Nar.⁴⁵

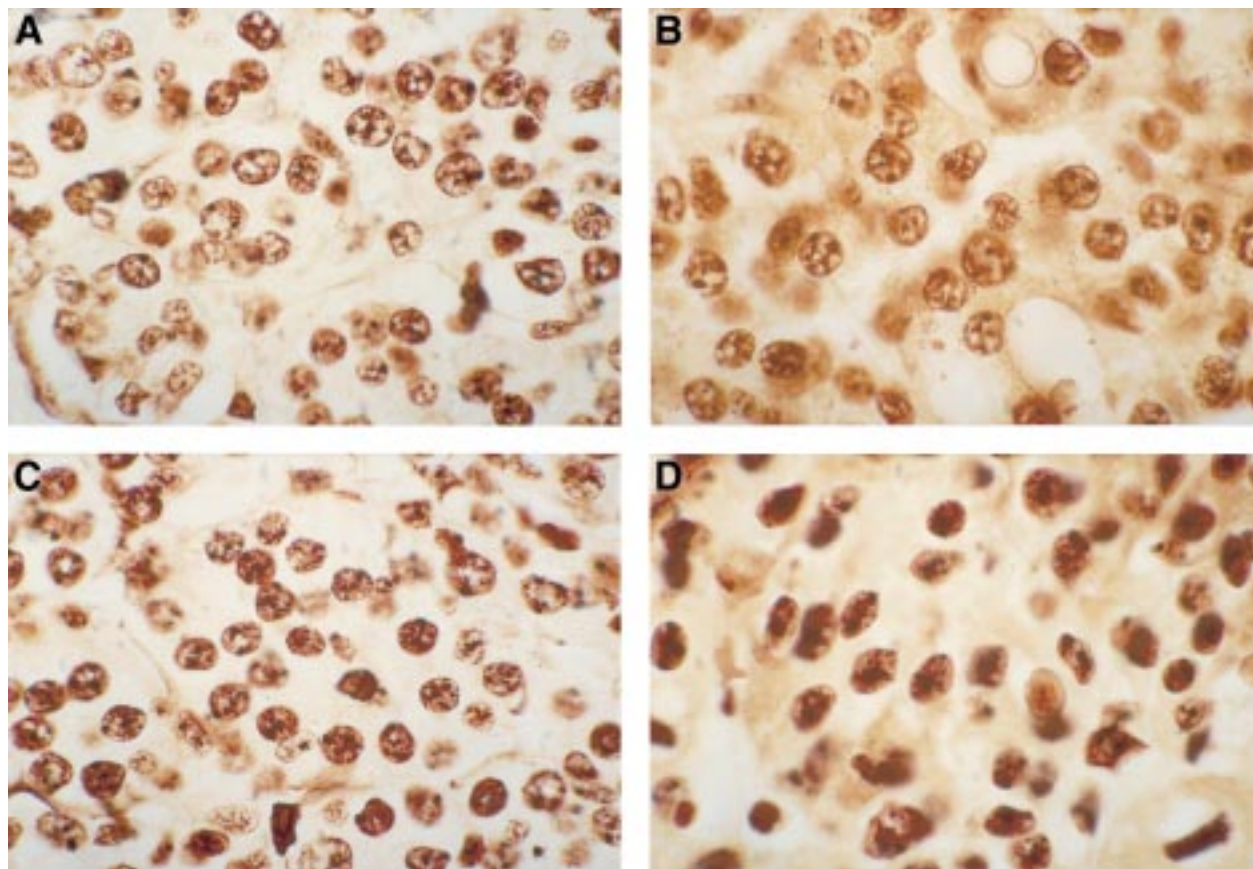


Figure 4 Silver stained nucleolar organiser regions (AgNORs) in gastric mucosa. (A) *H. pylori* negative chronic gastritis: small to medium-sized AgNORs in the nuclei. (B) *H. pylori* positive chronic gastritis: medium-sized irregular AgNORs in the nuclei. (C) *H. pylori* positive chronic gastritis, after eradication treatment. (D) Gastric cancer: large, irregular and sometimes unusually shaped AgNORs in neoplastic cells. Original magnification: A, B, C and D, $\times 1000$, oil immersion.

