

# Methylation of *CADM1* and *MAL* together with HPV status in cytological cervical specimens serves an important role in the progression of cervical intraepithelial neoplasia

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**Abstract.** Cervical cancer (CC) is the second most common type of cancer affecting the female population. The development of CC takes several years, and involves a precancerous stage known as cervical intraepithelial neoplasia (CIN). A key factor in the development of disease is the human papillomavirus (HPV) infection, which initiates carcinogenesis. Furthermore, CC is also impacted by epigenetic changes such as DNA methylation, which causes activation or exclusion of certain genes, and the hypermethylation of cytosines in promoters, thereby switching off previously active genes. The majority of DNA methylation events occur at cytosine-guanine nucleotides, which in the human genome are known as CpG islands. The aim of the present study was to investigate the methylation levels in intronic sequences of the two tumor suppressor genes cell adhesion molecule 1 (*CADM1*) and T-lymphocyte maturation associated protein (*MAL*) using cytological samples and to identify potential biomarkers involved in CIN by pyrosequencing. DNA was isolated from cervical smears from patients with CINs, with healthy patients serving as a control group. Samples were converted by treatment with sodium bisulfite and subsequent pyrosequencing to detect the methylation status of the selected genes. The presence of HPV DNA infection analyzed by the polymerase chain reaction, was detected in each sample. Of the total number of samples (n=91), the present study confirmed the presence of one or two high-risk subtypes of HPV in 39 cases (42.85%)

and HPV infection was significantly associated with CIN2+ lesions. For the two genes (*MAL* and *CADM1*) the present study confirmed that the median methylation was significantly higher in HPV positive patients [P=0.0097, 95% confidence interval (CI): (-0.030, -0.003)/P=0.0024, 95% CI: (-0.06, -0.01)] when compared with patients negative for HPV DNA infection, and the average methylation was demonstrated to be increased with the degree of cervical lesion. The present study used logistical regression to model the dependence between the case/control statuses (control group vs. Dg. 1-4). The area under the curve values for *MAL* were: 84% for cervical inflammation, 71% for CIN1, 73.4% for CIN2+ and 77% for squamous cell carcinoma (SCC); and for *CADM1* were: 88.6% for cervical inflammation, 68% for CIN1, 80% for CIN2+ and 89% for SCC. The present study confirmed that there were statistically significant differences between the methylation levels of individual CpGs and significantly higher median methylation in patients positive for HPV16/18. *CADM1* exhibited higher levels of methylation in almost every study group when compared with *MAL* during the transition of CIN and appeared to be a promising biomarker for future study.

## Introduction

Cervical cancer (CC) is the second most common type of cancer affecting the female population. The World Health Organization (WHO) and GLOBOCAN 2012 have recorded ~528,000 new cases of CC globally (1). The development of CC takes place over several years, and involves a precancerous stage known as cervical intraepithelial neoplasia (CIN), which is divided into three main stages (CIN1, CIN2, CIN3). The primary cause of this disease is infection by human papillomavirus (HPV). At present, over 100 types of HPV have been identified, of which the most prevalent types are HPV16/18, which are responsible for cervical carcinogenesis (2). According to the latest data the most common HPV virus identified in high-grade lesions is HPV16 (47.4%) followed by HPV31 (12.4%), HPV33 (7.1%) and HPV18 (7.1%) (3). The HPV genome is divided into three main regions; the long

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control region (LCR), which is composed of six open reading frames (ORFs); and the 'late region', with two ORFs coding for the viral structural proteins L1 and L2 (4). The major mechanism that engages HPV16/18 in cervical carcinogenesis is the manifestation of two early viral genes: E6 and E7 (known as viral oncogenes). E6 protein binds to the tumor suppressor gene p53 and causes its degradation while E7 inactivates the pRb gene. These mechanisms cause disruptions of cell cycle regulation (5).

Cytological screening has reduced the number of CC-associated mortalities; however, CC is still among the most prevalent oncological diseases, as many women underestimate the importance of regular gynecological examinations.

At present, classical cytology-based screening is being replaced in favor of diagnosing patients with high-risk HPV types, studies are also focused on additional co-factors, such as epigenetics and the search for suitable bio-markers identified via a triage test, PAP smear or HPV test. Epigenetic changes are stable alterations of gene expression without alteration in the DNA sequence itself, and can cause disease even in the absence of a mutation in the gene (6). These changes also include DNA methylation, which is characterized as a covalent chemical modification by the addition of a methylated group to the fifth carbon of the cytosine ring (5mC), thereby preventing access to proteins. DNA methylation is a typical mammalian cellular process and is one of the most well-established markers that defines a molecular landscape that is altered in cancer. Cytosine *methylation* in vertebrates is found primarily at CG *dinucleotides* (CpGs). CpGs are usually unmethylated in normal cells, while sporadic CpG sites in the other parts of the genome are methylated. This is associated with gene silencing. Aberrant DNA methylation occurs in the majority of types of cancer, and cause the silencing of some tumor-suppressor genes (TSG) leading to tumor cell growth (7). CpGs hypermethylation is not randomly distributed in carcinogenesis, and therefore may provide a useful signature for tumor diagnosis and prognosis (8).

