

Increased cell proliferation in chronic *Helicobacter pylori* positive gastritis and gastric carcinoma – Correlation between immuno-histochemistry and Tv image cytometry

Erika Szaleczky^a, László Prónai^{a,*},
Béla Molnár^a, Lajos Berczi^b, János Fehér^a
and Zsolt Tulassay^a

^a 2nd Department of Internal Medicine, Clinical Gastroenterology and Endocrinology Unit, Semmelweis University of Medicine, Budapest, Hungary

^b 1st Institute of Pathology and Experimental Cancer Research, Semmelweis University of Medicine, Budapest, Hungary

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Background: Epithelial cell proliferation activity has been reported both to be unaltered and increased in *Helicobacter pylori* (*H. pylori*) associated chronic gastritis. The proliferation rate decreased following *H. pylori* eradication, but results are controversial whether this change is dependent on the success of eradication. We compared the cell proliferation activity of *H. pylori* positive and negative gastric epithelial biopsies in chronic gastritis with and without intestinal metaplasia (IM) and gastric cancer by the expression of proliferation cell nuclear antigen (PCNA) and Tv image cytometry, and assessed the effect of *H. pylori* eradication on the cell proliferation rate in the gastric epithelium.

Methods: Brush smears and antral biopsies were taken from 70 patients (42 men, 28 women, mean age 58 ± 15 y.o.) on routine endoscopy. Patients were divided into four groups according to the histology; normal epithelia ($n = 10$),

chronic gastritis without IM ($n = 24$), chronic gastritis with IM ($n = 20$), and gastric carcinoma ($n = 16$). Thirty-three patients were *H. pylori* positive, and success of eradication was controlled in 24 cases. Cell proliferation was measured by immunohistochemistry using PCNA labeling index (LI) and by Tv image cytometry evaluating 12 morpho- and densitometric parameters of each nuclei and 6 additional parameters of each smear.

Results: PCNA LI, DNA index and S + G₂ ratio were all higher in chronic gastritis than in the normal epithelium, and were further increased in carcinoma. The lower PCNA LI observed in chronic gastritis with IM corresponds to the lower S phase ratio determined by Tv image analysis. In *H. pylori* positive cases, the proliferation activity was $69.3 \pm 13.05\%$ prior to the eradication and it decreased to $55.8 \pm 23.31\%$ after the successful eradication therapy. When immunohistochemistry was compared with Tv image cytometry, PCNA LI significantly correlated with the percentage of cells in G₁ phase ($r = -0.415$) and S phase ($r = 0.385$), Integrated Optical Density mean ($r = 0.598$), density maximum ($r = 0.608$), surface ($r = 0.670$), layers ($r = 0.638$), diameter minimum ($r = 0.619$), diameter maximum ($r = 0.730$) and perimeter ($r = 0.501$), respectively ($p < 0.05$).

Conclusions: Epithelial cell turnover is increased in chronic gastritis with or without IM, and in gastric carcinoma. The lower PCNA LI observed in chronic gastritis with IM corresponds to the lower S phase ratio determined by Tv image analysis. Cell proliferation decreases after successful *H. pylori* eradication. Both methods proved to be reliable for the determination of epithelial cell proliferation.

Keywords: *Helicobacter pylori*, chronic gastritis, gastric cancer, proliferating cell nuclear antigen, Tv image cytometry, DNA ploidy

*Correspondence to: Dr Prónai László, 2nd Department of Internal Medicine, Semmelweis University of Medicine, 1088 Budapest, Szentkirályi u. 46, Hungary. Tel.: +36 1 2660926; Fax: +36 1 2660816; E-mail: pronai@bel2.sote.hu.