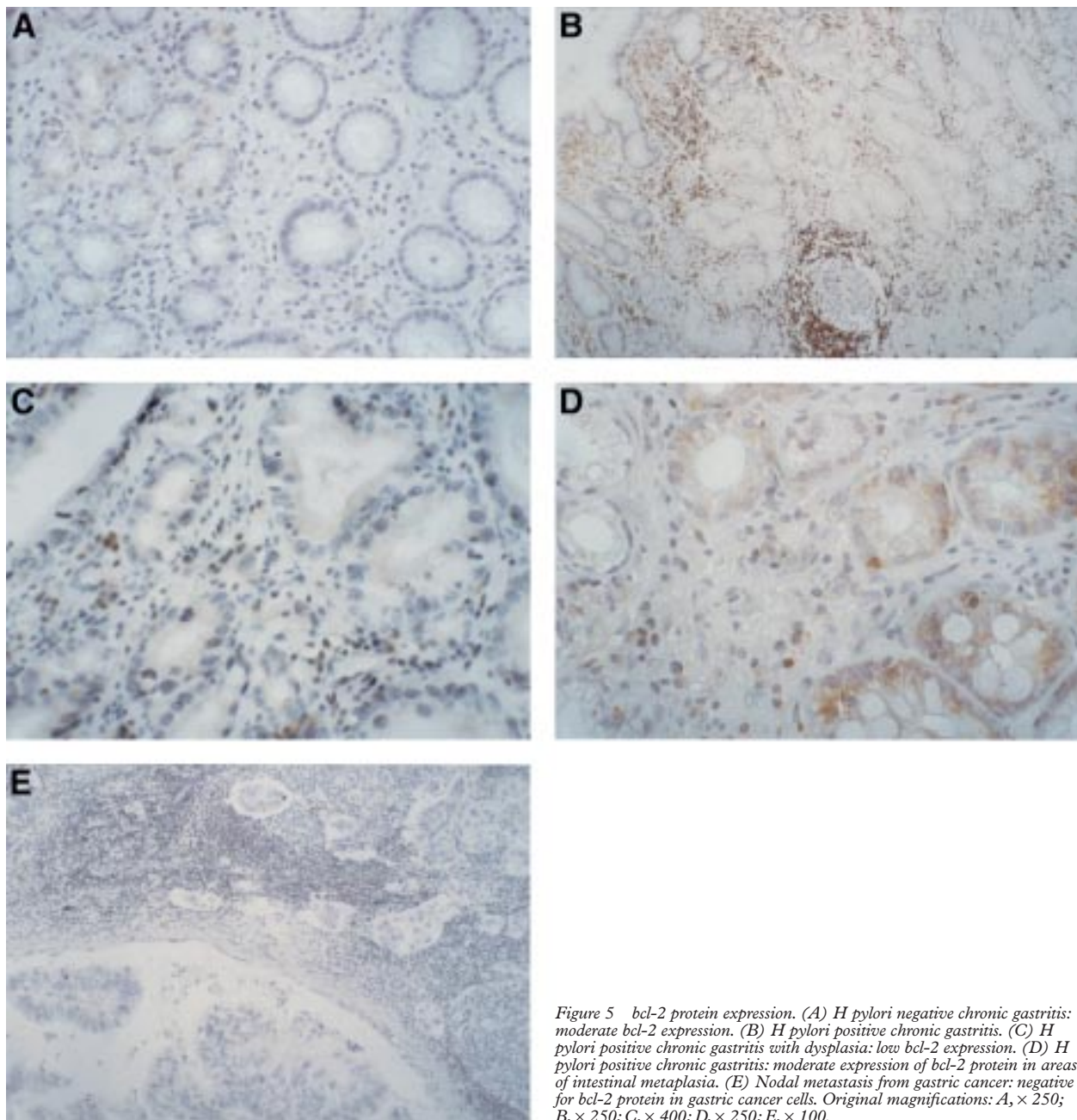


Figure 5 *bcl-2* protein expression. (A) *H pylori* negative chronic gastritis: moderate *bcl-2* expression. (B) *H pylori* positive chronic gastritis. (C) *H pylori* positive chronic gastritis with dysplasia: low *bcl-2* expression. (D) *H pylori* positive chronic gastritis: moderate expression of *bcl-2* protein in areas of intestinal metaplasia. (E) Nodal metastasis from gastric cancer: negative for *bcl-2* protein in gastric cancer cells. Original magnifications: A, $\times 250$; B, $\times 250$; C, $\times 400$; D, $\times 250$; E, $\times 100$.

