Expression of L1CAM in curettage or high L1CAM level in preoperative blood samples predicts lymph node metastases and poor outcome in endometrial cancer patients.

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ABSTRACT

Background: Several studies have identified L1CAM as a strong prognostic marker in endometrial cancer. To further underline the clinical usefulness of this biomarker, we investigated L1CAM as a predictive marker for lymph node metastases and its prognostic impact in curettage specimens and preoperative plasma samples. In addition we aimed to validate the prognostic value of L1CAM in hysterectomy specimen.

Methods: Immunohistochemical staining of L1CAM was performed for 795 hysterectomy and 1134 curettage specimen from endometrial cancer patients. L1CAM level in preoperative blood samples from 372 patients was determined using ELISA.

Results: Expression of L1CAM in curettage specimen was significantly correlated to L1CAM level in corresponding hysterectomy specimen ($P<0.001$). Both in curettage and preoperative plasma samples was L1CAM upregulation significantly associated with features of aggressive disease and poor outcome ($P<0.001$). L1CAM was an independent predictor of lymph node metastases, after correction for curettage histology, both in curettage specimen ($P=0.002$) and plasma samples ($P=0.048$). In the hysterectomy samples L1CAM was significantly associated with poor outcome ($P<0.001$).

Conclusions: We demonstrate that preoperative evaluation of L1CAM levels, both in curettage or plasma samples, predicts lymph node metastases and adds valuable information on patient prognosis.

Keyword: L1CAM, biomarker, endometrial cancer, curettage, blood sample

INTRODUCTION

Endometrial cancer is the most common gynaecologic malignancy and the fourth most common cancer among women in industrialized countries (Torre *et al*, 2015). Although the prognosis is good, fifteen to twenty percent of patients with presumed localized disease at diagnosis recur (Abeler & Kjorstad, 1991; Morrow *et al*, 1991). Identifying biomarkers that can select patient populations for optimal surgical and systemic treatment is important to improve outcome for these patients.

The diagnosis and treatment of endometrial cancer patients usually consists of a preoperative biopsy followed by imaging, surgery and adjuvant treatment depending on risk classification. A complete surgical staging of endometrial cancer patients involves both pelvic and para-aortic lymphadenectomy (Pecorelli, 2009). Worldwide there are however a wide variation in lymphadenectomy in endometrial cancer, and selection criteria for lymphadenectomy are not uniformly standardized (Maggino *et al*, 1998; Maggino *et al*, 1995). Metastases to the lymph nodes is known to be associated with poor prognosis and although lymphadenectomy provides diagnostic information that can help in selecting optimal adjuvant treatment, its effect on survival is uncertain, and it is associated with increased complication rates (Benedetti Panici *et al*, 2008; Morice *et al*, 2016; Wright *et al*, 2012). Improved identification of high-risk patients preoperatively is therefore needed to tailor the primary surgical strategy.

L1 cell adhesion molecule (L1CAM) is a cell adhesion molecule of the immunoglobulin superfamily (Moos *et al*, 1988). L1CAM consists of an extracellular domain, a transmembrane region and a highly conserved cytoplasmic domain (Moos *et al*, 1988). The extracellular domain of L1CAM can be cleaved by the metalloproteases ADAM10 and ADAM17 resulting in a soluble form of L1CAM (Maretzky *et al*, 2005; Mechtersheimer *et al*, 2001). L1CAM was initially identified in the nervous system, and plays an important role in

neurogenesis (Moos *et al*, 1988; Rathjen & Schachner, 1984; Schafer & Altevogt, 2010). Later L1CAM has also been shown to be involved in almost every aspect of cancer progression including promoting cell proliferation, migration, invasion and metastases of cancer cells (Altevogt *et al*, 2016; Raveh *et al*, 2009; Schafer & Altevogt, 2010).

Expression of L1CAM has been investigated in several cancer types, and is associated with poor outcome (Allory *et al*, 2005; Boo *et al*, 2007; Fogel *et al*, 2003; Hua *et al*, 2016; Schroder *et al*, 2009; Tischler *et al*, 2011; Tsutsumi *et al*, 2011; Wang *et al*, 2013). In endometrial cancer hysterectomy samples, L1CAM has been reported to be associated with aggressive disease characteristics including loss of hormone receptors and reduced survival (Bosse *et al*, 2014; Dellinger *et al*, 2016; Geels *et al*, 2016; Huszar *et al*, 2010; Kommoss *et al*, 2017; van der Putten *et al*, 2016; Zeimet *et al*, 2013). L1CAM is a strong prognostic marker within the subgroup of stage I endometrioid endometrial cancer (van der Putten *et al*, 2016; Zeimet *et al*, 2013), and within the subgroup of advanced stage endometrioid endometrial cancer (van der Putten *et al*, 2016), when evaluated postoperatively in hysterectomy samples. However, the expression of L1CAM in preoperative biopsies and its usefulness in preoperative prediction of lymph node metastases and outcome are not yet established. Also soluble L1CAM (sL1CAM) is suggested to be a valuable circulating biomarker in different cancers (Bondong *et al*, 2012; Zander *et al*, 2011). It has been detected in the culture medium from different cell lines including melanoma, breast and ovarian cancer (Beer *et al*, 1999; Gutwein *et al*, 2005; Li & Galileo, 2010), and also in serum and ascites of endometrial and ovarian cancer (Fogel *et al*, 2003; Wojciechowski *et al*, 2017), suggesting that also the soluble form of L1CAM may play a role in these cancers.

