Up-regulation of Lipocalin 2 Is Associated With High-Risk Human Papillomavirus and Grade of Cervical Lesion at Baseline but Does Not Predict Outcomes of Infections or Incident Cervical Intraepithelial Neoplasia

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Abstract

Our objective was to assess whether neutrophil gelatinase–associated lipocalin (NGAL)/lipocalin 2 (LCN2) expression in cervical human papillomavirus (HPV) lesions has implications on the outcome of HPV infections or disease progression. Cervical biopsy specimens from 225 women in the Latin American Screening study were analyzed for NGAL/LCN2 expression using immunohistochemical analysis, to assess associations with cervical intraepithelial neoplasia (CIN) grade, high-risk HPV, and in predicting outcomes of high-risk (HR)-HPV infections.

Expression of NGAL/LCN2 increased with lesion grade (odds ratio [OR], 3.86; 95% confidence interval [CI], 1.53-9.71; P = .001). Up-regulation was also related to HR-HPV detection (OR, 2.21; 95% CI, 1.15-4.24; P = .016) and showed a linear relationship to HR-HPV load (P = .002). NGAL/LCN2 expression was of no value in predicting the outcomes of HR-HPV infections or the surrogate end points (incident CIN 1+ and CIN 2+) of progressive disease. Because the SV40 large T antigen is a powerful up-regulator of this lipocalin, up-regulation of NGAL/LCN2 in CIN is probably induced by HR-HPV E6 oncoprotein, most likely by eliminating its normal transcription repression exerted by wild-type p53.

Cervical carcinomas (CCs) are caused by high-risk human papillomavirus (HR-HPV) infections, whereas the low-risk HPV (LR-HPV) types are rarely found in CC or its precursor (cervical intraepithelial neoplasia [CIN]) lesions.1-6 This divergent oncogenic potential of LR-HPV and HR-HPV is mainly attributable to the differences of the 2 major viral oncoproteins (E6 and E7) in interacting with the key regulatory cellular proteins p53 and pRb.1,4,7-9 Although E6 of the HR-HPV (but not LR-HPV) initiates degradation of the p53 tumor suppressor protein, HPV E7 of HR-HPV (but not LR-HPV) binds to pRb, resulting in G1/S transition of the cell cycle.1,4,7,11 In addition to p53 and pRb, a plethora of other cellular proteins are known or suspected to be involved as regulators in HR-HPV–driven cervical carcinogenesis.1,4,7,8,12 As such potential regulators, a group of proteins known as lipocalins deserves further attention because they have been recently shown to be up-regulated in many human carcinomas.13-15

The lipocalins are an ever-expanding group of proteins exhibiting great structural and functional diversity, within and between species.14,16 Although primarily classified as transport proteins, it is now clear that members of the lipocalin family execute a wide variety of functions. These include roles in retinol transport, cryptic coloration, olfaction, pheromone transport, and the enzymatic synthesis of prostaglandins. The lipocalins have also been implicated in the regulation of the immune response and cell proliferation, eg, in human cancer.14-17 With regard to human cancer, of particular interest is a predominant member of the lipocalin family, neutrophil gelatinase–associated lipocalin (NGAL).13-15,16,18

NGAL, also known as lipocalin 2 (LCN2) or human neutrophil lipocalin (HNL), was first identified as a gene (24p3) in mice, which was rapidly overexpressed by SV40

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tumor virus–induced mitosis in quiescent primary mouse kidney cells.\textsuperscript{19} In searching for a specific marker of neutrophils, a 45-kDa protein (HNL) was identified in the granules of human granulocytes.\textsuperscript{30} HNL/NGAL consists of 2 identical chains, each with a molecular weight of 24 kDa.\textsuperscript{13,14,20} Of all members of the lipocalin superfamily, it is NGAL that has been most consistently linked with human cancer.\textsuperscript{15,18}

During the past several years, NGAL/LCN2 has been extensively studied in experimental and human tumors.\textsuperscript{15,18} In addition to SV40,\textsuperscript{19} NGAL is known to be induced by several other tumor-promoting agents in vitro, including polyoma-virus, phorbol ester phorbol-12-myristate-13-acetate, transforming factor \textit{neu}, hepatocyte growth factor, retinoic acids, and glucocorticoids. Following the detection of NGAL in mammary cancer cells overexpressing neu (HER-2/c-erbB-2) in rats, NGAL was soon found in human primary breast cancers\textsuperscript{21} and subsequently in several other human malignancies, including colorectal,\textsuperscript{22} pancreatic,\textsuperscript{23} ovarian,\textsuperscript{24,25} urothelial,\textsuperscript{26} esophageal,\textsuperscript{27} gastric,\textsuperscript{28} and thyroid\textsuperscript{29} carcinomas. Until now, however, there is not a single study on NGAL/LCN2 in CC or CIN lesions, leaving this a completely unexplored field.