1. Introduction

Helicobacter pylori (*H. pylori*) infection is the main etiopathogenetic factor responsible for inflammatory and ulcerative changes in gastroduodenal mucosa. It may induce both antral and diffuse types of gastritis, and can be responsible for the development of gastric carcinoma [1]. In this latter case, intestinal metaplasia is the intermediary stage between gastritis and cancer. Cell proliferation in gastric foveolae is a physiological event with a constant turnover of the epithelial layer, which is essential for the effective mucosal barrier function. On the other hand, the high epithelial turnover may be the indicator of an increased risk of neoplastic changes, e.g., in long-standing, untreated *H. pylori* positive chronic gastritis [2]. Following *H. pylori* eradication therapy, the rate of proliferating cells in gastric epithelium has decreased [3], but results are controversial whether this change is detectable independently of the success of eradication [4].

The expression of proliferating cell nuclear antigen (PCNA) has previously been proven to be a reliable method for determination of proliferative activity of gastric epithelium [5]. PCNA is a 36 kDa molecule which is highly conserved in evolution. The entire gene for human PCNA has been isolated and sequenced. PCNA functions as a co-factor for DNA-polymerase delta in both the S phase and in the DNA synthesis associated with DNA repair. PCNA has been shown to be required for DNA nucleotide excision repair. It has a very long half-life such that on exit from M phase, cells will have detectable levels of PCNA for prolonged periods. In addition, very small levels of PCNA may be found even in non-cycling cells with upregulation occurring on entry into the cell cycle. PCNA expression can also be induced by growth factors.

The proliferating state of cells can be characterized by their DNA content. Recently, new Tv image cytometry systems appeared for quantitative DNA and morphometric analysis. It has been proved to be more sensitive compared to the flow cytometry method in detecting small DNA-aneuploid populations [6]. The Tv image analysis of gastric brush smears obtained directly from the gastric lesions has been shown to be highly specific but less sensitive method. Comparison between the mathematically determined G₁-S-G₂ phase fraction ratios and those determined by PCNA immunohistochemistry has not yet been performed.

We compared cell proliferation activity of *H. pylori* positive and negative gastric biopsies in chronic gastritis with and without intestinal metaplasia and gastric

cancer. The changes in cell proliferation following after *H. pylori* eradication therapy was also assessed. In a certain group of patients, Tv image cytometry analysis of brush smears obtained from the endoscopically pathological area was simultaneously performed. Correlation between the proliferation activity, determined by immunohistochemistry and morpho- and densitometric parameters, determined by Tv image analysis was also evaluated.

2. Patients and methods

Brush smears and multiple gastric biopsies – 3 from the antrum and 3 from the corpus – were taken from 70 subjects (42 men, 28 women, mean age 58 ± 15 year old) who underwent routine diagnostic endoscopy in the Endoscopy Laboratory of the 2nd Department of Medicine, Semmelweis University of Medicine. Biopsy specimen were placed in buffered formalin, routinely processed and stained. Modified Giemsa stain was used to detect *H. pylori*. Gastritis biopsy specimen was graded by the pathologist according to the histological activity using the number of mononuclear cells per power field: <5 MNC = normal, 6–20 MNC = mild, 21–40 MNC = moderate, >40 MNC = severe.

Patients were divided into four groups according to the routine histology: (1) normal epithelia ($n = 10$) (2) chronic gastritis without intestinal metaplasia ($n = 24$) (3) chronic gastritis with intestinal metaplasia ($n = 20$) (4) gastric carcinoma ($n = 16$). Thirty-three patients who were positive for *H. pylori* on all ¹³C urea breath test, rapid urease test and histology underwent *H. pylori* eradication therapy. Nine patients did not agree to control endoscopy, therefore, success of eradication could be controlled in 24 cases. One week triple therapy (2 × 1 g amoxicillin + 2 × 0.5 g clarithromycin + 2 × 20 mg omeprazole) was successful in 21 out of 24 patients, where all the ¹³C urea breath test, rapid urease test and histology were negative for *H. pylori*. A follow-up endoscopy was performed four weeks after the completion of the therapy.

