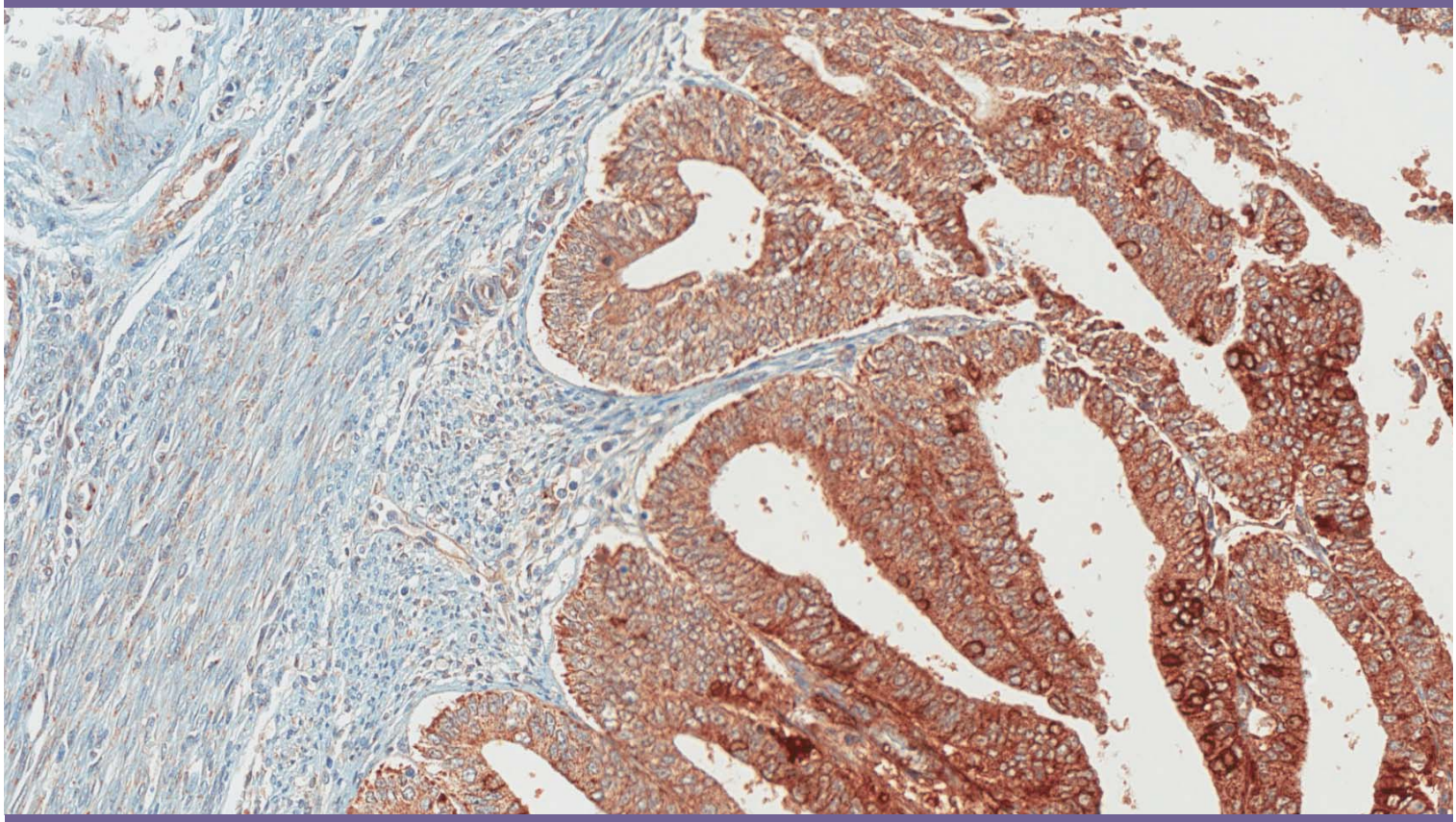
Universitat Autònoma de Barcelona

The role of activated leukocyte cell adhesion molecule (ALCAM) in endometrial cancer progression and dissemination

Laura Devis Jauregui



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Memoria presentada por

Laura Devis Jauregui

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Dr. Jaume Reventós (director) Dra. Eva Colás (directora) Dr. Antonio Gil (director)

Dra. Joaquima Navarro (tutora) Laura Devis Jauregui (estudiante)

A mi ama

A mi padre

A Angel

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Table of contents

Index of figures

Index of tables

Abbreviations & acronyms

ADAM17	ADAM metallopeptidase domain 17
AKT	V-Akt Murine Thymoma Viral Oncogene Homolog 1
ALCAM	Activated leukocyte cell adhesion molecule
ALCAMcytoless	ALCAM without its cytoplasmic tail
ANOVA	Analysis of variance
ANXA2	Annexin A2
ASR(W)	Age-world-standardized rate
AUC	Area under the curve
BDNF	Brain-derived neurotrophic factor
BM	Basal membrane
BMI-1	Polycomb group Ring finger protein 4
BRCA1	Breast cancer gene 1
BRCA2	Breast cancer gene 2
BSA	Bovine serum albumin
c-Met	Tyrosine-protein kinase Met, hepatocyte growth factor receptor
CAF	Cancer associated fibroblast
CAM	Cell adhesion molecule
CD6	Cluster of differentiation 6
CD9	Cluster of differentiation 9
CDH1	Cadherin 1, type 1, E-cadherin
CDH2	Neural cadherin, Cadherin 2, N-cadherin
CDK4/6	Cyclin-dependent kinase 4/6
CDKN2A	Cyclin-dependent kinase inhibitor 2A
cDNA	Complementary DNA
CEEA	Comité de Ética para la Experimentación Animal
Cherry (Ch)	mCherry fluorescent protein
CI	Confidence interval
CI5X	Cancer Incidence in Five Continents Volume X
cm	Centimetres
CMV	Cytomegalovirus
COX-2	Cyclooxygenase 2
CpG	Methyl-CpG-binding domain
CSR	Cumulative survival rate
Ct	Threshold cycle
CTNNB1	Catenin (cadherin-associated protein), beta 1 (β-catenin)
DAPI	4', 6-diamidino-2-phenylindole
ddCt	Delta-Delta-Ct
Dlg	Disc large
DMEM:F-12	Dulbecco's modified eagle medium: nutrient mixture F-12
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol

dTTP	Deoxythymidine triphosphate
EBRT	External beam radiation
EC	Endometrial carcinoma
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EEC	Endometrioid endometrial cancer
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EIN	Endometrial intraepithelial neoplasia
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial-to-mesenchymal transition
ER	Oestrogen receptor
ERM	Ezrin, Radixin, Moesin
ESGO	European Society of Gynaecologic Oncology
ESMO	European Society for Medical Oncology
ESTRO	European Society for Radiotherapy and Oncology
EtOH	Ethanol
Ets	Ets transcription factor family
ETV5	Ets variant 5
EUROCARE	EUROan CAncer REgistry based study on survival and care of cancer patients
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FBXW7	F-box/WD repeat-containing protein 7
FC	Fold change
FDR	False discovery rate
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FIGO	International Federation of Gynaecology and Obstetrics
FLNB	Filamin B
FYN	Tyrosine-protein kinase Fyn
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GEO	Gene Expression Omnibus
GFP	Green fluorescent protein
GLOBOCAN	GLOBOCAN 2012: Estimated Cancer Incidence, Mortality and Prevalence Worldwide
GSK3	Glycogen synthase kinase 3 beta
h	Hours
H&E	Haematoxylin and eosin
HBS	Hepes buffered saline
HEK 293T	Human embryonic kidney cells 293
Hep27	Dehydrogenase/reductase member 2
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER2	Human epidermal growth factor receptor 2
HGF	Hepatocyte growth factor
HNPCC	Hereditary non-polyposis colorectal cancer
HRP	Horseradish peroxidase

IARC	International Agency for Research on Cancer
ICAM-1	Intercellular adhesion molecule 1
ICAM-2	Intercellular adhesion molecule 2
IF	Immunofluorescence
Ig	Immunoglobulin
IGF-1	Insulin-like growth factor 1
IgSF	Immunoglobulin superfamily
IHC	Immunohistochemistry
IL-6	Interleukine 6
ILK	Integrin-linked kinase
IPA	Ingenuity pathway analysis
IRS	Immunoreactive scores
JNK	c-Jun N-terminal kinase
Ki67	Marker of proliferation ki67
KLF17	Kruppel like factor 17
KRAS	Kirsten rat sarcoma viral oncogene homolog LOXL2 Lysyl oxidase-like 2
L1CAM	L1 cell adhesion molecule
LAMC2	Laminin subunit gamma 2
LD	Low DNA
LH	Luteinizing hormone
LIV-1	Oestrogen-regulated protein LIV-1, Solute carrier family 39 member 6
LOH	Loss of heterozygosity
LPP	Lipoma-preferred partner
LVSI	Lymphovascular space invasion
mA	Milliamps
MAPK/ERK	Mitogen-activated protein kinase (ERK1/2)
MCAM	Melanoma cell adhesion molecule
MELF	Microcystic elongated and fragmented
µg	Micrograms
µl	Microliters
µm	Micrometres
mg	Milligrams
min	Minutes
ml	Millilitres
MLH1	MutL homolog 1
mM	Millimolar
mm	Millimetres
MMP-2	Matrix metalloproteinase 2
MMP-3	Matrix metalloproteinase 3
MMP-9	Matrix metalloproteinase 9
MMR	Mismatch repair system
MPA	Medroxyprogesterone acetate
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MSH2	MutS homolog 2

MSH6	MutS homolog 6
MSI	Microsatellite instability
MTA 3	Metastasis associated 1 family member 3
mTOR	Mammalian target of rapamycin
N.S	No statistically significant
NCAM	Neural cell adhesion molecule
NF-Kβ	Nuclear factor-kappa B
ng	Nanograms
NK4	Natural killer cells protein 4
nm	Nanometres
Notch	Notch receptor family
OR	Odds ratio
p-ERK	Phospho-p44/42 MAPK (Erk1/2)
p38	P38 mitogen-activated protein kinase
PBS	Phosphate‐buffered saline
PCA	Principal component analysis
PCOS	Polycystic ovary syndrome
PCR	Polymerase chain reaction
PCRD	Positive-charge-rich domain
PDGF	Platelet-derived growth factor
PEA3	Polyoma enhancer activator 3
PECAM-1	Platelet and endothelial cell adhesion molecule 1
PEG-PLL	Poly(ethylene glycol)-b-poly-L-lysine
PEI	Polyethylenimine
PFA	Paraformaldehyde
PI	Propidium iodide
PI3K	Phosphoinositide-3-kinase
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic
PIK3R1	Phosphatidylinositol 3-kinase regulatory subunit alpha
PKC	Protein kinase C
POLE	Polimerase ε
PPP2R1A	Serine/threonine-protein phosphatase 2A
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog
PVDF	Polyvinylidene difluoride
rcf	Relative centrifugal force
RIN	RNA integrity number
RIPA	Radioimmunoprecipitation assay buffer
RLP22	Receptor like protein 22
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
rpm	Revolution per minute
RT	Reverse transcription
RT-qPCR	Real-time quantitative PCR
sALCAM	Soluble ALCAM
SD	Standard deviation

SDF-1	Stromal derived factor 1
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEER	Surveillance, Epidemiology, and End Results. Program of the National Cancer Institute
SEGO	Spanish Society of Gynaecology and Obstetrics
SEM	Standard error of the mean
SF	Separation force
SHARP1	Basic helix-loop-helix family member e41
SLN	Sentinel lymph node
Smad2	Mothers against decantaplegic homolog 2
Smad3	Mothers against decantaplegic homolog 3
SNAIL	Snail family zinc finger
SPSS	Statistical Package for Social Science
SRCR	Scavenger receptor cysteine-rich domains
STAT-3	Signal transducer and activator transcription 3
TBS	Tris-buffered saline
TCGA	The Cancer Genome Atlas
TGF-β	Transforming growth factor beta
TMA	Tissue microarray
TMB	3,3',5,5' Tetramethylbenzidine
TNF-α	Tumour necrosis factor alpha
TP53	Tumour protein p53
TWIST	Twist basic helix - loop - helix transcription factor
TXNRD1	Thioredoxin reductase 1
UA	Uterine aspirate
UV	Ultraviolet
VCAM-1	Vascular cell adhesion molecule 1
VE-cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor
WB	Western blot
WHO	World Health Organization
WNT	Wingless-type MMTV integration site family
ZEB1	Zinc finger E-box binding homeobox 1
ZEB2	Zinc finger E-box binding homeobox 2

Chapter 1. Introduction

1.1 Endometrial cancer

1.1.1 Epidemiology

- **Incidence and mortality**

Endometrial cancer is the sixth most common cancer in women worldwide, the fourth in developed countries, and the third in Spain [1] (Table 1). Moreover, it is the most common gynaecologic malignancy of the female genital tract in western countries.

Based on the last Globocan data, the number of estimated cases in 2012 is 319,605 women in the world [2]. The estimated incidence ASR(W) (age-world-standardized rate) is 8.2/100,000 and the accumulated risk (0-74 years) is 0.97%. In Spain, the estimation is of 5,121 cases diagnosed in 2012 and the accumulated risk is 1.4%.

The geographic distribution of endometrial cancer in the world is considerably unequal (Figure 1). The highest incidence ASR(W) is registered in the more developed regions: 14.7 vs. 5.5 for the less developed regions. The accumulated risk in the more and less developed regions are 1.79% and 0.63%, respectively. The estimated incidence ASR(W) for the US is 19.5 and 13.9 for Europe.

World		Developed countries		Spain	
Breast	25.1	Breast	28.1	Breast	29.0
Colon	9.2	Colon	11.8	Colon	14.9
Lung	8.8	Lung	10.0	Corpus uteri	5.9
Cervix uteri	7.9	Corpus uteri	5.3	Lung	5.7
Stomach	4.8	Thyroid	4.3	Ovary	3.7
Corpus uteri	4.8	Stomach	3.3	Pancreas	3.5
Ovary	3.6	Non-Hodgkin lymphoma	3.3	Stomach	3.4
Thyroid	3.5	Pancreas	3.2	Non-Hodgkin lymphoma	3.2
Liver	3.4	Ovary	3.2	Melanoma	3.1
Others	28.9	Others	27.5	Others	27.7

Table 1. Ten leading cancers in women (type and percentage of diagnosed women) from Globocan 2012 (http://globocan.iarc.fr).

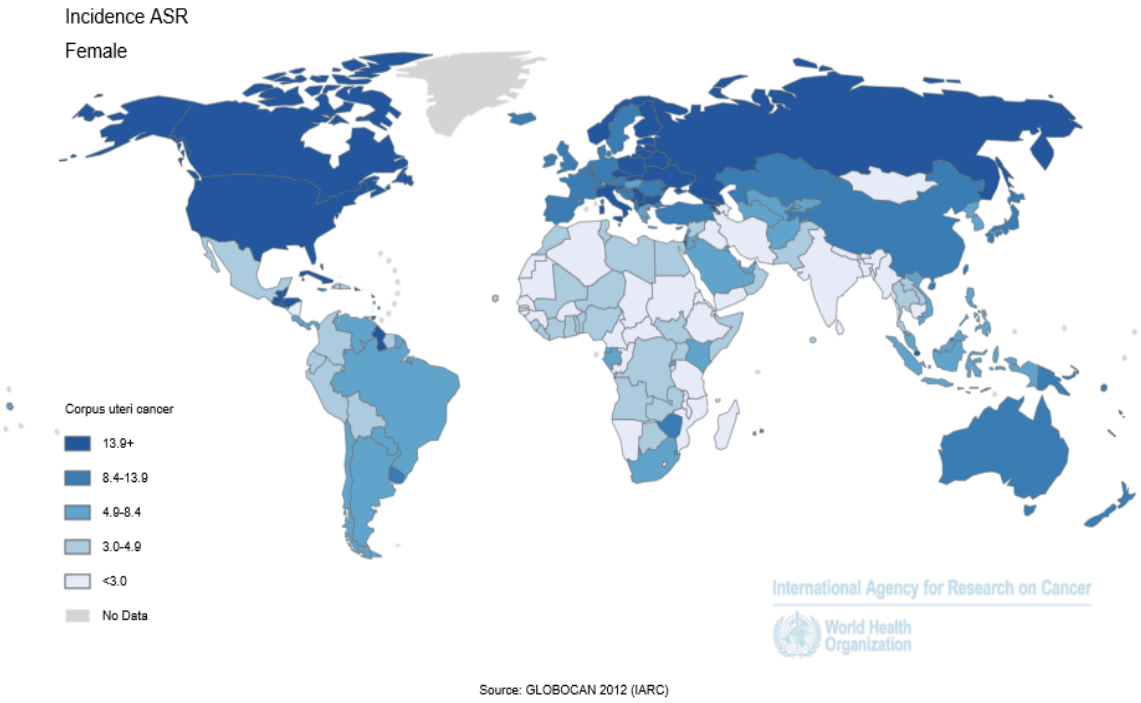


Figure 1. Estimated incidence ASR(W) of corpus uteri cancer per 100,000 person-year (from http://globocan.iarc.fr).

The latest available data (Cancer Incidence in Five Continents Volume X, CI5X, based on diagnoses made in the period 2003-2007), which allows comparing the structure of these ASR(W) incidence rates by age (Figure 2), shows very similar profiles between Spain, Europe and the US with a maximum incidence

reached at 65 years. However, we observed that the incidence for this age is slightly higher for Europe and the US compared to Spain.

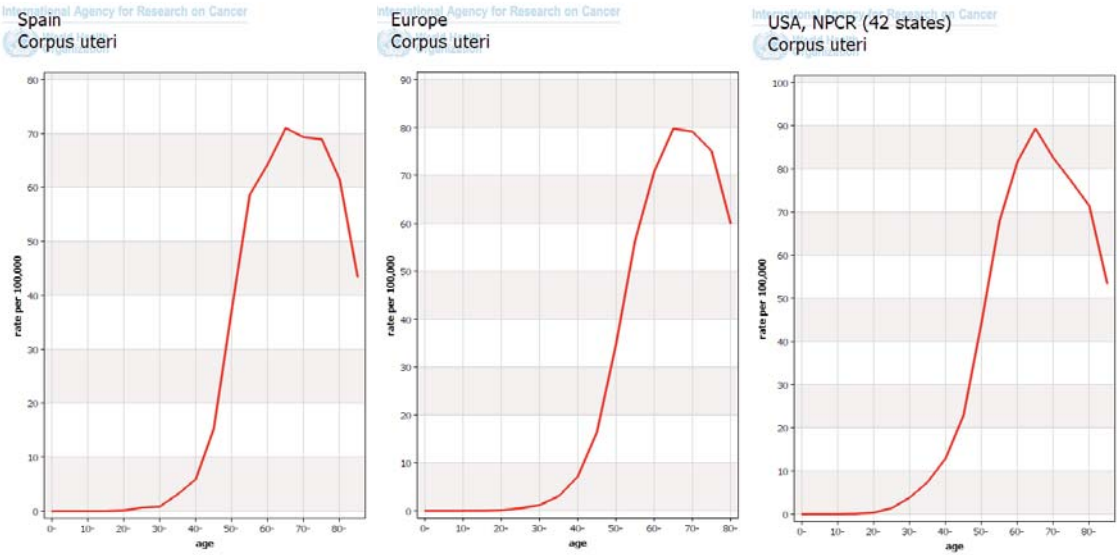


Figure 2. Incidence ASR(W) in Spain, Europe and USA by age from IARC.