The primary objective of this study was to assess the value of L1CAM in preoperative samples (curettage and plasma samples) as a predictive marker for lymph node metastases and

poor prognosis. In addition we aimed to validate the prognostic value of L1CAM in hysterectomy samples.

MATERIALS AND METHODS

Patient series

A population-based series was prospectively collected from 2001 to 2014 and included curettage specimen (n=600), primary tumours (n=795, of which 189 overlapped with cases published by van der Putten *et al.*(van der Putten *et al*, 2016)) and plasma (n=372) from patients diagnosed with endometrial cancer in Hordaland County (Norway). Within the MoMaTEC study (Molecular markers in treatment of Endometrial Cancer, NCT00598845) nine other centres contributed with preoperative biopsies from in total 534 patients treated for endometrial cancer prospectively included at their institutions. Formalin-fixed, paraffin-embedded (FFPE) tumour tissue from curettage specimen was collected from all participating institutions (Trovik *et al*, 2013). Clinical and histopathological data was collected, including age, FIGO stage (according to 2009 criteria), histologic type and grade and myometrial invasion from hysterectomy specimen. Follow up data were collected as previously described (Trovik *et al*, 2011). Preoperative histology reports were categorized as high risk (serous, clear cell, carcinosarcoma, undifferentiated carcinomas, endometrioid grade 3) or low risk (benign (diagnosed with endometrial cancer after hysterectomy), hyperplasia, endometrioid grade 1-2). Pelvic lymphadenectomy as part of surgical staging was conducted in the majority of cases (n=825 73%), of these additional para-aortic lymph node sampling was conducted for 6% (n=49). Fresh frozen tissue from hysterectomy samples was investigated for L1CAM mRNA expression in 232 patients, these overlapped with FFPE samples.

The study has been approved by Norwegian legislation, including the Norwegian Data Inspectorate, Norwegian Social Science Data Services and the Western Regional Committee

for Medical and Health Research Ethics (REK 2009/2315). All patients signed informed consent prior to inclusion in the study.

Immunohistochemical staining

FFPE tissue from curettage and hysterectomy samples were available in TMAs from 1134 and 795 patients respectively, for evaluation of L1CAM level. Tissue microarrays (TMA) were generated from FFPE tissue as previously described, with three tissue cylinders from each case (Stefansson *et al*, 2004). TMAs were dewaxed in xylene and rehydrated in graded ethanol series before microwave boiling in target retrieval buffer (pH9) for 15 minutes. L1CAM was detected using purified anti-CD171 (L1) antibody clone 14.10 (Biolegend, San Diego, CA, USA) diluted 1:100 for 60 minutes at room temperature, followed by 30 minutes incubation with secondary HPR-conjugated antibody. Diaminobenzidine was applied for 8 minutes before counterstained with haematoxylin. All slides were scored independently and blinded for clinical and pathological data by two authors using standard light microscopy. Inter-evaluator Kappa value was calculated to be 0.76 for L1CAM in two groups. The staining was evaluated as previously described (Engelsen *et al*, 2008). Briefly, both intensity and area of positive tumour cells were considered. The intensity of staining was graded from 0 (no staining) to 3 (strong), and the area from 0, 1 (< 10 %), 2 (10-50%) and 3 (51-100%). From this, a staining index (0-9) was calculated as the product of intensity and area. If heterogeneity was seen for the three tissue cylinders of each case, the three cylinders were given one overall averaged score. In subsequent statistical analysis indexes were grouped according to similarity in survival and considering the size of the subgroup and the number of events in each category. For L1CAM index 0-3 was considered low and 4-9 high (Supplementary Figure 1). Immunohistochemical (IHC) staining and staining evaluation of ER and PR has previously been described (Trovik *et al*, 2013). Using the same scoring system as above, ER staining index ≤3 and PR staining index 0 was defined as low expression/loss.