For immunohistochemistry, all biopsy specimen were fixed in buffered formalin and embedded in paraffin. 4 μm sections were cut and taken up on slides. After deparaffinization in xylene and rehydration through graded alcohol, sections were placed in citrate buffer pH 6.0 (Antigen Retrieval Citrate, Biogenex) and incubated in microwave oven (5 min at 750 W). After washing in phosphate-buffered saline (PBS), im-

munostaining was performed. Endogenous peroxidase was blocked by 3% H₂O₂ for 5 min. The slides were preincubated with 10% normal sheep serum diluted in 1% bovine serum albumin for 20 min. Sections were incubated with anti-PCNA antibody (Monoclonal Mouse Anti-PCNA Clone PC 10, DAKO) diluted 1 : 80 in normal serum at 37°C for 90 min. Sections were incubated for 30 min with biotinylated anti-rabbit and anti-mouse IgG, washed in PBS and incubated for 25 min with streptavidin conjugated to horseradish peroxidase (LSAB2 Kit Peroxidase, DAKO). After washing in PBS, slides were incubated with aminoethylcarbazole (AEC) for 15 min. Cell nuclei were counterstained with aqueous hematoxylin and slides were mounted with glycerin : PBS ratio 1 : 1. A known PCNA positive tissue section was used as positive control and included in each staining batch. A negative control, where the primary antibody was replaced by PBS, was carried out in parallel. The count was performed by light microscopy (40× objective) in well-oriented crypts using a grid placed in the ocular. The nuclei stained red completely and uniformly were considered as positive. The mean number of nuclei counted per specimen was 1000. The PCNA labeling index (LI) was defined as a percentage of the PCNA positive nuclei over the total nuclei counted.

For DNA ploidy measurements, brush smears were air-dried and fixed in Carnoy solution. Staining was performed with a modified Feulgen–Schiff method (pararosaniline). The measurements were carried out by the DNASK Tv image cytometry system [7]. DNASK hardware consists of and Olympus BH2 microscope with a 560 nm filter, a black and white CCD camera (Stemmer Datentechnik, Germany) a 512 × 512 pixel frame grabber (HRT 512 × 512, USA) with 256 gray values. Koehler illumination and 40× magnification was used for the measurements. Type and numerical aperture of the objective used was SPLAN 40PL, 0.70. DNASK software measures 12 different parameters of each nucleus after performing the correction of shading, glare and diffraction errors: morphometric parameters: surface (area of the nuclei), layers (ratio of the total nuclei area to the convex nuclei area), diameter minimum, diameter maximum, perimeter, form factor and densitometric parameters: integrated optical density (IOD), average density, sigma density, minimum density, maximum density, density range. Integrated optical density was calculated according to the Lambert–Beer law for the object and reference cells, respectively. From each smear further pa-

rameters can be determined in addition the mean values of the parameters mentioned above: DNA index, 2c deviation index, 5c exceeding rate (after the Böcking's algorithm) for quantification of aneuploidy, G₁, S, G₂ rate (after the Baisch method) [8]. In total, 29 parameters were determined for each smear. Because of the complexity of morphometry the detection of a large number of parameters is needed in order to overcome the limitation of the method and the difficulty of judgment. 200 cells were measured from every smear and 10 normal epithelial cells were used as internal reference (mean value of the CVs 6.1 ± 1.5 , maximal accepted value 7.5%) (Fig. 1). Preliminary studies disclosed that this number of cells are enough for the accuracy of the measurement as all cells are visualized. For standardization of the method, 200 Feulgen–Schiff stained lymphocytes nuclei of cytospin samples were analyzed by Tv image cytometry, and DNA measurements after standard propidium iodide staining of the same lymphocyte solution were carried out on a Facs-Star Plus flow cytometer system (Beckton Dickinson, USA). The CV of the DNA histograms obtained from the lymphocytes was used as reference of the internal error of the system. The spatial resolution of the image analysis system was quantified using a standardized Burkner chamber.

Statistical analysis: One-way ANOVA, paired *t*-test and correlation analysis of the CSS Statistica (Statsoft, USA, 1991) software were used for the evaluation of results. Null hypothesis was rejected if its probability was less than 0.05. For further evaluation post hoc LSD test was performed.