In the last few years, the incidence of endometrial cancer has increased, presumably related to different reasons such as increased life expectancy of the population, increased percentage of obese women and associated pathologies like diabetes and hypertension [3]. Although endometrial carcinoma presents a high incidence, this does not translate into high mortality rates (Figure 3). In fact, although endometrial cancer is the third most prevalent cancer, it is the ninth cause of death related to cancer in Spain, with a significant increase in hospital morbidity. The mortality ASR(W) for Spain is 1.9 and its 5-year prevalence is 95.5 per 100,000.

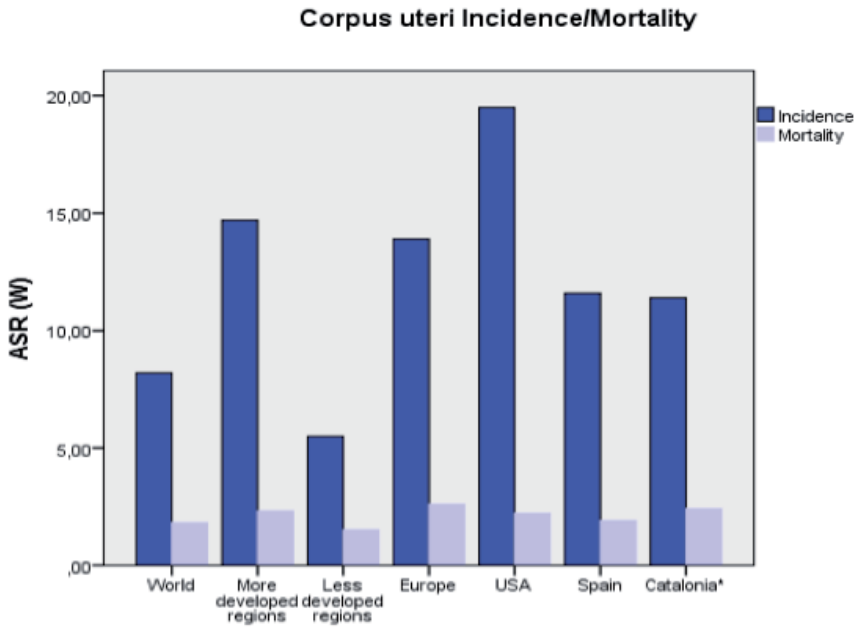


Figure 3. Incidence/Mortality ASR(W) per 100,000 (from http://globocan.iarc.fr).

In addition, as represented in the trend graph from 1980-2012, we observe a generalized increase in mortality in the cohorts over 65 years (Figure 4). This could be explained by the increased life expectancy of the Spanish population.

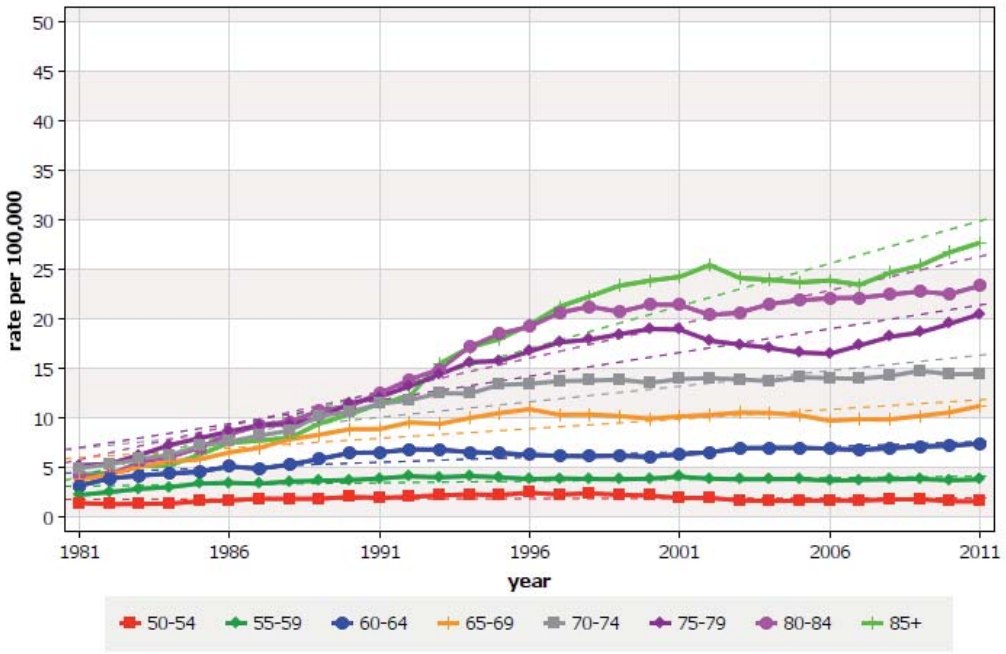


Figure 4. Evolution of mortality in Spain by age cohort and its trends from the IARC.

- **Survival**

The survival of patients with cancer is the main indicator of the effectiveness of the healthcare system. It is presented as the proportion of cases surviving 1-, 3- and 5-years from the time of diagnosis. The 1- and 5-year survival rates for endometrial cancer in Spain are 89.00% and 74.43%, respectively (data obtained from the European Cancer Registry Based Study on Survival and Care-EUROCARE 5 for the period 2000-2007) [4]. These survival rates are slightly below the European average (90.45% and 76.19%, respectively) (Table 2). Compared to the previous EUROCARE 4, for the period 1995-1999, the rate has increased by 1.30%.

Population	1-year	3-year	5-year
Northern Europe	93.08 %	85.97 %	83.16 %
Spain	89.00%	78.43 %	74.43 %
Southern Europe	89.98 %	79.56 %	75.34 %
European average	90.45 %	80.46 %	76.19 %
USA	-	-	81.8%*
*USA (SEER: 2006-2012)			

Table 2. Cumulative survival rate (CSR) from EUROCARE 5.

Survival is strongly influenced by the stage of the cancer at the time of diagnosis and the effectiveness of therapeutic procedures. Specific studies from data of the US shows that around 67% of cases are diagnosed when the tumour is still localized, 21% at regional stage and 8% at distant stage [1]. Depending on whether the cancer is diagnosed at local, regional, or distant stages, the 5-year survival rates are 95%, 68%, or 17%, respectively (Figure 5).

As seen in the graphs, endometrial cancer may be associated with race. The relative survival for Caucasians exceeds that of African Americans at every stage of diagnosis.

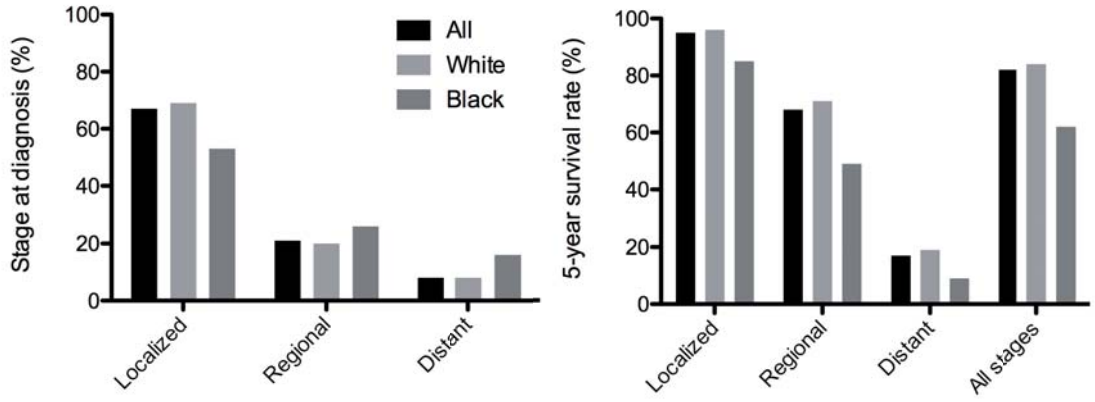


Figure 5. Endometrial cancer: stage at diagnosis and 5-year survival rate by race. On the left, distribution of endometrial cancer by race and stage at diagnosis (USA 2005-2011). On the right, 5-year survival rates among patients diagnosed with endometrial cancer by race and stage at diagnosis (USA 2005-2011), from Siegel et al. [1].

1.1.2 Risk factors

Although the aetiology of endometrial cancer is not clear, several risk factors have been identified: the association with long-term exposure to endogenous or exogenous oestrogen, obesity, hypertension, diabetes mellitus, some granulosa cell oestrogen-secreting tumours and genetic factors [5–7].

1.1.2.1 Long-term unopposed endogenous and exogenous oestrogen exposure

Endometrial cancer, type I (section 1.1.6.1), has been associated with an excess of oestrogen exposure. In fact, prolonged exposure to oestrogen (specially unopposed by progesterone) promotes uncontrolled cell proliferation of the endometrium and an increase in its thickness. In addition, it also inhibits apoptosis through a downstream cascade of transcriptional changes that include the modulation of tumour suppressor functions. Moreover, uncontrolled processes of cell determination and differentiation increase the risk of random mutations, DNA replication errors and as consequence an increase in the possibility of cancer development. Then, all these changes might lead to endometrial hyperplasia, which is known to be the precursor lesion of endometrial cancer (see section 1.1.5.1) (Figure 6) [6].

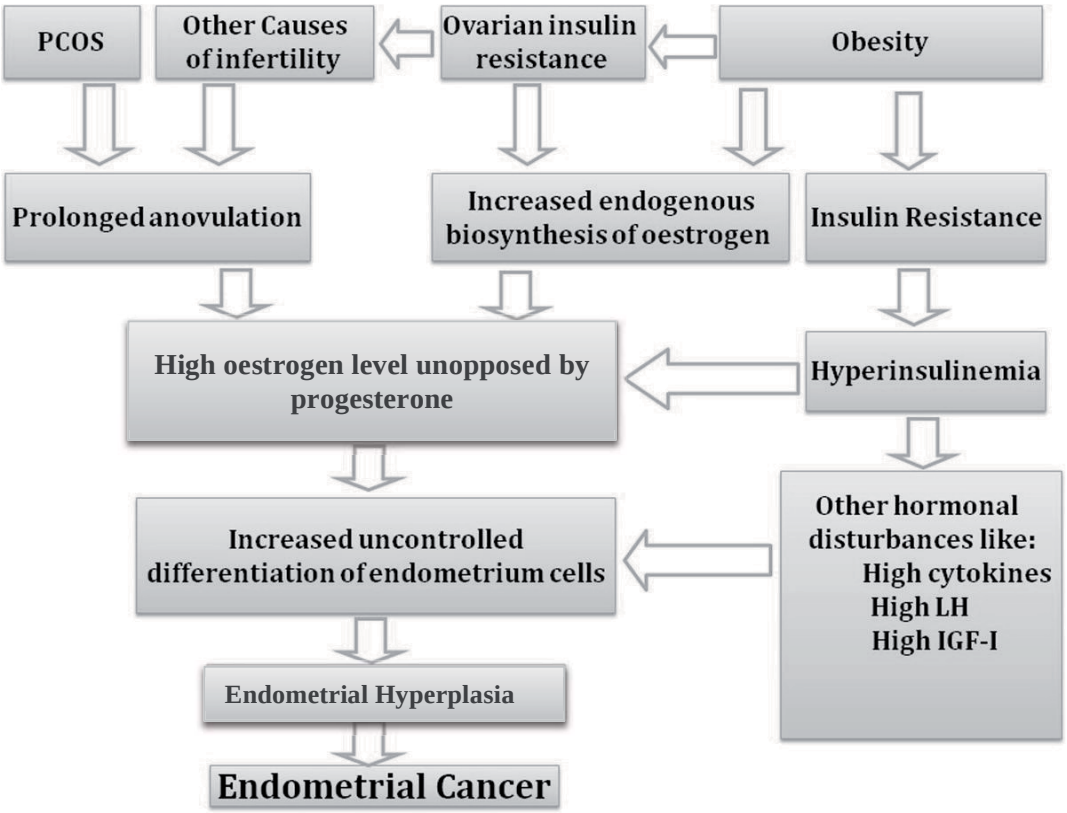


Figure 6. Proposed model for endometrial cancer type I, adapted from Ali AT. et al. [6].

A) Endogenous oestrogen exposure

The most well known conditions related to hyperoestrogenism and hence, to an elevated risk of endometrial carcinoma, are age, early menarche, late menopause, infertility, nulliparity and chronic anovulation, since all of these situations increase the lifetime exposure to oestrogen. These risk factors are briefly described below.

- **Age**

Endometrial cancer affects especially women older than 50 years, in more than 90% of cases. The mean age of detection is comprised between 62.6 and 68.7 years [8,9] presenting a maximum at 65 years. Only around 3-5% of the cases are presented in women <40 years. In fact, oestrogens have a larger effect after the menopause, since the compensatory levels of progesterone produced by the ovaries before the menopause have disappeared leading to an unopposed hyperoestrogenism.

- **Menarche and menopause**

Early menarche and late menopause are risk factors for the development of endometrial carcinoma [10–17]. Early menarche is associated with an earlier onset of ovulatory cycles and exposure to oestrogens. If this event is also accompanied by late age at menopause, the time of oestrogen exposure will be even longer because of the increased number of menstrual cycles [12].

- **Infertility and nulliparity**

Infertility is one of the main causes of endometrial cancer in women <40 years [16]. This is due to irregular menstrual periods or infrequent ovulation and chronic anovulation [17], both processes associated with increased oestrogen production and progesterone deficiency [6].

Also, nulliparity is associated with 2- to 3-fold increase in the risk of endometrial cancer due to a higher number of ovulatory menstrual cycles with absence of pregnancy and lactation [11].

- **Polycystic ovary syndrome (PCOS)**

Polycystic ovary syndrome (PCOS) is the most common ovulatory disorder that may cause chronic infertility when not treated. PCOS is characterized by a deficiency in progesterone levels that leads to the appearance of irregular menstrual cycles or even anovulation. Women diagnosed with PCOS have 3 times more risk of developing endometrial cancer [18–20]. This is due to a prolonged anovulation and consequential release of oestrogens. Unopposed oestrogen may enhance the development and growth of endometrial cancer, especially in young women.

B) Exogenous oestrogen exposure

- **Oestrogen therapy**

Oestrogen therapy is the use of oestrogen to balance the symptoms of the menopause [21]. The use of oestrogen alone increases the risk of endometrial cancer by 5-fold as it prolongs the exposure to oestrogen by delaying the age of the menopause. It has also been associated to an increase in the incidence of hyperplasia from 20-50% after one year of therapy without progesterone [22–24].

The risk is related to the dose and the duration of the exposure to oestrogen and even continues to be higher when women no longer use oestrogen.

- **Hormone replacement therapy**

Once the effects of using oestrogen alone were evidenced by an increase in the incidence of endometrial cancer, hormone replacement therapy was used as a substitute. This therapy consists of a combination of oestrogen and progestin, the last one used to attenuate the risk of unopposed oestrogen exposure [21,25]. The results concerning the study of the associated risk of hormone replacement therapy are still controversial. Although most studies have shown an increase in the risk of endometrial cancer, some have found a decrease in the risk, or even no reported association [11,26–28].

- **Tamoxifen**

Tamoxifen is the hormonal anti-oestrogen therapy used in pre-menopausal women with oestrogen-receptor-positive breast cancer, and a standard treatment in post-menopausal women with breast cancer. It is a selective oestrogen receptor modulator that, while presenting antagonistic effects in specific tissues, like for the breast, it presents agonistic effects in others tissues, including the uterus. The endometrial activity of tamoxifen appears to depend upon menopausal status [29]. The increased risk of developing endometrial cancer with the use of tamoxifen in postmenopausal women is well established.

1.1.2.2 Obesity

The association between obesity and the incidence of endometrial cancer has been demonstrated [30,31]. Obesity is one of the major contributors to the increasing occurrence of endometrial carcinoma in western countries [32]. Obese postmenopausal women have the propensity for a chronic oestrogenic stimulation that is not counterbalanced and this could lead to endometrial hyperplasia and endometrial carcinoma. Large-scale conversion of adrenal precursors into oestrone and oestradiol by the adipose tissues in women with obesity are the main reasons for excessive endogenous oestrogen levels [7]. Androgens produced by the adrenal cortex and postmenopausal ovaries are converted into oestrogens by aromatase enzymes that are also found in

adipose tissue [33]. In addition, increased fat accumulation has been associated with high levels of cytokines TNF-α, leading to stimulation of *de novo* synthesis of oestrogen [21]. Obesity is also associated with increased levels of insulin and insulin-like growth factor-1 (IGF-1). Both of them are ligands of the PI3K signalling pathway and could lead to the activation of the pathway, and as consequence, stimulating processes like cell proliferation and survival [31,34].

1.1.2.3 Diabetes and hypertension

Though the exact causes are not still well understood, the association of the incidence of endometrial cancer with hypertension and diabetes has been described [31,35].

1.1.2.4 Genetic factors

The hereditary component only represents approximately 5% to 10% of all reported endometrial cancer cases. Patients with inherited diseases such as Lynch II syndrome, Cowden syndrome or Peutz-Jeghers syndrome and *BRCA* mutation present an increased risk of developing endometrial cancer.

- **Lynch II syndrome**

Lynch II syndrome or hereditary non-polyposis colorectal cancer (HNPCC), is an autosomal dominant disease caused by pathogenic germ line mutations in DNA mismatch repair (MMR) genes [36]. Endometrial cancer represents the second most common cancer in families diagnosed with HNPCC. In addition to an increased risk of suffering endometrial cancer, these patients also present an increased risk of developing ovarian, colorectal and gastric cancers [37].

- **Cowden syndrome and Peutz-Jeghers syndrome**

Cowden syndrome is an autosomal dominant inherited disease, and part of the *PTEN* hamartoma tumour syndrome. Cowden syndrome patients have increased risk of both benign and cancerous tumours of breast, thyroid, colorectal, kidney, skin, and endometrium [38]. The cancer lifetime risk for endometrial cancer is 5-10% [39].

- ***BRCA***

Women with mutated breast cancer genes, *BRCA1* or *BRCA2*, have up to an 87% risk of developing breast cancer by age 70 and also a high risk of developing ovarian cancer. However, the link between having a *BRCA1* or *BRCA2* mutation and a higher risk of developing uterine cancer is not clear. A recent study suggests that women presenting a *BRCA1* mutation have a slightly higher risk of developing serous or serous-like endometrial cancer [40].

1.1.3 Protective factors

In contrast to the risk factors explained before, lower levels of oestrogen exposure are related to a decreased incidence of endometrial cancer [41]. Among the factors related to the lower levels of oestrogen exposure are, women with delayed menarche, with a high number of children and/or longer period of lactation. In addition, the use of oral contraceptives also decreases the risk of developing endometrial cancer [21,42]. In all aforementioned conditions, the levels of progesterone are increased resulting in the thinning and atrophy of the uterine glands.

Although smoking is clearly related to many adverse effects, it has been associated to a decrease in the risk of developing endometrial cancer [43,44]. Smoking presents an anti-oestrogenic effect, maybe involved in the absorption and metabolism of hormones, and it is also related with the loss of weight and earlier menopause.

Lastly, some practices such as physical activity also provide protection against endometrial cancer [45].

1.1.4 Diagnosis

1.1.4.1 Screening recommendations based on the assessed risk for endometrial cancer

- **Women at average and increased risk for endometrial cancer**

Women at average and increased risk for endometrial cancer, due to the use of unopposed oestrogen therapy, late menopause, undergoing tamoxifen

treatment, nulliparity, infertility, obesity, diabetes or hypertension, should be informed of the risks and symptoms of endometrial cancer and strongly advised to inform gynaecologists of any associated symptoms in order to diagnose the malignancy in an early stage and to receive the appropriate treatment [46]. There is no indication for a general population screening, as it does not present advantages in the early detection of endometrial cancer or reduction in mortality. The histological study will only be performed after presenting any symptomatology [3,47]. In fact, some studies lead to the conclusion that a general screening in asymptomatic women will increase the number of unnecessary biopsies because of false-positive test results, due to anxiety or complications from the biopsies [47,48].