Following this, several biomarkers (*CADMI*, *DAPK1*, *CDH1*, *EPB41L3*, *FAM19A4*, *MAL*, *PAX1*, *TERT*, *PRDM14*) have been studied which describes the hypermethylation that affect the expression of these genes, thus inducing or accelerating the carcinogenic mechanism, e.g., by deregulation of TSG (9). The present study focused on determining the mean intron region methylation of the two TSGs. They are known as T-lymphocyte maturation associated protein (*MAL*) and cell adhesion molecule 1 (*CADMI*).

*CADMI* was first observed in patients with non-small cell lung carcinoma (NSCLC) and mapped on chromosome 11q23 (10). This gene was proven to suppress tumor growth through anti-proliferative and pro-apoptotic activity, and the loss of *CADMI* expression leads to tumor formation and metastasis (11,12). Hypermethylation of *CADMI* is one of the principal causes of gene silencing (13) and was also detected in 40% cases of lung cancer, 32% of prostate cancer cases, 27% of pancreatic adenocarcinoma cases and 83% of cervical carcinoma cases (14,15). The *MAL* gene is located in the 2q11.1 region and the hypermethylation of its promoter region diminishes its tumor suppressor activity. In 90% of samples from spinocellular carcinoma patients and 93% of samples from patients with diagnosed adenocarcinoma,

hypermethylation of certain areas of this TSG has been demonstrated (16). In several studies, DNA methylation of the *CADMI/MAL* gene promoter has been shown to increase with the severity of cervical disorder (17-19) and that these epigenetic changes point to the presence of more severe high-grade dysplasias (CIN2+). The levels of average methylation in TSG is extremely high in cervical carcinomas and significantly increases in CIN3 lesions in women with high risk (HR)-HPV infection (20).

The aim of the present study was to investigate the methylation levels of *CADMI* and *MAL* using cytological smears by quantitative pyrosequencing analysis. The focus was on specific intronic regions have not been described in much detail previously, in order to examine the significance of the methylation levels of individual CpGs in *CADMI* and *MAL* in HPV16/18 positive patients and also in samples of patients with cervical inflammation.

## Materials and methods

*Specimens and study population.* Cervical specimens were obtained from 91 female patients in collaboration with the Department of Obstetrics and Gynecology at Jessenius Faculty of Medicine in Martin (Martin, Slovakia) and the Department of Molecular Biology and Division of Oncology (Biomedical Center Martin JFM CU, Slovakia). Cytological smears from the uterine cervix were collected and stored in a LBC/APTIMA transport fluid medium. The present study was approved by the Ethical Committee of Jessenius Faculty of Medicine in Martin and all patients provided written informed consent. The patient's clinical protocols were reviewed for clinical data, diagnosis and age of patients. The smears were divided into two groups (1st group: divided into 4 subgroups according to dysplasia, 2nd group: controls) according to the diagnosis of the patients. 1st group included patients who had been operated or diagnosed with cervical carcinoma or had visited an outpatient clinic for due to the presence or deterioration of a cervical lesion. Based on the condition and progression of cervical neoplasia, the following four subgroups were established diagnosis group (Dg).1: cervical inflammation without dysplasia, 20 samples, Dg.2: CIN1, 14 samples, Dg.3: 19 samples of CIN2+ [CIN2, CIN3, carcinoma *in situ* (CIS) included] and Dg.4, 7 patients with squamous cell carcinoma (SCC). Clinical diagnosis of individual samples were confirmed by histological examination. As the 2nd group: controls (Dg. 0), 31 samples were collected from patients without uterine cervical lesions, with normal onco-cytology outcome and who had not previously received cervical surgery. A total of 20 inflammation samples, 40 CIN1-CIN3/CIS or carcinomas and 31 control samples were collected. The highest median age (48.7 years) was in the SCC group (range, 32-68 years) and 41.5 years in patients with cervical inflammation (range, 22-64 years). The lowest median age was in the CIN2+ group (32 years) with range 21-65 years, followed by CIN1 (33 years) with a range of 19-67 years. The control group only has a slightly higher median age (38 years) than the CINs (range 23-75 years (Table I). In this case we did not confirm any statistical significance and therefore the differences in the median age between groups did not affect the results.

Table I. Median age of the patients and positivity rates for HPV16/18 in the cervical scrapes in regard to their most severe underlying histological diagnosis.

Dg.	Histology	Total n	Median age (range), years	HPV 16/18 positive, n (%)
0	Control	31	38 (23-75)	6 (19)
1	Inflammation	20	41.5 (22-64)	7 (35)
2	CIN 1	14	33 (19-67)	6 (42.8)
3	CIN 2+	19	32 (21-65)	13 (68.4)
4	SCC	7	48.7 (32-68)	7 (100)

Dg, diagnosis group; Dg. 0, controls; Dg.1, inflammation samples; Dg.2, CIN1; Dg.3, CIN2+; Dg.4, SCC; CIN, cervical intraepithelial neoplasia; SCC, squamous cell carcinoma; MAL, T-lymphocyte maturation associated protein; CADM1, cell adhesion molecule 1; HPV, human papillomavirus.