To define (for the first time) the role of NGAL/LCN2 in HR-HPV–associated cervical carcinogenesis, we analyzed a series of cervical biopsy specimens derived from 225 women included in the Latin American Screening (LAMS) study cohort (n = 12,114) in Brazil and Argentina.\textsuperscript{30,33} This study aimed to assess whether the expression of NGAL/LCN2 is associated with the grade of CIN and HR-HPV type at baseline and whether it has any influence on the outcome of these HR-HPV infections or the development of incident CIN 1+ and CIN 2+ in this longitudinal setting. In addition to exploring NGAL/LCN2 as a potential predictor (biomarker) of a prevalent (CIN or HR-HPV) event or an incident event (CIN progression or outcome of HR-HPV), we were interested in the potential conceptual significance of NGAL/LCN2 as one of the cellular proteins regulated by the oncoproteins of HR-HPV.

**Materials and Methods**

**General Study Design**

The ongoing LAMS study is a multicenter screening trial targeting the female populations at different risk for CC in 2 Latin American countries, Brazil and Argentina.\textsuperscript{30} At their baseline visit, a total of 12,114 consecutive women attending the 4 partner clinics, Campinas, Brazil; São Paulo, Brazil; Porto Alegre, Brazil; and Buenos Aires, Argentina, were screened for HPV and CIN using 8 different diagnostic tools, as detailed before.\textsuperscript{30,33} Women testing positive with any of these diagnostic tests were examined by colposcopy (and underwent biopsy) at their second visit. In addition, a 5% random sample of women with negative Papanicolaou (Pap) smears were recalled for a new Pap test at 12 months, as were 20% of women testing negative to the Hybrid Capture II (HC2) test (Digene, Gaithersburg, MD), to assess the rates of incident Pap smear abnormalities and HPV infections, respectively.\textsuperscript{32,33} The women with biopsy-confirmed low-grade CIN comprise the prospective cohort (n = 1,011), followed up for a minimum of 24 months. All high-grade lesions were promptly treated and followed up for the same period, using repeated Pap test, colposcopy, and HC2 assay at 12-month intervals. For the present analysis of NGAL/LCN2, baseline biopsy specimens taken from 225 of the women were available.

**Prospective Follow-up**

By using the aforementioned criteria we allocated women in the prospective cohort and scheduled them to be monitored in the clinic at 6-month intervals for a minimum of 24 months. A total of 1,011 women completed at least 1 follow-up visit, including examination by Pap smear, visual inspection with acetic acid and with Lugol iodine, colposcopy, and biopsy, in case of abnormalities.\textsuperscript{31,33} The mean follow-up time at this writing is 21.7 months (SD, 8.09 months; median, 24.2 months; range, 1-54 months).

**Outcomes and End Points of Cervical Lesions and HR-HPV Infections**

In the present study, biopsy specimens from 225 women included in this subcohort of 1,011 women were analyzed for the different surrogate end points of progressive disease: (1) progression to CIN 1+ and (2) progression to CIN 2+. Data were also analyzed for different outcomes of HR-HPV infections, including incident infections, virus persistence, and HPV clearance. Progression to CIN 1+ was based on detection (in baseline biopsy-negative women) of a biopsy-confirmed CIN 1+ lesion in any of the consecutive follow-up visits. Progression to CIN 2+ was defined as any case in which biopsy-confirmed progression from a baseline negative, a flat HPV lesion with no CIN, or a CIN 1 biopsy was established in the subsequent follow-up visits.\textsuperscript{34} Times to progression to CIN 1+ and CIN 2+ were calculated from the baseline visit to the respective follow-up visit when the progression event was first detected. Progression rates were calculated by dividing the numbers of progressed cases by woman-months at risk (WMR) and expressed per 1,000 WMR. Three outcomes of HR-HPV infections were recorded: incident, persistent, and clearance. As an incident HR-HPV infection, we recorded an appearance of a positive HC2 test (at 1 pg/mL relative light units/cutoff ratio) among baseline HR-HPV–negative women at any of the follow-up visits. HR-HPV was considered cleared if the HC2 assay was negative at the last follow-up visit. Persistent HR-HPV infections were considered present in women in whom 2 or more subsequent HC2 assays were
HR-HPV+ and in whom the infection was not cleared at the last follow-up visit. Times to these 3 outcomes were also calculated and expressed as cases per 1,000 WMR.