MORPHOMETRIC ANALYSIS

A Leica Quantimet 500C-Image analyser and processing system was used for morphometric analysis. Images were recorded by a JVC TK-1280E videocamera, connected to a Leitz Orthoplan light microscope. A QWIN V01.00 software package elaborated the data. Within the selected fields, a final number of 200 consecutive nuclei of cells from each case were measured with a $\times 40$ lens. Image acquisition and colour detection were performed in "RGB" (an option that allows the selection of red, green, and blue). Artefacts and image overlapping were corrected in Binary Edit. The AgNOR number for each nuclear area, the area of nucleus and each AgNOR, and the total area of AgNOR were calculated automatically, as were AgNOR length, breadth, perimeter, roundness, and the aspect ratio of each

nucleus.^{46 47} The perimeter was rectified using a correction factor of 1.064. Values are expressed in μm .

DNA PLOIDY

The Feulgen (sulphuric acid-fuchsin) technique was used for nuclear DNA staining of a section from each case, deparaffinised in xylene and rehydrated through decreasing concentrations of ethanol. Cellular DNA was quantified using a microprocessor-controlled image analysis system (Leica-Quantimet 500C analyser, a Sony Ccd camera, and a Leitz Orthoplan microscope). Leica-QWIN V0200A software was used to analyse the data. At least 200 cells from each patient were examined in non-consecutive random fields of representative areas of the lesions. The following indexes were determined for each

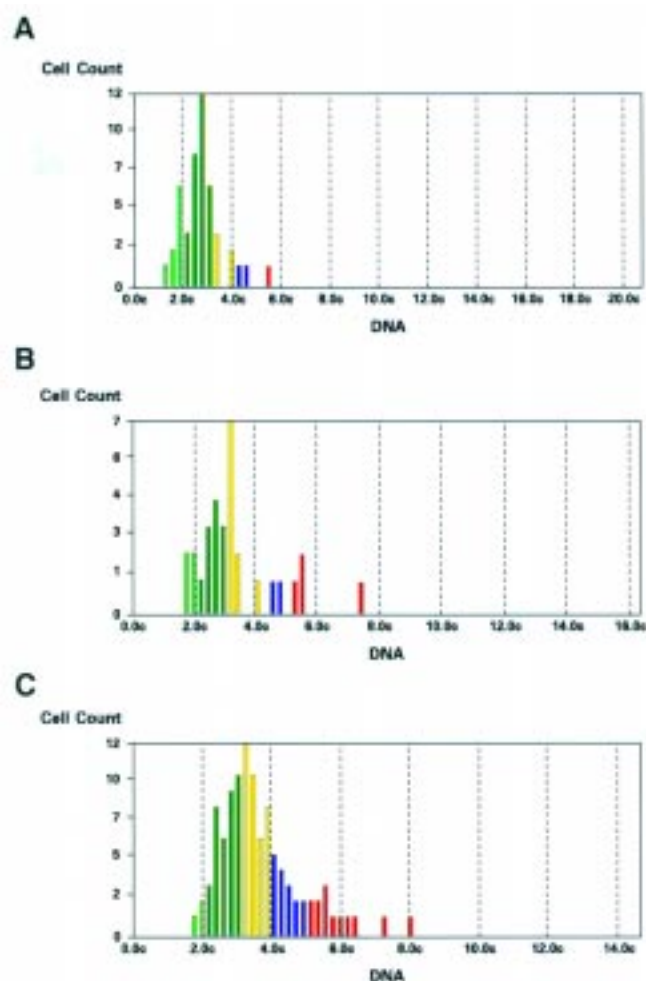


Figure 6 Representative DNA ploidy patterns in Feulgen stained sections of gastric mucosa. (A) *H. pylori* positive chronic gastritis with moderate dysplasia: mild degree of aneuploidy. (B) *H. pylori* positive chronic gastritis not responsive to eradication therapy without dysplasia: mild degree of aneuploidy. (C) Aneuploid pattern in gastric cancer (image analysis Quantimet 500C).

measurement: normal diploid DNA content (2c); 2c deviation index (2cDI), defined as the variance in DNA content of single cells around the normal diploid (2c) DNA peak; 5c exceeding events (5cEE), defined as the percentage of cells with DNA content higher than 5c; DNA malignancy grade (DNA-MG), a logarithmic transformation of the 2cDI value to produce a continuous scale ranging from 0 to 3.0 DNA content.⁴⁸ The methodology described here has been fully validated in gastric biopsy samples.⁴⁹

