## PAPER IV

Lipocalin 2 expression is associated with aggressive features of endometrial cancer.

*Manuscript*

IV

**Short Report**

# Lipocalin 2 expression is associated with aggressive features of endometrial cancer

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## Abstract

**Background.** Increased expression of lipocalin 2 (LCN2) has been observed in several cancers. The aim of our present study was to investigate LCN2 in endometrial cancer in relation to clinico-pathological variables, angiogenesis, markers of epithelial-mesenchymal transition (EMT) and patient survival. LCN2 has to our knowledge not been previously studied in endometrial cancer.

**Materials and Methods.** Staining was performed using a human LCN2 antibody on a population based series of endometrial cancer patients collected in Hordaland County (Norway) during 1981-1990 (n=256). Patients were followed from the time of primary surgery until death or last follow-up in 2007. The median follow-up time for survivors was 17 years.

**Results**. 125 (49%) of the endometrial cancers showed positive staining for LCN2, and expression was associated with non-endometrioid histological type (p=0.001), nuclear grade 3 (p=0.001), >50% solid tumor growth (p=0.001) and ER/PR negativity (p=0.028/0.006). LCN2 expression was not associated with any of the EMT-markers included in this study (P-cadherin, N-cadherin, E-cadherin, β-catenin). Of the angiogenic markers, LCN2 was significantly associated with VEGF-A expression (p=0.021), but not with VEGF-C, VEGF-D, bFGF2, vascular proliferation index or glomeruloid microvascular proliferation. LCN2 expression was significantly associated with distant tumor recurrences. Patients with tumors showing no LCN2 expression had the best outcome with a 81% 5-year survival compared to 73% for intermediate and 38% for strong LCN2 staining (p=0.007).

**Conclusion**. Increased LCN2 expression is associated with aggressive features and poor prognosis in endometrial cancer.

## Background

Lipocalin 2 (LCN2), also called NGAL, is a secreted glycoprotein belonging to the lipocalin protein family. LCN2 was first identified as a gene upregulated in mouse kidney cells infected by SV-40 tumor virus [1]. Members of the lipocalin family bind small molecules and cell surface receptors to form macromolecular complexes. They have been previously classified as transport proteins, but it is now clear that they have several different functions [2].

LCN2 protein is known to be secreted by epithelial cells, macrophages, neutrophils and tumor cells [3, 4]. Elevated levels of LCN2 have also been observed in plasma, serum and urine in various conditions such as metastatic breast cancer, colorectal liver metastasis, acute kidney injury, pancreatitis and preeclampsia [5-10]. In tumor tissue, increased expression of LCN2 has been observed in tumors like breast cancer, colorectal cancer, ovarian and pancreatic cancers [10-13].

Studies have indicated that LCN2 might be involved in the epithelial-mesenchymal transition (EMT) process. Colon carcinoma cells with high LCN2 expression were observed to have a decreased cell-cell adhesion due to a dissociation of β-catenin from E-cadherin [12]. E-cadherin expression was downregulated in breast cancer cell lines overexpressing LCN2 [10]. In contrast, mesenchymal markers were upregulated, and the cells showed an increased motility and invasiveness [10]. On the contrary, ovarian cancer cell

lines undergoing EMT showed a decreased expression of both LCN2 and E-cadherin [14]. Regarding angiogenesis, studies of pancreatic cancer cells showed LCN2 to block HUVEC tube formation and to reduce VEGF secretion [15]. Also, a breast cancer model showed LCN2 to inhibit tumor angiogenesis by suppressing RAS-induced VEGF expression in 4T1 tumor cells [16].

On this background, and since LCN2 expression has not been previously studied in endometrial cancer, the aim of our present study was to investigate LCN2 in these tumors in relation to angiogenesis, EMT markers, vascular invasion and patient survival.

## Methods

### Patient series.

All 316 patients diagnosed with endometrial carcinoma in Hordaland County (Norway) during the period 1981-1990 were studied. This endometrial cancer series and the variables histological type, histological grade, nuclear grade, solid growth, mitoses, FIGO stage, estrogen receptor, progeseterone receptor and HER-2 expression have previously been reported [17-20]. EMT-related markers P-cadherin, N-cadherin, E-cadherin and β-catenin are also recorded [18, 21]. Angiogenesis and vascular markers VEGF-A, VEGF-C, VEGF-D, bFGF2, blood vascular invasion, lymphatic vascular invasion, vascular proliferation index and glomeruloid microvascular proliferation has previously

been described and were studied in relation to LCN2 expression in this study [19, 22, 23].