**LAMS Methods**

Because the methods are detailed in a series of recent reports, the methods used in the LAMS study are described here only as pertinent to elaborating the data necessary for the present analysis.

**Epidemiologic Questionnaire**

All women who gave their consent to participate (n = 12,114) completed a detailed inquiry concerning the risk factors of HPV, CIN, and CC. This structured questionnaire contained questions exploring the reproductive history, sexual history, current sexual practices, sexual hygiene, medical history, smoking habits, and contraception.

**Pap Smears**

In the LAMS study, we compared the performance of 3 methods of cervical cytology: conventional Pap and 2 liquid-based cytology techniques (DNA-Citoliq, Digene Brazil, Sao Paulo; and SurePath, TriPath, Durham, NC).

In the present analysis, only the results of the conventional Pap test were used.

**Colposcopy and Directed Punch Biopsy**

All women testing positive with any of the screening tests were referred for colposcopy, performed by the colposcopists working at the partner clinics. Before the start of this trial, all colposcopists underwent a short training course, organized by the coordinator of the study, for consistency with the colposcopic procedures to be followed by all clinics. Lesions in the transformation zone (TZ) were assessed by applying a 5% acetic acid and iodine solution, under ×8 to ×12 magnification. If colposcopy was unsatisfactory, further exploration of the endocervix was systematically carried out under ×20 magnification using a Koogan speculum. The international nomenclature (of the International Federation for Cervical Pathology and Colposcopy) was used to classify the colposcopic patterns as follows: (1) normal, (2) abnormal TZ (ATZ) with minor changes (with or without features of HPV infection), (3) suggesting low-grade CIN (CIN 1), (4) ATZ with major changes suggesting CIN 2 or 3, and (5) cancer.

All abnormal colposcopies were confirmed by directed punch biopsy, following the common procedures. The number of biopsies was related to the volume of the ATZ, but 1 biopsy per quarter of ATZ was a minimum required. All acetowhite areas of the cervix and iodine-negative regions of the vagina were subjected to at least 1 biopsy. A cone biopsy by loop electrode excision procedure or cold knife conization was agreed to be performed in cases with the following: (1) biopsy-confirmed high-grade CIN, (2) Pap test showing high-grade squamous intraepithelial lesion and ATZ in colposcopy, and (3) regardless of the Pap test result, a large ATZ (≥50% of the TZ area). It is important to note that no loop electrode excision procedure or conization was performed on the basis of the HPV test result alone.

Directed punch and cone biopsy specimens were fixed in formalin, embedded in paraffin, and processed into 5-μm-thick H&E-stained sections for light microscopy, following the routine procedures. All biopsy specimens were examined among the daily routine in the pathology departments of the partner clinics in both studies and diagnosed using the commonly agreed-on CIN nomenclature. The pathologists were also asked to report the HPV-suggestive morphologic changes in flat lesions with no CIN, ie, HPV with no CIN (flat condyloma). The slides from 2 of the centers (Campinas and Sao Paulo) were subjected to reexamination by 2 pathologists of the EC partners (M.E. and K.S.). The reproducibility between the 2 observers was excellent (κ values >0.800), as previously reported. In all cases, the consensus diagnosis of the panel was considered the final diagnosis.

**Detection of HPV DNA by the HC2 Assay**

Primary HPV testing was done by using the HC2 assay, using cervical swabs (collected by a physician) and self-sampling devices (tampons), as described previously. The HC2 assay (n = 4,694 tests) was performed using the automated HC2 test system according to the manufacturer’s protocol. The samples were analyzed only for the presence of HR-HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68. The usual limit of 1 pg/mL of HPV-16 DNA was used as the positive control, ie, samples were classified as HR-HPV+ with a relative light units/cutoff ratio of 1.0 pg/mL or more.