Table 3 Gastric mucosa DNA ploidy variables in patients with gastritis and gastric cancer

Diagnostic groups	Patients (n)	DNA indexes							
		2c	p Value	2cDI	p Value	5cEE	p Value	MG	p Value
nCG with euploidy	10	1.36 (0.14)	} NS	0.95 (0.43)	} NS	—	} NS	0.49 (0.14)	} NS
pCG with euploidy	42	1.27 (0.09)		0.59 (0.23)		—		0.35 (0.10)	
pCG with aneuploidy	11	1.65 (0.25)	} <0.0001	2.46 (0.98)	} <0.0001	7.55 (4.53)	} NS	0.91 (0.22)	} <0.0001
Gastric cancer	11	2.26 (0.98)		10.71 (12.47)		22.76 (12.47)		1.60 (0.68)	

Results are expressed as mean (SD). p Value is calculated using the Mann-Whitney U test. nCG, *H. pylori* negative chronic gastritis; pCG, *H. pylori* positive chronic gastritis; 2c, normal diploid DNA content; 2cDI, 2c deviation index; 5cEE, 5c-exceeding event; MG, DNA malignancy grade.

ANTI-CagA SERUM

The *H. pylori* strain CCUG 17874 (CagA+) was denatured in Laemmli buffer⁵⁰ at 100°C for five minutes and electrophoresed in a 10% polyacrylamide gel with sodium dodecyl sulphate. Proteins were transferred to nitrocellulose sheets which were saturated with 3% defatted milk in phosphate buffered saline and 0.1% Triton X (Blotto). Strips were cut out, and serum samples were assayed at a dilution of 1:200 in Blotto. After overnight incubation at room temperature, strips were washed with Blotto, and then incubated with anti-human IgG conjugated with peroxidase at room temperature for 90 minutes. After washing, the reaction was visualised by addition of the substrate (H₂O₂ in a solution of 0.3% 4-chloro-I-naphthol in 0.05 M Tris/HCl buffer, pH 6.8). An anti-CagA antibody raised in rabbit against a recombinant CagA fragment served as a positive control.

STATISTICAL ANALYSIS

We set the power to 0.8, with type I error = 0.05 and control to case ratio 1:1. The aim was to detect a minimal difference of 30% between nCG and pCG groups. Data were analysed with the SPSS package for Windows. The categorical variables (*H. pylori* infection, cell infiltration, atrophy, intestinal metaplasia, dysplasia, DNA ploidy, p53, and c-Myc) were analysed using the χ^2 test or Fisher's exact test. Correlation between these variables was assessed using the Spearman correlation coefficient. The non-parametric Mann-Whitney U test was used to compare the DNA values (2c, 2cDI, 5cEE, and MG), AgNOR area, and PCNA expression in gastric crypt sections of patients with functional dyspepsia and gastritis with and without *H. pylori* infection. The Wilcoxon matched pair test was used to evaluate PCNA expression in gastric crypt sections of patients with gastritis with *H. pylori* infection before and after eradication therapy.

The analysis of the "before" and "after" data in the *H. pylori* positive group given eradication treatment was evaluated with the McNemar test.

Results

Table 1 shows the clinical, histological, and immunohistochemical variables of the population studied. Mean age increased in rank order from normal controls to patients with chronic gastritis and to those with gastric cancer; male sex was prevalent in *H. pylori* related disease

Table 4 Spearman correlation coefficient ($p < 0.0001$) of the main parameters analysed in the *H pylori* positive chronic gastritis group

	<i>H pylori</i>	PMN	Lymphocytes/ monocytes	Atrophy	IM	Dysplasia	DNA aneuploidy	PCNA	p53	bcl-2	c-Myc
<i>H pylori</i>	█	-	-	-	-	-	-	-	-	-	-
PMN	-	█	-	-	-	-	-	-	-	-	-
Lymphocytes/monocytes	-	-	█	-	-	-	-	-	-	-	-
Atrophy	-	-	-	█	-	-	0.61	-	0.57	-	0.49
IM	-	-	-	-	█	-	-	-	-	-	-
Dysplasia	-	-	-	-	-	█	-	-	-	-	-
DNA aneuploidy	-	-	-	0.61	-	-	█	-	0.58	-	0.70
PCNA	-	-	-	-	-	-	-	█	-	-	-
P53	-	-	-	0.57	-	-	0.58	-	█	-	-
bcl-2	-	-	-	-	-	-	-	-	-	█	-
c-Myc	-	-	-	0.49	-	-	0.70	-	-	-	█

