# Matrix Metalloproteinase-2 (MMP-2) and its Tissue Inhibitor (TIMP-2) are Prognostic Factors in Cervical Cancer, Related to Invasive Disease but not to High-risk Human Papillomavirus (HPV) or Virus Persistence after Treatment of CIN

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**Abstract.** *Objective: Matrix metalloproteinase-2 (MMP-2) and its tissue inhibitor (TIMP-2) are important regulators of cancer invasion and metastasis. Their associations to high-risk (HR) human papillomavirus (HPV) in cervical intra-epithelial neoplasia (CIN) and cervical cancer (CC) are unexplored and their prognostic significance in CC remains controversial. Materials and Methods: As part of our HPV-PathogenISS study, a series of 150 CCs and 152 CIN lesions were examined using immunohistochemical (IHC) staining for MMP-2 and TIMP-2 and tested for HPV using PCR with 3 primer sets (MY09/11, GP5+/GP6+, SPF). Follow-up data were available from all squamous cell carcinoma patients and 67 CIN lesions had been monitored with serial PCR for HPV after cone treatment. Results: MMP-2 increased with the grade of CIN, with major up-regulation upon transition to invasive cancer (OR 20.78) (95%CI 7.16-60.23) (p=0.0001). TIMP-2 retained its normal expression until CIN3, with dramatic down-regulation in invasive disease (p=0.0001 for trend). Thus, the MMP2:TIMP-2 ratio increased with progressive CIN, exceeding the value 1.0 only in invasive disease. Both MMP-2 and TIMP-2 are highly specific (TIMP-2; 100%) discriminators of CIN with 100% positive predictive value (TIMP-2), but suffer from low sensitivity and negative predictive value. Neither MMP-2 nor TIMP-2 showed any significant association with HR-HPV or virus persistence/clearance. TIMP-2 (but not MMP-2) was a significant predictor of survival in univariate (Kaplan-Meier) analysis (p=0.007), but lost its significance in multivariate (Cox) analysis. Conclusion: The activities of MMP-2 and TIMP-2 in cervical carcinogenesis seem to be unrelated to HR-HPV. The inverse MMP-2:TIMP-2 ratio is a sign of poor prognosis. A combination of a TIMP-2 assay with another test showing high SE and high NPV (e.g., HCII for HPV) should provide a potential screening tool capable of accurate detection of CIN.*

The high-risk human papillomavirus (HR-HPV) types are associated with cervical cancer (CC) and its precursor, cervical intra-epithelial neoplasia (CIN) lesions in practically all cases, unlike the low-risk HPV (LR-HPV) types that are rarely found in CIN and CC (1-5). These different oncogenic potentials of LR-HPV and HR-HPV are attributable to the different interactions of 2 major viral oncoproteins (E6 and E7) with the key regulatory cellular proteins, p53 and pRb (2, 3, 6-8). While the E6 of the HR-HPV (but not LR-HPV) initiates degradation of the p53 tumour suppressor protein, HPV E7 of HR-HPV (but not LR-HPV) binds to pRb resulting in G1/S transition of the cell cycle (3, 6, 8, 9). These

type-dependent functions of E6 and E7 are different from those of another HPV oncoprotein E5, which activates potent nuclear transcription factors through the ERK/MAPK signalling pathway, independent of HPV types (3, 8, 10, 11). Several different molecular pathways are interfered with by oncogenic HPV in a highly complex process leading to invasive CC (1-3, 7, 8, 11).

To progress from intra-epithelial neoplasia to invasive cancer, neoplastic cells must acquire properties making them capable of penetrating the basement membrane (BM) and degrading the underlying extracellular matrix (ECM), composed of several structural components, *e.g,*. collagen, elastin and fibronectin (12, 13). Matrix metalloproteinases (MMP) are a family of enzymes capable of degrading many of these ECM components and which are currently believed to play a major role in tumour invasion and metastases (14-17). There are at least 24 known MMPs, which are classified according to their substrate specificity as: a) interstitial collagenases, b) stromelysins, c) gelatinases, d) elastases and e) membrane-type MMPs (MT-MMP) (17, 18). One of the MMPs, MMP-2 (also known as 72 kDa type IV collagenase or gelatinase A), is able to degrade type IV collagen (12) and fibronectin components of the BM, thus facilitating the stromal and vascular invasion of tumour cells (15, 17-19). This degradative activity of MMP-2 is controlled by a specific tissue inhibitor of MMP-2, known as TIMP-2, a 22 kDa protein that forms a 1:1 stoichiometric complex with the active site of the enzyme (19-21). Activation of pro-MMP-2 requires the formation of a trimolecular complex with TIMP-2 and an MT1-MMP, resulting in cleavage of the $NH_2$-terminal prodomain of pro-MMP-2 and, further, to maturation of fully active MMP-2 (14, 15, 17, 19-21).

The expressions of MMP-2 and TIMP-2 have been studied in several types of human malignancies, including CC and its precursors (14, 19, 22-25). Usually, MMP-2 expression seems to be up-regulated in invasive cancer as compared with CIN lesions, but the role of TIMP-2 expression is less clear (22, 24). In these studies, surprisingly little attention was paid to the mutual associations between MMP-2 and TIMP-2 expressions and their relationships to HPV detection (19, 23, 25-27). Thus, further data are urgently needed on possible molecular interactions between HR-HPV and MMP-2/TIMP-2, as well as on their relationship to several intermediate end-point markers in cervical carcinogenesis. Similarly, only a few studies have attempted to elucidate the prognostic significance of MMP-2 or TIMP-2 in CC (19, 22, 26-30).

As a part of our ongoing systematic search for potential biomarkers in HPV-associated cervical carcinogenesis (HPV-PathogenISS study) (9, 10, 31, 32), a series of CIN lesions and invasive squamous cell carcinomas (SCC) were analysed to assess whether the expressions of MMP-2 and TIMP-2 might be of any use in predicting the intermediate

end-point markers in cervical carcinogenesis: a) the grade of CIN, b) HR-HPV detection, c) clearance of the virus after treatment of CIN, as well as d) survival of CC patients as the terminal event were examined. In the present study, the expressions of MMP-2 and TIMP-2 were studied in CIN lesions treated by conisation and monitored by serial PCR assays for HPV detection, and the survival data of CC patients were related to MMP-2 and TIMP-2 expressions in surgical samples.

## Materials and Methods

The material of this study comprised the retrospective component of the HPV-PathogenISS project (33) and was collected from the files of the Pathology Departments of 2 Italian hospitals (S. Orsola Malpighi Hospital, Bologna, and Maggiore Hospital, University of Trieste). Altogether, this prospective biopsy material comprised 302 patients with either an invasive cervical squamous cell carcinoma (CC) or cervical intra-epithelial neoplasia (CIN) diagnosed and treated in these 2 hospitals between 1986 and 2002. Of these 302 cases, 114 CIN and 38 CC cases were provided by Bologna, and 38 CIN lesions and 112 CCs were available from Trieste. The mean age of all CIN patients was 35.5 (range 18-79) years, and that of SCC patients 59.2 (range 27-89) years ($p=0.0001$).