ELISA

EDTA-blood was obtained from 372 patients with endometrial cancer before primary surgery. The blood samples were centrifuged at 1600 g for 15 minutes and the plasma was stored at -80^{o}C until measurement of sL1CAM. ELISA kits were bought from MyBioSource (Cat. No. MBS2023094, MyBioSource, San Diego, CA, USA) and the sandwich ELISA was performed according to the manufacturer´s instructions. Briefly, 100 µl plasma sample or standard was put into a 96-well microplate and incubated for two hours at 37^{o}C. Then, 100 µl biotin-conjugated L1CAM specific antibody was added and incubated for 1 hour at 37°C. After the well was washed three times with washing solution, 100 µl avidin conjugated to horseradish peroxidase (HRP) was added and incubated for 30 minutes at 37°C. The wells were washed five times and 90 µl substrate solution was added before 20 minutes incubation away from light followed by addition of 50 µl stop solution. The absorbance was measured in a microplate reader at the wavelength of 450 nm, and plasma concentration of sL1CAM calculated. For further statistical analysis the patients were grouped in four groups based on sL1CAM plasma level, and subsequently in high and low L1CAM based on similarities in survival (the three groups with lowest L1CAM level=low L1CAM level; the upper quartile=high L1CAM level) (Supplementary Figure 1). Plasma from healthy aged-matched female blood-donors (n=32) was used as control group.

Gene expression analysis

RNA was extracted from fresh frozen tissue from primary tumour from patients diagnosed with endometrial cancer. Hybridization to Agilent Whole Human Genome Microarray 44k (Cat. No. G4112F) was done according to the manufacturer´s instructions. The arrays were scanned and normalized as previously described (Krakstad *et al*, 2012).

Statistical analyses

Data were analysed using SPSS version 24 (SPSS Inc, Chicago, IL). Probability <0.05 was considered statistical significant, and all statistical tests were two-sided. Associations between groups were analysed using the chi-square test for categorical variable and the Mann-Whitney U test for continuous variables. Binary logistic regression was used to estimate odds ratios (OR) for lymph node metastases. Univariate survival analysis was performed using the Kaplan Meier (product-limit) method. Disease specific survival was defined as time from surgery to death from endometrial cancer. Survival between groups was compared using the log-rank (Mantel-Cox) test. The Cox proportional hazard regression model was used to evaluate the prognostic impact of L1CAM adjusted for other prognostic parameters.

RESULTS

Expression of L1CAM in endometrial cancer curettage predicts lymph node metastases and poor outcome.

L1CAM protein expression was evaluated by IHC in curettage samples from 1134 patients. Low expression was defined as staining index 0-3 and was found in 88% (n=1000) of the lesions, while 12% (n=134) expressed high levels of L1CAM. High L1CAM expression in curettage specimen was significantly associated with high age, loss of ER and PR expression and high-risk curettage classification and also high FIGO stage, non-endometrioid histology and high grade in the hysterectomy specimen, (all P<0.001) (Table 1). There was a highly significant correlation between L1CAM staining in curettage specimen and staining in the corresponding hysterectomy specimen (P<0.001) (Table 1). High expression of L1CAM in curettage specimen predicted poor disease specific survival both in the whole patient population (P<0.001) (Figure 1A), and within patients with low risk curettage histology (P<0.001) (Figure 1B). Expression of L1CAM also showed independent prognostic impact in Cox survival analysis after correction for age, FIGO stage, histologic subtype and grade

assessed in the hysterectomy specimens, with hazard ratio of 1.77 (95% CI 1.17-2.66, P=0.006) (Supplementary Table 1).

Interestingly, patients with high L1CAM expression in curettage specimen had significantly higher occurrence of lymph node metastases compared to patients with low expression of L1CAM, both in the whole patient population (33% vs. 10% respectively, P<0.001, Table 1), and in the subgroup with low risk histology classification (30% vs. 9% respectively, P<0.001, Supplementary Table 2). In a univariate model, curettage high-risk histology, combined loss of ER/PR (a marker previously shown to be a strong predictor of lymph node metastases in endometrial cancer patients (Trovik *et al*, 2013)), and high expression of L1CAM all predicted presence of metastatic lymph nodes (Table 2). When adjusting for curettage histology and preoperative ER/PR loss in a multivariate model, high expression of L1CAM predicted lymph node metastases with adjusted OR 2.51 (95% CI 1.41-4.64, P=0.002) (Table 2).

High sL1CAM level in preoperative blood samples is associated with lymph node metastases and poor survival in endometrial cancer patients.