The follow-up data were collected from the medical records and correspondence with the primary physicians, with respect to disease recurrence and deaths. Patients were followed from the time of primary surgery until death or last follow-up in 2007. The median follow-up time for the survivors was 17 years (range 6 – 23 years); 256 cases were available for this LCN2 study.

**Immunohistochemistry.**

Staining was performed with human LCN2 antibody (Clone #220310, MAB1757, R&D Systems, Minneapolis, MN, USA) on 5 μm sections of formalin-fixed and paraffin embedded tumor samples using tissue microarray arrays (TMA) slides. For antigen retrieval, sections were boiled in 10 mM citrate buffer for 10 minutes at 750W followed by 350W for 15 minutes. Sections were treated with goat serum diluted 1:4, and incubated for 1 hour at room temperature (RT) with LCN2 antibody diluted 1:25. Staining was done with 1:300 diluted goat anti-rat IgG-HRP (Santa Cruz, CA, USA) for 1 hour at RT, with diaminobenzidine peroxidase (DAB, Dako, Glostrup, Denmark) as substrate. The sections were counterstained with Dako REAL hematoxylin (Dako).

TMA-slides were evaluated in a standard light microscope (by M.M. and I.M.S.). Regarding LCN2, cytoplasmic staining intensity in tumor cells (graded 0-3) and staining area (0, no tumor cells positive; 1, <10%; 2, 10%-50%; 3, >50%) were recorded. A staining index (SI) was calculated as a product of staining intensity and positive area giving a staining index of 0-9. LCN2 cases were divided in two subgroups based on median. For survival, three subgroups were used (negative, weak/moderate and strong expression).

**Statistical Methods.**

Statistical analyses were performed by the PASW statistical software package version 17 (SPSS Inc., Chicago, IL). Associations between different categorical variables were assessed by Pearson's chi-square test. An association was considered significant if a p-value of <0.05 was obtained. Univariate survival analyses were performed using the Kaplan-Meier method (log-rank significance test). LCN2, together with standard clinico-pathological variables, were further analyzed by log-log plot to determine how these variables could be incorporated in the Cox' proportional hazards regression model (Lratio significance test).

## Results

**Distribution of staining**

125 (49%) of the endometrial cancers showed some staining for LCN2, while 131 (51%) of the cases were negative. Only 8 (3%) of the endometrial cancers had strong expression of LCN2 (SI=9) (Figure 1). Median SI was 0.

**Association with clinico-pathological markers**

LCN2 protein expression showed a significant association with non-endometrioid endometrial cancers, nuclear grade 3, >50% of solid growth and ER/PR negativity (Table 1).

**Association with EMT markers and angiogenesis**

EMT-markers P-cadherin, N-cadherin, E-cadherin and β-catenin did not show any significant associations with LCN2 expression.

There was a significant association between VEGF-A expression and LCN2 staining (p=0.021). However, LCN2 showed no significant associations with vascular invasion (lymphatic or blood vessels), vascular proliferation index, glomeruloid microvascular proliferation, VEGF-C, VEGF-D and bFGF2 expression.

**Metastatic spread**

Forty-one of the 256 patients examined (16%) showed recurrence of their primary endometrial cancer during the follow-up period. Regarding the site of

recurrent tumors, 34% were located in the vagina, 7% in the pelvic lymph nodes, 44% represented distant metastases (liver not included) and 15% were metastases to the liver; 174 cases did not show any spread of the disease. LCN2 expression was significantly associated with more advanced metastatic spread (Table 2).

**Patient survival**

Absence of LCN2 staining was associated with the best survival, cases with medium staining index (SI 2-6) showed an intermediate survival, while the small subgroup of patients showing strong LCN2 expression (SI 9) was associated with reduced survival (Figure 2). In multivariate survival analysis, standard clinico-pathological variables (histological type, histological grade, and FIGO stage) were included together with LCN2 expression in three groups. Strong LCN2 expression appeared to be an independent prognostic marker for survival, with Hazard ratio (HR) of 3.9, $p=0.027$ (Table 3). Histological grade (HR 2.8, $p<0.001$), and FIGO stage (HR 8.0, $p<0.001$) were independent prognostic factors in addition, but not histological type (HR 1.7, p NS) (Table 3).