*Available data.* All the cases from Bologna had had their HPV status determined by PCR, as reported before (34-36), whereas the samples from Trieste were tested for HPV in this study. Complete follow-up data were available for all 150 CC patients, with a mean follow-up of 51.7 months (range 1-218). Furthermore, all CIN cases from Bologna had been followed-up at 6-month intervals after cone treatment (for a mean of 10.5 months, range 2.4-27.6) and subjected to repeated colposcopy, PAP smear and biopsy (if residual suspected). A minimum of 2 (up to 7) serial PCR analyses were available from 67 cases, as recently reported (36).

*Biopsy.* Both the colposcopic biopsies and surgical samples were fixed in 10% buffered formalin, embedded in paraffin, and processed for 5-μm-thick paraffin sections stained with haematoxylin-eosin (HE) for routine diagnosis. All the slides were re-examined to confirm the diagnosis. On histological examination, the lesions were graded using the CIN nomenclature and categorised as CIN1, CIN2 or CIN3. The histological diagnosis of CC was confirmed in all cases and 2 adenocarcinomas present in the original cohort were excluded from this series.

*Immunohistochemistry for MMP-2 and TIMP-2.* Immunohisto-chemical (IHC) staining for MMP-2 and TIMP-2 was completed following standard IHC procedures. In brief, the 5-μm paraffin sections cut on poly-L-lysine-coated microscopy slides were first deparaffinised and rehydrated in graded alcohols. The sections were heated in citrate buffer (0.01 M, pH 6.0, DAKO Target Retrieval Solution) in a microwave oven (85-95°C, 3x5 min), followed by blocking the non-specific binding sites with normal rabbit serum. For MMP-2, the sections were incubated with the primary antibody, anti-human MMP-2 (CA4001/CA719E3C) (Abcam, Ltd., Cambridge, UK), in a humidified chamber for 1 h at room temperature (dilution 1:50). This purified mouse monoclonal (IgG1) antibody has been raised against the

N-terminal epitope (APSPIIKFPGD-VAPKTDK) of human MMP-2 and reacts with human, rat and mouse MMP-2. For TIMP-2, the primary antibody, anti-human TIMP-2 (ab4048) (Abcam) was used, at 1:50 dilution. This polyclonal rabbit (IgG) antibody has been raised against the synthetic peptide derived from the N-terminal half of human TIMP-2 and reacts with human TIMP-2 only. Both antibodies have been extensively tested in IHC staining of several different human tumours and were shown to perform well in paraffin-embedded sections. The primary antibody was followed by incubation with the biotinylated secondary antibody, polyclonal goat anti-mouse IgG (#6788, Abcam, dilution 1:250) and polyclonal goat anti-rabbit IgG (#6720, Abcam, dilution 1:200), for MMP-2 and TIMP-2, respectively. The slides were then processed with universal LSAB™-2 single reagents (peroxidase) kit (DakoCytomation) and the expressions of MMP-2 and TIMP-2 were localised by incubation with DAB (diaminobenzidine). As a final step, the slides were stained with a light haematoxylin counterstaining. Negative controls were similarly processed by omitting the primary antibody, while biopsies from breast and/or colon cancer were used as positive controls.

*Evaluation of the IHC staining.* IHC staining was examined using light microscope (Leitz Diaplan, Leitz Wetzlar, Germany), equipped with a digital camera (Leica DG300). In normal squamous epithelium, MMP-2 staining was regularly absent. In the CIN lesions and CC, MMP-2 immunostaining was confined to the cytoplasm (weak to strong in intensity), but occasional nuclear expression was also detected. In the original grading, only the cytoplasmic staining was graded, using a semi-quantitative scoring into 4 categories: 0=negative; 1=weak staining (few MMP-2-positive cells detectable or more diffuse weak staining); 2=moderate staining (positive cells easily detectable); and 3=strong staining (intense diffuse cytoplasmic staining throughout the lesion) (Figures 1-3).

TIMP-2 staining was abundant in normal squamous epithelium, and this normal pattern was used as reference for CIN lesions. The majority of the CIN lesions retained this normal expression until progression to CIN3. TIMP-2 immunostaining was also confined to the cytoplasm, but occasional expression in the underlying stroma was also detected. Only the cytoplasmic staining was graded, using 4 categories: 0=negative; 1=markedly reduced expression (large areas without staining); 2=moderately to slightly reduced expression (minor areas without staining); and 3=normal expression (staining equivalent to normal epithelium) (Figures 4-6). In some statistical analysis, the staining results were also treated as dichotomous categorical variables, where separately indicated: MMP-2 staining (negative-weak/moderate-intense) and TIMP-2 staining (negative-strongly reduced/moderately reduced-normal).

*HPV testing.* The 114 CIN and 38 SCC cases from Bologna had already been HPV tested for other purposes using PCR, as recently reported (34-36). In the present study, the 150 paraffin-embedded sections (112 SCC and 38 CIN) delivered from Trieste were subjected to HPV testing by PCR.

*Polymerase chain reaction.* To verify the extraction and the quality of DNA from the paraffin-embedded tissues, 5 μl of each sample were amplified with a primer set recognizing the *β-actin* gene (sense: 5'-GGCGGCACCACCATGTACCCT-3', anti-sense: 5'-AGGGGCCGGACTCGTCATACT-3'). The PCR mix contained

200 μM each dNTP, 1.5 mM MgCl$_2$, 1X PCR buffer, 40 pmol sense and anti-sense primer, 1.25 U AmpliTaq Gold (Applied BioSystem, Branchburg, USA). The PCR conditions were following: 94˚C, 10 min, for 1 cycle; 94˚C, 30 sec, 60˚C, 30 sec, 72˚C, 30 sec, for 25 cycles; finally, 72˚C for 7 min.

The samples were then amplified for the presence of HPV using different sets of degenerated primers as described separately for MY09/MY11 (37), GP5$^+$/GP6$^+$ (38) and biotinylated SPF primer mix located within the L1 region of the HPV genome (39), respectively. The PCR conditions for the My09/My11 were: 94˚C for 10 min, 1 cycle; 94˚C, 30 sec, 55˚C, 45 sec, 72˚C, 30 sec for 40 cycles; followed by an extension step at 72˚C for 7 min. The PCR mix contained 200 μM each dNTP, 40 pmol each primer, 2 mM MgCl$_2$, 1X PCR buffer and 1.25 U AmpliTaq Gold (29). For the GP5$^+$/GP6$^+$ primers, the following conditions were used: 94˚C for 10 min, 1 cycle; 95˚, 30 sec, 44˚C, 60 sec, 72˚C, 90 sec, for 40 cycles; then a final extension step at 72˚C for 7 min. Amplification with the SPF primer mix was carried out as follows: 94˚C, 10 min, 1 cycle; 94˚C, 30 sec, 52˚C,45 sec, 72˚C, 45 sec, for 40 cycles; final extension step at 72˚C for 7 min. Positive and negative controls were included in each amplification (37-39).

*HPV typing.* HPV typing was done using the reverse-hybridisation assay. The denatured biotinylated amplified product (10 ml) was hybridised with specific oligonucleotide probes, which are immobilised as parallel lines on membrane strips (InnoLiPA, Innogenetics, Ghent, Belgium) (39). After hybridisation and stringent washing, streptavidin-conjugated alkaline phosphatase was added and bound to any biotinylated hybrid previously formed. Incubation with BCIP/NBT chromogen yields a purple precipitate that can be visually interpreted. Based on the position of the visualised line, it is possible to determine the HPV genotype (39). The following HPV types were included in the test panel: HPV 6,11,16,18,31,33,34,35,39,40,42,43,44,45,51,52, 53,54,56,58,59,66,68,70 and 74.