The level of sL1CAM was also investigated in preoperative plasma samples from 372 patients with endometrial cancer. The plasma level of sL1CAM was significantly higher in endometrial cancer patients compared to healthy controls (P=0.001 (endometrial cancer patients: mean=997 pg/ml, SEM=27; healthy controls mean=684 pg/ml, SEM=29)). There was a significant correlation between sL1CAM level in plasma evaluated by ELISA, and L1CAM expression both in curettage specimen (P=0.007) (Supplementary Figure 2A) and hysterectomy specimen (P=0.015) (Supplementary Figure 2B) evaluated by IHC. High preoperative plasma levels of sL1CAM were significantly associated with high age, loss of ER and PR and high-risk histology in curettage, and also high FIGO stage and non-

endometrioid histology in the hysterectomy specimen (Table 3). High sL1CAM plasma level predicted poor disease specific survival both in the whole population (P<0.001) (Figure 1C) as well as in the group with low-risk curettage histology (P=0.04) (Figure 1D). sL1CAM level in plasma did not have independent prognostic impact when adjusting for age, FIGO stage, histologic type and grade (data not shown).

Patients with high sL1CAM plasma levels had significantly higher occurrence of lymph node metastases compared to patients with low sL1CAM level (23% vs. 9% respectively, P=0.003) when including the whole patent population, but not within the subgroup with low risk histology classification (Supplementary table 2). In a univariate model both high-risk histology in curettage and high plasma levels of sL1CAM were predictive of lymph node metastases. Also when correcting for curettage histology in a multivariate model, sL1CAM level in plasma was a predictor of lymph node metastases with OR 2.25 (95% CI 1.01-5.02, P=0.048) (Table 4).

Expression of L1CAM in hysterectomy specimen validates to be a strong predictor of poor outcome in endometrial cancer.

Several studies have identified L1CAM as a strong prognostic marker when examining hysterectomy specimens in endometrial cancer. In this study we investigated expression of L1CAM in 795 primary tumours. In 14% of the cases (n=110) expression of L1CAM was high while 86% (n=685) of the cases showed low L1CAM levels. There was a significant association between L1CAM protein levels and L1CAM mRNA levels (Supplementary Figure 3). We identified that high expression of L1CAM in hysterectomy samples is associated with features of aggressive endometrial cancer (Supplementary Table 3) and poor survival (Figure 1E). Expression of L1CAM also showed independent prognostic impact in Cox survival analysis when adjusting for age, FIGO stage, histologic subtype and grade with HR 2.7 (95% CI 1.8-4.3, P<0.001) (Supplementary Table 4). Within the subgroup of stage I

endometrioid endometrial cancer 5% of the cases expressed high L1CAM. Also within this subgroup expression of L1CAM was associated with characteristics of aggressive endometrial cancer (Supplementary Table 5) and poor survival (Figure 1F), but it did not have independent prognostic impact when adjusting for age and histologic grade (data not shown).

DISCUSSION

There are several studies suggesting L1CAM as a promising biomarker in endometrial cancer, and its expression has earlier been shown to be associated with aggressive disease and poor survival, and inversely correlated with expression of ER and PR (Bosse *et al*, 2014; Dellinger *et al*, 2016; Geels *et al*, 2016; Huszar *et al*, 2010; Kommoss *et al*, 2017; van der Putten *et al*, 2016; Zeimet *et al*, 2013). Current data points to an added prognostic value of L1CAM, and integrating L1CAM status as part of the molecular classification of endometrial cancer can be useful. We here validate that L1CAM is a strong prognostic marker in hysterectomy samples when including all samples in this prospectively collected population-based series. Also within the subgroup of patients with FIGO stage 1 endometrioid endometrial cancer, L1CAM expression is a predictor of poor survival, although not as strong as shown in other studies (van der Putten *et al*, 2016; Zeimet *et al*, 2013). The usefulness of L1CAM as a prognostic marker within this subgroup was also questioned in a recent study including 388 patients where L1CAM failed to be a clinically relevant marker of poor prognosis in FIGO stage 1 endometrioid endometrial cancer (Smogeli *et al*, 2016). These differences could be explained by different scoring methods and cut-offs for L1CAM, but also differences in the patient cohort with regard to proportion of patients where lymphadenectomy is performed, as well as the use of adjuvant treatment.

In this study TMAs were used to determine the expression of L1CAM, and the staining index was used as scoring method with high expression defined as 4-9 and low expression defined

as 0-3. Most other studies investigating the expression of L1CAM have used whole sections and a cut-off point of 10% (Bosse *et al*, 2014; Smogeli *et al*, 2016; van der Putten *et al*, 2016; Zeimet *et al*, 2013). The use of different scoring methods and cut-offs make the studies less comparable, however the fact that different scoring methods identifies L1CAM as a strong biomarker supports its robustness as biomarker in endometrial cancer. Although a interobserver κ-value of 0.76 was obtained for L1CAM in this study a scoring method like the staining index where both intensity and area is taken into account may be more subjectively influenced compared to the method only considering the area. Before potential clinical implementation of L1CAM as biomarker in endometrial cancer, the optimal staining protocol, scoring method and cut-off will have to be determined and validated. In an investigational setting the use of TMAs is both time and cost effective, and the method has been shown to yield reproducible results compared to full sections (Fons *et al*, 2007; Kononen *et al*, 1998). TMAs do however not provide the same morphological information as full sections, and should be used with caution, and depending on the research question. Although three tissue cores were used for each patient in this study, L1CAM positive areas of the tumor might be missed, which may result in underestimation of L1CAM expression. This is however also a challenge using full sections, and validation of markers is crucial before applied in a clinical setting.