- **Women at high risk for endometrial cancer**

Women diagnosed with HNPCC, women with a family history of the mutation, and women without genetic testing results but from families with a suspected autosomal dominant predisposition to colon cancer, are considered at high risk for endometrial cancer. For these women, an annual screening from 35 years of age is recommended. Due to the limited efficacy of screening, when women have no desire of having more children, the option of prophylactic hysterectomy and bilateral salpingo-oophorectomy should be considered [8,49].

1.1.4.2 Suspected diagnosis: clinical examination

- **Signs and symptomatology**

The most common early symptom of endometrial cancer is abnormal vaginal bleeding. The blood originates in the uterine cavity, where the tumour is located, and drains out of the vagina [8]. In fact, abnormal vaginal bleeding is present in around 90% of endometrial cancer patients. Women in the pre- and perimenopausal periods could experience irregular bleeding [50] due to hormonal changes [51] but when the bleeding occurs in postmenopausal women it should always be treated as a sign that deserves an evaluation by the clinician [52]. The probability of cancer in postmenopausal women which present irregular vaginal bleeding is comprised between 5-10%, the overall risk increases when women get older and with risk factors [53].

Some frequently reported symptoms of endometrial cancer are: lower abdominal pain or pelvic cramping, thin white or clear vaginal discharge in postmenopausal women, changes in bowel or bladder functions, anaemia and shortness of breath. However, most of them have been related to a more advanced stage of the disease [54] .

- **Pelvic examination**

During a pelvic examination, the gynaecologist inspects the vulva for irritations, lesions or abnormal vaginal discharge. Palpation of the vulva is performed and an examination of the internal organs is undertaken in order to evaluate if they are enlarged or tender. The use of a speculum into the vagina allows an examination of the cervix and the vaginal walls. In general, the results of a pelvic examination are normal in the size, shape and consistency of the uterus, until the disease is in an advanced stage.

- **Transvaginal ultrasound**

Transvaginal ultrasound is the diagnostic imaging technique of choice for the evaluation of the endometrium in patients presenting abnormal vaginal bleeding [3]. This technique leads the clinicians to discard others pathologies like myomas, polyps and also to evaluate the thickness of the endometrium. The use of ultrasound in premenopausal women presents increased difficulty because of the changes in the thickness of the endometrial wall due to cyclic hormonal variations. Transvaginal ultrasound normality is set to a cut-off of <4-5 mm, including both endometrial layers [55,56]. In a recent meta-analysis, the authors found a diagnostic accuracy characterized by a sensitivity of 95% and 98% with a specificity of 47% and 35%, respectively, at a cut-off of ≤4 mm and ≤3 mm. They reported that the use of transvaginal ultrasound is justified, and recommend decreasing the cut-off to ≤3 mm [57]. Although it presents a high sensitivity, a final definitive diagnosis will usually require endometrial sampling (Section 1.1.4.3).

Moreover, transvaginal ultrasound has been described as a potential tool to determine pre-operatively, myometrial infiltration (sensitivity 62-78%, specificity 81-94%) and cervical stroma infiltration (sensitivity 77-86%, specificity 85-99%) [58–61].

1.1.4.3 Confirmatory diagnosis: pathological examination

When there is a suspicion of endometrial cancer, the gold standard diagnosis is pathological examination of an endometrial biopsy (Figure 7), i.e. a sample of the endometrium is collected and analysed microscopically by the pathologist.

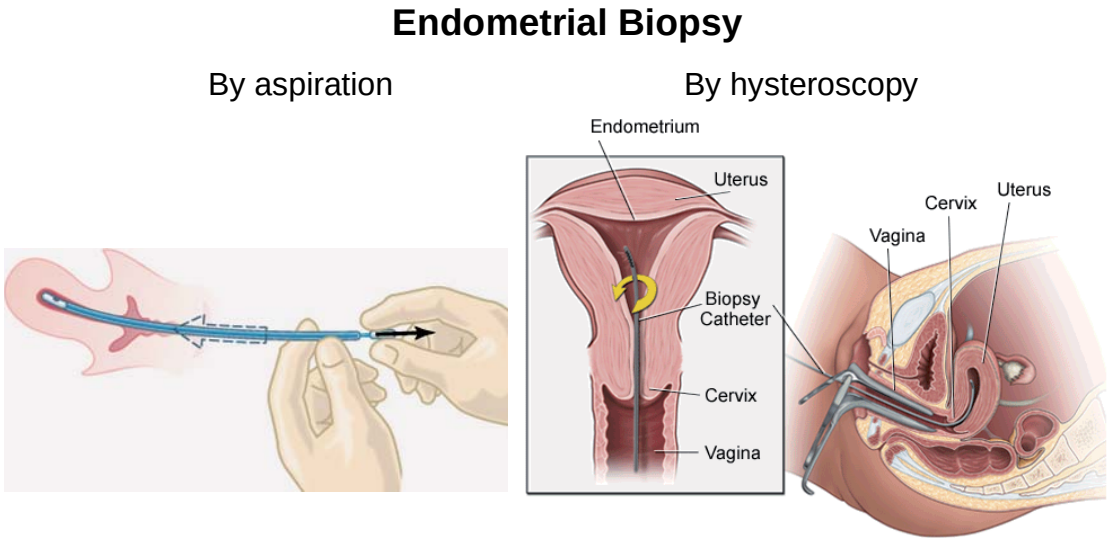


Figure 7. Biopsy by aspiration and by hysteroscopy.

Endometrial biopsies can be performed by aspiration, with a straw-like device (pipelle) that suctions in the uterine cavity, or by the use of a hysteroscope and a catheter. The hysteroscope is placed in the vagina to enable the visualization of the uterine cavity, and the catheter is introduced through the cervical opening to collect small pieces of selected endometrial tissue.

When biopsy by aspiration is not suitable for the patient, due to cervical stenosis or discomfort, or when the result from its analysis is not conclusive, a biopsy by hysteroscopy should be done [3,62]. Although both techniques are excellent diagnostic tools, the biopsy by aspiration presents some advantages (Table 3). The Spanish Society of Gynaecology and Obstetrics (SEGO) consider the endometrial biopsy by aspiration as the first method of choice for diagnosis [3].

Biopsy by aspiration	Biopsy by hysteroscopy
Less expensive	More expensive
Less painful	More invasive
Performed as an office procedure	Performed at the hospital
No anaesthesia	Previous anaesthesia
Faster	Previous blood testing required
No dissemination of endometrial cancer cells	Increased risk of dissemination of endometrial cancer cells in the peritoneal cavity [63]

Table 3. Features of the methods used for obtaining endometrial biopsies.

1.1.5 Endometrial preneoplastic lesions

1.1.5.1 Endometrial hyperplasia

Endometrial hyperplasia is defined as an increase in the gland to stroma ratio, greater than 1:1. Although the exact pathogenesis of the hyperplasia is not clear, this lesion is thought to result from excessive or unopposed oestrogen stimulation. However, it has also been described as an abnormal response by the endometrial glands to normal levels of oestrogen in some women [64].

The most commonly used classification system for endometrial hyperplasia is the World Health Organization (WHO) system, which is based on the architectural pattern of the endometrial glands and the presence or absence of cytologic atypia [65]. This classification leads to four possible categories: simple hyperplasia without atypia, complex hyperplasia without atypia, simple hyperplasia with atypia and complex hyperplasia with atypia [66] (Figure 8):

- Endometrial hyperplasia (simple or complex): irregularity and cystic dilatation of glands (simple) or crowding and budding of glands (complex) without atypia.
- Endometrial hyperplasia with atypia (simple or complex): simple or complex architectural pattern of endometrial glands, with atypical changes including cell stratification, tufting, loss of nuclear polarity, enlarged nuclei, and an increase in mitotic activity.

Kurman et al. found that, without treatment for a mean of 13 years, lesions with different degrees of complexity and atypia progressed to adenocarcinoma. Simple hyperplasia was associated with a 1% rate of progression to endometrial

cancer, complex hyperplasia was associated with a 3% rate, simple hyperplasia with atypia was associated with an 8% rate, and complex hyperplasia with atypia was the most significantly associated with cancer progression reaching a rate of 29% [67].

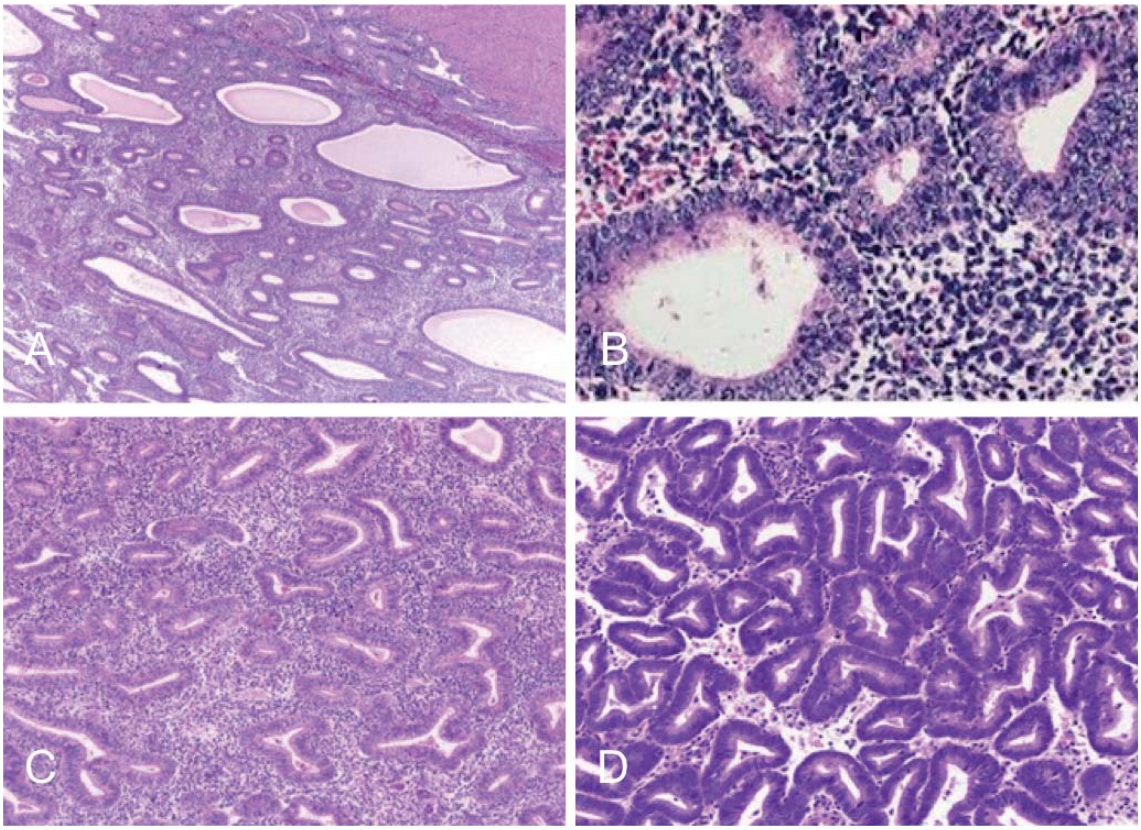


Figure 8. Endometrial hyperplasia (A) simple without atypia, (B) simple with atypia, (C) complex without atypia and (D) complex with atypia.

Despite the WHO classification having a good correlation in general with the risk of progression to cancer, this system presents limitations in the variability and the reproducibility of the diagnosis amongst specialized pathologists [68,69]. This is why an alternative classification, proposed in origin by the International Endometrial Collaborative Group, divides the lesion into benign hyperplasia and endometrial intraepithelial neoplasia (EIN) [64].

EIN is a premalignant clonal glandular proliferation with strict histologically defined criteria and improved prognostic value. By using computerized morphometric analysis, the stromal volume can be measured: epithelial crowding in precancers displaces stroma to a point at which the stromal volume is less than approximately half of the total tissue volume (stroma epithelium

gland lumen) [70,71]. Women with EIN who remain cancer free for the first year after diagnosis, have a 45-fold increased risk of eventual progression to endometrial cancer [72].

Although 85% of EIN lesions would be diagnosed as atypical hyperplasia in the WHO classification system, this means that the other 15% of EIN without atypia would not be properly classified at high risk of progression according to this classification [64]. This fact highlights the evident limitation of the WHO classification system.

1.1.6 Classification

Endometrial cancer is composed of biologically and histologically by diverse neoplasms with different pathogeneses. In order to include this heterogeneity on current classification systems, the clinicians estimate the histological type and grade of the tumours, as well as the anatomical site and the clinical and pathological extent of the disease.

1.1.6.1 Dualistic classification

In 1983, Bokhman et al. described a dualistic model of endometrial cancer [73]. This classification has been used to categorize this malignancy up to present. Based on clinical, pathological and molecular features, two main categories of endometrial carcinoma have been described: type I (endometrioid) and type II (non-endometrioid). This section will only address the clinical and pathological aspects. The molecular features of each type will be explained in section 1.1.7.1.

Type I or endometrioid adenocarcinomas represent around 80-90% of endometrial carcinomas [46]. They normally express oestrogen and progesterone receptors and are associated with excessive exposure to oestrogen. They occur in pre- or perimenopausal women and are usually preceded by endometrial hyperplasia with or without atypia [65]. They are usually well-differentiated tumours (low-grade) and are composed of glands that resemble in major measure the normal endometrium. Rare mucinous adenocarcinomas are also

considered type I carcinomas, since they usually express oestrogen and progesterone receptors and they are also basically low-grading tumours [74].

Type II or non-endometrioid carcinomas only represent around 10-20%. The most common non-endometrioid cancer is the serous carcinoma, followed by the clear cell carcinoma. They are high-grade tumours and present a significantly worse prognosis. By contrast with type I tumours, type II are hormone-independent tumours and related to EIN precursor lesions. When diagnosed, around 20% of patients present myometrial invasion and/or lymph node involvement, the main indicators associated with a poor prognosis and a decrease in the survival rate.

A summary of the principal characteristics of endometrioid and non-endometrioid tumours are listed in Table 4.

Features	**Type I**	**Type II**
Age	Pre-perimenopausal	Postmenopausal
Specific subtypes	Endometrioid-Mucinous	Serous-Clear cell
Prevalence	80-90% cases	10-20% cases
Diagnosis	Diagnosis in early stage	Diagnosis in advanced stage
Grade	Low grade	High grade
Oestrogen exposure	Associated with unopposed oestrogen exposure	Not associated with oestrogen exposure
Precursor Hyperplasia	Present	Absent
Hormone dependence	Hormone dependent	Hormone independent
Evolution	Slow evolution	Aggressive evolution
Prognosis	Better prognosis	Worse prognosis

Table 4. Clinicopathological classification of endometrial cancer.

1.1.6.2 Histological classification

The current classification of endometrial adenocarcinomas by the International Society of Gynaecological Pathologists and the WHO [65] divides endometrial cancers based on their histology and the features of the individual cancer cells (Table 5).

Histologic types
Endometrioid adenocarcinoma
Special variants:
- Variant with squamous differentiation
- Villoglandular variant
- Secretory variant
- Ciliated cell variant
Mucinous adenocarcinoma
Serous adenocarcinoma
Clear cell adenocarcinoma
Mixed adenocarcinoma
Squamous carcinoma
Transitional cell carcinoma
Small cell carcinoma
Undifferentiated carcinoma

Table 5. Histological types of epithelial endometrial carcinoma.

- **<u>Endometrioid adenocarcinoma</u>**

Endometrioid adenocarcinoma is the most common endometrial cancer (80-85%). It has been defined as a primary endometrial adenocarcinoma containing glands with resemblance to the normal endometrium glands (Figure 9).

Endometrioid adenocarcinoma is characterized by a diverse spectrum of histological differentiation going from a very well-differentiated carcinoma that resembles a complex hyperplasia with atypia to a poorly-differentiated carcinoma that can even be compared to an undifferentiated carcinoma [65]. In fact, they are graded based on the amount of solid growth of the glandular component, adjusted by nuclear features [75].

- Histologic Grade 1: adenocarcinoma with easily recognizable glandular pattern (< 5% solid growth).
- Histologic Grade 2: well-formed glands with interspersed solid sheets of neoplastic cells (<50% solid growth).
- Histologic Grade 3: solid sheets of cells with hardly recognizable glands, presenting nuclear atypia and higher mitotic activity (> 50% solid growth).

Severe nuclear atypia raises the grade by one.

A common feature of the endometrioid adenocarcinoma is the presence of glandular or villoglandular structures, lined by simple to pseudostratified columnar cells with their axes perpendicular to the basement membrane and slightly elongated nuclei polarized in the same direction.

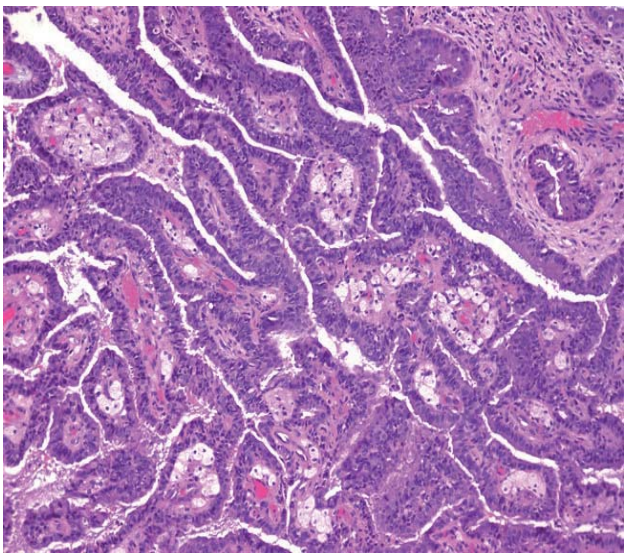


Figure 9. Endometrioid adenocarcinoma of the endometrium, grade 1.

This histology presents some variants comprising: adenocarcinomas with squamous, secretory or ciliated differentiation.

- **Mucinous adenocarcinoma**

Mucinous adenocarcinoma represents the 0.6-5% of endometrial cancer cases. It is characterized by the presence of intracytoplasmic mucin (Figure 10). It has been observed that both endometrioid and clear cell adenocarcinomas may have large amounts of intraluminal mucin, but the mucinous adenocarcinoma is the only one that contains mucin within the cytoplasm [65].

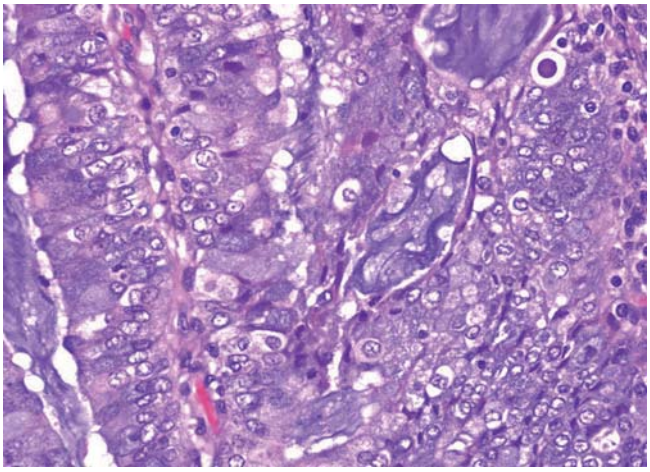


Figure 10. Mucinous adenocarcinoma containing mucin in the cytoplasm.

Mucinous adenocarcinoma presents some variants, presenting a microglandular pattern that could be confused with a microglandular hyperplasia [76,77]. These neoplasms have been reported as microglandular carcinomas. Rare intestinal differentiation can be observed in mucinous adenocarcinomas, containing goblet cells [65].