*Statistical methods.* Statistical analyses were performed using the SPSS® and STATA software packages (SPSS for Windows, Version 12.0.1, and STATA/SE 9.1). Frequency tables for categorical variables were analysed using the Chi-square test, with the likelihood ratio (LR) or Fisher's exact test to assess the significance of the correlation. Bivariate correlations between ordered variables were analysed using the Spearman correlation analysis (Spearman rho). Ratio statistics were used to calculate the ratio (mean and 95%CI, plus CV) for the MMP-2:TIMP-2, using lesion grade as the grouping variable. Differences in the means of continuous variables were analysed using non-parametric tests (Mann-Whitney) or ANOVA (analysis of variance). Logistic regression models using the step-wise backward approach and LR statistic for removal testing were used to analyse the power of different covariates as predictors of the outcome variables (CIN, HR-HPV), calculating crude ORs (and 95%CI). Performance indicators of MMP-2 and TIMP-2 as markers of CIN or HR-HPV were calculated using the contingency tables for sensitivity, specificity, positive (PPV) and negative predictive value (NPV), with 95%CI based on the F-distribution (±1.96xSE). Univariate survival (life-table) analysis for the outcome measure (HPV clearance/persistence, overall survival) was based on the Kaplan-Meier method. Multivariate survival analysis was run by using Cox's proportional hazards model in a backward step-wise manner with the log-likelihood ratio (L-R) significance test, and using the default values for entry and exclusion criteria. The
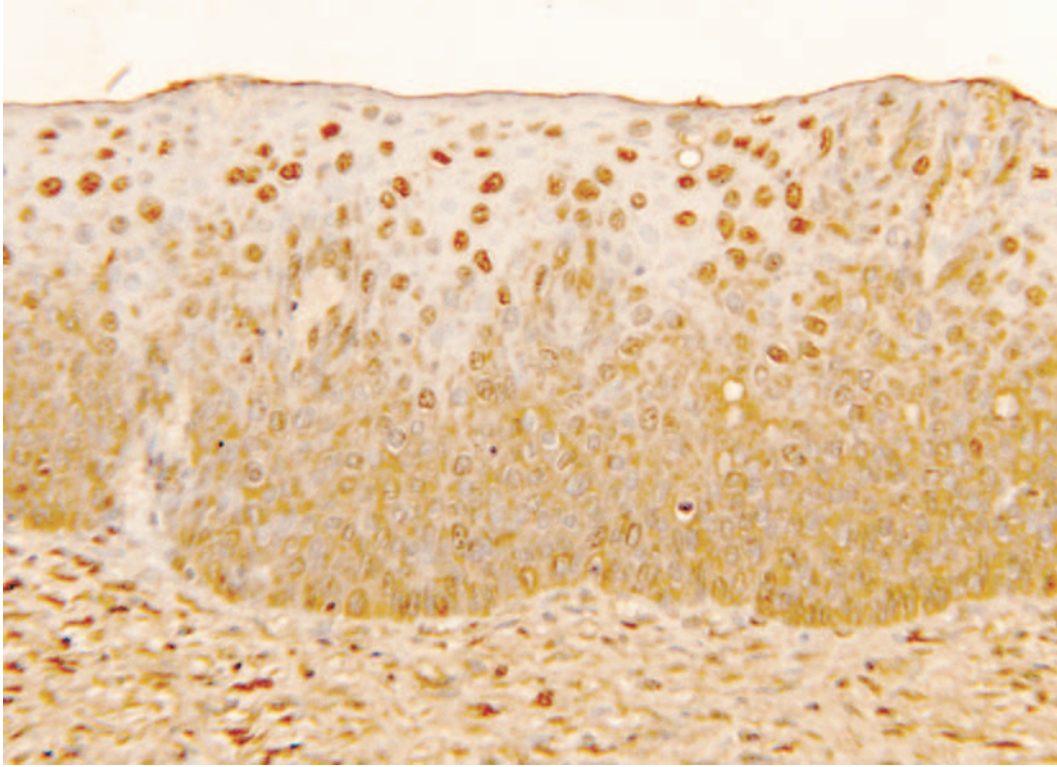
Figure 1. *A low-grade CIN1 lesion stained with MMP-2 antibody. Cytoplasmic MMP-2 expression was clearly detectable in the dysplastic cells within the lower one-third of the epithelium. Single cells in the upper one-third also demonstrated nuclear immunostaining. (IHC for MMP-2, original magnification x100).*
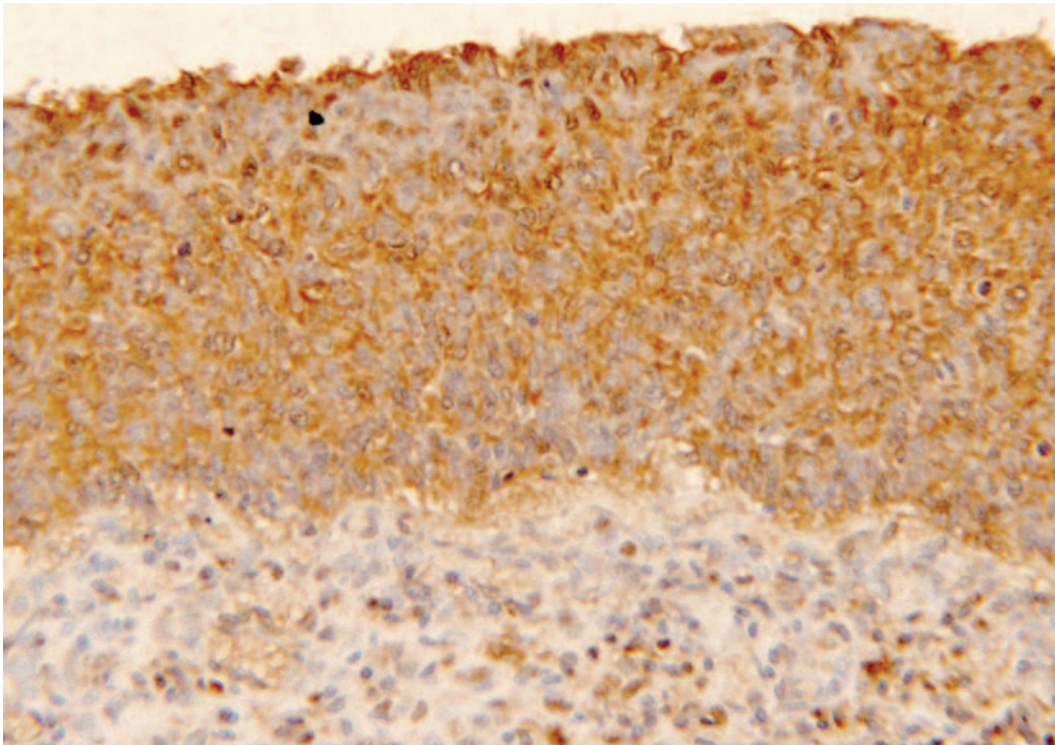