3. Results

Figure 1(a, b) demonstrates PCNA positive cells in HP positive gastritis and in gastric cancer, respectively. The overexpression of PCNA in the foveolae cells shows different pattern of gastric mucosa compared to the normal epithelium (Fig. 1a). In this case of gastric cancer, almost all cells are labeled (Fig. 1b).

Table 1 shows the percentage of PCNA positive cells observed. There is a higher level of PCNA positive cells in all groups when compared to that in the normal epithelium. LI in carcinoma cases is significantly higher than that in any other group. In cases of gastritis with intestinal metaplasia, the ratio of PCNA positive cells is decreased when compared to the gastritis cases without IM. This finding corresponds to the lower S phase ratio determined by Tv cytophotometry,

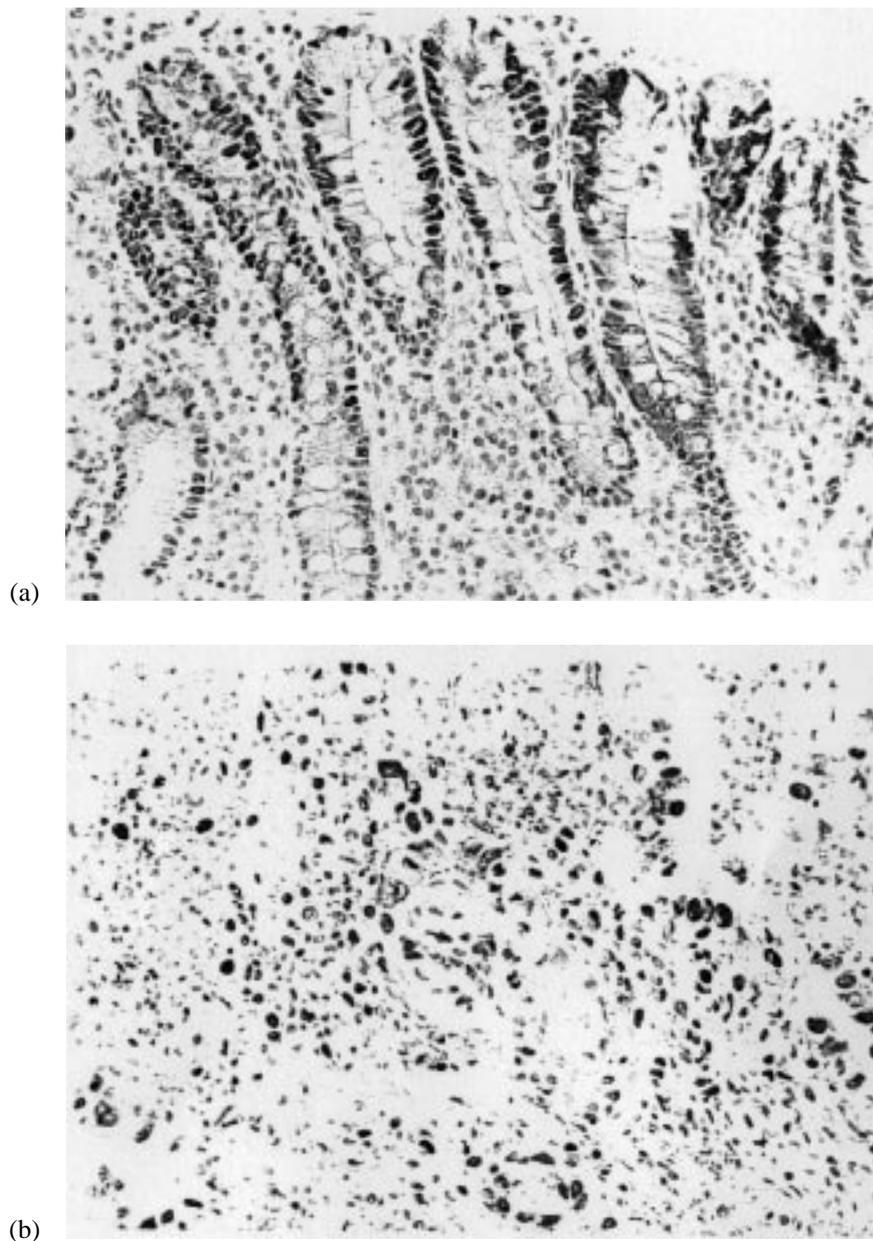


Fig. 1. PCNA immunostaining of *H. pylori* positive gastritis (a) and gastric cancer (adenocarcinoma) (b). Positive nuclei are stained red/black (counterstain in blue/gray, 200 \times) (2 pictures).

we found previously in gastritis with intestinal metaplasia.