PMN, polymorphonuclear leucocytes; IM, complete intestinal metaplasia; PCNA, proliferating cell nuclear antigen.

and gastric cancer. A gastric and/or duodenal erosive/ulcerative disease was detected in 30% of nCG cases and 55% of pCG cases. The inflammatory infiltrate consisted of neutrophils in pCG patients and mainly lymphomonocytes in nCG. Gastric atrophy was mild in 30% of nCG subjects and was generally restricted to the antrum. Atrophy was detected in 49% (26/53) of pCG patients; it was mild and restricted to the antrum in five patients, and moderate,

extending to the angulus, in 21 patients. Complete intestinal metaplasia, associated with atrophy, was found in 1/10 nCG and 16/53 pCG patients (restricted to the antrum in 10 of the latter). Incomplete metaplasia with atrophy was found in antral biopsy specimens from only 2/53 pCG patients. These two patients also had an abnormal DNA content and p53 and c-Myc overexpression. Gastric dysplasia, detected in the antrum of 3/53 pCG patients (one mild and two moderate), was associated with intestinal metaplasia (one complete and one incomplete), c-Myc and/or p53 overexpression in two cases, and abnormal DNA content in all three. Figure 1 gives the histological and immunohistochemical features and DNA content in pCG patients.

Cell proliferation, assessed by evaluation of PCNA expression (figs 2 and 3) and AgNOR silver staining, number, and morphometric variables, was increased (table 2 and fig 4) with respect to healthy controls in patients with gastritis regardless of *H pylori* infection, and associated with the inflammation. AgNOR expression was higher (about 3- to 5-fold) in patients with gastric cancer than in the other groups (table 2). PCNA expression was 2-fold higher in the lower basal portion of the gland in pCG patients with intestinal metaplasia compared with pCG patients without intestinal metaplasia (fig 2).

Expression of bcl-2 was preserved in controls and the gastritis groups, regardless of *H pylori* infection, intestinal metaplasia, and dysplasia (fig 5A-D). In gastric cancer, bcl-2 was expressed at very low levels in three early cancers and was not detectable in the remaining eight invasive cancers (fig 5E).

Image analysis of DNA content showed aneuploidy in 11/53 pCG patients, confined to biopsy specimens in which atrophy was present, and in all gastric cancer patients (fig 6). The DNA values (DNA indexes, 2cDI, 5cEE, and MG) were higher in gastric cancer than in pCG with aneuploidy (table 3). There were no significant differences in DNA values between the remaining groups (nCG with euploidy versus pCG with euploidy; table 3) or with regard to sex and age of patients (data not shown). Aneuploidy was significantly associated with atrophy (11/11) and expression of p53 (6/11) and c-Myc (8/11) oncoproteins (fig 1 and table 4).

The expression of c-Myc was low to moderate in 8/53 pCG patients (fig 7A, B); all these