**DNA extraction and bisulfite conversion.** Cervical cells (from all groups) yielded from swab smears were stored in LBC/APTIMA vials, and nucleic acids were extracted using a kit, subject to the type of medium in which the samples were stored. The first type contained dissolved cervical cells in the LBC medium, and cells were isolated by the DNase Blood and Tissue kit (Qiagen GmbH, Hilden, Germany). For the second type of DNA extraction a commercially available MasterPure™ Complete DNA and RNA Purification kit (Epicentre; Illumina, Inc., San Diego, CA, USA) was used, for the cells contained in 500  $\mu$ l of APTIMA transport medium. The concentration and quality of the sample was determined by measuring the sample purity in a UV spectrophotometer, and loaded onto 1.5% agarose gel. Subsequently, 1-2  $\mu$ g of DNA was bisulfite-treated using an EpiTect Bisulfite kit (Qiagen, Inc., Valencia, CA, USA). Up to 2  $\mu$ g of DNA were used in a total reaction volume of 20  $\mu$ l; the total amount of bisulfite reaction was 140  $\mu$ l (also containing 85  $\mu$ l bisulfite mix and 35  $\mu$ l of DNA Protect Buffer). The exact protocol for this reaction was described previously (21). Following bisulfite treatment genomic DNA was stored at -20°C until polymerase chain reaction (PCR) analysis.

**HPV DNA and detection.** A PCR reaction was used to diagnose the most common HPV genotypes (HPV16 and 18) by using primers that have been described and published in previous literature (22). The primers were designed according to the sequences from the PGMY09/11 primer set (23). The resulting sequence and the optimization of the PCR conditions were described previously (23).

**CpG assays and analysis of selected regions of CADM1 and MAL.** For the amplification of bisulfite-converted DNA a PyroMark PCR kit (Qiagen, Inc.) was used. The total PCR reaction volume was 25  $\mu$ l. For analysis of selected regions of CADM1 (3 CpG) and MAL (4 CpG) genes, commercially available CpG assays [PyroMark CpG Assay (200) Hs\_CADM1\_01\_PM (978746, PM00049686), PyroMark CpG Assay (200) Hs\_MAL\_01\_PM (978746, PM00011935, Qiagen GmbH)] (21) were used and with the exact sequence

region for analysis (Figs. 1 and 2). Briefly, the obtained PCR products were stored overnight in the refrigerator (+4°C), and 5  $\mu$ l of the product was removed for electrophoretic analysis (1.75% agarose gel with ethidium bromide staining). Subsequently, 10-20  $\mu$ l of PCR products were subjected to pyrosequencing by PyroMark Q96 ID (Qiagen, Inc). Completely methylated and unmethylated DNA were used as control samples (EpiTect Control DNA, methylated/EpiTect Control DNA, unmethylated; Qiagen GmbH). For the immobilization of the PCR product to the beads, a mixture of 10  $\mu$ l optimized biotinylated PCR product, 1.5  $\mu$ l streptavidin-coated sepharose beads, 40  $\mu$ l Binding buffer and 28.5  $\mu$ l deionized water was prepared. Bisulfite modified DNA was placed into a thermal cycler with the following program: Denaturation (95°C, 5 min), incubation (60°C, 25 min), denaturation (95°C, 5 min), incubation (60°C, 85 min), denaturation (95°C, 175 min), incubation (60°C, 25 min) and incubation (20°C). The total volume of a capture was pipetted into each well of the Pyromark plate low. Analysis was conducted according to the manufacturer's protocol, which was described previously (21).

**Statistical data analysis.** The two-sample test for equality of proportions with a continuity correction was used to examine the hypothesis of the equality of proportions of HPV+ and HPV- in patients and controls. Robust one-way analysis of variance was used to test the hypothesis of equality of medians in the patients groups for each CpG island. The test was followed by the Tukey honest significant difference post-hoc test. The effect size was quantified by the 95% CI. Univariate and multivariate logistic regression models were used to analyze the dependence between a response and predictor(s). The predictive ability was visualized by the receiver operating curve (ROC) and quantified by the AUC, the area under ROC. The cut-off on the class probability was determined by the Youden method, and translated into a cutoff on the median methylation. All analyses were performed using R version 3.2.3 (24). P<0.05 was considered to indicate a statistically significant difference.

## Results

**HPV 16/18 detection and genotyping.** The present study confirmed the presence of one or two high-risk genotypes of HPV in 39 cases (42.85%); however, the number of positive subtypes of HPV DNA per patient were not discerned. HPV DNA was present in 19% (6/31) of controls, in 35% (7/20) of patients with inflammation, in 42.8% (6/14) of CIN1 cases, in 68.4% (13/19) of CIN2+ cases and in 100% (7/7) of SCC (Table I). Logistic regression was used to analyze the dependence between the status and age in the patient and control groups. However, the association was not statistically significant (P=0.636). The two-sample test for equality of proportions with a continuity correction was used to examine the hypothesis of the equality of proportions of HPV+ and HPV- in patients and controls. HPV infection has been significantly present in CIN2+ cases (P=0.001528), with a 95% CI of -0.78 and -0.19, and in SCC cases (P=0.0002933), 95% CI: (-1, -0.58).