In endometrial cancer the tumour is easily accessible for biopsy prior to surgery. Histologic type and grade are routinely investigated in the preoperative biopsies, and this provides prognostic information relevant for the extent of the surgery. However the correlation between preoperative assessment of curettage and postoperative evaluation of hysterectomy specimens varies (Eltabbakh *et al*, 2005; Frumovitz *et al*, 2004; Lampe *et al*, 1995; Leitao *et al*, 2008; Werner *et al*, 2013). Therefore, identification of reliable markers in curettage material that could predict lymph node metastases would provide a better basis for treatment

selection. There are different molecular markers that have been studied in preoperative biopsies, and could serve as predictive markers for metastatic disease (Salvesen *et al*, 2012). One example is combined loss of ER and PR for prediction of lymph node metastases and poor survival in endometrial cancer (Trovik *et al*, 2013). Its usefulness in tailoring surgical treatment is currently tested in a clinical trial (Momatec2, NCT02543710), where the decision to perform lymphadenectomy in low and intermediate risk patients is dependent of the preoperative hormone receptor status. In the present study we investigate L1CAM level in curettage and preoperative blood samples, and its potential for predicting lymph node metastases and poor prognosis. Only few studies have previously investigated the expression of L1CAM in curettage samples. Bosse *et al.* (Bosse *et al*, 2014) compared expression of L1CAM in curettage and hysterectomy samples from 42 patients and Fogel *et al.* (Fogel *et al*, 2003) from 14 patients. Both studies reported a good concordance between staining in curettage and hysterectomy samples (Bosse *et al*, 2014; Fogel *et al*, 2003). We here confirm that the L1CAM staining in the curettage and hysterectomy sample are significantly associated. More importantly, we report the novel finding that high L1CAM expression in curettage samples is a significant predictor of lymph node metastases, both in a univariate analysis and in a multivariate analysis correcting for curettage histology and preoperative ER/PR expression. Our large study shows that L1CAM expression in curettage specimens is associated with features of aggressive endometrial cancer disease and poor survival both in the whole patient population and within the subgroup of patients with a preoperative low-risk histology classification. These findings suggest that evaluation of L1CAM in curettage samples could be a valuable supplement to the currently performed preoperative assessment for determination of surgical extent. However, this is the first study investigating L1CAM as a predictive marker for lymph node metastases and its prognostic impact in curettage

specimens, and these findings would have to be validated in other studies before its place in the preoperative assessment can be determined.

The soluble form of L1CAM has earlier shown to be present in sera of endometrial cancer patients. In a study by Fogel et. al. nine out of ten patients with L1CAM positive endometrial tumours also had detectable concentrations of sL1CAM in preoperative serum samples (Fogel *et al*, 2003). A recent study investigating serum levels of sL1CAM in 35 endometrial cancer patients found it to be lower in patients compared to healthy controls, and no correlations between soluble L1CAM concentration and histopathology, stage or grade were found (Wojciechowski *et al*, 2017). The latter is contradictory to our results as we find that the level of sL1CAM is significantly increased in endometrial cancer patients compared to healthy controls, and that a high level of sL1CAM is associated with aggressive disease characteristics, and poor survival. In addition we report that a high level of sL1CAM in preoperative blood samples predicts of lymph node metastasis both in a univariate analysis, and also multivariate analysis correcting for preoperative histology. Increased level of sL1CAM has been found to be associated with poor prognosis also in other cancer types, such as in gastrointestinal stromal tumours (Zander *et al*, 2011) and ovarian cancer (Bondong *et al*, 2012). Whether the soluble form of L1CAM also in addition to serving as a prognostic biomarker, has a role in tumour development and progression is not clear, but studies investigating its function have suggested that sL1CAM is involved in stimulating cell motility and contribute to cell survival through activating anti apoptotic pathways (Bondong *et al*, 2012; Mechtersheimer *et al*, 2001).