PCNA LI was not different between *H. pylori* positive and negative cases.

The percentage of PCNA positive cells observed in mild, moderate and severe gastritis, is shown in Table 2. In severe gastritis, a significantly higher LI could be detected while LI did not statistically differ between mild and moderate gastritis.

The percentage of PCNA positive cells decreased in the same patients after the successful *H. pylori* eradication therapy, however, the difference observed did not reach statistical significance (prior to $69.27 \pm 13.05\%$ vs. after *H. pylori* eradication $55.81 \pm 23.31\%$, $p = 0.084$).

Three typical DNA histograms of normal, gastritis and carcinoma cases are shown in Fig. 2. In case of normal epithelium, 8–12% of the cells are in the S +

Table 1

Ratio of PCNA positive cells in different gastric lesions

Histology	LI (%)
Normal ($n = 10$)	$45.8 \pm 11.07^{*,+}$
Chronic gastritis ($n = 19$)	$63.3 \pm 18.25^{*,\hat{}}$
Gastritis with intestinal metaplasia ($n = 19$)	$52.0 \pm 21.65^{\#}$
Carcinoma ($n = 11$)	$76.2 \pm 12.35^{+,\hat{},\#}$

Results are expressed as mean \pm SD.Significant differences between groups are marked ($p < 0.05$):*between normal and chronic gastritis; + between normal and carcinoma; $\hat{}$ between chronic gastritis and carcinoma; # between gastritis with intestinal metaplasia and carcinoma.

LI%, PCNA labeling index.

 n , Number of cases.

Table 2

Ratio of PCNA positive cells in different degree of gastritis

Gastritis	LI (%)
Mild ($n = 41$)	$50.0 \pm 23.48^*$
Moderate ($n = 14$)	$49.8 \pm 24.23^{\hat{}}$
Severe ($n = 9$)	$71.3 \pm 13.59^{*,\hat{}}$

Results are expressed as mean \pm SD.Significant differences between groups are marked ($p < 0.05$):*between mild and severe chronic gastritis; $\hat{}$ between moderate and severe gastritis.

LI%, PCNA labeling index.

 n , Number of cases.

G₂ phase. When gastritis is present, an increased cell proliferation with wider DNA histograms can be seen, and the percentage of cells in S + G₂ phase is higher. In these cases, no aneuploid cells are found about 5c. In carcinoma, an aneuploid population and aneuploid cells above 5c could also be detected (Fig. 2).

When normal epithelia, gastritis with intestinal metaplasia and carcinoma cases were compared, analysis of variance revealed significant differences in various densito- and morphometrical parameters. Object cells CV, DNA index, 2cDI, the ratio of cells in S phase and IOD mean were all increased in malignant smears. The result of post-hoc comparisons is shown in Table 3.

A significantly higher DNA index (1.08 ± 0.06 vs. 1.05 ± 0.05) and higher average density of nuclei were found in *H. pylori* positive cases. This correlates to the DNA amount.

Comparison of results obtained by immunohistochemistry and Tv image cytophotometry revealed that PCNA LI correlated with percentage of cells in G₁ phase ($r = -0.4147$) and S phase ($r = 0.3851$). Concerning the cases of gastritis with intestinal metaplasia, the following parameters showed correlation with the LI: IOD mean ($r = 0.5982$), density maximum ($r =$

0.6079), surface ($r = 0.6596$), layers ($r = 0.6377$), diameter minimum ($r = 0.6194$), diameter maximum ($r = 0.7302$) and perimeter ($r = 0.5010$).