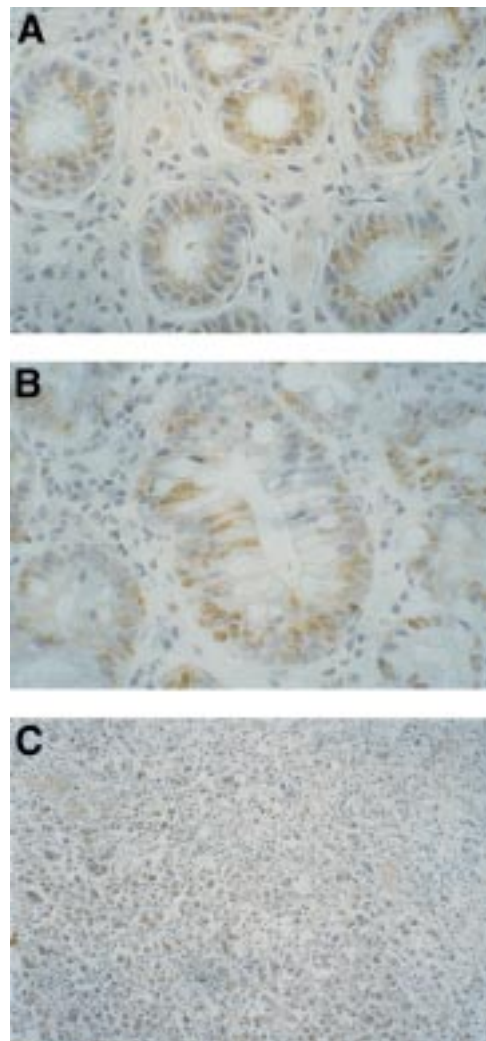


Figure 7 c-Myc expression. (A) *H pylori* positive chronic gastritis: low to moderate positivity for c-Myc expression. (B) *H pylori* positive chronic gastritis with intestinal metaplasia: moderate degree of positivity for c-Myc protein. (C) Gastric cancer: positivity for neoplastic cells for c-Myc protein. Original magnifications: A, $\times 400$; B, $\times 400$; C, $\times 100$.

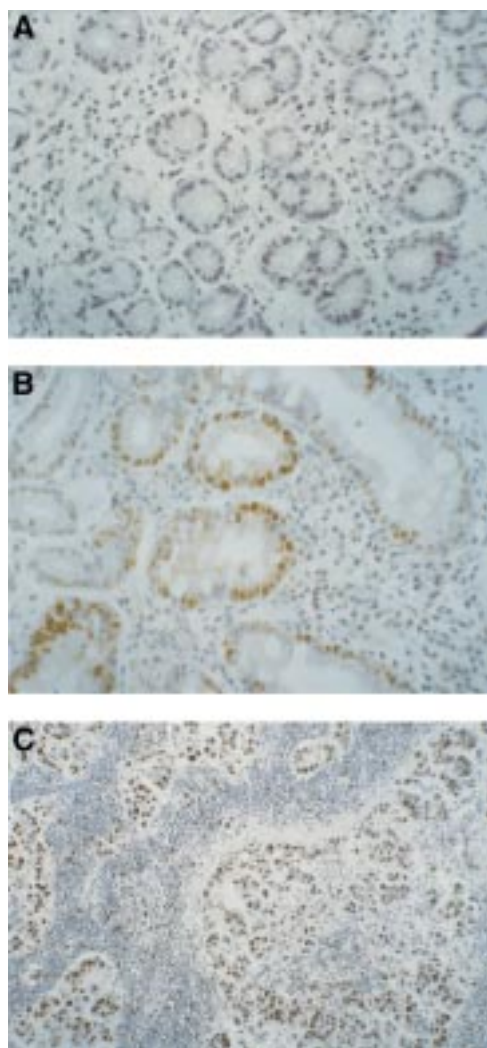


Figure 8 p53 protein expression. (A) *H pylori* positive chronic gastritis: sparse low positivity for p53 protein. (B) *H pylori* positive chronic gastritis with intestinal metaplasia: positivity for p53 protein in areas of intestinal metaplasia. (C) Gastric cancer: high positivity for p53 protein. Original magnifications: A, $\times 250$; B, $\times 250$; C, $\times 100$.

patients also had an abnormal DNA content, and two had glandular dysplasia (fig 1). A moderate to strong c-Myc positivity was detected in 4/11 gastric cancers (fig 7C), while c-Myc was undetectable in control subjects and nCG patients.

Table 5 Histological and genomic parameters in 53 *H pylori* positive patients with chronic gastritis before and after eradication therapy

	Before therapy (n=53)	After therapy		p Value
		<i>H pylori</i> eradicated (n=45)	<i>H pylori</i> persisted (n=5)	
Atrophy	26 (49)	16 (35)	5 (100)	NS
Intestinal metaplasia	18* (34)	10 (22)	4† (80)	NS
Dysplasia	3 (6)	0	3 (60)	NS
DNA aneuploidy	11 (21)	0	3 (60)	0.01
p53	8 (15)	0	2 (40)	0.05
c-Myc	8 (15)	0	3 (60)	0.05

Values in parentheses are percentages. p Value was calculated using the McNemar test comparing before and after therapy regardless of the outcome of treatment. The eradication treatment consisted of 15 days on omeprazole 20 mg twice a day, clarithromycin 500 mg twice a day, and tinidazole 500 mg twice a day. Three subjects dropped out during follow up.

*16 complete and two incomplete intestinal metaplasias; †two complete plus two incomplete intestinal metaplasias.

Finally, p53 was overexpressed in 63% (7/11) of gastric cancers (fig 8C) and in 15% (8/53) of pCG (fig 8A, B); six of the latter also had abnormal DNA content and gastric metaplasia (fig 1). No control subject nor nCG patient showed p53 expression.