Mucinous adenocarcinomas are graded like endometrioid adenocarcinomas, but they are usually grade 1.

- **Serous adenocarcinoma**

Serous carcinoma is the major type II or non-endometrioid carcinoma (5-10%). It has been described as a primary adenocarcinoma of the endometrium, which comprises a complex pattern of papillae with cellular budding and could contain psammoma bodies.

The papillae usually have fibrovascular cores, secondary and tertiary papillary processes and sloughing of the cells [78–80] (Figure 11). The nuclei are generally rounded and non-perpendicular to the basement membrane. The nuclei present poor differentiation and are more often apically localized. They usually present eosinophilic macronucleoli, solid cell nests and foci of necrosis. Psammoma bodies are found in about 30% of cases and can be found in high quantity. When the tumour grows in a glandular pattern, the glands are generally complex and "labyrinthine".

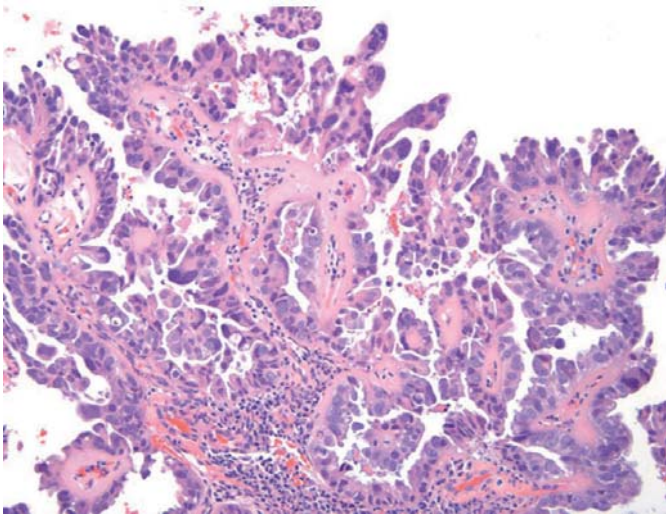


Figure 11. Serous adenocarcinoma. Papillae covered by cuboid and cylindrical cells with pleomorphic nucleus.

Serous carcinoma could come from an endometrial intraepithelial neoplasia [81,82]. In contrast with endometrioid adenocarcinomas, serous carcinoma is a high-grade carcinoma and is not graded [83]. Moreover, it has been observed in older patients, frequently diagnosed at advanced stages and highly related to recurrence and a poor outcome.

- **Clear cell adenocarcinoma**

Clear cell adenocarcinoma is the second most common type II or non-endometrioid carcinoma (1-4%).

Clear cell adenocarcinoma is composed of clear or hobnail cells arranged in solid, tubulocystic or papillary patterns or a combination of both. Histologically, it is composed of clear, glycogen-filled cells and hobnail cells that project individually into lumens and papillary spaces (Figure 12). It contains large, highly pleomorphic nuclei and multinucleated forms. The architectural growth pattern could be tubular, papillary, tubulocystic or solid and more frequently a mixture of them. Occasionally the neoplastic cells present granular eosinophilic cytoplasm [65,84].

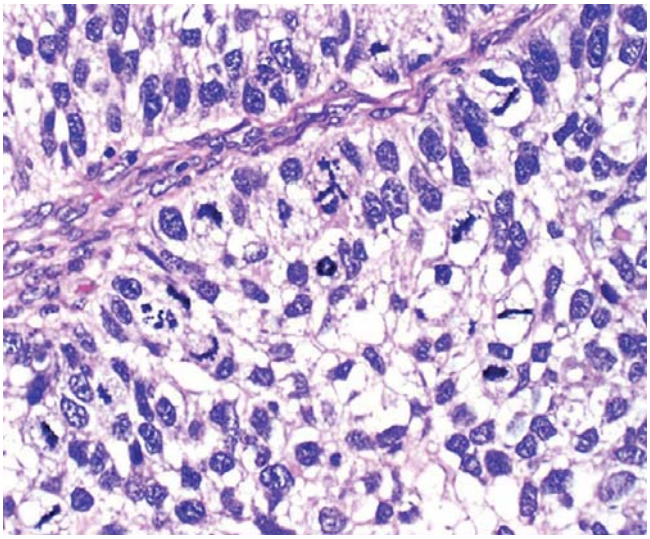


Figure 12. Clear cell adenocarcinoma.

Like serous carcinoma, it is present in older patient populations, diagnosed at advanced stages and not graded.

- **Mixed cell adenocarcinoma**

Mixed adenocarcinoma (Figure 13) is composed of both type I and type II carcinomas in which the less represented type comprises at least 10% of the tumour volume. The pathologist will report the percentage of the minor component and it has been found that ≥25% of a type II tumour suggests a worse prognosis [83].

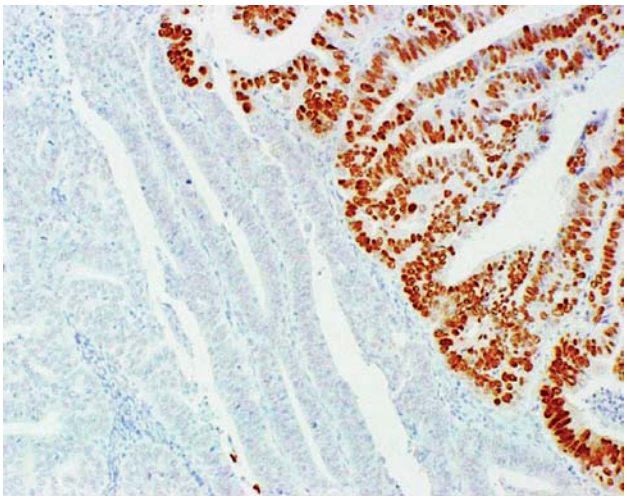


Figure 13. Mixed carcinoma of endometrioid (left) and serous (right) carcinomas. Only the serous component presents positive p53 nuclear staining.

- **Squamous cell carcinoma**

Squamous cell carcinoma is composed of squamous cells of varying degrees of differentiation [65] (Figure 14). It occurs in postmenopausal women and it is associated with cervical stenosis and pyometra, however it is really uncommon (0.1-0.5%).

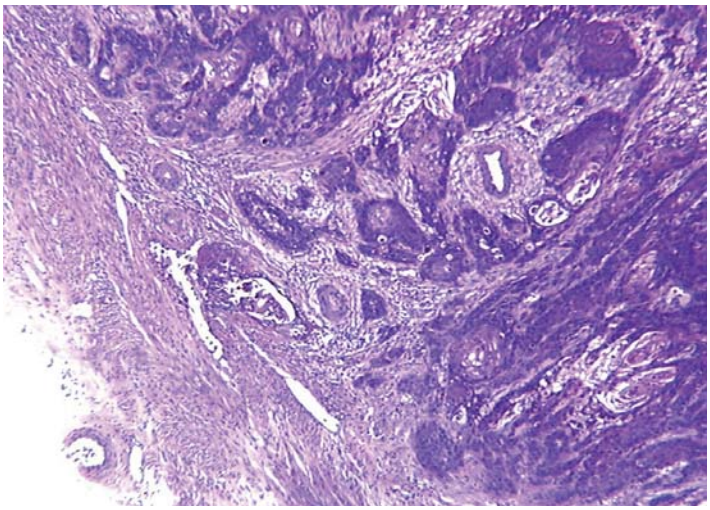


Figure 14. Squamous cell carcinoma. Atypical squamous cells involving the myometrium.

Its appearance is essentially identical to squamous cell carcinoma of the cervix, including a rare verrucous variant [85,86]. The prognosis of this endometrial cancer is really poor, whereas the verrucous variant is more favourable.

- **Transitional cell carcinoma**

Transitional cell carcinoma is an extremely rare endometrial carcinoma. It is composed of at least 90% of cells resembling urothelial transitional cells (Figure 15). If the percentage of transitional cells is smaller, the tumour would be diagnosed as mixed carcinoma with transitional cell differentiation [65].

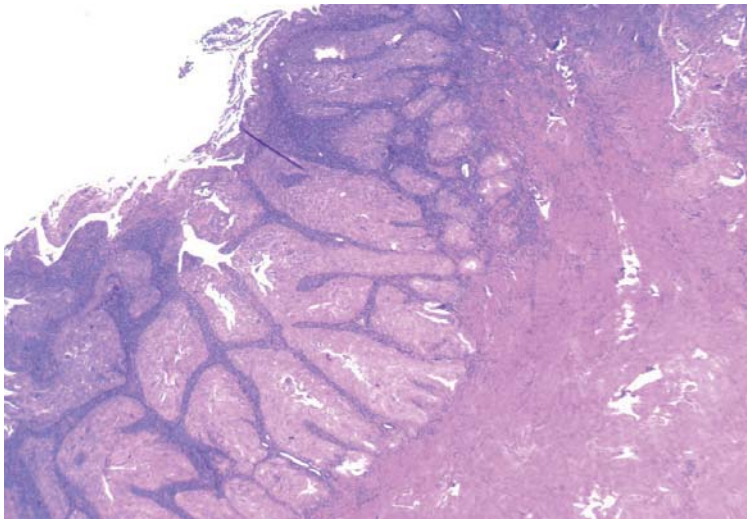


Figure 15. Transitional disposition of neoplastic cells.

The tumours are often polypoid or papillary and the transitional component is graded 2-3. All endometrial transitional cell carcinomas are cytokeratin-20 negative and cytokeratin-7 positive [87,88]. Human papillomavirus type 16 has been described in 22% of studied cases, suggesting an etiologic role [87].

- **Small cell carcinoma**

Small cell carcinoma is a rare tumour <1% of all endometrial carcinomas. It resembles the small lung cell carcinoma (Figure 16). Small cell carcinomas are positive for cytokeratin and frequently positive for neuroendocrine markers, while one half of all cases are positive for vimentin.

The prognosis is better than all small cell carcinoma in other organs with a 5-year survival of around 60% [89].

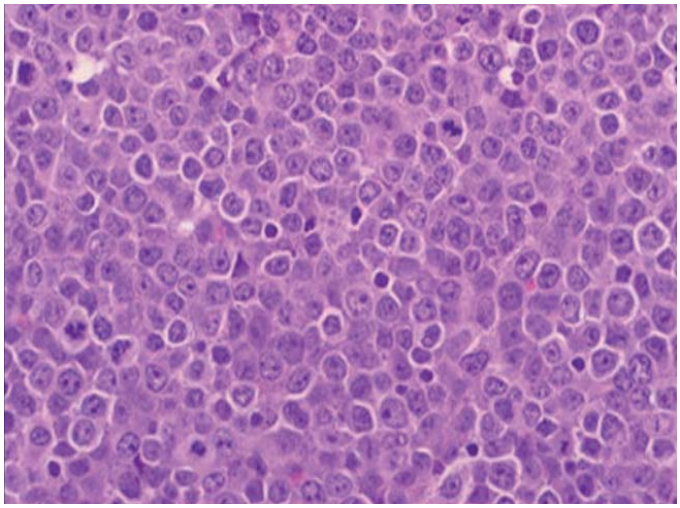


Figure 16. Small cell carcinoma. Sheet distribution of small atypical cells.

- **Undifferentiated carcinoma**

Undifferentiated carcinomas are characterized by a lack of differentiation.

1.1.6.3 FIGO staging

The International Federation of Gynaecology and Obstetrics (FIGO) developed its classification and staging system for endometrial cancer in 1958. The staging of endometrial cancer was changed from clinical to surgico-pathologic in 1988. The surgical staging was updated in 2009 (Table 6), to solve the problems observed in reproducibility, accuracy, and predictive value noticed during previous years [90,91]. The FIGO staging provides relevant information to assess the spread of the tumour in the body (Figure 17). It uses surgical and pathological staging. For the pathological assessment all these parameters are evaluated:

- Myometrial invasion
- Cervical involvement
- Tumour size and location
- Extension of tumour to Fallopian tubes and ovaries
- Tumour grade and histology
- Lymphovascular space invasion (LVSI)
- Lymph node status

Stage	
I	Tumour confined to the corpus uteri
IA	No or <50% invasion of the myometrium
IB	Invasion ≥50% of the myometrium
II	Tumour invades cervical stroma but does not extend beyond the uterus
III	Local and/or regional spread of the tumour
IIIA	Tumour invades serosa of the corpus uteri and/or adnexae
IIIB	Vaginal and/or parametrial involvement
IIIC1	Positive pelvic lymph nodes
IIIC2	Positive para-aortic lymph nodes with or without pelvic nodes
IV	Tumour invades bladder/bowel mucosa, and/or distant metastases
IVA	Tumour invasion of bladder and/or bowel mucosa
IVB	Distant metastases including intra-abdominal and/or inguinal lymph nodes

Table 6. Revised FIGO staging, adapted from Plataniotis et al. [92].

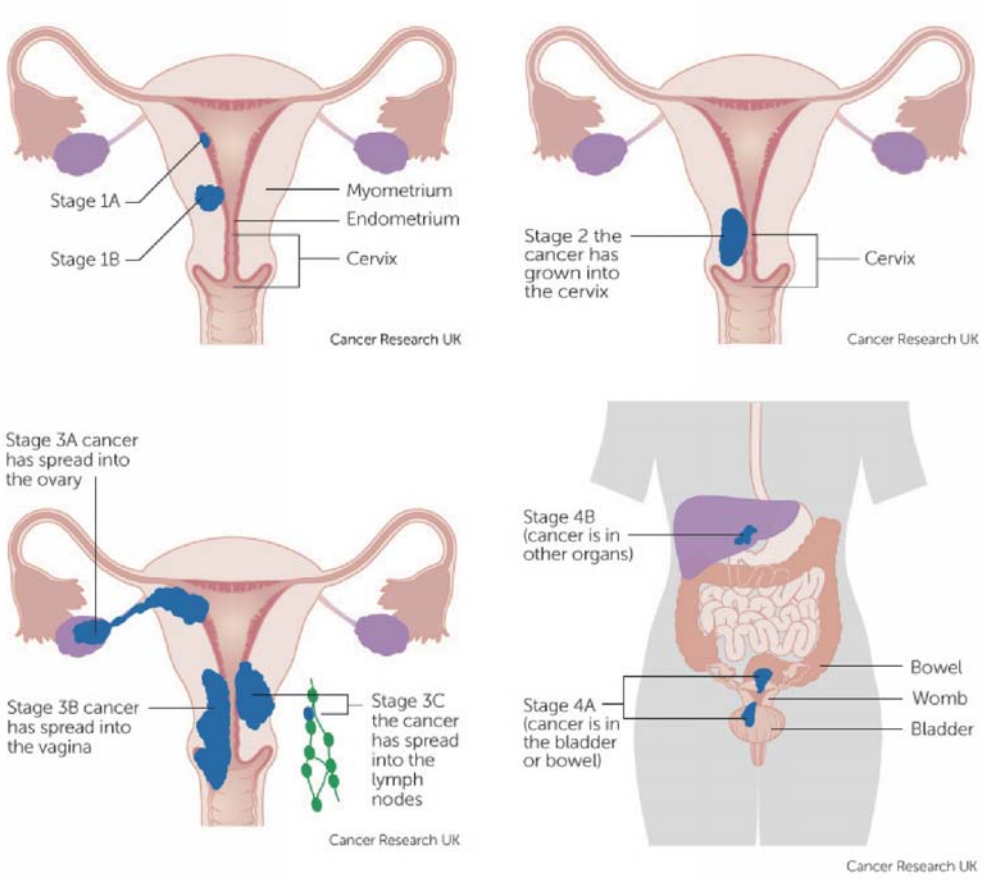


Figure 17. FIGO staging from Cancer Research UK (CRUK) CC BY-SA 4.0, via Wikimedia commons.

1.1.7 Molecular bases

Tumour development comprises genetic, epigenetic and functional changes at the cell metabolism, regulation of gene expression and cell division. Oncogenes and tumour suppressor genes are two categories of genes that play a key role in cancer development. Proto-oncogenes are involved in processes of growth and maintenance of tissues and organs. They stimulate cell division, stop apoptosis and control cell differentiation. Gain-of-function mutations change proto-oncogenes in oncogenes. At the cellular level, the oncogenes act as dominant, under activation or aberrant increased expression, one mutated copy (allele) is sufficient to alter the phenotype of the cell to malignant [93]. By contrast, tumour suppressor genes promote tumorigenicity through a loss-of-function in both alleles. Damage in tumour suppressor genes allows tumour growth and cell death escape.

1.1.7.1 Dualistic model

In addition to the clinical and pathological aspects (section 1.1.6.1) the dualistic classification of endometrial cancer [73] divides type I and type II cancers based on genetic alterations [94,95]. The predominant molecular alterations for each type are given in Table 7 and in the following sections.

Genetic Alteration	Type I (%)	Type II (%)
***PTEN* inactivation**	50-80	10
***KRAS* mutation**	15-30	0-5
***β-catenin* mutation**	20-40	0-3
Microsatellite instability	20-40	0-5
***p53* mutation**	10-20	80-90
HER2/neu	10-30	40-80
***p16* inactivation**	10	40
E-cadherin reduced	10-20	60-90
ER and PR expression	70-73	19-24

Table 7. Genetic alterations in endometrial cancer by type (%), adapted from N. Bansal et al. [95]. ER and PR: oestrogen and progesterone receptors.

A) Type I endometrioid endometrial cancer

- ***PTEN* silencing**

Phospatase and TENsin homolog (*PTEN*) gene codes a 47 KDa protein with tyrosine kinase activity that acts as a tumour suppressor gene. *PTEN* is altered in around 80% of endometrioid endometrial cancers [96]. Inactivation of *PTEN* is due to mutations that lead to a loss of expression [71], and to a lesser extent due to a loss of heterozygosity (LOH) [97] or promoter hypermethylation. Loss of PTEN has been found in precancerous lesions and as a consequence linked to an early event in endometrial cancer, which probably originated in response to hormonal associated risks [98].

PTEN has been observed to have both lipid and phosphatase activity, leading to different functions [95]. The lipid phosphatase activity negatively regulates the level of phosphatidylinositol (3,4,5)-triphosphate and partially, in co-operation with increased p27, causes cell cycle arrest at the G1/S stage [99]. Mutation of *PTEN* increases the activation of phosphatidylinositol 3-kinase (PI3KCA) leading to AKT phosphorylation [100]. The PTEN phosphatase activity is involved in inhibition of focal adhesion formation, cell spread and migration, as well as MAPK signalling inhibition. Consequently, altered PTEN expression leads to tumour cell growth, escape to apoptosis, and atypical cell spreading and migration.

- **Microsatellite instability**

The human genome is divided into DNA coding sequences (1.5%) and noncoding sequences (98.5%). Around half of noncoding DNA consists of different types of repetitive sequences, whose function is to maintain the chromosomal structure and is involved in the evolution of genes and genomes [101]. Microsatellite DNA are sections of 2-5 nucleotides in various places of the genome, which due to their repetitive structure are susceptible to replication errors. Accumulated mutations in these sequences during DNA replication and defects in the mismatch repair system (MMR) lead to microsatellite instability (MSI) [102]. The most well known members of the MMR are: MLH1, MSH2 or MSH6. MSI has been described in around 20% of sporadic endometrioid endometrial carcinomas [103]. In endometrial cancer, *MLH1* inactivation due to

hypermethylation of CpG islands is the most common mechanism leading to MSI [104].

MSI has been reported to be more common in endometrioid than non-endometrioid cancers [105]. In fact, an association between MSI and *PTEN* mutation has been reported for endometrioid endometrial carcinomas. In patients presenting MSI the ratio of mutation in *PTEN* increases up to 60-80% compared to a 24-35% in tumours without MSI [106]. This suggests that *PTEN* could be a target gene for mutations in a deficient DNA repair scenario.