4. Discussion

The assessment of cell proliferation activity may supply information about the regeneration process or malignant transformation of the epithelia. A consecutive quantitative DNA and morphometric analysis of the epithel cells may yield further quantitative diagnostic and prognostic data. Both quantitative DNA analysis performed by flow cytometry and PCNA labeling rates were shown to be independent prognostic factors in gastric cancer [9]. Until now, most of the data on DNA-aneuploidy and cell proliferation kinetics were obtained by flow cytometry analysis. In recent years, new microscopic image analysis systems were developed. The Tv image cytometry systems were shown to be more sensitive in the detection of low grade DNA-aneuploidy compared to flow cytometry [6]. In addition to the DNA content, these systems can determine several other densito- and morphometric parameters of one nuclei. We have previously shown that the image analysis of gastric imprint smears might yield important additional parameter to the histological diagnosis [10].

Here we found that both morpho- and densitometrical parameters show significant differences between the normal gastritis and carcinoma cases. As the results of this study, a multivariate statistical approach can be developed for the automated classification of the samples.

Böcking et al. showed previously, that gastric inflammatory epithelia show non-malignant polyploidisation. Therefore the 5c exceeding rate could not be accepted, as a marker of malignancy [11]. Its role as diagnostic criteria should be evaluated in multiparametric study.

The obtained difference in DNA Index between the *H. pylori* positive and negative group requires further justification.

As our data concerns only the measurement of 200 nuclei per case, the correlation between PCNA and proliferation parameters should be considered as preliminary. DNA measurement with higher number of cells (several thousands) should be done using automated scanning microscopes.

PCNA appears to be a reliable method that has been showed to correlate well with autoradiography and