Figure 2. *A high-grade CIN3 lesion, with full thickness expression of MMP-2. As compared with the low-grade lesion in Figure 1, the staining intensity was clearly increased and the MMP-2 expression was almost exclusively confined to the cytoplasm. (IHC for MMP-2, original magnification x100).*
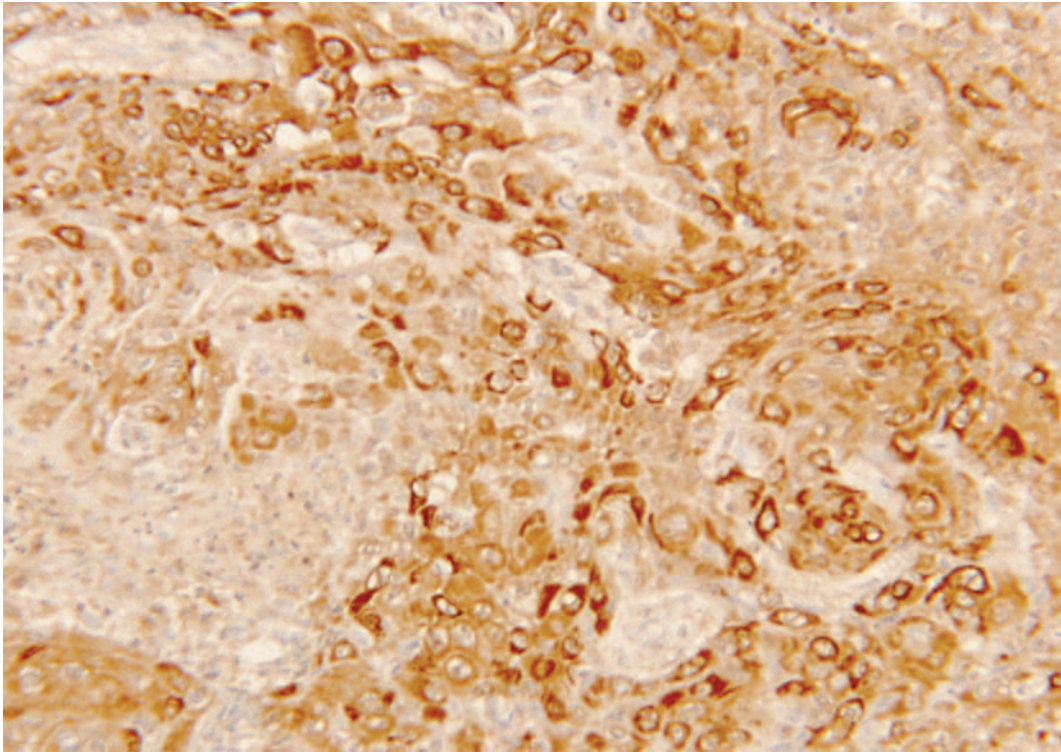
Figure 3. *Detail of an invasive cervical carcinoma. MMP-2-positive cells were found throughout the lesion, showing characteristic cytoplasmic expression. Bare nuclei were surrounded by a cytoplasmic ring of MMP-2-positive immunostaining in most of the cancer cells. The staining intensity varied from weak to intense dark brown, indicating that the expression levels of MMP-2 varied from cell to cell. (IHC for MMP-2, original magnification x100).*
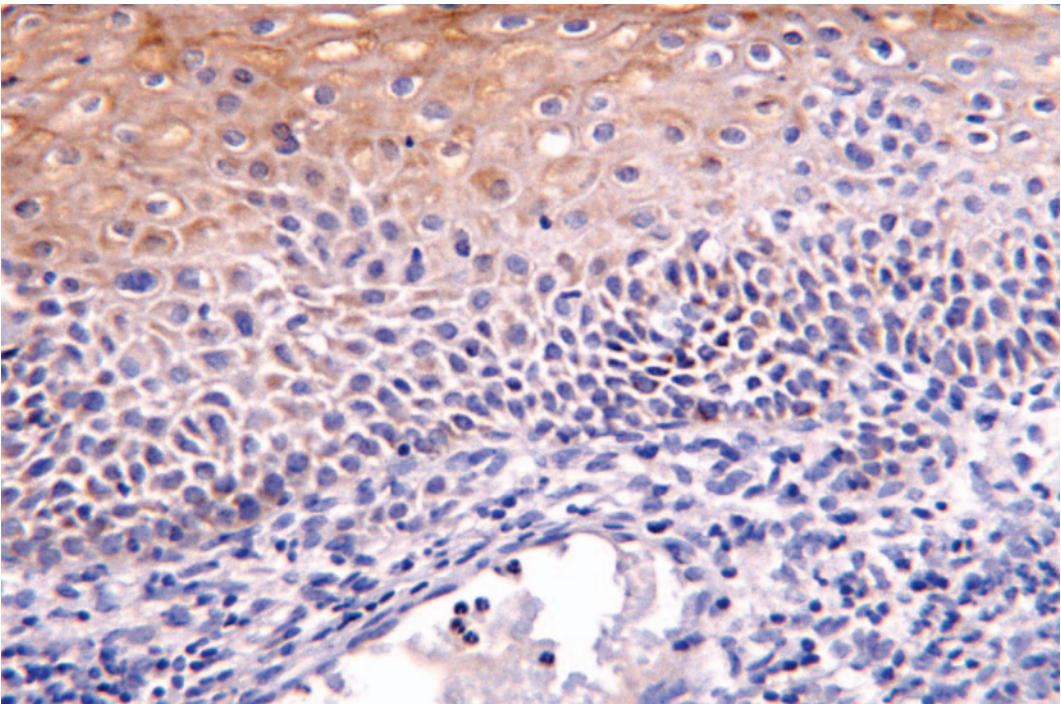