- ***KRAS***

KRAS oncogene codes a 21 KDa protein involved in the signal transduction pathway of cell proliferation and differentiation. Mutation in *KRAS* results in an activation of the pathway leading to unregulated proliferation and reduced cell differentiation [99].

KRAS mutations have been found in 10–30% of endometrial carcinomas, predominantly in endometrioid tumours [107,108]. The *KRAS* mutation has also been found in 16% of endometrial hyperplasia cases [109]. Consequently, this mutation is related to an early event in endometrial cancer.

- ***CTNNB1***

The *CTNNB1* gene codes the β-catenin protein, a component of the adherens junctions and of the E-cadherin complex. As a consequence it has a relevant role in tissue architecture maintenance, cell differentiation, and signal transduction. Moreover, β-catenin is a downstream member of the WNT signalling pathway, which is related to embryogenesis and tumorigenesis [110]. Mutation in this gene leads to protein stabilization and as a consequence, protein accumulation in the cytoplasm, the nucleus and constitutive target gene activity [111].

β-catenin nuclear accumulation has been widely reported in endometrioid compared to non-endometrioid endometrial carcinomas, and also in hyperplasia with atypia. Thus, the *CTNNB1* mutation is linked to an early event in endometrial carcinogenesis [112].

- **Oestrogen and progesterone receptors**

Oestrogen (ER) and progesterone (PR) receptors belong to a group of nuclear receptors. They act as transcription factors by binding to specific locations on the DNA. ER belongs to the group of receptors under 17β-oestradiol activation. It presents two different subtypes, ERα and ERβ, encoded by different genes. While ERα is the main receptor in the endometrium and leads to increased proliferation, ERβ has an antiproliferative effect and modulates the ERα mediated functions [99]. The balance of the two different isoforms is crucial in type I endometrioid carcinoma. In fact, significant differences in the *ERα/β* mRNA ratios and protein expression between normal endometrium and endometrial carcinoma were evidenced. Decreasing levels of ERα have been described in both well-differentiated and poorly-differentiated tumours compared to control postmenopausal women [113].

The PR is an intracellular receptor that binds to progesterone. Two isoforms of PR have been observed, PR-A and PR-B, each one with a different molecular weight. In the endometrium, PR-A downregulates the effects of ERα activity while PR-B acts as an oestrogen agonist. As in the case of the ERs, the imbalance of the PR isoforms ratio is critical in endometrial tumorigenesis [114]. The absence of ER and PR has been related to tumour aggressiveness and bad prognosis [113,115].

A summary of the molecular events associated to the development of endometrioid endometrial carcinoma is presented in Figure 18.

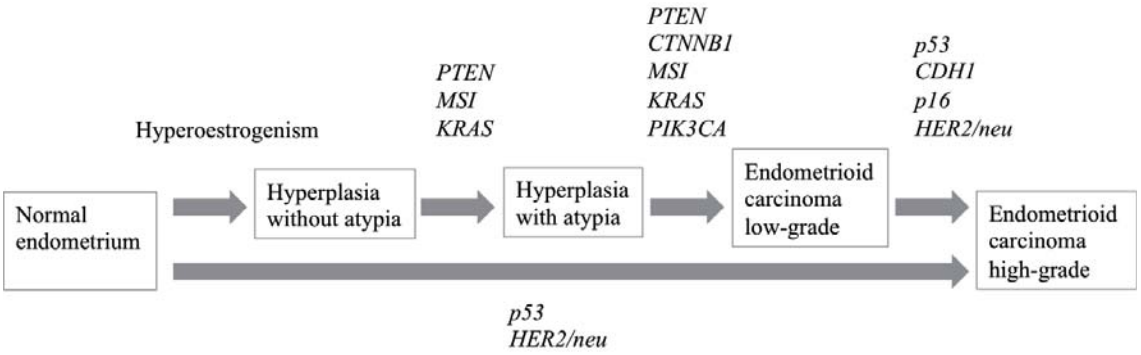


Figure 18. Molecular events associated with endometrioid endometrial carcinoma.

B) Type II non-edometrioid endometrial cancer

- **Aneuploidy *p53* mutation**

The *p53* gene encodes a tumour suppressor protein containing transcriptional activation, DNA binding, and oligomerization domains. It has a crucial function by stopping the propagation of cells with DNA damage. Mutation in the *p53* tumour suppressor gene is the most common mutation in type II (non-endometrioid) endometrial cancer, and it is present in around 80-90% of these tumours. After DNA damage, p53 accumulates in the nucleus and causes cell cycle arrest and apoptosis [111]. Mutated *p53* leads to a non-functional protein that accumulates within the cell and acts as a double negative inhibitor of the wild-type p53 [95].

- ***Her2/neu* amplification**

Her2/neu (*c-erbB2*) is an oncogene, that codes for a transmembrane glycoprotein receptor, tyrosine kinase, which is involved in cell growth, survival, adhesion, migration and differentiation [99]. Amplification of *Her2/neu* has been described in 10-30% of all endometrial carcinomas and in 40-80% of serous endometrial cancer [116].

- ***CDH1***

Cadherin 1 or epithelial cadherin (E-cadherin) is a protein that in humans is encoded by the *CDH1* gene. It is a transmembrane cell adhesion molecule, composed of 5 extracellular domains and one cytoplasmic tail, which lead to linkage to the actin cytoskeleton. Reduced E-cadherin has been related to a decrease in cell-cell adhesion and as a previous step in cell migration. Negative or low E-cadherin staining has been found in 62% and 87% of serous and clear cell carcinoma, respectively [117].

- **p16**

The *CDKN2A* tumour suppressor gene codes for two proteins, one of them is the p16 protein, whose function is to regulate cell cycle. By binding to cyclin dependent kinases 4 and 6 (CDK4 and CDK6), p16 blocks their abilities to

stimulate cell cycle progression. Inactivation of this gene occurs in around 40% of type II endometrial cancers and leads to uncontrolled cell growth [95,102].

The molecular features associated with the development of a non-endometrioid carcinoma are represented in Figure 19.

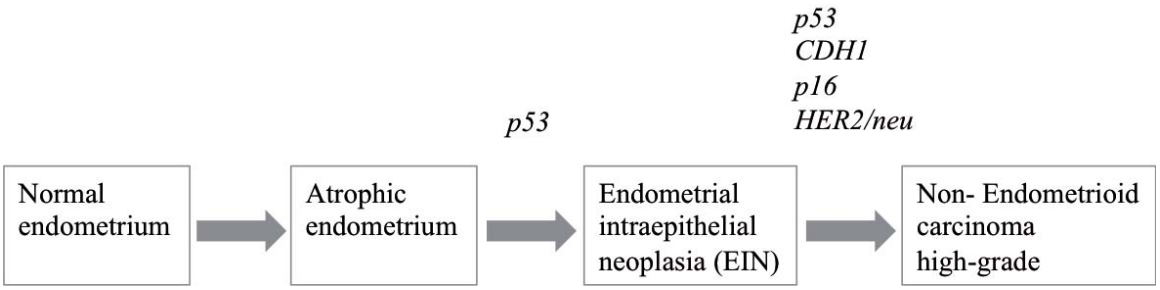


Figure 19. Molecular events associated with non-endometrioid endometrial carcinoma.

1.1.7.2 TCGA model

Although the dualistic classification has been broadly used, it is not entirely accurate since some endometrial cancers present shared characteristics of both type I and type II groups. Thus, a molecular classification was pursued in order to develop a more accurate subtype classification of endometrial cancer.

The Cancer Genome Atlas (TCGA) Research Network proposed a novel classification based on an integrated genomic characterization of endometrial carcinoma [118].The authors performed a genomic, transcriptomic and proteomic characterization of 373 endometrial carcinomas using array and sequencing based technologies. Thanks to this extensive characterization and data analysis, the authors described a novel classification that divides endometrial carcinomas into 4 subtypes and also identifies similarities between endometrial cancer and others types of cancers.

The four groups can be observed in Figure 20, and are listed as follows:

- ***Pole* (ultramutated)**: this group classifies around 10% of endometrioid endometrial cancers. It is characterized by an ultrahigh somatic mutation frequency and a common hotspot mutation in the exonuclease domain of *POLE*. It comprises few copy-number aberrations, increased frequency of C→A transversions, and mutations in *PTEN*, *PIK3R1*, *PIK3CA*, *FBXW7* and *KRAS*. It presents an excellent progression free-survival.

- **Microsatellite instability (MSI)**: this group is characterized by *MLH1* promoter methylation. It also presents frameshift deletions in *RLP22*, frequent non-synonymous *KRAS* mutations, and few mutations in *FBXW7*, *CTNNB1*, *PPP2R1A* and *p53*.
- **Low copy-number**: this group is composed basically of endometrioid tumours, with high mutation frequency in *CTNNB1*.
- **High copy-number**: this group is composed essentially of serous-like tumours. Most of these tumours have *p53* mutations and a high frequency of *FBXW7* and *PPP2R1A* mutations. It presents a worse progression free-survival.

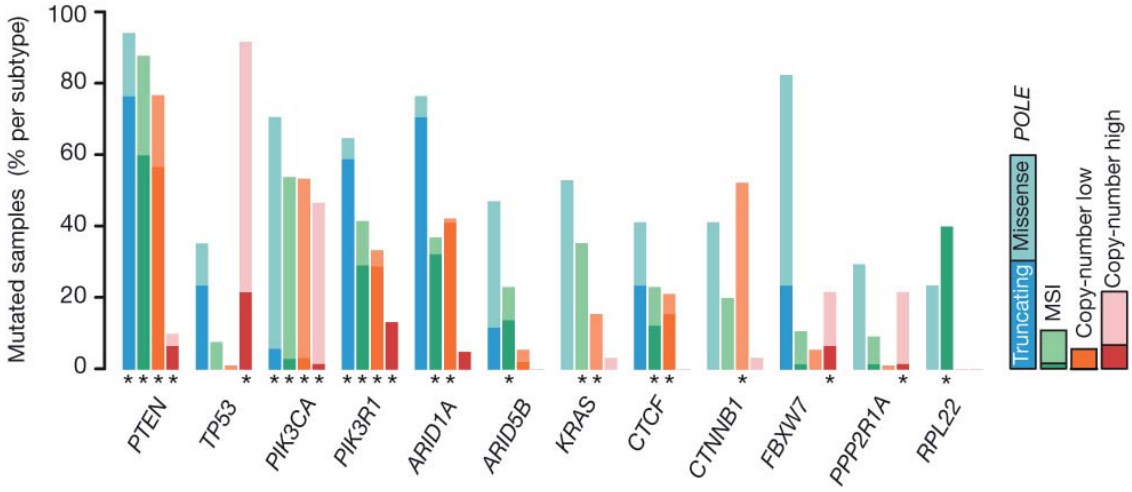


Figure 20. TCGA molecular classification of endometrial cancer. Recurrently mutated genes in the four groups. Figure from G. Getz et al. [118].

The molecular data demonstrated that around 25% of tumours classified as high-grade endometrioid have similarities with serous carcinomas, including *p53* mutations and a high copy-number. The genomic-based classification might lead to a better understanding and facilitate the development of treatments tailored to specific disease groups and thus, improve the management of these patients.

1.1.8 Prognostic factors

It is generally well accepted that endometrial cancer presents a favourable prognosis, due to its early detection. However, there are still relevant subgroups

of patients presenting poor prognosis. Thus, it is important to identify accurate predictive and prognostic factors that may envisage the development of recurrent disease, in order to improve the choice of primary and adjuvant therapy.

Several studies demonstrated that the prognostic factors for endometrial cancer could be divided into uterine and extra-uterine factors [119–123]. The uterine factors are: the histological type and grade of the tumour, the depth of myometrial infiltration, vascular invasion, the presence of endometrial hyperplasia with atypia, cervical involvement, DNA ploidy and S-phase fraction, and expression of ER and PR. By contrast, the extra-uterine factors include: a positive peritoneal cytology, adnexal involvement, presence of pelvic and para-aortic lymph node metastasis, and/or peritoneal metastasis. Among those, the most significant factors are the histological type and grade, the depth of myometrial invasion, and lymphovascular invasion [124]. The FIGO stage is the single most important prognostic factor and the most used as a reference, with a significant reduction in survival in advanced stages [1]. In fact, the 5-year survival rate decreases from 81% in stage I to 69% for stage II, 51% for stage III, and 16% for stage IV [123,125,126]. Although the spread to regional lymph nodes is an important prognostic factor, the role of lymphadenectomy in women with early-stage tumours still remains controversial [127].

Prognostic factors are assessed before and after surgical treatment to define the staging of the tumour and the risk of recurrence of each patient. Apart from medical history, clinical examination, and endometrial biopsy, the initial preoperative evaluation includes blood count, liver and renal functions tests and chest X-ray [92]. Magnetic resonance imaging (MRI) should be requested to determine the cervical and nodal involvement as well as the myometrial invasion. Before surgery, the preoperative staging relies on the type and grade of the tumour, the depth of myometrial infiltration and the extent of cervical involvement, and the medical condition of the patient. The pre-operative staging will guide the extent of the surgery, which is always the primary treatment of endometrial cancer. Accurate staging of the tumour is always achieved after surgery, and it is called clinical staging.

Clinicians are able to divide patients diagnosed with endometrial cancer based on their risk of recurrence into the following categories (Table 8).

Risk group	Description
Low	Stage I endometrioid, grade 1-2, <50% myometrial invasion, LVSI negative
Intermediate	Stage I endometrioid, grade 1-2, ≥50% myometrial invasion, LVSI negative
High-intermediate	Stage I endometrioid, grade 3, <50% myometrial invasion, regardless of LVSI Stage I endometrioid, grade 1-2, LVSI unequivocally positive, regardless of invasion depth
High	Stage I endometrioid, grade 3, ≥50% myometrial invasion, regardless of LVSI status Stage II Stage III endometrioid, no residual disease Non-endometrioid
Advanced	Stage III residual disease and stage IVA
Metastatic	Stage IVB
FIGO 2009 used; molecular factors were considered but not included; tumour size considered but not included; nodal status may be considered for treatment recommendations LVSI lymphovascular space invasion	

Table 8. Risk group classification to guide adjuvant treatment, from Colombo et al. [46].

The stage I intermediate and high-intermediate risk groups include patients with grade 1 or 2 adenocarcinoma with ≥50% myometrial infiltration or grade 3 tumours with superficial infiltration and the presence of extensive lymph vascular space invasion in the uterine specimen [128]. The 5-year risk of recurrence for patients in this group has been described as between 20% and 25% [129–133].

The high-risk and advanced groups include stage I patients G3 with ≥50% myometrial infiltration and all patients at stages II-III, IV endometrioid adenocarcinomas and all non-endometrioid endometrial cancers. The 5-year risk of recurrence for patients in this group has been described between 30% and 65% [134–137]. Although non-endometrioid endometrial cancers only represent

around 10% of diagnosed endometrial cancers, they account for more that 50% of total recurrences and deaths [78,79,138,139].

1.1.9 Treatment

1.1.9.1 Surgery

Following the recommendations adopted by the European Society for Medical Oncology (ESMO), the European Society of Gynaecology and Obstetrics (ESGO) and the European Society for Radiotherapy and Oncology (ESTRO) consensus conference [46], a work-up is mandatory before surgery and must include: family history, assessment and list of comorbidities, geriatric assessment, clinical examination including pelvic examination, transvaginal or transrectal ultrasound, pathology assessment including histologic subtype and histological grade of the tumour of an endometrial biopsy or curettage sample.

The primary treatment of endometrial cancer is surgery, traditionally by laparotomy. The final extent of the surgery should be adjusted to the patient's medical conditions. Within the last few years, the use of minimally invasive techniques has been widely accepted. In fact, the same results of both disease-free and overall survival have been found for laparoscopy, with further benefits: shorter hospital stay, less use of pain killers, lower rate of complications, and improved quality of life [5,140]. Moreover, the use of the robotic approach has incremented the potential of laparoscopy, especially in obese women [141].

The recommended surgery depending on the stage of the disease is listed in Table 9 [5].

Stage I	IA G1-G2	Hysterectomy with bilateral salpingo-oophorectomy
	IA G3	Hysterectomy with bilateral salpingo-oophorectomy +/- bilateral pelvic-para-aortic lymphadenectomy
	IB G1-G3	Hysterectomy with bilateral salpingo-oophorectomy +/- bilateral pelvic-para-aortic lymphadenectomy
Stage II		Hysterectomy with bilateral salpingo-oophorectomy and bilateral pelvic-para-aortic lymphadenectomy
Stage III		Maximal surgical cytoreduction with a good performance status
Stage IV	IVA	Anterior and posterior pelvic exenteration
	IVB	Systemic therapeutical approach with palliative surgery
Serous and Clear cell		Hysterectomy with bilateral salpingo-oophorectomy, pelvic and para-aortic lymphadenectomy, omentectomy, and peritoneal biopsies

Table 9. Surgical procedures depending on staging, adapted from Colombo et al. [5].

- **Stage I**

When a patient is diagnosed in FIGO stage I, the standard surgical approach consists of total hysterectomy and bilateral salpingo-oophorectomy with or without lymphadenectomy. Although some authors state that lymphadenectomy could help in determining the prognosis of the patient and in adapting the adjuvant therapies, the results about its benefits are still controversial. Many studies suggest a complete surgical staging for intermediate- high-risk endometrioid endometrial cancers (stage IA G3 and IB).

In Benedetti et al., the authors studied the effect of lymphadenectomy (excluding stage IA-B G1 and non-endometrioid tumours) in a randomized trial in 514 patients. The results did not evidence any improvement either in disease-free survival or in overall survival [142]. Moreover in the ASTEC trial [143] the results in a randomized study, in 1,408 patients with tumours confined to the uterus, support the idea that lymphadenectomy in these patients did not show compelling evidence on overall or recurrence-free survival. However, these studies were not focused on the possible benefits of lymphadenectomy in high-risk patients. In 2010, the SEPAL retrospective study compared systemic pelvic lymphadenectomy and systemic pelvic and para-aortic lymphadenectomy. The

overall survival in patients undergoing pelvic and para-aortic lymphadenectomy increased significantly [144].

In addition, sentinel lymph node (SLN) mapping has also been studied in early stage endometrial cancer [145] with positive results. SLN is defined as the first chain node in the lymphatic basin that receives primary lymphatic flow. When the SLN is found negative for metastatic disease, then other nodes are expected to be disease-free. More studies are needed for both the use of lymphadenectomy for high-risk patients and its impact on tailoring the subsequent treatment and the use of SLN biopsy.

- **Stage II**

The standard surgical approach for patients diagnosed with stage II consists in hysterectomy with bilateral salpingo-oophorectomy and systemic pelvic lymphadenectomy with or without para-aortic lymphadenectomy. Lymphadenectomy is recommended in order to guide surgical staging and posterior adjuvant treatment.

- **Stage III-IV**

In patients diagnosed with stage III-IV of the disease, the recommended surgery is maximal surgical debulking in patients with a good performance and resectable tumour. In the cases when optimal cytoreduction can be performed, a significant increase in both disease-free and overall survival has been observed [146]. In cases presenting distant metastatic disease, palliative surgery could be considered in patients with a good performance status.

- **Serous and clear cell carcinomas**

Serous and clear cell carcinomas are more aggressive and present a higher rate of recurrence and metastatic disease. Thus, for their treatment complete staging with total hysterectomy, bilateral salpingo-oophorectomy, pelvic and para-aortic lymphadenectomy, omentectomy, and peritoneal biopsies are required.