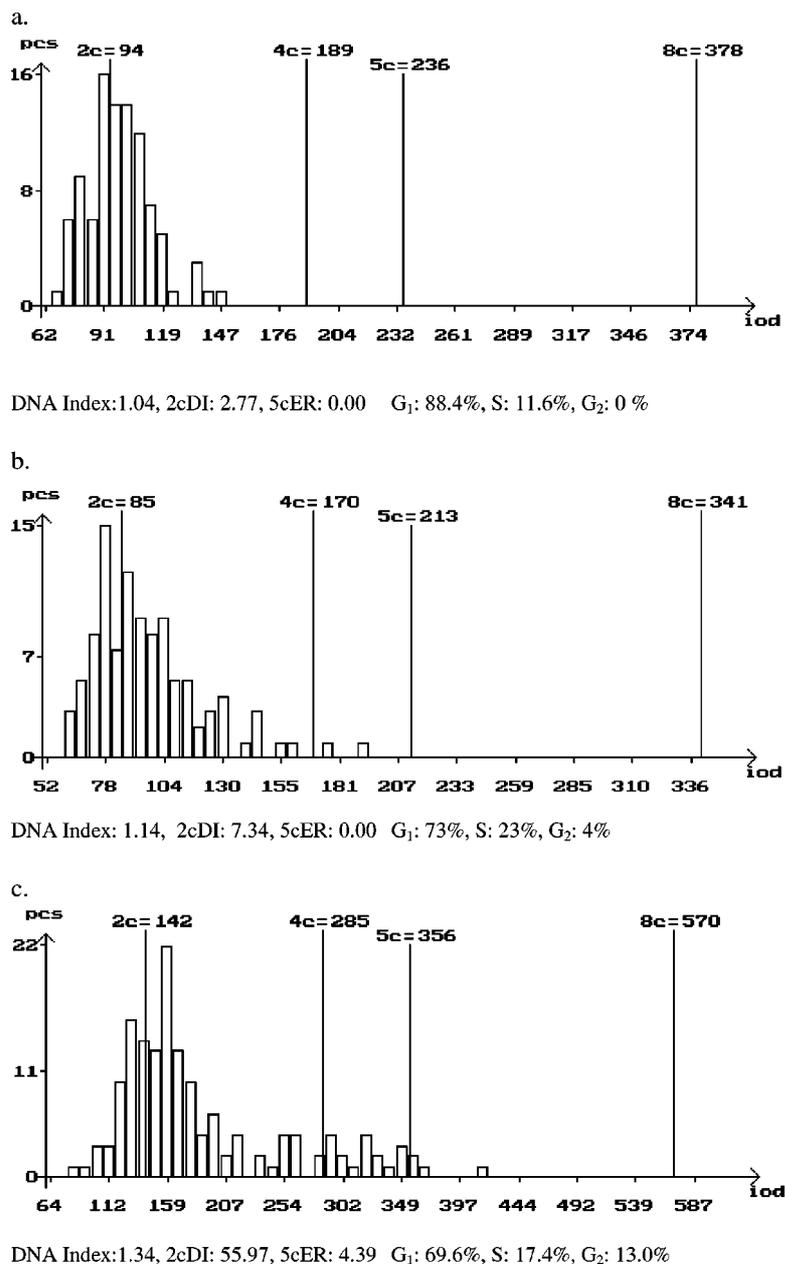


Fig. 2. DNA histograms of a normal (a), a chronic active gastritis (b), and a carcinoma (c) case.

bromodeoxyuridine (BrdU) immunostaining, although not all authors are convinced of the validity of the method for measuring cell proliferation. According to Lynch, PCNA detected by PC10 does not accurately reflect the S-phase fraction in gastric mucosa as determined by BrdU labeling [12]. However, researches on a variety of malignant tumors demonstrated a good correlation between PCNA immunoreactivity and cell proliferation data obtained by other techniques such

as ^3H thymidin incorporation and BrdU. The value of PCNA LI in assessing cell proliferation is also supported by studies of carcinoma of the stomach, showing a good correlation with S + G_2 phase of flow cytometric analysis and with Ki67. When PCNA LI was compared with BrdU and flow cytometry it has been shown a higher percentage of proliferating cells that might be due to the long half life of PCNA (about 20 h). Strongly positive nuclei has been reported to

Table 3
DNA parameters of cytological brush smears

	Normal (n = 8)	Gastritis with IM (n = 18)	Carcinoma (n = 9)
Object cells CV	19.8 ± 5.35*	23.7 ± 8.26 [^]	31.6 ± 10.54* [^]
DNA Index, s	1.03 ± 0.02*	1.07 ± 0.07 [^]	1.27 ± 0.22* [^]
2cDI, s	6.9 ± 11.82*	4.9 ± 3.65 [^]	29.2 ± 31.97* [^]
5cER, s	0.06 ± 0.15	0.44 ± 1.21	2.42 ± 3.04
G ₁	85.5 ± 8.54*	82.5 ± 12.48 [^]	66.1 ± 16.23* [^]
S	14.2 ± 8.06*	15.3 ± 9.49 [^]	27.5 ± 12.49* [^]
G ₂ , s	0.2 ± 0.679	2.2 ± 3.89	6.4 ± 6.06
IOD mean, s	82.7 ± 33.73*	107.9 ± 26.48	120.5 ± 47.01*
Average density	111.1 ± 9.46	117.6 ± 8.30	111.1 ± 5.52
Sigma density	6.0 ± 2.53	6.0 ± 2.49	4.7 ± 1.20
Maximum density	175.2 ± 9.99+	184.0 ± 8.79+ [^]	172.7 ± 6.51 [^]
Density range	49.1 ± 10.43	51.0 ± 9.31	47.3 ± 6.58
Surface	47.4 ± 21.87*	63.7 ± 19.36	69.2 ± 22.60*
Layers	2.0 ± 0.51	2.3 ± 0.39	2.4 ± 0.40
Diameter minimum	5.4 ± 1.75*	6.4 ± 1.23	7.0 ± 1.33*
Diameter maximum	8.5 ± 1.96+,*	10.0 ± 1.54+	10.3 ± 1.64*
Perimeter	32.0 ± 9.03	38.9 ± 9.78	40.9 ± 7.61
Form factor	1.6 ± 0.13	1.6 ± 0.28	1.7 ± 0.16

Results are expressed as mean ± SD; n: number of cases.