**Immunohistochemical Detection of NGAL/LCN2**

A total of 225 slides from the same number of women were available for immunohistochemical analysis of NGAL/LCN2. In brief, 4-μm-thick sections were cut on ChemMate Capillary Gap Microscope slides (DAKO, Glostrup, Denmark) and kept overnight at 55°C, deparaffinized in xylene, and rehydrated in graded alcohol. Before immunohistochemical analysis, NGAL/LCN2 antigen retrieval was done by heating the tissue sections in a buffer of 10 mmol/L tris(hydroxymethyl)aminomethane and 1 mmol/L EDTA (pH 9.0) in a microwave oven for 10 minutes (600 W). Immunohistochemical staining for NGAL/LCN2 was performed with the DAKO TechMate 500 Plus Autostainer using the monoclonal NGAL/LCN2 antibody (MAB1757; R & D Systems, Minneapolis, MN) diluted 1:15 and reagents from the DAKO REAL kit (DAKO). The sections were washed with distilled water and Tris-buffered saline buffer. Then the sections were stained with the primary antibody and the secondary biotinylated antibody (antimouse...
IgG) for 30 minutes. Endogenous peroxidase activity was blocked by using 5% hydrogen peroxide, 3 times for 2.5 minutes each. This was followed by incubation with streptavidin peroxidase for 30 minutes. Counterstaining was performed with hematoxylin for 1 minute, and the immunoperoxidase reaction was developed using 3,3′-diaminobenzidine 3 times for 5 minutes each. Finally, the sections were washed with distilled water and mounted with Aquamount (BDH Laboratory Supplies, Poole, England). Negative control samples were similarly processed by omitting the primary antibody, and biopsy specimens from breast and pancreatic cancer were used as positive control samples.

### Evaluation of Immunohistochemical Staining for NGAL/LCN2

In normal, mature squamous epithelium, NGAL/LCN2 was typically present as weak cytoplasmic staining in the basal layer cells and also in cells close to the epithelial surface. In metaplastic epithelium, NGAL/LCN2 expression was more diffuse and also usually increased in intensity. In low-grade CIN 1, NGAL/LCN2 expression was not dramatically different from that in metaplastic epithelium. In CIN 2 and particularly in CIN 3, marked up-regulation of NGAL/LCN2 was typically observed, with expression found throughout the full thickness of the epithelium. In most biopsy specimens, NGAL/LCN2-expressing cells (granulocytes) were also encountered among the inflammatory cell infiltrates in the connective tissue underlying the epithelial lesion. In the original grading of the NGAL/LCN2 staining, semiquantitative scoring into 4 categories was used: 0, no expression; 1, weak staining (equivalent to normal squamous epithelium); 2, slightly to moderately increased staining (intermediate cells are stained); and 3, strongly increased staining (all layers show positive NGAL/LCN2 staining). In statistical analysis, the staining results were also treated as a dichotomous variable (normal vs increased) to calculate the risk estimates (odds ratio [OR]). In this study, no attempt was made to enumerate the cells expressing NGAL/LCN2 among the subepithelial inflammatory cell infiltrates.

### Statistical Methods

Statistical analyses were performed using the PASW Statistics for Windows, version 17.0 (SPSS, Chicago, IL) and Stata SE 10.1 (Stata, College Station, TX) software packages. Frequency tables for categorical variables were analyzed by using the c² test, with the likelihood ratio or Fisher exact test to assess the significance. Differences in the means of continuous variables were analyzed by using nonparametric tests (Mann-Whitney) or analysis of variance. Performance indicators (sensitivity, specificity, positive predictive value, negative predictive value, and their 95% confidence intervals [CIs]) for NGAL as a predictor of baseline CIN 2/3 or HR-HPV and the longitudinal predictive values for the 3 viral outcomes and incident CIN 1+/CIN 2+ were all calculated by using Stata SE software and the algorithm of Seed and Tobias, which also calculates the area under the receiver operating characteristic curve (AUC). In all tests, P values of less than .05 were considered statistically significant.

### Results

Expression of LCN2 in cervical biopsy specimens as related to lesion grade is summarized in Table 1. There was a significant linear trend of increasing up-regulation of NGAL/LCN2.

### Table 1

<table>
<thead>
<tr>
<th>LCN2 Expression</th>
<th>Negative-Normal Expression</th>
<th>Slight-Moderate Up-regulation</th>
<th>Strong Up-regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lesion Grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal or HPV-NCIN (n = 94)</td>
<td>46 (49)</td>
<td>33 (35)</td>
<td>15 (16)</td>
</tr>
<tr>
<td>CIN 1 (n = 64)</td>
<td>24 (38)</td>
<td>23 (36)</td>
<td>17 (27)</td>
</tr>
<tr>
<td>CIN 2 (n = 22)</td>
<td>7 (32)</td>
<td>8 (36)</td>
<td>7 (32)</td>
</tr>
<tr>
<td>CIN 3 (n = 36)</td>
<td>6 (17)</td>
<td>16 (44)</td>
<td>14 (39)</td>
</tr>
<tr>
<td>SCC (n = 1)</td>
<td>0 (0)</td>
<td>1 (100)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

CIN, cervical intraepithelial neoplasia; HPV-NCIN, flat human papillomavirus lesion with no CIN; LCN2, lipocalin 2; SCC, squamous cell carcinoma.