**Pyrosequencing.** Samples modified with sodium bisulfite were subsequently used in the PCR reaction. Using

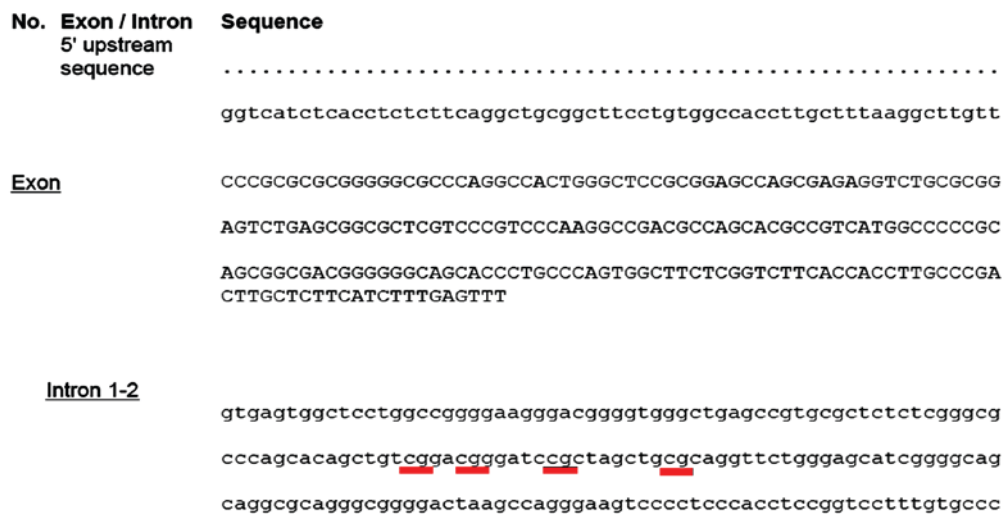


Figure 1. Map of CpG methylation sites in the first intron of T-lymphocyte maturation associated protein. Chromosomal location: Chr2: 95691554-95691815. Individual CpGs are underlined in red. CpG, CG dinucleotides.

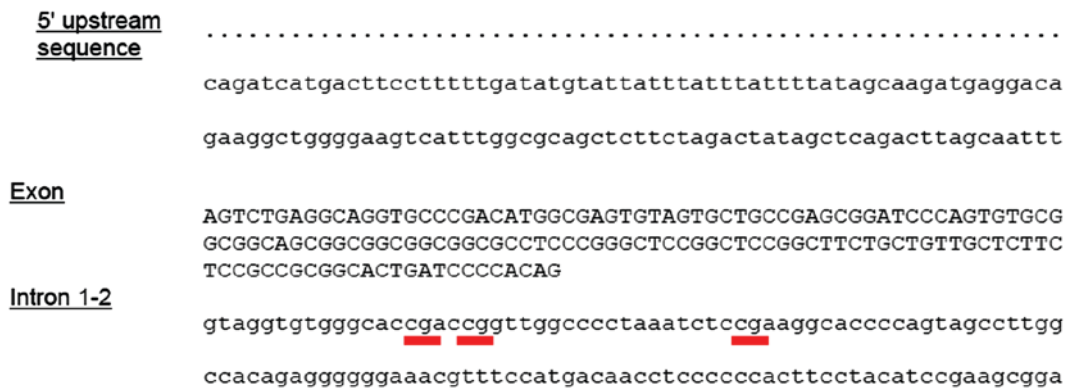


Figure 2. Map of CpG methylation sites in the first intron of cell adhesion molecule 1. Chromosomal location: Chr11: 115374815-115374991. Individual CpGs are underlined in red. CpG, CG dinucleotides.

methylate-specific primers, we converted cytosine to uracil, respectively thymine in the PCR product while methylated cytosine remained unchanged. The present study focused on the detection of methylated regions in the sequence of two TSGs (*MAL* and *CADMI*) by pyrosequencing. By logistic regression it was identified that the *MAL/CADMI* methylation status in the control group was not associated with age of patients (P-values are not presented). By the Welch two-sample test with the one-side alternative the median methylation in HPV+/- DNA in all patient groups were compared. For *MAL* and *CADMI* the median methylation was identified as significantly higher in HPV positive patients [P=0.0097, 95% CI: (-0.030, -0.003) for *MAL*/P=0.0024, 95% CI: (-0.06, -0.01) for *CADMI*] than in the patients negative for HPV DNA (Fig. 3).

*Average methylation and determining the degree of methylation of individual CpG islands.* By the Robust One-way ANOVA the hypothesis of equality of medians in the patients groups for each CpG island were examined. The ANOVA P-values were P=0.0002 for *MAL* (1st CpG), P=0.0066 for *MAL* (2nd CpG), P=0.1099 for *MAL* (3rd CpG), P=0.0207 for *MAL* (4th CpG)/P=0.0074 for *CADMI* (1st CpG), P=0.0063 for *CADMI* (2nd CpG), P=0.0007 for *CADMI* (3rd CpG).

Tukey's post-hoc HSD test was used to perform the multiple comparisons testing. Pairs (CpG islands) with significantly different methylations are presented in Table II.

An ANOVA with Tukey's post hoc test, was used to test the hypothesis of equality of medians in the patient groups, for averaged 4 resp. 3 CpG islands. The ANOVA P-values were: P=0.0026 for *MAL* and P=0.0001 for *CADMI*. Fig. 4 illustrated the pairs with significantly different methylations. *MAL* was demonstrated to have a significantly lower median methylation compared with *CADMI* for each group (Dg.0: P=0.010145, 95% CI: -0.036, -0.003; Dg.1: P=0.0001447, 95% CI: -0.050, -0.0180; Dg.2: P=0.001147, 95% CI: -0.0378, -0.003; Dg.3: P=0.0003808, 95% CI: -0.0745, -0.0235; Dg.4: P=0.04694).