The frequency of DNA aneuploidy, and p53 and c-Myc expression in relation to histological findings shows that aneuploidy and p53 and c-Myc expression were invariably associated with atrophy and were more pronounced in patients with intestinal metaplasia (fig 1).

The *H pylori* CagA status, investigated in 25/53 patients, was positive in 22 and negative in three. Atrophy was present in 54% of the CagA positive patients, intestinal metaplasia in 36%, dysplasia in 4%, DNA aneuploidy in 22%, and c-Myc and p53 expression in 22% and 18% respectively. However, the small number of subjects with CagA negative *H pylori* prevented statistical evaluation.

One year after antibiotic treatment, the study protocol was repeated in the 53 pCG patients (table 5). Three patients dropped out. *H pylori* was eradicated in 90% of patients (45/50), in whom cell proliferation, PCNA expression (fig 3E) and AgNOR staining (fig 4C) decreased in parallel with the reduction in inflammatory infiltrate, whereas no significant difference was detected in patients with intestinal metaplasia (fig 2). The intensity of neutrophil infiltration was directly related to the rate of mucosal cell proliferation in the patients who were responsive to *H pylori* eradication treatment ($p < 0.02$). Atrophy disappeared completely in five patients (all with mild atrophy restricted to the antrum); it regressed from moderate to mild only in the antrum in nine and remained unchanged in seven. Complete metaplasia associated with atrophy disappeared in four of 14 patients. Finally, DNA aneuploidy and p53 and c-Myc expression, previously detected in eight, six, and five of the 45 patients respectively, disappeared.

In the five subjects in whom *H pylori* infection persisted after eradication therapy, atrophy, metaplasia, dysplasia, and markers of genomic instability remained unchanged.

Discussion

Concurrent or previous *H pylori* infection is associated with a 2.7- to 12-fold risk of gastric cancer.² Carcinogenesis invariably starts with cell hyperproliferation.^{2 3 51 52} The relation between *H pylori* infection, gastric mucosal damage, and the cell proliferation rate is a matter of debate. *H pylori* urease activity and leucocyte infiltration have a mitogenic effect,^{3 19 53-55} but gastric cell hyperproliferation also occurs independently of *H pylori* infection.^{56 57} In our study, the gastric epithelial proliferative rate, evaluated using PCNA and AgNOR analyses, was increased in gastric cancer and chronic gastritis, regardless of *H pylori* infection (figs 2-4 and table 2). Therefore mucosal cell hyperproliferation appears to be a constant finding of chronic gastric damage whatever the aetiology, but it is related to inflammatory infiltrates which were prevalently lympho-monocytic in nCG and made up of

polymorphonuclear leucocytes in pCG (table 1). Mucosal hyperproliferation is linked to apoptosis, and there is mounting evidence that *H pylori* induces apoptosis.^{58 59} The bcl-2 proto-oncogene is involved in controlling apoptosis and is related to the initial phase of cancer.^{60 61} The expression of the bcl-2 gene was unaltered in our patients with chronic gastritis, regardless of *H pylori* infection, which supports the idea that *H pylori* induced apoptosis occurs through a mechanism that is independent of bcl-2 gene expression.⁶² bcl-2 gene expression was also preserved in our patients with early gastric cancer, but was undetectable in advanced cancer. This finding contrasts with reports of bcl-2 overexpression in intestinal type gastric cancer.^{37 38} It is conceivable that the discrepancy is related to differences in populations studied and in the type, staging, and grading of cancer.

A chronic hyperproliferative state may also favour cellular DNA damage.^{3 17-22 24 25} We detected a relation between chronic hyperproliferation and cellular DNA damage only in *H pylori* positive patients with active gastritis. Therefore the cytotoxic effect of *H pylori* infection and the related polymorphonuclear leucocyte infiltration may play important roles in the development and progression of mucosal damage. Aberrant DNA is a prognostic indicator for cancer^{49 63-65} and seems to be independent of other clinical pathological factors.⁶⁶ We detected aneuploidy in all our cancer patients, but we also identified a novel subset of pCG patients with aneuploidy (11/53) (table 3). Aneuploidy was significantly associated with c-Myc and/or p53 expression (fig 1 and table 4), which are the most widely used markers of genomic instability.