Figure 4. *Normal epithelium stained with TIMP-2 antibody. This picture demonstrates a characteristic TIMP-2 expression in normal cervical epithelium, to which the expression in CIN lesions and cancer was compared. Immunostaining was exclusively cytoplasmic and strictly limited to epithelial cells, whereas the underlying stroma remained TIMP-2-negative. The staining intensity was quite homogeneous, indicating that the expression levels of TIMP-2 did not vary markedly from cell to cell. (IHC for TIMP-2, original magnification x100).*
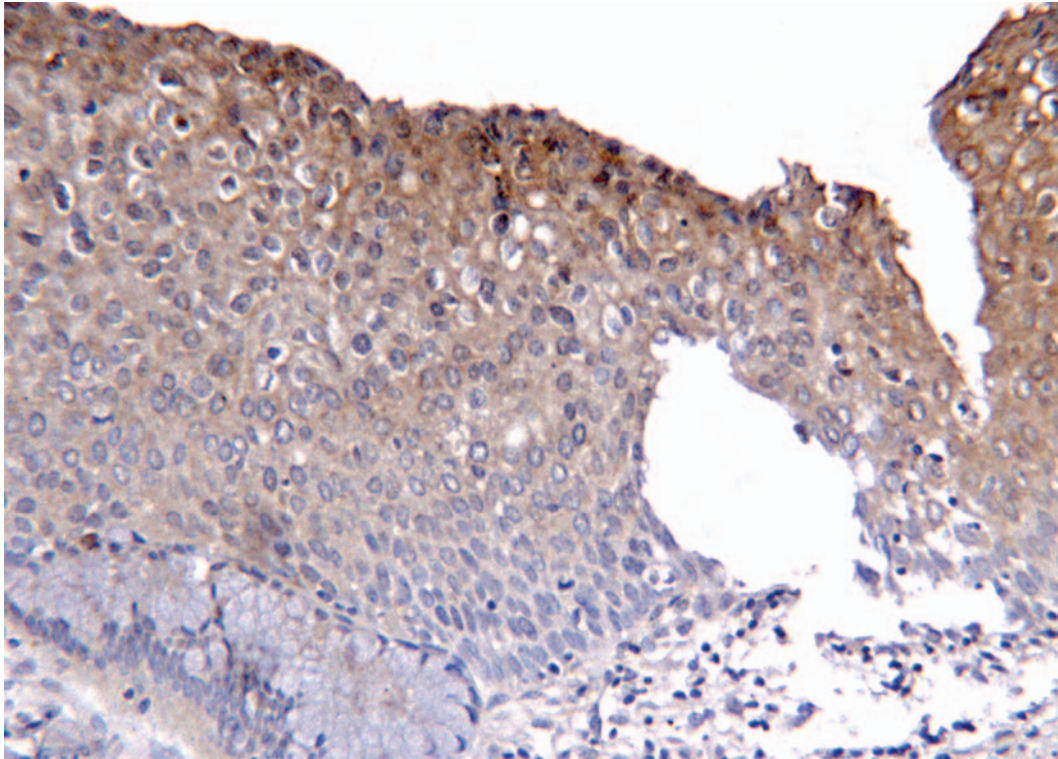
Figure 5. *A CIN2 lesion with typical HPV-induced morphological changes (koilocytes) in the uppermost one-third of the epithelium. This lesion had retained TIMP-2 expression equivalent to that of the normal epithelium. Cytoplasmic TIMP-2 staining was detectable almost throughout the lesion, except for the lowermost few layers of dysplastic cells, where TIMP-2 expression was absent or very weak. (IHC for TIMP-2, original magnification x100).*



Figure 6. *Detail of a well-differentiated squamous cell carcinoma, stained with TIMP-2 antibody. As compared with the normal epithelium, the TIMP-2 expression was significantly reduced, but not entirely absent, however. A few TIMP-2-positive cells were detectable in the vicinity of the invasive fronts, demonstrating weak cytoplasmic staining, whereas the majority of cancer cells remained completely TIMP-2-negative. (IHC for TIMP-2, original magnification x100).*

Table I. *Expressions of MMP-2 and TIMP-2 as related to the grade of cervical lesions.*

| Lesion grade | MMP-2 expression | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Negative | | Weak | | Moderate | | Strong | |
| | No. | % | No. | % | No. | % | No. | % |
| Negative for CIN | 6 | 85.7 | 1 | 14.3 | 0 | 00.0 | 0 | 00.0 |
| CIN1 | 13 | 72.2 | 5 | 27.8 | 0 | 00.0 | 0 | 00.0 |
| CIN2 | 5 | 25.0 | 11 | 55.0 | 3 | 15.0 | 1 | 5.0 |
| CIN3 | 12 | 14.0 | 32 | 37.2 | 20 | 23.3 | 22 | 25.6 |
| SCC | 6 | 4.4 | 23 | 17.0 | 37 | 27.4 | 69 | 51.1 |
| Total (n=266)* | 42 | 15.8 | 72 | 27.1 | 60 | 22.2 | 92 | 34.6 |

*Fisher's exact test, $p=0.0001$ (also for linear trend).

| Lesion grade | TIMP-2 expression | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Negative | | Markedly reduced | | Slightly-moderately reduced | | Equivalent to normal epithelium | |
| | No. | % | No. | % | N | % | No. | % |
| Negative for CIN | 0 | 00.0 | 0 | 00.0 | 0 | 00.0 | 9 | 100.0 |
| CIN1 | 0 | 00.0 | 2 | 10.0 | 2 | 10.0 | 16 | 80.0 |
| CIN2 | 0 | 00.0 | 1 | 6.7 | 2 | 13.3 | 12 | 80.0 |
| CIN3 | 4 | 4.9 | 10 | 12.3 | 18 | 22.2 | 49 | 60.5 |
| SCC | 18 | 13.1 | 45 | 32.8 | 35 | 25.5 | 39 | 28.5 |
| Total (n=262)** | 22 | 8.4 | 58 | 22.1 | 57 | 21.8 | 125 | 47.7 |

** Fisher's exact test, $p=0.0001$ (also for linear trend).

assumption of proportional hazards was checked by log-minus-log (LML) survival plots. In all tests, values $p<0.05$ were regarded as being statistically significant.

## Results

The expressions of MMP-2 and TIMP-2 as related to the grade of the cervical lesion are provided in Table I. There was a linear relationship between the increasing grade of CIN and the intensity of MMP-2 expression ($p=0.0001$ for linear trend). Using the 2-tier category of staining (negative-weak/moderate-intense), the latter was associated with CIN3/cancer with overall response (OR) 20.78 (95%CI 7.16-60.23)($p=0.0001$). TIMP-2, in turn, retained its expression equivalent to that of the normal cervical epithelium until high-grade lesions (60% in CIN3), with dramatic down-regulation upon transition to invasive disease, however (28.5% in CC). This gradual decrease in TIMP-2 expression was also significant across the strata ($p=0.0001$). Using the 2-tier category of staining (negative-strongly reduced/moderately reduced-normal), the former was associated with CIN3/cancer with OR 7.46 (95%CI 2.23-24.89) ($p=0.0001$).

The MMP-2:TIMP-2 ratio was calculated using the ratio statistics (95%CI and CV) (data not in Tables). There was a linear increase in the MMP-2/TIMP-2 ratio in parallel with the progressive lesion as expressed by weighted mean, as follows: negative for CIN (NCIN), 0.056 (95%CI –0.87-0.198); CIN1, 0.100 (95%CI 0.017-0.183); CIN2, 0.390 (95%CI 0.217-0.564); CIN3, 0.686 (95%CI 0.583-0.724); SCC, 1.200 (95%CI 1.061-1.339)(ANOVA, $p=0.0001$). The mean centred CVs were: 244.9%, 169.7%, 79.4%, 72.6% and 62.2%, respectively.

Of the CIN lesions, 70.5% were HR-HPV-positive, in contrast to only 11.1% of those without CIN. HR-HPV types were even more prevalent in CC cases, 77.6%, the remaining 22.4% being either negative (4.9%) or the HPV type could not be determined (17.5%). HR-HPV detection was associated with CC with OR 27.25 (95%CI 3.28-226.09) and with CIN with OR 19.12 (95%CI 2.31-157.81).

The expressions of MMP-2 and TIMP-2 as related to the detection of HR-HPV in the lesions are provided in Table II. Neither MMP-2 nor TIMP-2 showed any significant relationship with the detection of HR-HPV. For MMP-2, the association was close to borderline significance ($p=0.051$), whereas TIMP-2 expression was practically identical in the presence or absence of HR-HPV. Using the 2-tier grading did not disclose any significant link between HR-HPV and MMP-2 or TIMP-2 expressions either.