Logistic regression models using the most promising markers were investigated to see whether marker combinations could be used to improve the discrimination between cases and the control group. Multinomial logistic regression was used to examine whether HPV status impacted DNA methylation at specific CpG islands or if there is any dependence between age of patient and increasing risk of neoplasia or HPV. For *CADMI* the present study confirmed that the last CpG (3rd) was significantly more methylated in samples with

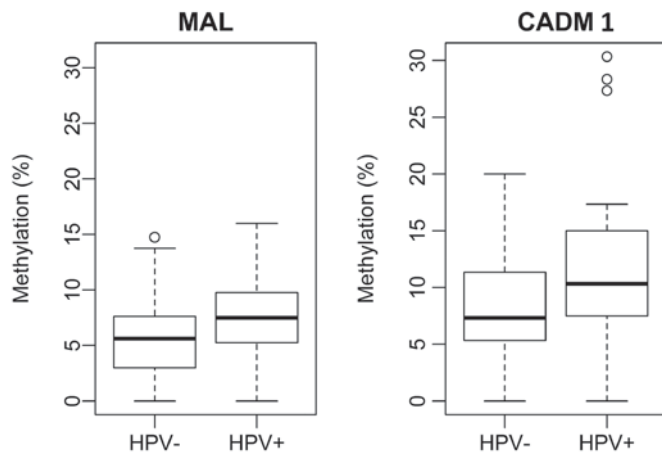


Figure 3. Welch's two-sample test with one-side alternative comparisons of the median methylation of *MAL* and *CADM1* in HPV+/- DNA. The boxplot represents the median and the interquartile range. *MAL*, T-lymphocyte maturation associated protein; *CADM1*, cell adhesion molecule 1; HPV, human papillomavirus.

inflammation ( $P=0.001$ ), CIN1 ( $P=0.086$ ), CIN2+ ( $P=0.038$ ) compared with the other examined CpGs (1st, 2nd). In *MAL* it was also discovered that the first CpG was more significantly methylated than other CpGs ( $P=0.043$  for inflammation,  $P=0.017$  for CIN1,  $P=0.155$  for CIN2+). For SCC (Dg.4) the second and third CpGs for the gene *MAL* were significantly more methylated ( $P=0$  for 2nd CpG,  $P=0.012$  for 3rd CpG) than in other groups. For *CADM1* no statistically significant differences were identified in the SCC (Dg.4) group (Table III).

**Determination of the methylation cut-off using logistic regression (*MAL*, *CADM1*).** Logistic regression was used to examine the dependence between the case/control statuses (control group vs. Dg. 1-4). The AUC values for *MAL* were: 84% for cervical inflammation, 71% for CIN1, 73.4% for CIN2+, 77% for SCC; and for *CADM1* were: 88.6% for cervical inflammation, 68% for CIN1, 80% for CIN2+ and 89% for SCC. The ROC graph is shown in Fig. 5 (left) for *MAL* and Fig. 5 (right) for *CADM1*. The cut-off on the class probability was determined by the Youden method. The cutoff for *MAL* and *CADM1* on the median methylation corresponding to the cutoff on the class probabilities was 0.09. Using this cutoff value, *MAL* had the highest sensitivity and specificity in inflammation samples (80 and 83.9% respectively) followed by SCC samples (71.4 and 77.4% respectively). CIN1 was less sensitive (57.1%) but still had high specificity (83.9%). However, for the *MAL* gene CIN2+ group had the highest sensitivity (94.7%) but the lowest specificity (41.9%). *CADM1* had the highest sensitivity (100%) and also specificity (80.6%) in SCC and also in CIN2+ where the sensitivity was 73.7% with an even higher specificity (80.6%). In patients with inflammation a high sensitivity (90%) and specificity (77.4%) were observed. The lowest sensitivity (57.1%) but still high specificity (80.6%) for the median methylation was found in CIN1. The dependence between women with inflammation and CIN1 was also calculated (AUC=68% for *MAL*, AUC=74.4% for *CADM1*), CIN1 and CIN2+ (AUC=50.5% for *MAL*, AUC=66.5% for *CADM1*), CIN1 and SCC (AUC=56% for *MAL*, AUC=78.5% for *CADM1*).

## Discussion

Epidemiological and molecular studies have demonstrated that persistent infection of HR-HPV is one of the causes of cervical carcinogenesis (25,26) and the major mechanism that engages HPV16/18 in cervical carcinogenesis is the manifestation of two early viral genes E6 and E7 (4). Only a minority of all persistent HR-HPV infections alters the expression of viral genes E6 and E7, consequently resulting in an increased expression of oncoproteins (26). This process is histologically known as CIN2/3 and, without treatment can ultimately lead to CC. Recent studies have demonstrated that the inclusion of testing to detect the presence of HR-HPV DNA in cytology after 6 months of patient treatment has significantly increased the sensitivity of CIN2+ detection (27,28). HR-HPV testing is an attractive modality for primary screening due to its extremely high sensitivity for CIN3+ lesions. However, the specificity of HR-HPV testing is lower compared with cytology (approximately 4-6%) (29). In order to manage the increased number of colposcopy referrals, HR-HPV positive women should be further stratified by secondary triage tests such as p16/Ki67 immunostaining and methylation markers (29). In the present study, HPV DNA was detected in 42.8% CIN1, 68.4% CIN2+ and 100% of SCC samples. From these results, an increase in the incidence of HPV DNA positive samples was observed as the severity of the lesion increased.