1.1.9.2 Adjuvant treatment

The majority of patients diagnosed with endometrial cancer present a low-risk and thus, are treated by surgery alone [8]. Prognostic factors are used to classify patients according to their risk of recurrence (see section 1.1.8) in order to determine the indication for adjuvant therapy (Table 10). These clinicopathological prognostic factors include: age, FIGO stage, depth of myometrial invasion, tumour grade and type and LVSI [122].

Risk group	Recommended adjuvant treatment
Low and Intermediate	No required adjuvant radiation therapy
High-intermediate	Adjuvant brachytherapy for negative nodes Adjuvant EBRT for LVSI unequivocally positive
High	EBRT Platinum-based chemotherapy can be considered in stage I G3 with adverse risk factors and in patients with stage II–III
Advanced	Radiotherapy and/or chemotherapy
Metastatic	Chemotherapy or hormonal therapy depending on the histological type and the clinical features of the patient EBRT in locoregional recurrence
Serous and clear cell	Platinum-based adjuvant chemotherapy

Table 10. Recommended adjuvant treatment based on the assessed risk.

- **Low and intermediate-risk**

Low-risk disease (Stage I G1-2 with no or superficial myometrial invasion) does not required adjuvant radiation therapy [90,147]. In fact, a randomized trial in patients diagnosed at stage IA G1-2, comparing vaginal brachytherapy and observation, showed no differences in survival, recurrence rate and late toxic effects [5]. Three large randomized trials (PORTEC-1, GOG-99 and ASTEC MRC-NCIC CTG EN.5) [133,148,149] did not demonstrate the benefits of external beam radiation (EBRT) in overall and disease-free survival. Moreover, EBRT was associated with risk of long-term morbidity.

The PORTEC-2 trial studied the effects of EBRT versus vaginal brachytherapy, in a randomized trial including 427 patients with intermediate-risk, and found

that although both radiations were effective, the patients treated with vaginal brachytherapy have a better quality of life [20].

- **High-intermediate-risk endometrial cancer**

Following the consensus between ESMO-ESGO-ESTRO, when nodes are negative, adjuvant brachytherapy is recommended to decrease vaginal recurrence. Adjuvant EBRT is recommended for LVSI unequivocally positive. No benefits on systemic therapy have been reported. In fact, on-going or recent trials are focused on the study of EBRT alone or combined with chemotherapy for patients with high-intermediate- and high-risk (GOG-249). However, the first results seem to evidence no differences in relapse-free survival or overall survival [90].

- **High-risk endometrial cancer**

EBRT is the standard therapy used for high-risk patients to maximize pelvic control. Platinum-based chemotherapy can be considered in stage I G3 with adverse risk factors and in patients with stage II–III.

In the RTOG 9708 phase II study (N=46), patients received concurrent pelvic RT and two cycles of cisplatin (50 mg/m2 days 1 and 28) followed by four additional courses at 28-day intervals of cisplatin (50 mg/m2) and paclitaxel (175mg/m2). The results evidenced that the 4-year overall survival rates increased for the whole group (85%) and for stage III patients (77%). The recently completed trials PORTEC-3 and GOG-258 investigated the role of combined cisplatin-based chemoradiation plus adjuvant chemotherapy compared with either RT alone or chemotherapy alone, respectively, in those patients [46].

- **Advanced disease**

There is no agreement on the standard treatment of patients diagnosed with advanced endometrial cancer. The treatment consists in a combination of surgery, radiotherapy and/or chemotherapy. In fact, the GOG-122 trial tested the effect of abdominal radiation versus doxorubicin-cisplatin chemotherapy in stage III-IV. The results evidenced a significant improvement in the patients treated with chemotherapy, in both progression-free and overall survival [137].

- **Locoregional recurrence**

For vaginal relapse the standard treatment consists in EBRT, which presents high rates of local control and a 5-year survival of 50%. When there is pelvic relapse, the treatment is surgery or radiation for central pelvic relapse and radiation associated if possible with chemotherapy for regional pelvic relapse [46].

- **Metastatic disease**

The majority of patients diagnosed with advanced or recurrent disease will be candidates for systemic palliative therapy. The choice between chemotherapy and hormonal therapy depends on the histological type and the clinical features of the individual patient. Hormonal therapy is recommended for endometrioid histologies only and involves mainly the use of progestational agents; tamoxifen and aromatase inhibitors are also used. It is the preferred front-line systemic therapy for grade 1-2 tumours without rapidly progressive disease. The status of the hormone receptor should be determined before hormone therapy, as it may be more successful in patients presenting positive PR and ER. The overall response to progestins is ~25%. Other endocrine therapies have demonstrated efficacy in phase II trials among patients with advanced or recurrent disease, like tamoxifen, anastrozole and fulvestrant. They have been associated with a ~10% rate of response [150–153]. Up to a 40% response has been reported by using single cytotoxic agents in chemotherapy-naïve metastatic patients. No specific regimen can be recommended for second-line chemotherapy.

Regarding the progestagen therapy, in the Cochrane review [154] seven trials assessing 4,556 women were identified and studied in order to evaluate the effects of progestagens in endometrial cancer. Three trials included women with stage I disease, whereas four included women with more advanced disease. Meta-analysis of four trials showed that there was no significant difference in the risk of death at 5-years between adjuvant progestagen therapy and no further treatment. Only relapse of disease appeared to be reduced by progestagen therapy in one trial. All together suggesting that the role of progestagens is not supported. However, the tumour progesterone receptor status was not assessed in the studies.

- **Serous and clear cell carcinomas**

Platinum-based adjuvant chemotherapy has been shown to improve the progression-free and overall survival rate in stage I and II [155]. The same adjuvant chemotherapy is recommended for more advanced stages of the disease (stage III-IV).

1.1.9.3 Targeted therapy

Understanding the molecular mechanisms altered in cancer have led to the development of drugs, targeting specific processes like angiogenesis, DNA repair and apoptosis.

The most deregulated pathway in endometrial cancer corresponds to PI3K-AKT-mTOR. Decreased transcription in *PTEN*, due to mutations, leads to decreased PI3K inhibition and increased activity of AKT and mTOR [156]. Some mTOR inhibitors (temsirolimus, deforolimus and everolimus) have been tested in phase II trials and they have been found to stabilize the disease in 44% of metastatic or recurrent patients [157,158]. Everolimus and letrozole have demonstrated a clinically beneficial rate in women with recurrent disease [159].

Angiogenesis is one of the key processes of cancer. Angiogenic factors are produced by the cancer cells; they promote new vessel formation and recruit supporting cells [160]. Vascular endothelial growth factor (VEGF) overexpression has been associated with poor outcomes in most gynaecologic malignancies, one of them being endometrial cancer [160]. The function of drugs targeting angiogenesis, like bevacizumab or tyrosine kinase inhibitors, is being studied in endometrial cancer. Bevacizumab has been tested in patients with recurrent or persistent endometrial cancers [161] in a phase II trial and presented a response and stable disease in 13.5% and 40.4% of patients, respectively. Bevacizumab combined with paclitaxel and cisplatin, or temsirolimus has also been described [162,163]. The bevacizumab, paclitaxel and carboplatin regimen is active and tolerable in advanced and recurrent disease but its impact awaits the results of the recently completed randomized phase II trial. For the association of bevacizumab and temsirolimus, first result indicate a high rate of toxicity and further research needs to be done.

A phase II trial concerning the use of tyrosine kinase inhibitors, evaluated gefitinib in 26 patients with persistent or recurrent disease, however the treatment did not demonstrate satisfactory efficacy [164].

One of the important steps in cancer growth comes from the deregulation of the cell cycle. In that sense, palbociclib is a reversible oral inhibitor of CDK4/6. A phase II trial of letrozole associated or not with palbociclib is ongoing in Europe [90].

Some groups have studied the use of targeted therapies to HER2/neu in aggressive endometrial cancer but without promising results [165,166].

1.1.10 Myometrial invasion

Endometrial cancer spreads by (1) direct extension through the myometrium, (2) exfoliation of cells that are shed through the fallopian tubes, (3) lymphatic dissemination, and (4) haematogenous dissemination [62].

Direct extension of the tumour through the myometrium is the most common form of spread. In fact, myometrial invasion is considered one of the main prognostic factors of endometrial cancer and has been described to directly influence the risk of recurrence with an increased risk of lymphatic, pelvic and aortic metastases [167]. Thus, improving our knowledge of the molecular mechanisms responsible for promoting or controlling the invasive process of endometrial carcinoma remains of crucial importance. Further research is expected to lead to the development of targeted therapies against this process, consequently improving the overall survival of patients [168].

The initial steps associated with the myometrial invasion include tumour cell dissociation from the epithelial layer of the endometrial glands, and penetration of those cells through the basement membrane, into the adjacent tissue, the myometrium [62]. To succeed in this invasive process, one of the main mechanisms describes that epithelial tumour cells undergo an epithelial to mesenchymal transition (EMT) [169,170].

1.1.10.1 EMT phenotype

EMT is a complex process that is crucial in physiological processes, such as embryonic development, but also in pathogenic processes. In cancer, EMT is responsible for inducing a more invasive and aggressive phenotype in tumour cells. During the EMT process, epithelial cells lose their basal apical polarity and cell-cell contacts, undergo a dramatic remodelling of the cytoskeleton, change to a spindle shape morphology, and acquire a migratory phenotype.

The EMT process could be initiated by several extracellular signals and secreted soluble factors that lead to the activation of specific signalling pathways and transcription factors [171] (Figure 21, explained in detail in section 1.1.10.2). In addition, some authors have suggested the initiation of EMT caused by dynamic interactions between the tumour microenvironment and the cancer cells [172].

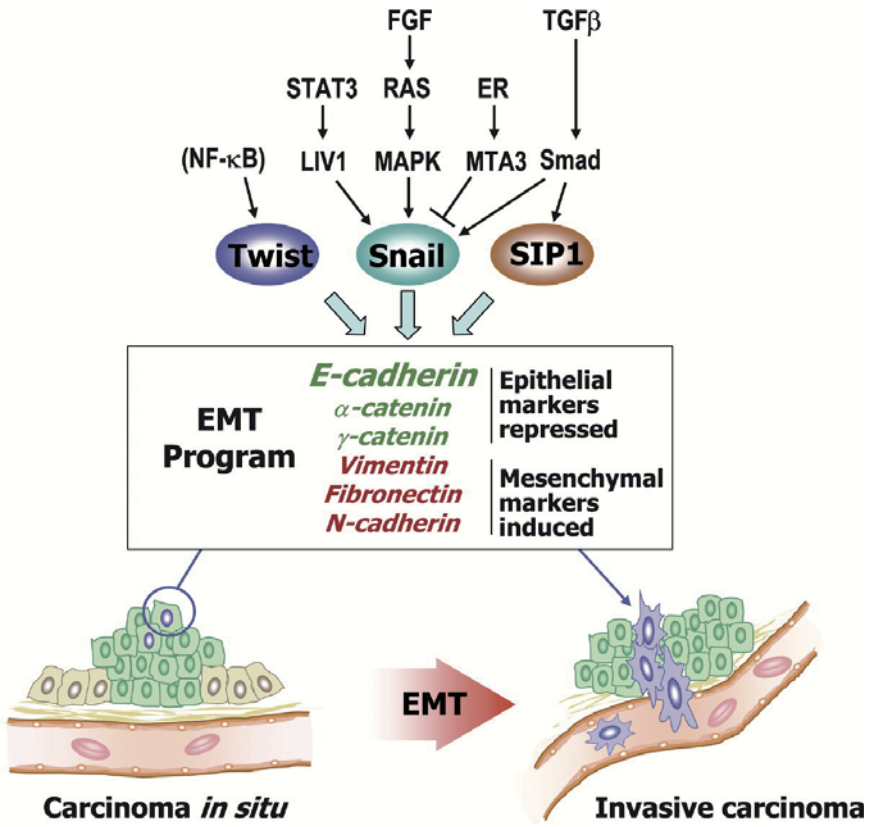


Figure 21. Drivers and mediators of EMT, from Kang and Massague [173].

A hallmark of EMT is the loss of E-cadherin expression coupled with an overexpression of mesenchymal markers like N-cadherin and cadherin 11. This

process is known as “cadherin switch” and leads to the disassembly of the cell-cell junction [173]. In addition, cells acquire markers like smooth muscle actin, vimentin, fibronectin and increased metalloproteinase activity (MMP-2, MMP-3 and MMP-9) [174].

Loss of E-cadherin has been demonstrated to be crucial for endometrial cancer progression. In fact, a decrease in E-cadherin expression has been associated with tumour dedifferentiation, myometrial invasion and patients with advanced stage [175,176]. Moreover, negative E-cadherin expression has been found in tumours with poor prognostic factors, such as grade 3 and non-endometrioid tumours [168,177].

Several developmentally important genes that induced EMT have been described as E-cadherin repressors. One of them is the zinc finger protein SNAIL, a DNA binding factor that recognizes E-box motifs in target promoters, including the E-cadherin promoter [178]. SNAIL has been observed to participate in endometrial progression and dedifferentiation [179]. In fact, SNAIL and E-cadherin protein expression presented an inverse correlation in both primary tumours and metastasis. Moreover, an association between reduced expression of E-cadherin, nuclear expression of SNAIL and lymph node metastasis and death risk has also been established [180].

Another known E-cadherin repressor is TWIST [181]. In fact, TWIST has been shown to promote EMT in endometrioid endometrial cancer by direct repression of E-cadherin or by upregulation of the EMT inducer BMI-1 [182]. TWIST helps EMT and is responsible for a more infiltrating cell phenotype, promoting myometrial invasion and worse patient survival.

Kruppel like factor 17 (KLF17) has been observed to promote EMT through the regulation of TWIST, leading to endometrioid endometrial cancer progression [183].

The zinc finger protein ZEB1 has also been shown to control endometrial cancer motility in the Ishikawa cell line. ZEB1 expression in this cell line results in decreased E-cadherin and increased migration [184]. Moreover, a correlation between the ZEB1 expression and the progression of gynaecological carcinomas has been found [185].

1.1.10.2 Molecular factors that induce EMT in endometrial cancer

There are multiple factors responsible for producing EMT in endometrial cancer, some of which are described below.

- **ETV5**

In recent years, in our laboratory, a crucial role for the Ets translocation variant 5 (*ETV5*) in endometrial cancer progression has been proposed. ETV5 is a transcription factor, that belongs to the PEA3 subfamily of the Ets family, characterized by a sequence of 85 amino acids in an evolutionarily conserved DNA-binding domain that regulates the expression of diverse genes by binding to its promoter in a central A/GGAA/T core motif [186,187]. ETV5 is overexpressed in endometrioid endometrial cancer with a significant increase in patients diagnosed in stage IB (FIGO 2009), associated with myometrial infiltration [188]. ETV5 was found to correlate with metalloproteinases MMP-2 and -9 in endometrioid endometrial cancer tissues [189]. ETV5 transcriptionally activates the zinc finger E-box-binding transcription factor ZEB1, resulting in decreased E-Cadherin. Overexpression of ETV5 in Hec1A led to an EMT. These included a modulation of cell-cell adhesion, cell-cell contact, cellular junctions and actin cytoskeleton reorganization. In addition, cellular functions like cell-to-cell signalling, cell movement, and adhesion to substrates were also altered [190].

In an orthotopic murine model, ETV5 overexpression induces a more aggressive and infiltrative pattern of myometrial invasion. ETV5 function is mediated both *in vitro* and *in vivo* through the increased activity of MMP-2 [191]. We have also evidenced the role of ETV5 as a protective factor against oxidative stress induced by the Hep27 as well as its ETV5-dependent mitochondrial location in Hec1A cells [192].

Moreover, we identified the role of the lipoma-preferred partner (LPP), which acts as a novel co-regulatory partner of ETV5 in the transcriptional regulation of the EMT process [190]. When ETV5 promoted EMT, LPP is reorganized from cell-cell contacts to focal adhesions and after an external stimuli, it translocated to the nucleus establishing a crosstalk between the tumour cells and their microenvironment. All of this demonstrates its validity as an *in vitro* and *in vivo*

model that mimics invasive endometrial cancer cells in the invasive area of the tumour.

- **Oestrogen and progesterone receptors**

The role of ER and PR in endometrial cancer is commonly accepted. Oestrogen has been proposed to stimulate endometrial cancer invasion by interactions between the cancer and stromal cells [171]. It stimulates tumour necrosis alpha (TNF-α), which induces stromal hepatocyte growth factor (HGF) expression and as a consequence an increase in NK4, resulting in invasion of endometrial cancer cells [193]. ERα loss has been associated with increased EMT, vascular invasion and myometrial invasion [194] while ERβ has been described as an important factor in progression of myometrial invasion [195]. Van der Horst et al. proposed that a loss of progesterone throughout the disease might promote EMT [196]. In this study, PR modulated cell lines were treated with medroxyprogesterone acetate (MPA), resulting in decreased migration and downregulation of EMT markers. A downregulation of EGF, IGF-1, IL-6, integrin/ILK, PDGF, TGF-β, VEGF and Wnt/β-catenin signalling were found in the PR modulated cell lines.

- **TGF-β :**

TGF-β members have been described as promoters of cancer, and involved in cell proliferation, survival, migration, and invasion and as important inducers of EMT in both development and cancer [197]. TGF-β1 promoting EMT was associated with the initial steps of endometrial cancer [198]. TGF-β binds to a heteromeric complex of transmembrane serine/threonine kinase, the type I (RI) and type II receptors (RII). RI phosphorylates Smad2 and Smad3, which then form a heteromeric complex with Smad4, translocate into the nucleus and regulate TGF-β-responsive gene transcription [199]. The annulment of TGF-β receptor signalling produced apoptosis and reduced the aggressive phenotype of endometrial cancer cells by reversal of autocrine TGF-β induced EMT [200].

- **Other factors**

Other growth factors such as, EGF, IGF-1, VEGF, PDGF and FGF, and signalling pathways like Notch and Wnt/β-catenin play a major role in E-

cadherin repression [170,201]. The Notch pathway induces EMT by activating nuclear factor-Kβ (NF-Kβ) pathway or by modulating the activity of TGF. In endometrial cancer, overexpression of SHARP1 in Ishikawa cell line suppresses EMT and metastasis by attenuating NOTCH1 signalling [202]. The Wnt pathway leads to EMT through the inhibition of phosphorylation of β-catenin mediated by glycogen synthase kinase 3 beta (GSK3) [203]. In high-grade endometrial cancer, the nuclear accumulation of β-catenin and its association with E-cadherin loss was related to the acquisition of an aggressive phenotype [204]. EGFR was found overexpressed in endometrial cancer [205] and shown to stimulate EMT through SNAIL upregulation and consequent E-cadherin downregulation [206].

1.1.10.3 MELF type invasion

Recently, a new pattern of myometrial invasion named MELF has been observed which appears in low-grade endometrioid endometrial cancers and is related to a poorer prognosis. MELF changes were more frequently found in tumours with local mucinous differentiation and often associated with vascular invasion and fibromyxoid stromal alteration. They may represent a specific tumour-stromal reaction within the myometrium and are localized specifically at the deep invasive front of tumours.

The MELF pattern is characterized by the loss of standard glandular architecture, attenuation of neoplastic epithelium and infiltration of stroma by small nests of cells and individual tumour cells [207,208]. It is characterized by the presence of microcystic, elongated and fragmented invasive glands (MELF) [209,210] that have been shown to be accompanied by a decrease in E-cadherin levels [211]. In addition to the reduced E-cadherin, they are also described by a strong cytokeratin-7 staining, increased cyclin-D1 and p16, loss of membranous β-catenin, frequent negative staining for hormone receptors, and they are more often present in patients presenting *KRAS* mutation [212]. Another finding that supports the hypothesis that MELFs represent active areas of the tumour is the increase in fascin expression described by Stewart et al. [213].