The c-Myc oncogene is implicated in the transformation and progression of mutated cells.⁶⁷⁻⁶⁹ In this study it was expressed in 36% of gastric cancer (4/11) and 15% of pCG (8/53) patients. This is a novel finding because the expression of c-Myc has not previously been investigated in chronic gastritis or in relation to *H pylori* status. c-Myc expression is more frequent in gastric adenocarcinoma than in adenoma and has also been proposed as an aid to differentiate between the two conditions.³⁹ Half of the patients with c-Myc expression and aneuploidy had p53 overexpression, which was also detected in 63% of gastric cancer patients. The mutation of the p53 gene, which we found in 15% of pCG patients, is a common occurrence in colorectal carcinoma where it is related to a poor prognosis and distant metastasis.³⁰⁻³² It has been reported in gastric cancer^{34 35} and precancerous gastric lesions³⁶ but never in relation to *H pylori* infection.

Interestingly, aneuploidy and c-Myc and p53 expression were not detected in the absence of *H pylori* contamination or in patients with *H pylori* infection but without gastric atrophy. Therefore, in our population, the appearance of genomic instability required the presence of both gastric atrophy and chronic *H pylori* infection (fig 1).

Several studies have shown that patients with preneoplastic and neoplastic gastric epithelial lesions are more likely to be infected by CagA positive strains⁷⁰⁻⁷⁴ which possess a genomic insertion called "cag pathogenicity island" which includes genes involved in virulence.⁴¹ Infection by CagA positive *H pylori* strains increases the risk of developing atrophy and intestinal metaplasia possibly because of the enhanced inflammatory potential shown by these strains.⁷⁰⁻⁷³ Although investigated only in about 50% of pCG patients, the high prevalence of positivity for CagA (23 of 25 subjects studied) may help to explain the high incidence of atrophy (49%) and intestinal metaplasia (34%) observed in our patients. Furthermore, the enhanced prevalence of DNA aneuploidy and p53 and c-Myc expression in our patients is in agreement with the observation of an increased cancer risk in individuals infected by CagA positive *H pylori* strains compared with individuals infected by Cag negative *H pylori* strains⁷⁴ and, more in general, in individuals who live in areas with a high rate of CagA positive *H pylori* strains.⁷⁰ Unfortunately, we were not able to verify this hypothesis directly because of the small number of CagA negative patients in this study.

The effects of *H pylori* eradication on atrophy and related intestinal metaplasia are controversial and there is no consensus among gastrointestinal pathologists as to the identification and grading of these lesions. In a recent study, no changes in intestinal metaplasia and atrophy were detected after *H pylori* eradication irrespective of *H pylori* CagA status.⁹ In contrast, we found that *H pylori* eradication may be followed, one year after treatment, by the disappearance of complete metaplasia and/or reduction of atrophy (table 5), when both are present in the antrum. Other groups have reported similar results.^{11 12} The patchy nature of the lesions and the subjective nature of the interpretation may account for these controversial findings.^{75 76} However, according to a recent stringent definition of atrophy, "true irreversible" atrophy is differentiated from "apparent" atrophy which is reversed by removal of inflammation.⁷⁷ Our finding of disappearance or regression of atrophy, mainly from the antrum, after *H pylori* eradication suggests that inflammatory infiltration plays a role in this controversial histological finding, even though we cannot rule out the involvement of other factors such as age, sex, diet, genetic makeup of the host, nature of the lesions, biopsy sampling, *H pylori* strains, and environmental toxic factors. Long term studies and a larger number of stratified patients in relation to age of infection are needed to establish definitely the reversibility of *H pylori* related lesions and the predictive value of genetic markers for *H pylori* eradication.

Our data confirm the significant link between such precancerous features as incomplete metaplasia and dysplasia and genomic instability. However, we found genomic instability in four cases not showing these morphological aspects (fig 1). It is noteworthy that in Barrett's oesophagus, p53 overexpression

precedes dysplasia in patients followed for about three years.^{78, 79}

In conclusion, chronic *H pylori* infection seems to be responsible for genomic instability in a subset of cases of *H pylori* positive chronic atrophic gastritis and also in the absence of notorious precancerous lesions like metaplasia and dysplasia; eradication of *H pylori* infection can reverse inflammation and related atrophy, metaplasia, and genomic instability. Finally, we suggest that pCG patients with atrophy should receive eradication treatment for *H pylori*, while pCG patients with *H pylori* infection who do not respond to antibiotic treatment should be examined for markers of genomic instability and closely monitored.

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