Recent results suggest that other factors, such as genetic and epigenetic changes (CpG DNA methylation), are required for the onset of carcinogenesis (30). Approximately ten human genes have consistently elevated methylation in cervical pre-cancers including *CADM1*, *EPB41L3*, *FAM19A4*, *MAL*, *miR-124*, *PAX1* and *SOX1*. Methylation testing is still in the early stages of development; however, it is exhibiting promise as an accurate molecular classifier (31).

Methylation of CpG regions of multiple TSGs occurs at different stages of development of CIN and in the process of transition to invasive cervical carcinoma, of which *CADM1* and *MAL* are the most commonly methylated genes (17,32,33). The principal effect of DNA methylation in promotor/intronic regions of *CADM1* and *MAL* is their downregulation. *MAL* was identified to be an essential component of the glycolipid-enriched membrane (GEM) rafts, which have been implicated in the polarized sorting of apical proteins (34,35). This down-regulation may disturb regular apical sorting, thereby disrupting cellular polarity, which is a phenomenon observed in epithelial cells infected with HPV. The down-regulation of *CADM1* may result in the loss of the Rb tumor suppressor pathway signaling, which represents a relatively common event in cervical carcinogenesis (36). However, the molecular mechanism of *CADM1/MAL* involvement in cervical carcinogenesis has not yet been thoroughly investigated and the definitive confirmation that the down-regulation of these genes in primary CCs will require further study.

A previous study reported that there was a correlation between methylation and the progression of neoplasia to a higher stage. There were no difference in the median methylation between the control group and CIN1 or between CIN1 and CIN2+. Based on these results they considered that the methylation degree of individual CpG islands in the promoter

Table II. Multiple comparisons testing of each CpG island in the two tumor-suppressor genes.

Dg.	<i>MAL</i> 1.	<i>MAL</i> 2.	<i>MAL</i> 3.	<i>MAL</i> 4.	<i>CADMI</i> 1.	<i>CADMI</i> 2.	<i>CADMI</i> 3.
0 vs. 1	0.00000 <sup>c</sup>	0.00000 <sup>c</sup>	0.01914 <sup>a</sup>	0.00031 <sup>c</sup>	0.00010 <sup>c</sup>	0.00006 <sup>c</sup>	0.00002 <sup>c</sup>
0 vs. 2	0.00632 <sup>b</sup>	0.01790 <sup>a</sup>	0.11492	0.46309	0.57563	0.46030	0.03467 <sup>a</sup>
0 vs. 3	0.00692 <sup>b</sup>	0.00179 <sup>b</sup>	0.02417 <sup>a</sup>	0.01624 <sup>a</sup>	0.00147 <sup>b</sup>	0.02081 <sup>a</sup>	0.00170 <sup>b</sup>
0 vs. 4	0.01825 <sup>a</sup>	0.06606	0.95381	0.12388	0.02095 <sup>a</sup>	0.04557 <sup>a</sup>	0.01732 <sup>a</sup>
1 vs. 2	0.36317	0.06729	0.60254	0.03027 <sup>a</sup>	0.02851 <sup>a</sup>	0.05680	0.17161
2 vs. 3	0.57294	0.83631	0.88263	0.24036	0.02276 <sup>a</sup>	0.21970	0.80962
2 vs. 4	0.41027	0.59283	0.18941	0.25177	0.03047 <sup>a</sup>	0.06590	0.30761

<sup>a</sup>P<0.05, <sup>b</sup>P<0.01, <sup>c</sup>P<0.001. Dg, diagnosis group; Dg.0, controls; Dg.1, inflammation samples; Dg.2, CIN1; Dg.3, CIN2+; Dg.4, SCC; CIN, cervical intraepithelial neoplasia; SCC, squamous cell carcinoma; MAL, T-lymphocyte maturation associated protein; CADM1, cell adhesion molecule 1.

region of the *CADMI* gene was lower in normal squamous epithelium than in CINs (37). However, when CIN passed to the invasive cervical cancer (ICC), the average methylation of specific CpG regions increased significantly, which could lead to increased or complete inhibition of gene expression and result in a loss of cell adhesion and tumor-suppressor function of the gene and thereby inducing malignant transformation cells (37). The authors in the above-mentioned study also confronted the results of the median methylation in TSG and progression of disease with results reported by other study (17). These publications demonstrated that the frequency of average methylation in the promoter region of the gene increases with the severity of disease and that the presence of methylation in the ICC was significantly different from the degree of methylation occurring in other types of lesions.

Based on these results the present study hypothesized that progression of disease was dependent on the degree of the median methylation or HPV infection and not on the age of the patient. The same analysis was also applied in an additional study (38) and confirmed statistical significance (P=0.05). A previous study monitored the degree of methylation of the *CADMI/MAL* genes from the uterine cervix specimens using the quantitative methylation-specific PCR method and demonstrated that the median methylation of these genes is a significant predictor of the CIN1 status: positive patients (35%), CIN2: positive patients (32.8%), CIN3: positive patients (59%). They also identified a statistically significant association between the HR-HPV infection positivity and the transition of the disease to a more advanced stage (39).