The calculated performance indicators for MMP-2 and TIMP-2 as markers of CIN, high-grade CIN (CIN3 and above) and HR-HPV are shown in Table III. Moderate-

Table II. *Expression of MMP-2 and TIMP-2 as related to high-risk HPV types in the lesions.*

| HR type* | MMP-2 expression | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Negative | | Weak | | Moderate | | Strong | |
| | No. | % | No. | % | No. | % | No. | % |
| Present | 23 | 12.2 | 55 | 29.3 | 42 | 22.3 | 68 | 36.2 |
| Absent | 18 | 26.9 | 16 | 23.9 | 15 | 22.4 | 18 | 26.9 |
| Total (n=255)** | 41 | 16.1 | 71 | 27.8 | 57 | 22.4 | 86 | 33.7 |

*includes cases that were HPV-negative; **Chi-Square test; L-R statistics, $p=0.051$.

| HR type* | TIMP-2 expression | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Negative | | Markedly reduced | | Slightly-moderately reduced | | Equivalent to normal epithelium | |
| | No. | % | No. | % | No. | % | No. | % |
| Present | 17 | 9.1 | 38 | 20.4 | 42 | 22.6 | 89 | 47.8 |
| Absent | 5 | 7.2 | 18 | 26.1 | 14 | 20.3 | 32 | 46.4 |
| Total (n=255)*** | 22 | 8.6 | 57 | 22.0 | 56 | 22.0 | 121 | 47.5 |

*includes cases that were HPV-negative; ***Fisher's exact test, $p=0.798$.

Table III. *Performance indicators of MMP-2 and TIMP-2 as markers of CIN and HR-HPV type.*

| Outcome variable | *MMP-2 expression (95%CI) | | | |
|---|---|---|---|---|
| | Sensitivity | Specificity | PPV | NPV |
| CIN*** | 36.6% (28.1-45.1) | 87.5% (64.6-100) | 97.8% (93.6-100) | 8.2% (2.3-14.1) |
| CIN3[#] | 66.9% (60.8-73.2) | 91.1% (82.8-99.4) | 97.3% (94.8-99.9) | 35.9% (27.1-44.8) |
| HR-HPV | 58.5% (51.5-65.6) | 50.7% (38.8-62.8) | 76.9% (70.2-83.8) | 30.3% (21.8-38.8) |

| Outcome variable | **TIMP-2 expression (95%CI) | | | |
|---|---|---|---|---|
| CIN*** | 14.8% (8.3-21.3) | 100% (100-100) | 100% (100-100) | 9.2% (3.8-14.7) |
| CIN3[#] | 35.3% (28.9-41.7) | 93.2% (85.7-100) | 96.3% (92.1-100) | 22.5% (16.5-28.6) |
| HR-HPV | 29.5% (23.0-36.1) | 66.7% (55.5-77.7) | 70.5% (60.4-80.6) | 25.9% (19.5-32.4) |

*MMP-2 staining (negative-weak/moderate-intense); **TIMP-2 staining (negative-strongly reduced/ moderately reduced-normal);
***any grade of CIN (SCC cases excluded); [#]CIN3 cut-off (SCC cases included).

intense MMP-2 expression was an 87.5% specific indicator of CIN, with 97.8% PPV, but NPV is only 8.2%. With the CIN3 cut-off, there was a slight gain in specificity and a significant increase in sensitivity and NPV, without compromising PPV. TIMP-2 expression was a 100% specific discriminator between CIN and non-CIN, with 100% PPV, but it suffered from low sensitivity and low NPV. However, lifting the cut-off to CIN3 changed these indicators only slightly and, like MMP-2, TIMP-2 was of no value as a predictor of HR-HPV.
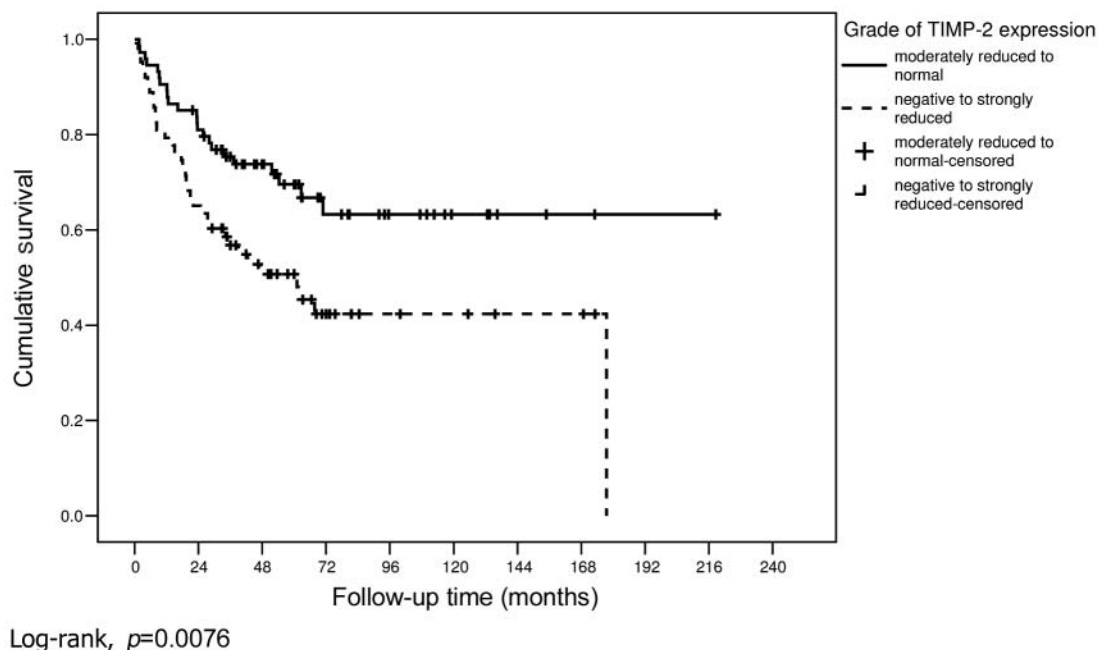
Figure 7. *TIMP-2 expression as a predictor of overall survival in Kaplan-Meier analysis.*

Of the HPV-positive women treated for CIN and controlled by serial PCR, 41/67 (61.2%) had been cleared of their HR-HPV infection by the last PCR assay, for a total of 705 women-months at risk (WMR), with a monthly clearance rate of 5.8% (58/1.000 WMR). Clearance (64.3%) (18/28) in cases with moderate-intense MMP-2 expression was identical (63.0%) (17/27) as in the cases with negative-weak MMP-2 staining ($p=0.919$). The corresponding figures for TIMP-2 were 0% (0/3) and 62.0% (31/50) (Fisher's exact test, $p=0.066$). In univariate (Kaplan-Meier) survival analysis, MMP-2 or TIMP-2 were not significant predictors of HPV clearance/persistence after treatment of CIN (log-rank; $p=0.143$ and $p=0.654$, respectively).