Significant differences are marked: s, $p < 0.05$.

Significant differences between groups are marked ($p < 0.05$): *between normal and carcinoma; [^]between chronic gastritis with intestinal metaplasia (IM) and carcinoma; +between normal and chronic gastritis with intestinal metaplasia.

show a better correlation with BrdU and flow cytometry indexes.

In this study, we compared data obtained by two different methods: Tv image cytometry analysis and PCNA immunostaining. When compared with some previous studies [2,3] the absolute values of cell kinetic results are different on both normal mucosa and in case of gastritis. However, according to our knowledge, no studies using real comparable methods have been published yet.

PCNA LI, DNA index and S + G₂ ratio are all higher in chronic gastritis than those in the normal epithelium, and are further increased in carcinoma. In most studies, gastritis inevitably leads to increased epithelial cell proliferation. Results are controversial whether *H. pylori* has a causative role in hyperproliferation. In an *in vitro* study, *H. pylori*-products impaired the physiological process required for mucosal repair and Vac A inhibited cell proliferation specifically [13]. On the contrary, most of the previous *in vivo* studies on gastric epithelial turnover have shown that *H. pylori*-induced chronic active gastritis is associated with hyperproliferation [2,14–17]. Other authors, however, found no difference in PCNA LI, antrum mucosal cell proliferation determined by BrdU [18], or ³H-thymidin in-

corporation [19] between *H. pylori* positive and negative patients. In our study, the DNA index is higher in *H. pylori* associated gastritis than that in *H. pylori* negative ones, whereas PCNA LI is not different between the two groups. The distribution of PCNA positive cells in chronic gastritis is different from that in normal epithelia. In case of gastritis, we found PCNA positive cells in the upper part of gastric foveolae, which in normal conditions does not represent the site of epithelial renewal. This “superficialization” of the proliferative compartment was already shown in *H. pylori* positive patients [16].

Several mechanisms have been suggested for *H. pylori* involvement in gastric carcinogenesis. Increased cell proliferation induced by *H. pylori*, might play a role in initiation and promotion of the process [20]. In chronic gastritis, hyperproliferation supposed to be present for many years. Genetic mutations are facilitated in the proliferating cells and they are also more sensitive to exogen mutagenic factors such as N-nitroso compounds formed in the stomach. Panella et al.'s data showed a progressive increase of epithelial proliferation in the successive stages of *H. pylori* infection, ranging from gastritis to the development of incomplete intestinal metaplasia, a well-

known precancerous condition [2]. In contrast with his and Fraser's [4] results which show that IM is associated with higher rates of epithelial cell proliferation, we observed a lower PCNA LI in chronic gastritis with IM which corresponds to the lower S phase ratio in gastritis with IM as determined by Tv cytometry. We detected a significantly higher LI in severe gastritis, as suggested by Peek, who found that mucosa proliferation in gastritis correlated with the severity of acute gastritis. He observed no parallel increase in apoptosis which might contribute to a higher risk for carcinoma in these cases [21].

The percentage of PCNA-positive cells in *H. pylori* positive patients decreased after the successful eradication therapy, however, the difference did not reach statistical significance. Results in the literature are controversial. Gastric mucosal cell proliferation determined by BrdU has been reported to be increased in *H. pylori* associated gastritis and it returned to normal after eradication therapy [22,23]. PCNA LI was significantly decreased after successful eradication, or even following ineffective therapy [4]. In this latter case, the lower proliferation rate could be explained by the reduced inflammation. On the contrary, PCNA LI of patients who remained *H. pylori* positive was unaltered, while it was reduced in patients who cleared *H. pylori* [3].

Controversies in the literature might origin from the relative small number of cases, but also suggest that *H. pylori* is not the only factor responsible for the increased cell turnover, and this regulation is more complex than previously estimated. Because of this complexity, parallel measurements are needed. Our results with parallel measurements of both PCNA-LI and Tv cytometry, support the view that cell proliferation is enhanced if *H. pylori* infection is present. However, further and larger studies are needed to strengthen this hypothesis.

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