The MassARRAY technique EpiTYPER DNA was used for analysis of the 15 methylated regions of the *CADMI* in ICC, CIN1, CIN2/3 tissue samples and a control group. In 9 CpGs they exhibited an increased methylation status in ICC compared with the control group, and the average degree of methylation (2, 3, 4. and 5. CpG) in the selected promoter region was higher than in the remaining CpG islands. However, CpGs 9 and 10 were less methylated. This provides evidence for varying degrees of methylation in the individual CpG regions in the *CADMI* gene (37). In our selected area the final CpG located behind the first exon was significantly more methylated in each group than the first two CpGs (P-values: 0.001 for inflammation samples, 0.086 for CIN1, 0.038

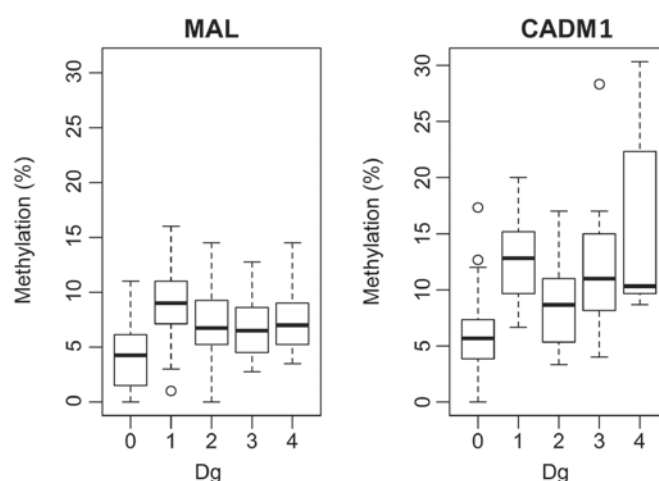


Figure 4. Boxplot of the methylation of the controls and the four levels of lesion (*MAL/CADMI*). *MAL*, T-lymphocyte maturation associated protein; *CADMI*, cell adhesion molecule 1; Dg, diagnosis group.

for CIN2+). The present study also identified that one CpG (1st CpG) was significantly more methylated in gene *MAL* than other 3 CpGs (P-values: 0.043 for inflammation samples, 0.017 for CIN1, 0.155 for CIN2+).

In several studies, DNA methylation of the *CADMI/MAL* gene promoter region has been demonstrated to increase with the severity of cervical disorders (17-19) and that these epigenetic changes suggest the presence of CINs. These findings are also supported by the results of an additional study, which demonstrated that levels of the median methylation in tumor suppressor genes are extremely high in cervical carcinomas and significantly increase in CIN3 lesions in women with HR-HPV infections that persists over a period of time longer than 5 years (20). The present study confirmed in both genes (*CADMI/MAL*) that the median methylation is significantly higher in HPV positive patients than in HPV negative patients. A higher median methylation and more HPV positive patients were identified in SCC than in CIN1 cases.

In a previous study, by analysis (pyrosequencing) of the promoter regions of the genes on tumor cell lines (C33A HPV negative, HeLa HPV18 positive, SiHa and CaSki HPV16 positive)

Table III. Multinomial logit model of diagnosis as a function of age, HPV, MAL 1-4 and CADM1 1-3.

Dg. no.	Age	HPV	MAL 1	MAL 2	MAL 3	MAL 4	CADM1 1	CADM1 2	CADM1 3
1	0.054	0.622	0.043	0.697	0.111	0.315	0.989	0.678	0.001
2	0.321	0.093	0.017	0.195	0.875	0.043	0.091	0.584	0.086
3	0.423	0.028	0.155	0.721	0.867	0.451	0.183	0.261	0.038
4	0.584	0.000	0.909	0.000	0.012	0.227	0.346	0.233	0.316

Dg, diagnosis group; Dg.1, inflammation samples; Dg.2, CIN1; Dg.3, CIN2+; Dg.4, SCC; CIN, cervical intraepithelial neoplasia; MAL, T-lymphocyte maturation associated protein; CADM1, cell adhesion molecule 1; HPV, human papillomavirus.