Of the 150 CC patients, 91 (60.7%) were alive and 59 had died during the follow-up. The expression of MMP-2 was related (albeit not significantly) to survival in that the probability of being alive with moderate-intense MMP-2 was only 55.7% as compared to 72.4% in women with negative or weakly MMP-2-positive tumours ($p=0.098$). Importantly, TIMP-2 expression proved to be significantly related to survival in CC patients, in that 68.9% of women with normal-slightly/moderately reduced TIMP-2 were alive in contrast to only 46.0% of those whose tumours were TIMP-2-negative or weakly positive ($p=0.007$). Similarly, the MMP-2:TIMP-2 ratio was significantly higher (1.79) among women who died of their disease than that (1.34) among survivors (Mann-Whitney, $p=0.014$).

In Kaplan-Meier analysis, MMP-2 expression (2-tier grading) did not predict survival in CC patients (log-rank, $p=0.208$). Importantly, the TIMP-2 expression was a powerful predictor of survival ($p=0.0076$), which was markedly inferior in women with negative-weak TIMP-2 expression in their tumours (Figure 7). Similarly, the MMP-2:TIMP-2 ratio was a significant predictor of survival in univariate (Cox) survival analysis ($p=0.018$). Of all variables analysed, the FIGO stage was the most powerful predictor of survival in Kaplan-Meier analysis ($p=0.0001$).

In multivariate survival (Cox) analysis, TIMP-2 expression lost its significance as an independent prognostic predictor, however. It was removed from the model, when adjusted for age, tumour grade, FIGO stage, HR-HPV and MMP-2, in a forward step-wise approach. The same was true with the MMP-2:TIMP-2 ratio. In the final Cox model, the FIGO stage ($p=0.0001$) and age ($p=0.016$) were the only independent predictors of patient survival. When FIGO stage 1 was used as the reference, the HR (Hazard Ratio) for dying of the disease in Stage 2 was 2.97 (95%CI 0.63-13.42), in Stage 3, the HR was 7.35 (95%CI 1.89-28.66) and in Stage 4, the HR was 80.11 (95%CI 14.35-447.12). The mean age of women who were alive was 54.2 years as compared to 66.7 years for those who died of their CC ($p=0.0001$).

## Discussion

Because of the paucity of data on MMP-2 and TIMP-2 expressions in CIN and CC and their relationships with HPV detection in particular, MMP-2 and TIMP-2

expressions were analysed as predictors of several intermediate end-point markers in cervical carcinogenesis and their prognostic value in CC, as part of our systematic search for prognostic markers in CC (9, 10, 31-33, 40, 41). Consistent with the published data (22-26, 42-44), we found biopsies with normal epithelium negative for MMP-2. In CIN lesions, the intensity of expression increased linearly with the progressive lesion, reaching the maximum in invasive cancer (Table I). Much less data are available on TIMP-2 expression (22, 24-46), which seems to be intense in normal squamous epithelium (24), but less intense in CIN lesions (22, 45). The results for invasive carcinomas are more controversial, both strong (22, 47) and weak (45) expressions having been reported in the literature. The present results clearly confirmed the detection of intense TIMP-2 expression in normal cervical epithelium and this (reference) pattern was retained up to CIN3, with a major down-regulation upon transition to invasive cancer (Table I). Some reports also described TIMP-2 expression in stromal cells (22, 24, 46), which was confirmed in some of our lesions.

In several previous studies, the expression of MMP-2 was shown to increase with the increasing grade of CIN and further in invasive cancer (22-26, 42-44). This was confirmed in our study, where MMP-2 expression showed a linear increase with progressive CIN, being most intense in CCs (Table I). Indeed, the major up-regulation took place upon progression from CIN2 to CIN3, and further to invasive disease. Indeed, moderate-intense MMP-2 expression was associated with CIN3/cancer with OR=20.78 (95%CI 7.16-60.23) ($p$=0.0001) implicating a strong association of MMP-2 activity with progressive disease, as suggested by many of the previous studies (19, 22-28, 42-44, 48).

TIMP-2, on the other hand, was shown to retain its normal expression pattern until late in disease progression, when 60% of CIN3 lesions still expressed TIMP-2 approximately at the level of normal epithelium (Table I). This confirmed the recent observations of Nair *et al.* (24), as to the expression of TIMP-2 in normal epithelium, LSIL and HSIL, but did not agree with their statement that 95% of invasive carcinomas show intense TIMP-2 expression (24). One of the reasons for these divergent observations might be the use of different antibodies and slightly different IHC protocols. Our results are more consistent with those reported by Sheu *et al.* (45), who detected only low levels of TIMP-2 in CCs. This differential expression of MMP-2 and TIMP-2 in cervical lesions resulted in a significant change in the MMP-2:TIMP-2 ratio, increasing in favour of MMP-2 with disease progression. Using the ratio statistics, this increase was linear from normal epithelium to invasive cancer ($p$=0.0001), where the MMP-2:TIMP-2 ratio exceeded the value 1.0 for the first time. In

other terms, one of the markers of progression to invasive disease seems to be this inverse balance between MMP-2 and its inhibitor (TIMP-2). As long as the lesion was intraepithelial, the MMP-2:TIMP-2 ratio was below 1.0, which is consonant with their known biological behaviour, *i.e.*, TIMP-2 counterbalancing the biological activity of MMP-2 (14, 15, 17, 19-21). The eventual prognostic implications of this disturbed balance will be discussed later (27, 45, 46).

This up-regulation relatively late in carcinogenesis makes MMP-2 different from some other markers analysed in our studies to date (9, 10, 31, 40, 41). Intense-moderate expression of MMP-2 distinguished CIN with 87.5% specificity and 97.8% PPV, but had low sensitivity (36.6%) (Table III). These figures are slightly inferior to those calculated for p16[INK4a] (9), ERK1 (10), Survivin (40) and VEGF-C (31), but similar to those obtained for nm23-H1 (41). A close correlation was recently reported between VEGF-C (marker of angiogenesis) and MMP-2 in CC (49), which was also confirmed in the present series (Spearman rho=0.381, $p$=0.0001) (data not in Tables). It is of great interest that MMP-2 expression seems to be inversely related to the level of nm23-H1 (Spearman rho=-0.167, $p$=0.008), whereas TIMP-2 shows a direct linear relationship to this anti-metastatic gene product (Spearman rho=0.339, $p$=0.0001). Further calculations for these mutual interrelationships between the different markers will await the completion of our work with all the biomarkers to be analysed in this material (32, 33).

These performance indicators for MMP-2 in predicting CIN were not calculated in any of the previous studies (19, 22-28, 42-44, 48). IHC is a technique readily applicable to cytology samples and, as a test with >90% SE and 97% PPV in detecting CIN3, IHC analysis of MMP-2 might be a promising marker in CC screening. In this respect, the prospects for TIMP-2 might be even better, since it was shown to be a 100% specific discriminator of CIN, with 100% PPV. In theory, a combination of MMP-2 or TIMP-2 assays with another test showing high SE and high NPV, *e.g.,* HPV testing by Hybrid Capture 2 (HCII), might provide an ideal screening tool for CIN lesions in PAP smears. Unfortunately, we were unable to directly test this concept in the present series, because PAP smears were not available for study. The viability of this concept remains to be seen in carefully designed population studies testing optional screening tools.