1.1.10.4 Tumour microenvironment

Malignant tumours are complex structures that consist of cancer cells and the surrounding tumour stroma [214]. For tumour growth and systemic dissemination dynamics, interactions between the cancer cells and the local/distant host environment are required [215].

The extracellular matrix (ECM) and the stromal cells compose the endometrial tumour microenvironment [216]. The ECM suffers deep reorganization, to provide structural support to the cells, with increased collagens, proteoglycans, and glycosaminoglycans [217], whereas the stromal cells, comprising stem and progenitor cells of the stroma, cancer-associated fibroblasts (CAFs), pericytes, endothelial cells, and inflammatory cells, supply the epithelium with abnormal paracrine factors, which enable EMT and metastatic progression through interactions with the cancer cells [218].

The role of the tumour microenvironment has been evidenced in endometrial cancer. Some authors described that secretions from normal endometrial fibroblasts could inhibit proliferation, through inhibition of PI3K signalling, in the Ishikawa cell line [219,220]. Endometrial cancer cells evidenced an increase in cell motility and invasiveness in response to CAFs secretion, suggesting a pro-tumorigenic role in cancer progression. In fact, the use of inhibitors for PI3K/Akt and MAPK/ERK demonstrated a suppression of CAF mediated endometrial cancer proliferation [221].

Although the mechanisms by which CAFs regulate invasion are not widely known, they have been found to promote cancer progression and metastasis, through the release of stromal derived factor 1 (SDF1) [222,223]. Hepatocyte growth factor (HGF) and c-Met compose another important ligand/receptor pair, which is known to produce mitogenic and motogenic effects on various cell types. Moreover, c-Met positive staining has been correlated to advanced stages [224]. In addition to these findings, some authors have evidenced the interactions between tumour cells and the microenvironment, demonstrating how factors released by the surrounding stroma, play an important role in activating the expression of transcription factors in those cells undergoing an EMT [225–227]. In this way, cancer cells at the invasive front of certain carcinomas

can be seen to have undergone EMT, suggesting that these cancer cells were under different microenvironmental stimuli from those received by cancer cells at the cores of these lesions. More research to deepen the understanding of the dialogue between CAFs and tumour cells is necessary.

1.2 Adhesion molecules and tumour dissemination

Metastasis is a major clinical problem and one of the main causes of cancer death. The metastatic pathway is a multistep process by which cancer cells give rise to metastatic lesions in a new tissue or organ. Five sequential steps describe the metastatic pathway, including (1) cell proliferation in the primary tumour and angiogenesis, (2) local cell invasion, (3) intravasation and dissemination, (4) extravasation and (5) colonization and proliferation in new tissue or organ [228]. Moreover, cancer cells have to escape from the immunological attack to successfully metastasize. As explained in the previous section, the detachment of tumour cells from the primary site is accompanied by the acquisition of an invasive phenotype through EMT and a switch in the cell-cell adhesion repertoire. Cell migration of tumour cells is prone to a dynamic regulation of adhesion and de-adhesion processes, evidencing the necessity of cellular plasticity of adhesion molecules to enable metastatic spreading.

The main types of cell adhesion molecules (CAMs) are: cadherins, integrins, immunoglobulin superfamily members and selectins (Figure 22). These proteins are typically transmembrane receptors and are composed of three domains: an intracellular domain that allows interactions with the cytoskeleton, a transmembrane domain, and an extracellular domain that interacts with other CAMs. They mediate cell-cell adhesion, cell-extracellular matrix adhesions, and translate external stimuli within the cell by interacting with a complex network of cytoskeletal signalling molecules [229]. They are regulated by several mechanisms, including transcriptional regulation, post-translational changes, and cleavage from the cell surface [230], allowing a rapid response to external signals like growth factors or cytokines.

Much has been written about cadherins, integrins and their role in cancer metastasis, however the immunoglobulin superfamily (IgSF) has received less attention. Nevertheless, the IgSF plays a key role in cell-cell adhesion and several members among this family have been linked to cancer progression.

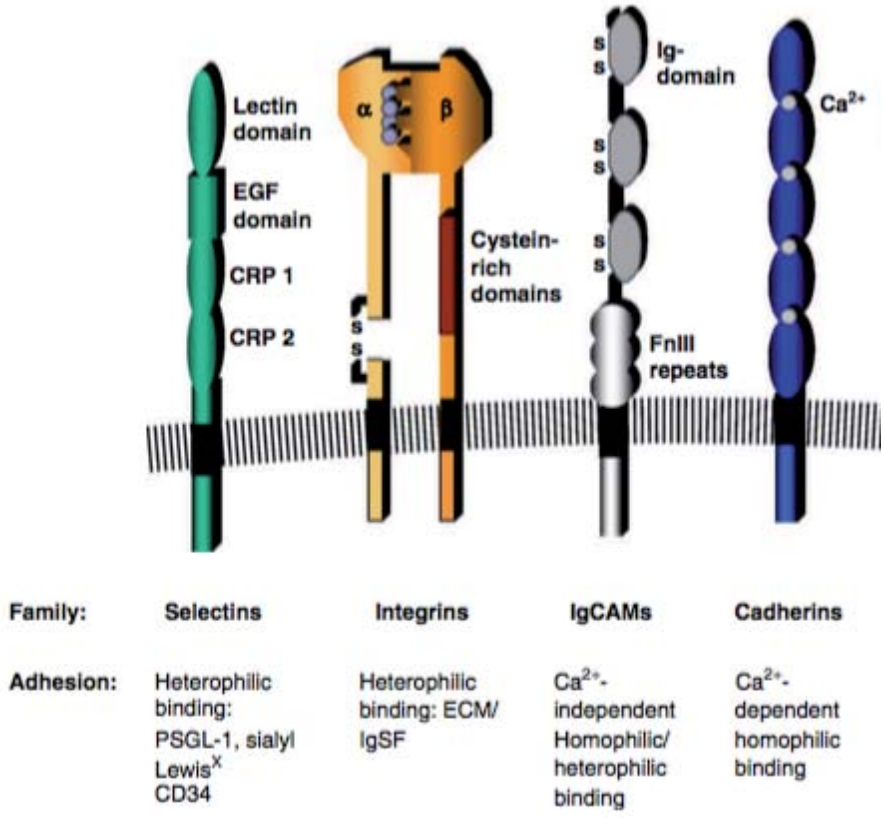


Figure 22. Schematic representation of the principal cell adhesion molecules, from K. Reiss et al. [229].

1.2.1 The immunoglobulin superfamily

The immunoglobulin superfamily (IgSF) is composed of over 765 members, comprising a different range of proteins, including cell surface glycoproteins, proteins of the T cell receptors complex, virus receptors and major histocompatibility complex class I and II molecules [101,231,232]. IgSF members are characterized by the presence of variable immunoglobulin Ig-like domains. Each one composed of a sandwich structure with two opposed antiparallel β-pleated sheets stabilized by a disulphide bridge and fibronectin type III repetitions [233]. The majority of the IgSF members present the same structure: an extracellular

domain, a single transmembrane domain and a cytoplasmic tail. However, some are linked to the cell surface through a glycosylphosphatidylinositol anchor [234]. Through their N-terminal domains, they mediate both homotypic and heterotypic calcium-independent adhesion [235]. The extracellular interactions of the IgSF can lead to signalling within the cell, due to their C-terminal cytoplasmic tail, which is linked to cytoskeletal or adaptor proteins.

Some of the most important members of the IgSF are the intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), platelet endothelial cell adhesion molecule 1 (PECAM-1), neural cell adhesion molecule (NCAM), activated leukocyte cell adhesion molecule (ALCAM), L1 cell adhesion molecule (L1CAM) and melanoma cell adhesion molecule (MCAM). Several of the IgSF members have been described as biomarkers for cancer progression in different cancer types: glioma, melanoma, breast, endometrial, ovarian, prostate and colon cancer [236]. The specific functions associated with each IgSF member in the metastatic process are listed in Table 11.

Stage in metastasis	Known role	Potential role
(1) Cell proliferation in primary tumour		
(i) Apoptotic evasion	NCAM, ALCAM	MCAM
(ii) Angiogenesis	PECAM-1, ICAM-1	VCAM
(2) Local cell invasion		
(i) Cell-cell interactions	MCAM, ALCAM, L1CAM	
(ii) Directional cell migration and cell polarity	MCAM	ICAM-1, VCAM-1, PECAM-1, NCAM, MCAM, L1CAM
(iii) Matrix degradation	MCAM, NCAM	ALCAM
(3) Intravasation and dissemination		MCAM, ALCAM
(4) Extravasation		MCAM, ALCAM, NCAM, L1CAM, PECAM-1
(5) Colonization and proliferation	As for (1) and (2)	As for (1) and (2)
(6) Immunological scape		MCAM, ALCAM, NCAM

Table 11. The role of the IgSF members in the steps of metastasis, adapted from Wong et al. [236].

Thanks to its role in neurite outgrowth, axon guidance, and long-term potentiation, NCAM is one of the most studied members of the IgSF. NCAM

binds to FGFR, through its fibronectin type III domains, prevents FGFR endocytosis and leads to its activation. NCAM also associates with other signal-transduction molecules, like FYN and FAK [237], resulting in sustained MAPK activation together with increased cell motility, increased cell-substrate adhesion, cell migration, and invasion [238]. The N-cadherin binding to FGFR is dependent on NCAM and the union of the complex, leading ultimately to the activation of β1-integrin mediated cell matrix adhesion (Figure 23).

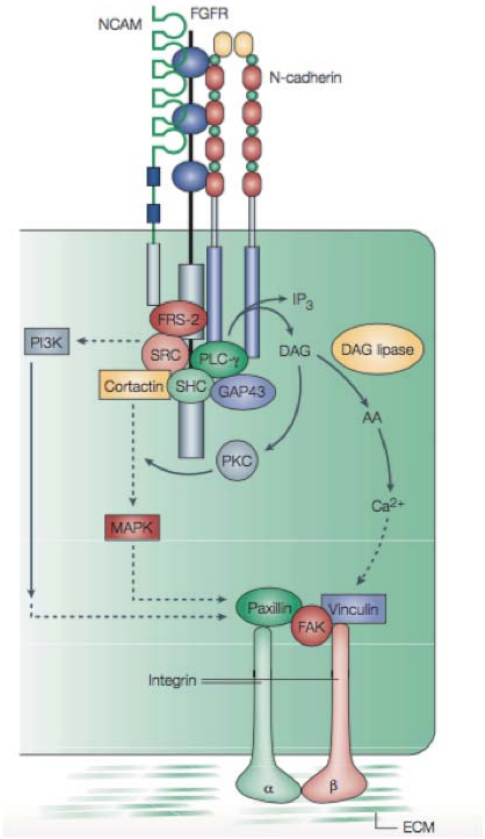


Figure 23. NCAM and N-cadherin signalling in neurons, from Cavallaro et al. [234].

MCAM has also been found to recruit the protein tyrosine kinase FYN to its cytoplasmic tail, leading to FAK activation [239]. The same was found for L1CAM, whose expression in ovarian cancer has been linked to sustained FAK phosphorylation and resistance to apoptosis [240].

Several members of the IgSF have been associated with an increase in apoptosis evasion and in angiogenesis. In fact, the expression of NCAM seems to be associated with the activation of NF-Kβ [241]. In addition, MCAM has been described as an activator of the NF-Kβ pathway via the upstream p38 mitogen-

activated protein kinase (MAPK) [242]. After acquiring apoptotic resistance, tumour progression depends on new blood vessel formation. As regards to the role of the IgSF members in angiogenesis, the vascular endothelial growth factor (VEGF), which is secreted by tumour cells and stroma, has been shown to stimulate the expression of ICAM-1, VCAM-1 and PECAM-1 in endothelial cells. The IgSF has also been associated with the promotion of local invasion, by increasing MCAM, ALCAM and L1CAM homophilic cell-cell interactions. In fact, they have been shown to control directional cell migration and regulate the expression of metalloproteinases responsible of the degradation of ECM and tumour cells dissemination [236].

Although much remains to be investigated regarding the exact mechanisms, the relation of the IgSF members to the metastatic cascade seems evident. Specifically, ALCAM has been implicated in the progression of several cancer types, but its role in tumorigenesis or its specific signalling pathways still remain unclear.

1.2.2 Activated leukocyte cell adhesion molecule (ALCAM)

1.2.2.1 Introduction

ALCAM is a member of the IgSF and was identified by expression cloning based on its ability to bind to CD6 [243,244]. The human gene for ALCAM is located on chromosome 3 (3q13.1q13.2), is composed of 16 exons and has a size of over 200 kb. ALCAM orthologs are expressed in many species with alternative names such as ALCAM, CD166, MEMD, SB10 antigen and HCA in humans; HB2, KG-CAM and F84.1 antigen in rat; MuSC in mouse; DM GRASP, SC1 and BEN in chicken; and Neurolin and E21 antigen in zebrafish [245,246].

ALCAM is a type I transmembrane glycoprotein of 105 kDa (69 kDa after deglycosylation) composed of an extracellular domain of 500 amino acids, a transmembrane domain of 22 amino acids, and a short cytoplasmic domain of 34 amino acids. The ALCAM extracellular domain consists of five Ig-like domains (2 NH_2-terminal, membrane-distal variable-V-type and 3 membrane-proximal-constant –C_2-type Ig folds) [243,245] (Figure 24).

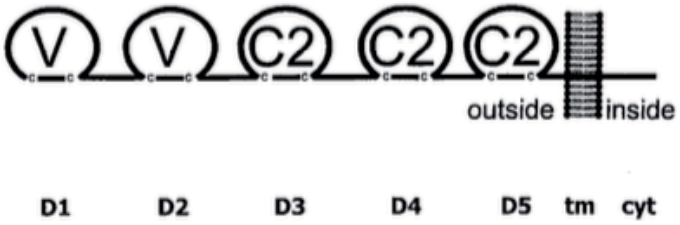


Figure 24. Schematic representation of the cell adhesion molecules VVC2C2C2 subgroup in the immunoglobulin superfamily. Members are type I transmembrane proteins with five extracellular NH2-terminal immunoglobulin domains (D1, D2, D3, D4 and D5), a transmembrane domain, and a C-terminal cytoplasmic tail, from Swart et al. [245].

The fact that the cytoplasmic tail of ALCAM is highly conserved suggests that ALCAM-related functions could be partially explained by conveying extracellular signals into the cytoplasm. Despite the participation of ALCAM in many different biological processes, ALCAM knockout mice are fertile and have no visible defects [247]. However, an axon fasciculation and a neuromuscular synapse defects have been identified [248].

1.2.2.2 Molecular basis of the cell adhesion mediated by ALCAM: structure-function analysis

Heterophilic and homophilic interactions of ALCAM with its partners involve different parts of its structure and present significant differences regarding strength and function. In fact, the affinity of the homophilic interactions (Kd=29-48 μM, koff ≥5.3 s^{-1}) is two orders of magnitude lower than the ALCAM-CD6 interaction (Kd=0.4-1 μM, koff ≥0.4-0.63 s^{-1}) [249].

- **ALCAM homophilic adhesion**

The extracellular region of ALCAM is composed of two functional and structural modules, a ligand binding module composed of the membrane distal V-type Ig loops (D1-D2) that mediates receptor *trans-trans*-interactions between opposing cells, and an avidity module composed of the three membrane proximal C2-type Ig-loops (D3-D5). The strength of the avidity is controlled by recruitment of ALCAM molecules to the site of cell-cell contact and by receptor *cis*-oligomerization via the C-type immunoglobulin domains [250]. Coordination of both modules is necessary for stable ALCAM-ALCAM interaction, leading to the

formation of a tight bilayered ALCAM network (Figure 25) [251].

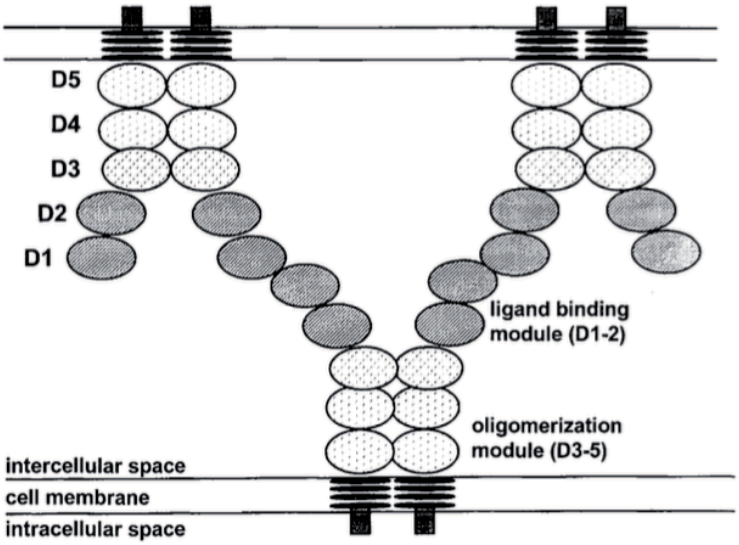


Figure 25. ALCAM-ALCAM interactions between cells [245,250], from Van Kempen et al. and Swart et al. The extracellular structures of ALCAM consist of two functional modules. Immunoglobulin domain D1 is an essential part of the ligand-binding module and in *trans*-interactions between ALCAM molecules on the neighbouring cells. The D4-D5 domains are involved in *cis*-oligomerization at the cell surface.

Zimmerman et al. described the interaction between ALCAM and the actin cytoskeleton [252]. This interaction could be allowed by the presence in its short cytoplasmic tail of a positive-charge-rich domain (PCRD) as well as a PDZ-binding motif KTEA placed at the C-terminus [253,254]. The inhibition of actin polymerization by low levels of cytochalasin D toughly stimulates homotypic ALCAM–ALCAM interactions, increases protein lateral mobility, and the formation of ALCAM clusters in the cell surface, demonstrating that cytoskeleton regulates ALCAM-mediated adhesion [255]. Although little is known about its cytoplasmic partners and how it interacts with the actin, ALCAM has been observed to form a supra-molecular complex with Syntenin-1 and Ezrin [256]. PKCα also plays a role in the cytoskeleton-dependent avidity modulation of ALCAM [252] and regulates the supra-molecular complex formed by ALCAM-Ezrin [254].

In summary, activation of ALCAM-mediated adhesion is dynamically regulated through actin cytoskeleton-dependent clustering. Furthermore, it has been associated with cell motility and metastasis in different tumours [257].

- **ALCAM heterophilic adhesion**

Among the heterophilic interactions mediated by ALCAM, the most studied is the one between ALCAM and CD6. However, additional interactions with other proteins have been suggested.

CD6 is a surface receptor present on T-lymphocytes, thymocytes and a subset of B cells. It belongs to the SRCR family, characterized by the presence of scavenger receptor cysteine-rich domains. ALCAM has been identified as the only known ligand for CD6 [243,250]. Cell clustering promoted by ALCAM-CD6 heterophilic interaction, comprises the NH2-terminal Ig domain (D1) of ALCAM and the membrane-proximal SRCR domain of CD6 [258,259] with a stoichiometry of 1:1. These interactions are significantly stronger than ALCAM homophilic interactions [249,260]. In addition, these interactions are needed for the binding of thymocytes to thymic epithelial cells and of T-cells to activated leukocytes. They are thought to be relevant for T-cell proliferation and maturation [261].

ALCAM has been shown to directly associate with another tetraspanin, CD9, in a protein complex on the leukocyte surface that also includes the metalloproteinase ADAM 17. Through these interactions, CD9 upregulates both homophilic and heterophilic ALCAM interactions [262].

ALCAM has also been seen to interact with L1CAM and this interaction seems to target retinal axons during development [248,263,264].