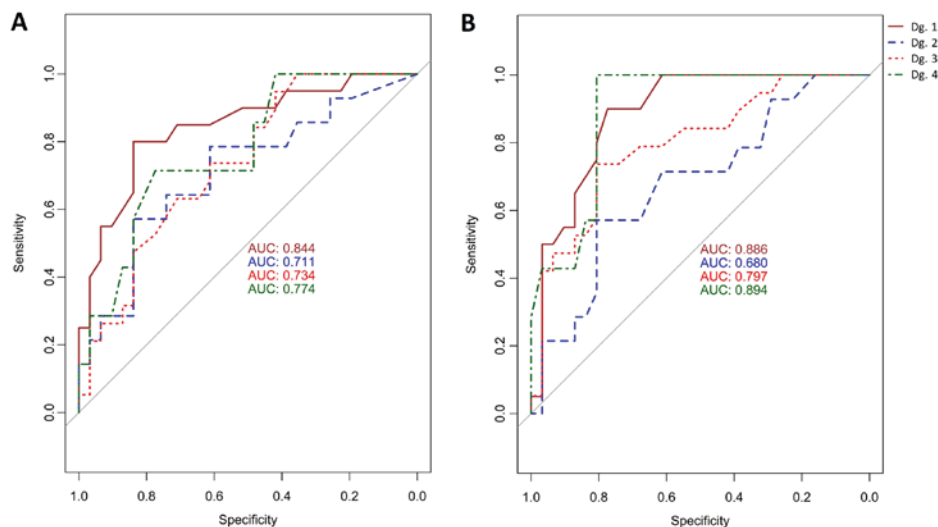


Figure 5. (A) ROC curve with AUC values (logistic regression) for samples from the control group and Dg. 1 (brown), 2 (blue), 3 (red) and 4 (green) depending on the median methylation of the *MAL*. (B) ROC curve with AUC values (logistic regression) for samples from the control group and Dg. 1 (brown), 2 (blue), 3 (red) and 4 (green) depending on the median methylation of the *CADM1*. ROC, receiver operating curve; AUC, area under the curve; *MAL*, T-lymphocyte maturation associated protein; *CADM1*, cell adhesion molecule 1; Dg, diagnosis group.

the hypermethylation of the *CADM1* gene in the CaSki, SiHa and HeLa cell lines were detected; however, not in the C33A cell line. The methylation status of the *MAL* was positive for the CaSki and SiHa cell lines but not for the HeLa and C33A cell lines. Based on these results, hypermethylation of the *CADM1* gene was demonstrated to be associated with HR-HPV infection. Hypermethylation of the *MAL* gene was also associated with infection with high-risk HPV types. Although the methylation stage indicates that the HeLa and C33A cell lines are not statistically significant ( $P=0.148$ ) (40). In an additional study the methylation state of the promoter region of the *CADM1* gene (nucleotides -444 to -305) were analyzed by pyrosequencing. They demonstrated that methylation was significantly increased in HPV positive HeLa cell lines (71.7%), SiHa (84.8%), CaSki (95.2%) and significantly decreased in HPV negative cell lines C33A (2.6%) (41). For further analysis cell lines were not used; however, tissue taken from patients with L-SIL and H-SIL as well as from patients with cervical carcinoma were analyzed. The degree of average methylation for the *CADM1* was 3.5 (95% CI, 3.0-4.0) in the L-SIL group, 5.6 (95% CI, 4.0-4.7) in the H-SIL group and 17.7 (95% CI, 10.8-29.1) in carcinomas. For the *MAL* gene, the average methylation rate was 2.7 (95% CI, 2.5-3.0) in the L-SIL group, 3.7 (95% CI, 3.0-4.6) in the H-SIL and 13 (95% CI, 7.6-22) in carcinomas. For both genes  $P<0.001$  (40).

The degree of average methylation increased significantly in carcinoma samples as well as in pre-invasive cervical lesion samples (39). The present study also confirmed that the median methylation significantly increased from CIN1 cases through CIN2+ cases to carcinomas in both genes, while the median methylation of *CADM1* was higher through each group than the median methylation of the gene *MAL*.

In the present study, statistically significant differences were identified in the inflammation samples when compared with the control or CIN1 group, which may suggest that hypermethylation of the TSGs in inflammatory samples is not random and that may be indicative of the early stages of carcinogenesis. Previous studies generally did not contain an inflammatory group, which could lead to an underestimation of at-risk patients. It is well-established that inflammation is a possible precursor of CIN1, and may culminate in CC (38), therefore it would be appropriate to include a cervical inflammation group in further methylation studies.

In conclusion, the present study focused on the detection of methylation in the region of two TSGs (*MAL* and *CADM1*). Several major studies have confirmed a significant increase of the median methylation in the specific promoter region of these genes tested on cell lines, while the percentage of methylation increased with the progression of neoplasia

to cervical carcinoma (39,40). However, in the majority of studies, pyrosequencing was not used as a sensitive tool for quantitative analysis of CpG methylation. Since many patients underestimate the importance of regular medical check-ups, it is important during examinations to provide them with the necessary comfort, not to exacerbate their stress and try to capture the dysplasia at an early stage. The specimens used in the present study therefore constituted of cytological smears from the cervix. *CADMI* was confirmed to be more methylated in almost every study group compared with *MAL*, and that third CpG in *CADMI* exhibited higher methylation levels during the transition of CIN and has the potential to serve as a promising biomarker for future study. There was also a significantly higher median methylation in HPV+ patients than in HPV-patients which supports the suitability of this combination (methylation levels/HPV positivity) as an early detection tool for this severe oncological disease.

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### Availability of data and materials

The datasets generated/analyzed during the current study are available from the corresponding author on reasonable request.

### Authors' contributions

SM and ZL designed the study. SM and VH performed the experiments. MG conducted the statistical analysis. JV, MN, EK and TB obtained and handled cervical specimens and clinical data. SM analyzed the data and was a major contributor in writing the manuscript. MK performed the histological examination of the samples and clinical data. ZL, PZ and JD supervised the entire study, and participated in study design and coordination. All authors read and revised the manuscript, and approved the final version to be published.

### Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Jessenius Faculty of Medicine in Martin. All patients provided written informed consent prior to their inclusion in the present study.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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