## Discussion

In this study we found that LCN2 expression appears to be associated with aggressive features of endometrial carcinoma like the non-endometrioid histological type, high nuclear grade and ER/PR negativity. This has been

shown in other tumor forms like breast cancer [10], but has to our knowledge not been reported for endometrial cancers. About 50% of the tumors in this study showed LCN2 expression, and this is comparable to breast cancers with positive staining in 33% of the cases [24].

LCN2 expression has been reported to be associated with ER and PR negative and HER-2 positive breast cancers [10, 24-26]. In endometrial cancer, we found an association between LCN2 expression and ER/PR negative tumors. Regarding HER-2 status and LCN2, a similar relationship was indicated, although of borderline statistical significance only.

Many studies report LCN2 to be involved in the EMT-process and metastatic spread [10, 14, 15]. We did not find any significant associations between LCN2 expression and the cadherins or β-catenin staining. Also, LCN2 expression showed no clear association with our angiogenesis markers except VEGF-A expression. However, cases with strong LCN2 expression showed a poorer prognosis than cases with no expression also in multivariate analysis, and LCN2 staining showed an association with the most aggressive metastases.

## Conclusions

In conclusion, LCN2 expression is likely to be involved in metastatic spread and tumor progression in endometrial cancer, although the mechanism is presently not clear, and further studies are needed.

## Competing interests

The authors declare that they have no competing interests.

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## Figure text

**Figure 1**

**LCN2 protein expression**: Immunohistochemical staining showing A) strong and B) no expression of LCN2 in endometrial cancer (magnification x 400).

**Figure 2**

**Survival analysis for LCN2:** Univariate survival analysis (Kaplan-Meier method, log-rank significance test) for LCN2 in endometrial cancers.

**Table 1**

LCN2 protein expression by various clinico-pathological variables among 256 endometrial cancers

| Variable | | LCN2 SI 0 N (%) | LCN2 SI 1-9 N (%) | P-value[a] |
|---|---|---|---|---|
| Histological type | Endometrioid | 126 (55%) | 103 (45%) | 0.001 |
| | Non-endometrioid | 5 (19%) | 22 (81%) | |
| Histological grade | Grade 1 and 2 | 89 (56%) | 71 (44%) | 0.066 |
| | Grade 3 | 42 (44%) | 54 (56%) | |
| Nuclear grade | Grade 1 and 2 | 101 (59%) | 71 (41%) | 0.001 |
| | Grade 3 | 30 (36%) | 54 (64%) | |
| Solid growth | <50% | 104 (56%) | 82 (44%) | 0.013 |
| | ≥50% | 27 (39%) | 43 (61%) | |
| Mitoses[b] | Low | 103 (54%) | 89 (46%) | NS |
| | High | 28 (44%) | 36 (56%) | |
| FIGO stage[c,d] | I/II | 109 (53%) | 97 (47%) | NS |
| | III/IV | 22 (45%) | 27 (55%) | |
| ER[e,f] | Negative | 25 (40%) | 38 (60%) | 0.028 |
| | Positive | 103 (56%) | 82 (44%) | |
| PR[g,h] | Negative | 28 (38%) | 46 (62%) | 0.006 |
| | Positive | 98 (57%) | 74 (43%) | |
| HER-2[i,j] | Weak | 115 (54%) | 100 (46%) | 0.084 |
| | Strong | 11 (37%) | 19 (63%) | |
| VEGF-A[k,l] | Weak | 114 (55%) | 94 (45%) | 0.021 |
| | Strong | 17 (36%) | 30 (64%) | |

[a]P-value from $\chi^2$ test. Missing data in [c]one case for FIGO stage, [e]8 cases for ER, [g]10 cases for PR, [i]11 cases for HER-2/neu and one case for [k]VEGF-A. [d]FIGO stage according to 1998 criteria. Cut-off points used, [b]median for mitosis, [e,h]lower quartile for ER/PR and [j]median for HER-2, [l]upper quartile for VEGF-A.