The primary treatment for endometrial cancer patients is surgical, which typically includes hysterectomy and bilateral salpingo-oophorectomy with or without lymphadenectomy. Although lymphadenectomy is part of the complete staging procedure and important for risk stratification of endometrial cancer patients, no survival benefit is shown in randomized

clinical trials, and it is associated with increased complication rates and prolonged operation time in a co-morbid and obese patient population (Benedetti Panici *et al*, 2008; Morice *et al*, 2016; Pecorelli, 2009; Wright *et al*, 2012). Identifying markers that preoperatively can predict prognosis and lymph node metastases is important. Biomarkers can aid in tailoring the surgical treatment through identifying those patients with advanced disease who would benefit from extensive surgery, but also aid in preventing over-treatment in low-risk patients. Both L1CAM expression evaluated by IHC in curettage and the level of sL1CAM evaluated by ELISA in plasma seem to be promising biomarkers in endometrial cancer. Although L1CAM expression in curettage is a stronger predictor of both lymph node metastases and disease specific survival in multivariate analysis compared to sL1CAM in plasma, and may be the preferred method, the fact that no tissue, only a blood sample is necessary for the sL1CAM analysis is an advantage. However, additional studies and in particular prospective randomized trials would be important to evaluate the effect of implementing L1CAM expression in curettage samples and sL1CAM level in blood samples into routine clinical practice.

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Figure 1. Expression of L1CAM is a prognostic marker in curettage samples, blood samples and hysterectomy samples of endometrial cancer patients.

High expression of L1CAM in curettage samples was significantly associated with poor outcome, in the whole patient population (A), and in the subgroup of patients with low-risk histology in curettage (B). In plasma from endometrial cancer patients high level of L1CAM was predictive of poor outcome both in the whole patient population (C) and in the subgroup of patients with low-risk histology in curettage (D). High expression of L1CAM in hysterectomy samples is significantly associated with poor survival, both in the whole population (E), and in the subgroup with stage 1 endometrioid endometrial cancer (F). * Curettage histological risk classification, low risk (benign, hyperplasia or endometrioid grades 1-2) or high risk (non-endometrioid or endometrioid grade 3). Number in brackets: number of patients in the group/number of events in the group.

Supplementary Figure 1. Disease specific survival according to staining index and sL1CAM level.

L1CAM expression was scored using the staining index, and high staining index was significantly associated with poor disease specific survival (A). Patients were grouped in four groups based in sL1CAM plasma level, and the group with high sL1CAM level had significantly poorer survival compared to the groups with intermediate and low sL1CAM levels (B).

Supplementary Figure 2. Correlation between L1CAM protein and plasma levels.

Protein expression of L1CAM investigated by IHC in curettage (A) and hysterectomy (B) samples were significantly correlated to sL1CAM levels in plasma evaluated by ELISA.

Supplementary Figure 3. Correlation between L1CAM protein and mRNA levels.

In hysterectomy samples there is a significant correlation between protein levels of L1CAM investigated by IHC and L1CAM mRNA levels.