* Data are given as number (percentage). P = .019 (Fisher exact test); P = .0001 for linear trend.
LCN2 (P = .0001) in parallel with increasing grade of CIN. When dichotomized (negative-weak vs moderate-strong), up-regulated NGAL/LCN2 expression predicted CIN 3+ with an OR of 3.86 (95% CI, 1.53-9.71; P = .001) and CIN 2+ with an OR of 2.81 (95% CI, 1.41-5.63; P = .002).

Table 2 depicts the association of NGAL/LCN2 expression with HPV detection and semiquantitative viral load detected with the HC2 assay. NGAL/LCN2 was up-regulated more often in HPV+ lesions than in those remaining HPV– (P = .054). Dichotomized expression was associated with HR-HPV detection with an OR of 2.21 (95% CI, 1.15-4.24; P = .016). The log-transformed semiquantitative HR-HPV viral loads were also directly related to up-regulation of NGAL/LCN2 (P = .002). When the performance indicators were calculated, NGAL/LCN2 was not a particularly good predictor of HR-HPV, with an AUC of 0.594 (95% CI, 0.517-0.671).

Expression of NGAL/LCN2 in baseline biopsy specimens was related to outcome of the HPV infection as shown in Table 3 and Table 4. Up-regulation of NGAL/LCN2 was a 100% sensitive marker in predicting incident HR-HPV (AUC, 0.736) but was less sensitive in predicting persistent HR-HPV infection or virus clearance, using the longitudinal performance indicators. The association with any of these events was not significant. All of these calculations were hampered by the small number of events.

Table 5 and Table 6 give the data on NGAL/LCN2 as a predictor of the 2 surrogate end points of progressive disease (incident CIN 1+ and CIN 2+). The 3-tier grading of NGAL/LCN2 expression was practically identical in the baseline biopsy specimens that subsequently progressed to incident CIN 1+, with no significant difference. Longitudinal performance indicators did not provide any useful values, with an AUC of 0.497. The same was true with NGAL/LCN2

### Table 2
Expression of LCN2 as Related to Detection of HR-HPV and Its Viral Load*

<table>
<thead>
<tr>
<th>HC2 Assay</th>
<th>Negative-Normal Expression (n = 63)</th>
<th>Slight-Moderate Up-regulation (n =59)</th>
<th>Strong Up-regulation (n = 49)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV+ (n =112)</td>
<td>34 (30.4)</td>
<td>43 (38.4)</td>
<td>35 (31.3)</td>
</tr>
<tr>
<td>HPV- (n = 59)</td>
<td>29 (49)</td>
<td>16 (27)</td>
<td>14 (24)</td>
</tr>
<tr>
<td>Mean (± 95% CI) viral load</td>
<td>1.42 (0.66-2.69)</td>
<td>3.13 (2.28-3.98)</td>
<td>3.37 (2.39-4.35)</td>
</tr>
</tbody>
</table>

CI, confidence interval; HC2, Hybrid Capture II; HR-HPV, high-risk human papillomavirus; LCN2, lipocalin 2.

* Data are given as number (percentage) unless otherwise indicated. For HPV+ vs HPV–, P = .054 (χ², log rank); P = .043 for linear trend. For viral load, P = .002, analysis of variance; P = .004, Kruskal-Wallis (Monte-Carlo simulation with the 10,000 sample and 99% CI). The semiquantitative viral load was determined by the relative light units/ cutoff ratio in the HC2 assay, log-transformed. The performance indicators of dichotomous (increased vs normal) LCN2 expression were as follows: sensitivity, 69.6% (95% CI 60.2%-78.0%); specificity, 49.2% (95% CI 35.9%-62.5%); positive predictive value, 72.2% (95% CI, 62.8%-80.4%); negative predictive value, 46.0% (95% CI, 33.4%-59.1%); and area under the receiver operating characteristic curve, 0.594 (95% CI, 0.517-0.671).