**Table 2**

Associations between LCN2 expression and metastatic spread among 215 endometrial cancers.

| Variable | Site of tumor recurrence | LCN2 SI 0<br>N (%) | LCN2 SI 1-9<br>N (%) | P-value[a] |
|---|---|---|---|---|
| Recurrent disease[b] | No tumor recurrence | 96 (55%) | 78 (45%) | 0.029 |
| | Vaginal cuff | 11 (79%) | 3 (21%) | |
| | Pelvic lymph nodes | 2 (67%) | 1 (33%) | |
| | Distant metastasis (not liver) | 9 (50%) | 9 (50%) | |
| | Liver | 0 (0%) | 6 (100%) | |

[a]P-value from $\chi^2$ test, [b]Missing data for 41 pateints.

**Table 3.**

Multivariate survival analysis (Cox' proportional hazards regression model) of clinico-pathological variables and LCN2 expression in patients with endometrial cancer (n=255).

| Variables | Categories | HR[a] | 95% CI[b] | P-value[c] |
|---|---|---|---|---|
| LCN2 | Negative | 1.0 | | 0.027 |
| | Weak/moderate | 1.1 | 0.6-1.9 | |
| | Strong | 3.9 | 1.4-10.8 | |
| Histological type | Endometrioid | 1.0 | | NS |
| | Non-endometrioid | 1.7 | 0.9-3.2 | |
| Histological grade | Grade 1 and 2 | 1.0 | | <0.001 |
| | Grade 3 | 2.8 | 1.6-4.9 | |
| FIGO stage | I/II | 1.0 | | <0.001 |
| | III/IV | 8.0 | 4.8-13.6 | |

[a]Hazard ratio, [b]95% confidence interval, [c]Lratio test.

**Figure 1**

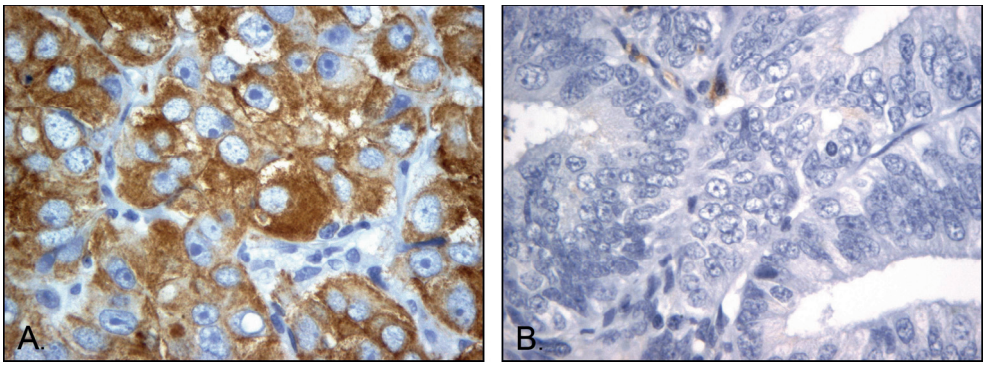
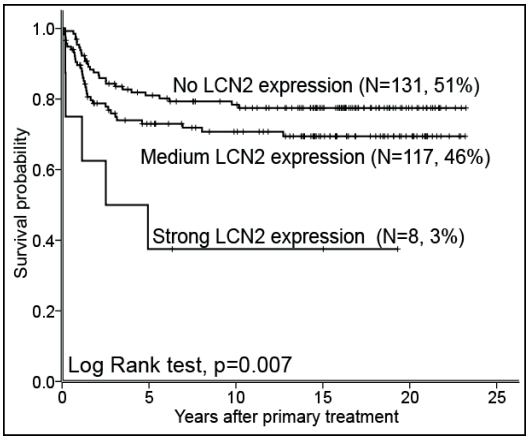


**Figure 2**


1.0
0.8
0.6
0.4
0.2
0.0
Survival probability
No LCN2 expression (N=131, 51%)
Medium LCN2 expression (N=117, 46%)
Strong LCN2 expression (N=8, 3%)
Log Rank test, p=0.007
0
5
10
15
20
25
Years after primary treatment