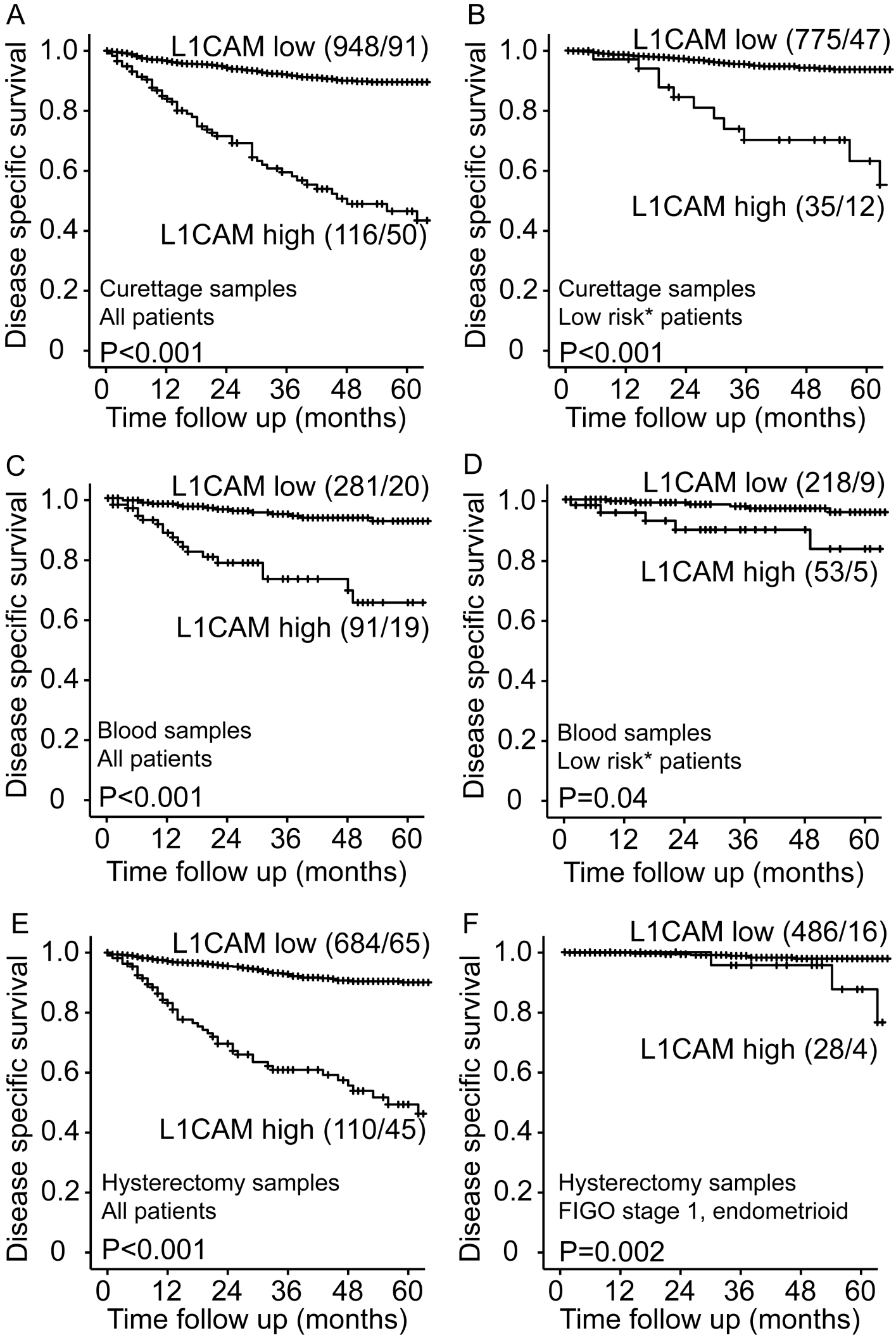
A
Disease specific survival
1.0
0.8
0.6
0.4
0.2
0
L1CAM low (948/91)
L1CAM high (116/50)
Curettage samples
All patients
P<0.001
0
12
24
36
48
60
Time follow up (months)
B
Disease specific survival
L1CAM low (775/47)
L1CAM high (35/12)
Curettage samples
Low risk* patients
P<0.001
Time follow up (months)
C
Disease specific survival
L1CAM low (281/20)
L1CAM high (91/19)
Blood samples
All patients
P<0.001
Time follow up (months)
D
Disease specific survival
L1CAM low (218/9)
L1CAM high (53/5)
Blood samples
Low risk* patients
P=0.04
Time follow up (months)
E
Disease specific survival
L1CAM low (684/65)
L1CAM high (110/45)
Hysterectomy samples
All patients
P<0.001
Time follow up (months)
F
Disease specific survival
L1CAM low (486/16)
L1CAM high (28/4)
Hysterectomy samples
FIGO stage 1, endometrioid
P=0.002
Time follow up (months)

Table 1. Clinico-pathological variables related to L1CAM status in curettage specimens for women operated for endometrial cancer			
	L1CAM		
Variable	Low (0-3) n (%)	High (4-9) n (%)	*P*-value
Age			<0.001
<66	520 (95)	30 (5)	
≥66	480 (82)	104 (18)	
Information available preoperatively			
Curettage histology*			<0.001
Low risk	811 (95)	40 (5)	
High risk	170 (64)	94(36)	
PR curettage			<0.001
Positive	795 (96)	36 (4)	
Negative	191 (69)	88(31)	
ERα curettage			<0.001
Positive	746 (95)	43 (5)	
Negative	228 (72)	89 (28)	
Information available postoperatively			
FIGO-09 stage			<0.001
I-II	872 (91)	84 (9)	
III-IV	128 (72)	50 (28)	
Histologic type			<0.001
Endometrioid	874 (96)	36 (4)	
Adenosquamous	10 (91)	1 (9)	
Clear cell	29 (71)	12 (29)	
Serous papillary	39 (36)	70 (64)	
Carcinosarcoma	35 (76)	11 (24)	
Undifferentiated/other	13 (77)	4 (23)	
Histologic grade**			<0.001
Grade 1/2	754 (97)	21 (3)	
Grade 3	120 (86)	19 (14)	
Metastatic nodes			<0.001
Negative	657 (92)	60 (8)	
Positive	77 (72)	30 (28)	
Myometrial infiltration			<0.001
<50%	608 (92)	51 (8)	
≥50%	309 (84)	60 (16)	
Ploidy			<0.001
Diploid	288 (94)	17 (6)	
Aneuploid	49 (58)	35 (42)	
L1CAM hysterectomy specimen			<0.001
Low	431 (96)	16 (4)	
High	26 (36)	46 (64)	

*Curettage histological risk classification, low risk (benign, hyperplasia or endometrioid grades 1-2) or high risk (non-endometrioid or endometrioid grade 3).
**only endometrioid

Missing information on curettage histology classification for 19 patients, PR status in curettage for 24 patients, ERα status in curettage for 28 patients, grade for 9 patients, metastatic nodes for 310 patients, myometrial infiltration for 106 patients, ploidy for 745 patients and L1CAM status in hysterectomy specimen for 615 patients.