### Table 3
Expression of LCN2 as a Predictor of Different Outcomes of HR-HPV Infection*

<table>
<thead>
<tr>
<th>HR-HPV End Point</th>
<th>LCN2 Expression</th>
<th>Negative-Normal Expression</th>
<th>Slight-Moderate Up-regulation</th>
<th>Strong Up-regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incident Yes (n = 1)</td>
<td>0 (0)</td>
<td>1 (100)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>No (n = 55)</td>
<td>26 (47)</td>
<td>18 (33)</td>
<td>11 (20)</td>
<td></td>
</tr>
<tr>
<td>Yes (n = 14)</td>
<td>5 (36)</td>
<td>5 (36)</td>
<td>4 (29)</td>
<td></td>
</tr>
<tr>
<td>No (n = 42)</td>
<td>21 (50)</td>
<td>14 (33)</td>
<td>7 (17)</td>
<td></td>
</tr>
<tr>
<td>Persistence Yes (n = 11)</td>
<td>4 (36)</td>
<td>6 (55)</td>
<td>1 (9)</td>
<td></td>
</tr>
<tr>
<td>No (n = 45)</td>
<td>22 (49)</td>
<td>13 (29)</td>
<td>10 (22)</td>
<td></td>
</tr>
</tbody>
</table>

HR-HPV, high-risk human papillomavirus; LCN2, lipocalin 2.

* Data are given as number (percentage). For incident HR-HPV, P = .537 (Fisher exact test); P = .727 for linear trend. For cleared HR-HPV, P = .526 (Fisher exact test); P = .537 (Fisher exact test); P = .526 (Fisher exact test). For viral load, P = .273 for linear trend. For cleared HPV persistence, P = .307 (Fisher exact test); P = .981 for linear trend.

### Table 4
Longitudinal Performance Indicators of Dichotomous (Increased vs Normal) Lipocalin 2 Expression*

<table>
<thead>
<tr>
<th>High-Risk Human Papillomavirus End Point</th>
<th>Sensitivity, %</th>
<th>Specificity, %</th>
<th>Positive Predictive Value, %</th>
<th>Negative Predictive Value, %</th>
<th>AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incident</td>
<td>100 (2.5-100)</td>
<td>47.3 (33.7-61.2)</td>
<td>3.3 (0.1-17.2)</td>
<td>100 (88.8-100)</td>
<td>0.736 (0.000-1.000)</td>
</tr>
<tr>
<td>Cleared</td>
<td>64.3 (35.1-87.2)</td>
<td>50.0 (34.2-65.8)</td>
<td>30.0 (14.7-49.4)</td>
<td>80.8 (60.6-93.4)</td>
<td>0.571 (0.420-0.722)</td>
</tr>
<tr>
<td>Persistence</td>
<td>63.6 (30.8-89.1)</td>
<td>48.9 (33.7-64.2)</td>
<td>23.3 (9.9-42.3)</td>
<td>84.6 (65.1-96.6)</td>
<td>0.563 (0.396-0.729)</td>
</tr>
</tbody>
</table>

AUC, area under the receiver operating characteristic curve.

* Values in parentheses are the 95% confidence intervals.
as a predictor of incident CIN 2+ (AUC, 0.467). However, the negative predictive value approached 90%, implicating that negative-weak NGAL/LCN2 precludes progression to CIN 2+ with high accuracy (95% CI, 77.8%-96.6%). Times to progression to CIN 1+ or CIN 2+ were identical in different categories of NGAL/LCN2 expression, with no significant differences (data not shown).

Discussion

During the past several years, mounting evidence implies that NGAL/LCN2 and several other related lipocalins are overexpressed in a variety of human cancers.13-15,21-29 The most convincing evidence on NGAL/LCN2 comes from the studies on breast cancer.13,18 with several lines of evidence currently suggesting that NGAL/LCN2 expression may represent a predictor of poor prognosis in breast cancer. As suggested in a recent review, NGAL/LCN2 expression may provide prognostic information for risk assessment and potential identification of a subset of patients with breast cancer who may benefit from more aggressive adjuvant therapy.18

Much of the data on possible mechanisms explaining these unfavorable effects of NGAL/LCN2 on the outcome of breast cancer come from vitro studies on cell lines.18 During the past several years, mounting evidence implies that NGAL/LCN2 and several other related lipocalins are overexpressed in a variety of human cancers.13,15,21-29 The most convincing evidence on NGAL/LCN2 comes from the studies on breast cancer.13,18 with several lines of evidence currently suggesting that NGAL/LCN2 expression may represent a predictor of poor prognosis in breast cancer. As suggested in a recent review, NGAL/LCN2 expression may provide prognostic information for risk assessment and potential identification of a subset of patients with breast cancer who may benefit from more aggressive adjuvant therapy.18

Thus, overexpression of NGAL/LCN2 in MCF-7 human breast cancer cells resulted in increased growth rates, proliferation, angiogenesis, and increased levels of matrix metalloproteinase (MMP)-9.36 NGAL is known to complex with MMP-9, thereby preventing MMP-9 degradation and increasing MMP-9 enzyme activity in vitro,37 as well as in patients with breast cancer.36 Activity of all MMPs promotes cancer progression by degrading the basement membrane and extracellular matrix, liberating vascular endothelial growth factors (VEGFs), all of which contributes to increased angiogenesis, invasion, and metastasis. It is interesting that Branca et al38,39 recently showed that MMPs (MMP-2 and its inhibitor tissue inhibitor of metalloproteinase-2) and VEGFs (VEGF-C) seem to be intimately involved also in the process of HR-HPV–associated cervical carcinogenesis.