Persistent HR-HPV infections have recently achieved increasing attention as a cause of significantly increased risk of treatment failures in CIN (1, 2, 4, 5, 36). Monitoring for this increased risk of disease recurrence after cone treatment using a suitable marker would be of considerable clinical value, and we were interested to see whether MMP-2 or TIMP-2 expression might be of any predictive value for

HR-HPV clearance (persistence) after CIN treatment. Unfortunately, no such evidence could be obtained and MMP-2 and TIMP-2 add 2 other members to the growing list of markers analysed in this material (p16[INK4a], ERK-1, VEGF-C, Survivin, nm23-H1, 67 kDa laminin receptor, topo IIα, NF-κB, telomerase) (9, 10, 31, 32, 40, 41), none of which has proved to possess any predictive value for either clearance or persistence of HR-HPV in the cone-treated cervix (36).

To date, few studies have addressed the relationship between MMP-2/TIMP-2 expression and detection of HPV in CIN or CC, and the results are contradictory (19, 23, 25-27, 29). Thus, Garzetti *et al.* (29) reported a significantly higher MMP-2 index in HPV-positive CC, which was also related to the risk of lymph node metastases. On the other hand, no association was established between MMP-2 expression and HPV in cervical cancer cell lines (CaSki and HeLa) (27). Similarly, a recent IHC study did not disclose any relationship between MMP-2 expression and HPV detection in CIN lesions (26). This is consonant with the data of Ahmed *et al.* (19), who found MMP-2 to predict recurrence of CC, independently of the HPV status (19). Similarly, Garzetti *et al.* (23) described marked overexpression of MMP-2 in microinvasive carcinomas as compared with CIN, but this up-regulation was unrelated to the HPV status in these lesions. Recently, in a small series of CIN lesions (n=29) and CC (n=15), all MMP-2-positive cases proved to be HPV-positive with HCII (25).

The present results are in line with those previous reports failing to disclose any direct association between MMP-2 (or TIMP-2) and HPV (Table II). Accordingly, neither MMP-2 nor TIMP-2 showed any significant relationship with the detection of HR-HPV. These observations make MMP-2 and TIMP-2 different from all the other markers analysed in this series so far, shown to be correlated with HR-HPV, at least in univariate analysis (9, 10, 31, 40, 41). The contrast is particularly striking for p16[INK4a] (9) and Survivin (40), which proved to be independent predictors of HR-HPV even in multivariate analysis. This implies that MMP-2 and TIMP-2 are not functionally regulated by the HR-HPV oncoproteins unlike, *e.g.,* 16[INK4a], Survivin and VEGF-C (9, 31, 40). Some of the recently discovered molecular regulatory mechanisms of MMP-2 and TIMP-2 are discussed later.

The prognostic value of MMP-2 and/or TIMP-2 expression in CC has been addressed in a series of previous studies (19, 26-30, 46-49), but the data are incomplete. Indeed, MMP-2 as a predictor of OS has been assessed in one study only (49), where Ueda *et al.* failed to establish any prognostic value for MMP-2 in a series of 52 CCs (49), despite its significant relationship to the depth of invasion, pelvic lymph node metastases and microvascular density. Basically, similar data have been provided by several other studies, where up-regulated

MMP-2 expression has been linked with many of these adverse clinical parameters (19, 26-30, 46-48). We also assessed whether MMP-2 (or TIMP-2) up-regulation is a marker of more aggressive behaviour by correlating the expression to histological grade and FIGO stage, but failed to find any such evidence (data not shown). The present study is the first where systematic evidence is provided on the prognostic value of MMP-2 and its inhibitor TIMP-2 in CC. In the present series of 150 patients, survival was adversely effected by up-regulated MMP-2, although the difference (55.7% *vs.* 72.4%) did not reach statistical significance. This confirmed the data of one previous report (49). On the other hand, TIMP-2 expression proved to be a powerful predictor of survival, in that 68.9% of women with normal-slightly/moderately reduced TIMP-2 were alive compared to only 46.0% of those whose tumours were TIMP-2-negative or weakly positive (*p*=0.007). This was also confirmed in univariate (Kaplan-Meier) survival analysis (Figure 7).

For the first time, direct evidence is provided implicating a significant prognostic value for TIMP-2 in CC. Failure to establish such evidence for MMP-2 does not necessarily exclude the possibility that this MMP also plays an important prognostic role, as suggested by several studies (19, 26-30, 46-49). As previously discussed (27, 45, 46), it is most probably the dynamic balance between MMP-2 and its inhibitor, TIMP-2, which is a key determinant of tumour invasion. The present study provided additional evidence that this, indeed, seems to be the case. First, the MMP-2:TIMP-2 ratio increased in parallel with the progression of CIN, but inverted in favour of MMP-2 only in invasive cancer. This could implicate that, as long as the lesion is intraepithelial and the MMP-2:TIMP-2 ratio is below 1.0, the tissue inhibitor (TIMP-2) is capable of counterbalancing the biological activity of MMP-2 (14, 15, 17, 19-21). Second, we observed that the MMP-2:TIMP-2 ratio was significantly higher (1.79) among women who died of their disease than that (1.34) among those who survived (Mann-Whitney, *p*=0.014). In univariate survival analysis, the MMP-2:TIMP-2 ratio was a significant predictor of survival (*p*=0.018). Similar observations were recently reported in 2 studies, where an increased MMP-2:TIMP-2 ratio was a sign of poor prognosis (50, 51). In our study, this significance was lost in multivariate (Cox) analysis, however. These data indicate that the MMP-2:TIMP-2 ratio is an important prognostic determinant in CC, but its predictive power is inferior to that of the 2 most potent classic prognostic factors, FIGO stage and age. Even so, these data have important implications, *e.g.,* in predicting stage-specific survival in CC.

There is no doubt that MMP-2 and TIMP-2 are important regulators of tumour invasion and metastasis (14, 15, 17, 19-21). This was confirmed in the present

study, where normal expression of TIMP-2 had a favourable effect on overall survival (Figure 7). Interestingly, the functions of these 2 proteins, promoting invasion (MMP-2) and counteracting it (TIMP-2), seem to be unrelated to HR-HPV. Some recent studies elucidated the highly complex control mechanisms of MMP-2 and TIMP-2 in cervical carcinogenesis (52-57). Thus, in SiHa cells, surface $\alpha_2\beta_1$ and $\alpha_v\beta_3$ integrins seem to regulate MMP-2 expression, most probably mediated by the FAK (focal adhesion kinase) signalling pathway and implicating a membrane-dependent activation of pro-MMP-2 (52, 53). A strong co-expression of MMP-2 and VEGF-C was recently reported in CC (49)(also confirmed in our series), as was a co-expression of the angiogenesis-inhibitor TSP-2 (trombspondin-2) and MMP-9 (54). MMP-2 expression also bore a close correlation with another angiogenic stimulator, thymidine phosphorylase (dThdPase) (55), conferring an extremely high metastatic potential to CC cell lines. Finally, aberrant hypermethylation of TIMP-2 was shown to favour the development of CC (56) and a highly specific MMP inhibitor, MMI-166, proved to be a potent inhibitor of MMP-2 activity in CC cells (57). It is obvious that further work is necessary to elucidate the eventual molecular links (if any) between MMP-2/TIMP-2 and HR-HPV in cervical carcinogenesis.

## Acknowledgements

## Appendix

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