Table 2. Prediction of lymph node metastases based on curettage histology, status of L1CAM and ER/PR in curettage specimen in 763 lymph node sampled endometrial cancer patients.

Variable	N*	Uni-variate OR	95% CI	P	Multi-variate OR	95% CI	P	Sensi-tivity	Speci-ficity	PPV	NPV
Curettage histology**											
Low-risk	590	1			1						
High-risk	173	3.39	2.19-5.23	<0.001	1.94	1.16-3.25	0.011	0.49	0.75	0.22	0.91
L1CAM expression											
Low	681	1			1						
High	82	4.49	2.69-7.50	<0.001	2.51	1.41-4.64	0.002	0.28	0.92	0.33	0.90
ER/PR expression											
Normal	606	1			1						
Loss***	157	3.26	2.10-5.07	<0.001	1.91	1.15-3.17	0.013	0.42	0.82	0.26	0.90

* Only patients with data available for all variables included in the multivariate logistic regression analysis are included in the univariate analysis (N=763).

** Curettage histological classification, low risk (benign, hyperplasia or endometrioid grades 1-2) or high risk (non-endometrioid or endometrioid grade 3).

*** Patients with double loss of ER/PR expression.

Table 3. Clinico-pathological variables related to sL1CAM status in preoperative blood samples from women treated for endometrial cancer

Variable	sL1CAM Low n (%)	sL1CAM High n (%)	*P*-value
Age			<0.001
<66	165 (89)	20 (11)	
≥66	116 (62)	71 (38)	
Information available preoperatively			
Curettage histology*			<0.001
Low risk	217 (80)	54 (20)	
High risk	58 (62)	36 (38)	
PR curettage			0.027
Positive	160 (81)	37 (20)	
Negative	37 (67)	18 (33)	
ERα curettage			0.023
Positive	157 (81)	37 (19)	
Negative	38 (67)	19 (33)	
Information available postoperatively			
FIGO-09 stage			<0.001
I-II	252 (80)	61 (20)	
III-IV	29 (49)	30 (51)	
Histologic type			<0.001
Endometrioid	238 (80)	61 (20)	
Adenosquamous	4 (100)	0 (0)	
Clear cell	9 (75)	3 (25)	
Serous papillary	18 (55)	15 (45)	
Carcinosarcoma	10 (59)	7 (41)	
Undifferentiated/other	2 (29)	5 (71)	
Histologic grade**			0.95
Grade 1/2	192 (80)	48 (20)	
Grade 3	47 (80)	12 (20)	
Metastatic nodes			0.003
Negative	225 (83)	44 (17)	
Positive	22 (63)	13 (37)	
Myometrial infiltration			0.027
<50%	175 (80)	42 (20)	
≥50%	106 (71)	44 (29)	
Ploidy			0.15
Diploid	145 (78)	42 (22)	
Aneuploid	31 (67)	15 (33)	

* curettage histological risk classification, low risk (benign, hyperplasia or endometrioid grades 1-2) or high risk (non-endometrioid or endometrioid grade 3).

**only endometrioid

Missing information on curettage histology classification for 7 patients, PR status in curettage for 120 patients, ERα status in curettage for 121 patients, grade for 4 patients, metastatic nodes for 68 patients, myometrial infiltration for 5 patients and ploidy for 139 patients.

Table 4. Prediction of lymph node metastases based on curettage histology, and status for sL1CAM in preoperative blood samples in 299 lymph node sampled endometrial cancer patients.

Variable	N*	Univariate OR	95% CI	P	Multivariate OR	95% CI	P
Curettage histology**							
Low-risk	224	1			1		
High-risk	75	5.83	2.79-12.22	<0.001	5.21	2.46-11.06	<0.001
sL1CAM blood level							
Low	242	1			1		
High	57	2.96	1.38-6.31	0.005	2.25	1.01-5.02	0.048

* Only patients with data available for all variables included in the multivariate logistic regression analysis are included in the univariate analysis (N=299).

** Curettage histological risk classification, low risk (benign, hyperplasia or endometrioid grades 1-2) or high risk (non-endometrioid or endometrioid grade 3).