Although NGAL/LCN2 expression has not been previously studied in cervical cancer and its precursors, the closest parallel could be found in studies of esophageal squamous cell carcinoma (SCC), which is another human malignancy recently linked to HR-HPV.1,3,4 Indeed, in a recent report on 30 esophageal SCCs, strong NGAL/LCN2 expression was disclosed in cancer cells, and, in addition, tissue extracts demonstrated an increase in NGAL/MMP-9 enzymatic activity that correlated closely with tumor invasion.27 Another line of data on NGAL/LCN2 that could bear intriguing links to HR-HPV is derived from the early experiments of Hraba-Renevey et al,19 who demonstrated in mouse kidney cell cultures that the wild-type (wt) SV40 large T antigen was required for the increase of 24p3 (NGAL homologue) messenger RNA (mRNA) levels.19 In fact, it is this analogy between SV40 large T antigen and HR-HPV E6 oncogene that led (in 1989) to the discovery of the E6-p53 interaction and opened the way to understanding how HR-HPV induces malignant transformation.1,4

There is little reason to doubt that once shown for SV40 large T antigen in the mouse model,19 HR-HPV E6 also could increase the mRNA expression of NGAL/LCN2 in human squamous cells, as already shown in esophageal SCC.27 Under these circumstances, one would expect to see some differences in NGAL/LCN2 expression as related to the grade of CIN and HR-HPV detection and to the outcome of the HR-HPV infections and development of incident CIN 1+ and CIN 2+. Instead of NGAL/LCN2 mRNA, we analyzed

**Table 5**

Expression of LCN2 as a Predictor of the Two Surrogate End Points of Disease Progression

<table>
<thead>
<tr>
<th>LCN2 Expression</th>
<th>Negative-Normal Up-regulation</th>
<th>Slight-Moderate Up-regulation</th>
<th>Strong Up-regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incident CIN 1+</td>
<td>9 (50)</td>
<td>7 (39)</td>
<td>2 (11)</td>
</tr>
<tr>
<td>No (n = 81)</td>
<td>40 (49)</td>
<td>25 (31)</td>
<td>16 (20)</td>
</tr>
<tr>
<td>Incident CIN 2+</td>
<td>5 (56)</td>
<td>2 (22)</td>
<td>2 (22)</td>
</tr>
<tr>
<td>No (n = 90)</td>
<td>44 (49)</td>
<td>30 (33)</td>
<td>16 (18)</td>
</tr>
</tbody>
</table>

CIN, cervical intraepithelial neoplasia; LCN2, lipocalin 2.

* Data are given as number (percentage). For incident CIN 1+, P = .688 (Fisher exact test); P = .642 for linear trend. For incident CIN 2+, P = .809 (Fisher exact test); P = .688 (Fisher exact test). For incident CIN 1+, P = .934 for linear trend.

**Table 6**

Longitudinal Performance Indicators of Dichotomous (Increased vs Normal) Lipocalin 2 Expression

<table>
<thead>
<tr>
<th>End Point</th>
<th>Sensitivity, %</th>
<th>Specificity, %</th>
<th>Positive Predictive Value, %</th>
<th>Negative Predictive Value, %</th>
<th>AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incident CIN 1+</td>
<td>50.0 (26.0-74.0)</td>
<td>49.4 (38.1-60.7)</td>
<td>18.0 (8.6-31.4)</td>
<td>81.6 (68.0-91.2)</td>
<td>0.497 (0.366-0.628)</td>
</tr>
<tr>
<td>Incident CIN 2+</td>
<td>44.4 (13.7-78.8)</td>
<td>48.9 (38.2-59.7)</td>
<td>8.0 (2.2-19.2)</td>
<td>89.8 (77.8-96.6)</td>
<td>0.467 (0.287-0.646)</td>
</tr>
</tbody>
</table>

AUC, area under the receiver operating characteristic curve; CIN, cervical intraepithelial neoplasia.

* Values in parentheses are the 95% confidence intervals.
the expression of NGAL/LCN2 in CIN lesions using immunohistochemical analysis to assess whether the NGAL/LCN2 expression patterns in cervical lesions have any associations with these 4 outcomes.

In our series, NGAL/LCN2 expression could be identified in practically all cervical biopsy specimens, including normal epithelium and all grades of CIN (Table 1). NGAL/LCN2 expression in normal squamous epithelium was present as weak cytoplasmic staining in the basal layer cells and also in cells close to the epithelial surface. In many biopsy specimens with squamous metaplasia, NGAL/LCN2 expression was more diffuse and also increased in intensity. Unfortunately, there are no previous reports to make direct comparisons for these NGAL/LCN2 staining patterns, but a large number of representative illustrations of normal uterine cervix and CC are found at the Web site of the Human Protein Atlas.40 Our observations are compatible with the NGAL/LCN2 expression patterns presented in those illustrations.

By using the staining pattern of the normal squamous epithelium as a reference, we noticed that the expression of NGAL/LCN2 seemed to increase almost in parallel with the increasing grade of CIN (Table 1). Because of the lack of any published observations on CIN and CC for direct comparison, we needed to refer to a recently reported parallel in which NGAL/LCN2 expression was shown to increase with increasing proliferation of skin keratinocytes. Lee et al41 studied the expression of NGAL/LCN2 in several skin diseases and found NGAL expression to be highly increased in psoriasis-like inflammatory disorders (eg, lichen planus) and skin cancers (keratoacanthoma and SCC), implying that NGAL expression is related to hyperplasia of the skin. Similarly, Moniaux et al42 demonstrated up-regulated NGAL/LCN2 expression in low-grade pancreatic intraepithelial neoplasia-1, suggesting that it is an early marker of pancreatic carcinogenesis.

In our series, up to 30% of CIN 2 lesions still retained only weak expression of NGAL/LCN2, equivalent to that in the normal epithelium. NGAL/LCN2 expression reached the peak in CIN 3 lesions, of which only 17% retained the baseline profile and almost 40% showed significant overexpression (Table 1). It is tempting to speculate that this obvious up-regulation on transition from CIN 2 to CIN 3 might bear some association with HR-HPV. In such a case, up-regulated NGAL/LCN2 in these high-grade lesions could imply increasing up-regulation by HR-HPV E6 oncoprotein (an analogue of the SV40 large T antigen), favoring progressive cervical disease.

Indeed, this concept seems to hold true, at least in part, as evidenced by the close association of NGAL/LCN2 expression with the detection of HR-HPV in these lesions (Table 2). Dichotomized expression (increased vs normal) was associated with HR-HPV detection with an OR of 2.21 (95% CI,
would expect that the abundant expression of this lipocalin should have some unfavorable impact on the outcome of HR-HPV infections or progression to CIN. This concept was challenged in the present study, however, when we failed to provide any confirmatory evidence to support the view that NGAL/LCN2 expression could modify these viral and clinical outcomes (Tables 3 and 4). Similarly, there was practically no difference in the baseline of NGAL/LCN2 expression patterns among progressive and nonprogressive lesions (Tables 5 and 6), and NGAL/LCN2 was of no value in discriminating the incident CIN 1+ and CIN 2+ cases from cases that did not progress. It is to be emphasized, however, that, like all studies with similar design, the present study does not provide any longitudinal data from the biopsy specimens obtained at follow-up visits.32,33 The expression of lipocalins is a highly dynamic process,13,14 being regulated by a multitude of local and systemic stimuli and inhibitors that are impossible to record without a longitudinal study.

This is the first study to examine the expression of a dominant member of the lipocalin family, NGAL/LCN2, in CIN lesions.18 NGAL/LCN2 up-regulation in the baseline biopsy specimens was most consistently associated with high-grade CIN. The expression profile of this lipocalin was also intimately related to detection of the HR-HPV and viral load. To our disappointment, however, NGAL/LCN2 expression was not a useful biomarker (with practical predictive value) of prevalent or incident CIN 2+ or any of the HR-HPV outcomes. On the other hand, NGAL/LCN2 might prove to be conceptually interesting as one of the novel cellular proteins regulated by oncogenic HPV. Indeed, using the analogy to the experimental data on SV40 large T antigen model19 and given the close similarities between NGAL/LCN2 and survivin in their associations with high-grade CIN and HR-HPV,43 it is tempting to speculate that the up-regulation of NGAL/LCN2 in high-grade CIN could be orchestrated by the HR-HPV E6 oncoprotein, possibly by eliminating its normal transcriptional repression exerted by wt p53. However, at this stage, this remains speculation, and experimental data on HPV+ CC cell lines are needed to elucidate the interactions between HR-HPV and NGAL/LCN2.

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