Several reports indicate a functional link between ALCAM and cadherins. In fact, ALCAM expression has been localized in cell-cell contact areas, where it may interact with other cell–cell adhesion molecules. In a panel of prostate cancer cells that lacked the gene encoding α-catenin, upon reconstitution of the α-catenin/E-cadherin complex by α-N-catenin transfection, ALCAM relocalized from the cytoplasm to the cell membrane where it co-localized with E-cadherin [265]. Ofori-Acquah et al. [266] reports the existence of a complex formed by ALCAM with vascular endothelial (VE)-cadherin and N-Cadherin in pulmonary microvascular endothelial cells. In the same cells, discs-large (Dlg), a component of adherens junctions in a number of epithelial tissue types [267] was immunoprecipitated together with ALCAM.

1.2.2.3 Regulation of ALCAM concentration in the cell

Transcriptional regulation, endocytosis, and/or metalloproteinase shedding can control the concentration of ALCAM in the cell.

ALCAM clustering in the plasma membrane induces its rapid internalization via dynamin- and clathrin-dependent endocytosis, which is controlled by PI3K and mitogen-activated protein kinase ERK. The ALCAM cytoplasmic tail has been observed to interact directly with ubiquitin and this ubiquitination seems to be responsible for ALCAM endocytosis, affecting its function in axon navigation [268,269]. ALCAM is continuously recycled through endocytic pathways and is detectable in early endosomes.

In addition to ubiquitination, ALCAM concentration in the cell surface can be controlled by metalloproteinase cleavage. In a panel of ovarian cancer cell lines, ALCAM was shed by ADAM 17, leading to the generation of a soluble ALCAM composed of most of its extracellular domain [270]. Soluble ALCAM has also been found in ascites and sera of patients, suggesting that a similar process might occur *in vivo*.

1.2.2.4 General functions

Although ALCAM was initially identified and primarily expressed in activated leukocytes, it is widely expressed in human tissues and cells, including neuronal, immune, epithelial, and stem cells of hematopoietic and mesenchymal origin [251]. ALCAM contribution has been described in T-cell activation and proliferation, axon fasciculation, angiogenesis and hematopoiesis. However, the pathologic state of ALCAM expression has been associated with different cancers, such as melanoma, colon, prostate, breast, ovarian, bladder and oesophageal squamous cell cancers [271].

- **ALCAM in hematopoietic cells**

ALCAM and CD6 are actively recruited at the T-cell-APC (antigen-presenting cell) interface and enable immunological synapse stabilization [272]. ALCAM-CD6 interaction contributes to the early and later stages of dendritic cell induced T-

cell activation and proliferation [261]. In addition, ALCAM plays a role in mediating the transmigration of T-cells and monocytes across the blood-barrier [273,274]. ALCAM-CD6 interaction results in activation of three mitogen-activated protein kinase cascades, ERK1/2, p38 and JNK [275].

- **ALCAM in vertebrate development**

ALCAM is expressed on endometrial luminal and glandular epithelial cell surfaces and on the blastocyst cell surface [127] but not in embryos, at the 8-cell or morula stages. However, ALCAM expression presents a distinctive, temporal and spatial distribution in development of several tissues [276,277]. In a Xenopus model, loss of ALCAM results in lack of cardiac morphogenesis [278]. Moreover, ALCAM is strongly associated with haematopoiesis and vasculogenesis [279].

The knockdown of Alcama (the zebrafish ortholog of mouse and human ALCAM) in zebrafish presented imperfections in neural crest differentiation, resulting in cartilage defects [280]. Alcama played a crucial, non-autonomous role in pharyngeal endoderm during zebrafish cartilage morphogenesis. Moreover, double knockdown of Alcama and Nadl1.1 (the zebrafish ortholog of mouse and human L1CAM) evidenced that the two proteins interact during cartilage morphogenesis.

ALCAM has also been described as a guidance molecule in migration and neuronal outgrowth during development [276,281].

- **ALCAM in multipotent and stem cells**

ALCAM has been used, with other markers, to define multipotent cells from a wide spectrum of tissues like umbilical cord blood [282], testes [283], foetal lung [284], bone marrow [285], dental pulp [286] and intervertebral disc [287]. However, the exact function of ALCAM in the multipotent capacity of these cells still remains not fully understood.

- **ALCAM in neuronal network**

ALCAM has been involved in the extension of axons [263,264] as well as in axonal guidance and mapping [248]. Moreover, as explained before, ALCAM knockout mice presented irregularities in axon fasciculation and a delay in maturation of neuromuscular junctions [247,248].

- **ALCAM in cancer**

ALCAM was first identified as MEMD in melanoma cell lines [288]. Since then, ALCAM has been associated with tumorigenesis of many cancers comprising melanoma [289], prostate [290], colorectal [291], breast [292,293,294], ovarian [295], oral [296], pancreatic [297] and thyroid cancer [298].

In those cancers, the potential use of ALCAM as a biomarker of recurrence was evidenced, but it has been linked to contradictory results, greatly limiting its value to predict a clinical outcome. In fact, even for the same type of malignancy ALCAM has been described as a marker of good prognosis, of poor prognosis, or even completely unrelated to survival (Table 12).

Tumour type	Level of ALCAM in studied malignancies
Melanoma	Increased expression in vertical phase growth
Prostate cancer	Up-regulated in low-grade tumours compared to high-grade tumours
Breast cancer	Reduced expression is associated with poor prognosis (nodal involvement, higher grade, higher TNM stage, and worse NPI) and clinical outcome (local recurrence and death)
Breast cancer	Intraductal and invasive carcinomas have high ALCAM expression; high cytoplasmic ALCAM expression is associated with shortened patient disease-free survival
Colorectal cancer	Up-regulation is an early event in malignant transformation; membranous ALCAM expression correlates with shortened patient survival
Colorectal cancer	Loss of membranous ALCAM indicated worse patient prognosis
Colorectal cancer	No relation with patient survival
Bladder cancer	Increased expression in bladder cancer with correlation between ALCAM expression and stage/grade
Oesophageal squamous cell carcinoma	Overexpression of ALCAM is associated with poor prognosis (late clinical stage, enhanced tumour invasiveness, and nodal metastasis)

Table 12. ALCAM levels in various malignancies, adapted from Ofori et al. [300].

Also, ALCAM localization within the tumour (membranous or cytoplasmic staining) has been linked to different outcomes. The role of ALCAM in cancer seems related to the specific context and its function appears induced by the

tumour tissue of origin. Some authors have associated these contradictory results to the heterogeneous shedding of the protein within the same tumour [299]. Serum levels of ALCAM have recently been explored as a diagnostic tool in ovarian, oesophageal, pancreatic, breast, thyroid and colon malignancies [299,301–305].

Although the exact function of ALCAM still remains not fully understood, its participation in cancer progression has been extensively studied. Interestingly, *in vivo* mouse model studies [257,306] based on the upregulation of a truncated ALCAM, lacking the D1 domain, reflected reduced subcutaneous tumour growth but were accompanied by an accelerated spontaneous lung metastasis in a melanoma transplant tumour model. On the contrary, a secreted variant of ALCAM containing only the V-type domain 1 (D1) has been observed to confer ligand binding and attenuated invasion in the highly metastatic BLM melanoma cells [307].

ALCAM-mediated cell-cell adhesion has also been shown to contribute to MMP-2 activation. BLM melanoma cells transfected with a dominant-negative ALCAM mutant (ΔN-ALCAM) presented inhibition of MMP-2 activation, but increased invasive behaviour, suggesting less dependence on MMP-2 activity. The authors evidenced the role of ALCAM as a cell density sensor and initiator of a signal toward MMP-2 activation [308].

Further research is needed to evaluate the contribution of ALCAM in each tumour type, as well as to study its interactions with other cellular intermediates, and to determine whether ALCAM could be a potential therapeutic target to treat primary or metastatic disease.

Chapter 2. Objectives

Around 80% of endometrial cancer patients are diagnosed when the tumour is still localized and confined to the uterus, which is usually associated with a better prognosis. The recurrence risk assessment is defined based on the evaluation of clinical prognostic factors like FIGO staging, grade, and tumour histology, to guide the management of patients. Early-diagnosed patients are categorized following this classification and receive the established treatment according to their assessed risk. However, a small but consistent number of these patients diagnosed continue to relapse or even die of the disease. Therefore, it is of vital importance to find markers to predict recurrence for all those patients for whom a sufficiently robust risk factor is still not known.

Regarding the other 20% of patients, diagnosed at a more advanced stage of the disease, they present myometrial invasion and/or lymph node affectation, which are both related to a poor prognosis and patient survival. In particular, myometrial invasion is one of the most relevant risk factors, being strongly correlated with high-risk cancer and being also associated with an increase in the rate of recurrence and a decrease in the 5-year survival rate. Despite myometrial invasion supposing a critical step in cancer dissemination, the molecular mechanisms involved in the acquisition of migratory and invasive

capabilities of tumour cells, which allow the infiltration of the myometrium, still remain unclear.

Cancer progression combines multiple processes, which involve many cell adhesion molecules. Aberrant expression of these molecules has been related to loss of cell-cell adhesion, disaggregation of tumour cells from the lamina propria, and acquisition of an invasive and migratory phenotype. ALCAM is a member of the immunoglobulin superfamily, which participates in both homotypic and heterotypic interactions between adjacent cells. In the last few years, ALCAM expression has been related to the tumorigenesis of many cancers, but its exact function and mechanisms are still not fully understood.

Under this scenario, this thesis has focused on two specific objectives:

(i) The study of ALCAM as a marker of recurrence in endometrioid endometrial cancer and its function in tumour progression.

(*ii*) The characterization of the molecular mechanisms associated with ALCAM in the superficial and the invasive front of the tumour and its relation to myometrial infiltration.

Both objectives aimed to improve survival of endometrial cancer patients. The former will enhance the accuracy of current methods for risk assessment; and the latter will permit the identification of key molecules in the myometrial invasion process to advance personalized treatment.

The tasks of these two main research objectives, are presented as follows:

(i) The study of ALCAM as a marker of recurrence in endometrioid endometrial cancer and its function in tumour progression.

a. Retrospective multicentre study to evaluate the immunohistochemical expression of ALCAM in endometrioid endometrial tumours, and specifically in the early stage of the disease, as a reliable marker of recurrence.

b. The lentiviral silencing, by using two specific hairpins, of ALCAM in Hec1A and Ishikawa endometrial cancer cell lines and its validation at mRNA and protein level.

c. A behavioural study of the Hec1A and Ishikawa endometrial cancer

cell lines and their stable populations with ALCAM downregulation, examining cell migration on a 2D and 3D model, invasion, cell-cell adhesion by measuring the strength with a dual micropipette technique and cell proliferation.

d. An orthotopic murine model, by inoculating Hec1A shControl and shALCAM cells directly into the uterus of mice, to evaluate the effects of ALCAM depletion in primary tumour and metastasis formation.

e. A microarray study profiling to elucidate ALCAM-dependent gene expression in Hec1A cell line and the identification of the principal altered functions and pathways by using IPA and PANTHER software.

f. Validation of the ALCAM-dependent genes by using RT-qPCR in Hec1A ALCAM-depleted and ALCAM-overexpressing cells. This is conducted with the aim of finally validating, by western blot, the selected target genes presenting a coherent behaviour between the ALCAM loss- and gain-of-function.

(ii) The characterization of the molecular mechanisms associated with ALCAM in the superficial and the invasive front of the tumour and its relation to myometrial infiltration.

a. The immunohistochemical study of ALCAM and its relation with a set of epithelial and mesenchymal markers, which are of relevant importance in endometrial cancer dissemination, at the superficial and the invasive front of endometrioid endometrial tumours.

b. The detection of soluble ALCAM in uterine aspirates by ELISA and its evaluation to discriminate patients presenting myometrial infiltration.

c. A study of ALCAM cleavage by MMP-9. The MMP-9 expression and activity in uterine aspirates were measured by using the zymography and correlated with soluble ALCAM in the same biological fluid.

d. An orthotopic murine model, by inoculating Hec1A luciferase and Hec1A-ETV5 luciferase overexpressing cells directly into the uterus of the mice, to evaluate the immunohistochemical expression and localization of ALCAM in the inner tumour and at the invasive front. In addition, this *in vivo* model, which mimics the first steps of myometrial infiltration, leads to the study of the relationship between ALCAM, ETV5 and MMP-9.

e. A behavioural study of the Hec1A-ETV5 mesenchymal endometrial cell and the effects of full-length and cytoplasmic-truncated ALCAM recovery, by examining cell migration using an aggregate spreading on a fibronectin coated surface and cell-cell adhesion using the dual micropipette technique.

f. Characterization of the ERK1/2 pathway involved in ALCAM rescue in Hec1A-ETV5 overexpressing cells by western blot.

Chapter 3. Materials and methods

The materials and methods used in this thesis are described in this section including the individual specifications for each objective (i) and (ii).

3.1 Human endometrial cancer samples

Ethical approval was obtained by each participating institution. Samples were obtained after informed consent was signed.

3.1.1 Tissues

Objective (i):

For the 10-year retrospective multicentre study, a total of 174 EEC formalin fixed paraffin embedded tissue samples were recruited from: Vall d'Hebron Hospital (Barcelona, Spain), Arnau de Vilanova Hospital (Lleida, Spain), Hospital del Mar (Barcelona, Spain), University Hospital of Santiago de Compostela (Santiago de Compostela, Spain), MD Anderson Cancer Center Madrid (Madrid, Spain) and Virgen del Rocio Hospital (Sevilla, Spain). The clinicopathological parameters of these patients are described in Table 13.

Variable	Σ (%)
Age	172 (100.0)
<64 years	76 (44.2)
≥64 years	96 (55.8)
FIGO Stage	172 (100.0)
Stage IA	85 (49.4)
Stage IB	49 (28.5)
Stage II	25 (14.5)
Stage III	13 (7.6)
Grading	172 (100.0)
Grade 1	99 (57.6)
Grade 2	46 (26.7)
Grade 3	27 (15.7)
Recurrence	174 (100.0)
No	122 (70.1)
Yes	52 (29.9)

Table 13. TMA description (i).

Objective (ii):

A total of 116 endometrioid endometrial carcinomas (EEC) formalin-fixed, paraffin-embedded tissue samples were recruited from the Pathology Department of the Hospital del Mar (Barcelona, Spain). The Clinicopathologic characteristics of these patients are detailed in Table 14.

Variable	Σ (%)
FIGO Stage	116 (100.0)
Stage IA	56 (48.3)
Stage IB	41 (35.3)
Stage II	15 (12.9)
Stage III	4 (3.4)
Grading	116 (100.0)
Grade 1	79 (68.1)
Grade 2	24 (20.7)
Grade 3	13 (11.2)
Myometrial Infiltration	116 (100.0)
No	58 (50.0)
Yes	58 (50.0)

Table 14. TMA description (ii).

3.1.2 Uterine aspirates

A total of 40 uterine aspirates from patients diagnosed with endometrial cancer were collected in the Vall d'Hebron Hospital (Barcelona, Spain). Uterine aspirates were collected by aspiration with a Cornier Pipelle (Eurogine Ref. 03040200) in the office of the gynaecologist or prior to surgery in the operating room and transferred to 1.5 ml microtubes. The clinical and pathological characteristics of the patients are described in Table 15.

Variable	Σ (%)
FIGO Stage	40 (100.0)
Stage IA	15 (37.5)
Stage IB	12 (30.0)
Stage II	7 (17.5)
Stage III	3 (7.5)
Stage IV	3 (7.5)
Myometrial Infiltration	40 (100.0)
No	17 (42.5)
Yes	23 (57.5)

Table 15. Clinicopathological parameters of selected uterine aspirates.

3.1.2.1 Uterine aspirates processing

Phosphate buffer saline 1x (PBS) was added in a 1:1 (v/v) ratio to uterine aspirates, and centrifuged at 2,500 rcf for 20 min in order to separate the soluble fraction (supernatant) from the solid fraction (pellet). The separated fractions were kept at −80°C until use.

3.2 Protein detection

3.2.1 Immunohistochemistry

Immunohistochemistry (IHC) is based on the utilization of monoclonal and/or polyclonal antibodies for the detection of specific antigens in tissue sections. IHC is an important application to determine the tissue distribution of an antigen of interest in health and disease. It is widely used for diagnosis of cancers because specific tumour antigens are expressed *de novo* or upregulated in certain cancers. For visualizing the antibody-antigen interaction, the secondary antibody is conjugated to an enzyme such as peroxidase that will catalyse a colour-producing reaction.

Objective (i):

Two different tumour areas of each patient tissue block were selected for tissue microarray (TMA) construction. Sections were incubated with the primary antibody for 1 h at room temperature using 1:100 mAb ALCAM (MOG/07; Abcam, Cambridge, USA). Epitope retrieval was performed in citrate buffer pH 9. After incubation, the reaction was visualized with the EnVision FLEX Detection Kit (DAKO, Glostrup, Denmark) using diaminobenzidine chromogen as a substrate. Sections were counterstained with haematoxylin & eosin. A pathologist evaluated ALCAM expression using two criteria: an intensity score (ranging from 1-3) and a % of positive staining cells [0-100%]. The product of the two assessments yielded final values on a scale ranging from 0-300. The cut-off for the dichotomization corresponds to the first quartile of the distribution of the ALCAM expression value in the cohort: ALCAM positive ≥ 30.

Objective (ii):

For each patient, 2 spots from the superficial area and 2 spots from the invasive front were selected for TMA construction. ALCAM, E-cadherin, β-catenin, ETV5, COX-2, MMP-2 and MMP-9 immunohistochemical expressions were analysed in both areas of the tumour. To detect extracellular ALCAM ectodomain, antigen retrieval and primary antibody incubation were performed as in objective (i). To detect MMP-2, MMP-9, and ETV5, citrate buffer pH 9 was used for antigen retrieval. Then the sections were incubated with 1:100 rAb ETV5 (H-100; Santa Cruz Biotechnology, CA, USA), 1:50 mAb MMP-2 (CA-4001; Abcam, Cambridge, MA, USA) and 1:50 rAb MMP-9 (3852; Cell Signalling Technology, Beverly, MA, USA) for 1h (MMPs) or 2h (ETV5) at room temperature. Detection of COX-2 was performed with antigen retrieval citrate pH 6 buffer and incubation was performed overnight using 1:100 gAb COX-2 (M-19; Santa Cruz Biotechnology, CA, USA). Primary antibodies mAb E-cadherin and mAb β-Catenin (36 and 14; Roche, Basel, Switzerland) were used with Ventana Benchmark automated slide stainer, 24 min at 36ºC.

A pathologist evaluated the expression of each protein using immunoreactive scores (IRS). ALCAM, E-Cadherin and COX-2 expressions were evaluated from the product of the intensity (ranging from 1-3) and the percentage [0-100%] of neoplastic cells with positive staining. The product of the two assessments yielded final values on a scale ranging from 0-300. ETV5, β-catenin, MMP-2 and MMP-9 were only evaluated based on the “percentage”, and so, in scale IRS [0-100%]. For the selected candidates, the IRS was dichotomized (positive/negative), by using the average of their staining (calculated with both the superficial and the invasive staining) and the cut-off value was established with the first quartile of the distributions of the ALCAM, ETV5 and MMP-9 expressions values (Tukey’s first hinge). The cut-off for each protein was: ALCAM positive ≥ 65, ETV5 positive ≥ 25, MMP-9 positive >5.

3.2.2 Western blot and protein cell extraction

Western blot (WB) is a technique used to detect specific proteins in

homogenate tissue samples or cellular extracts. Gel electrophoresis is used in order to separate denatured proteins by length and electric charge. Then, the proteins are transferred to a nitrocellulose or PVDF membrane. Finally, specific antibodies are used to target the desired protein.

Before protein extraction, cultured cells were washed with PBS 1x and then scraped with 1 ml of PBS 1x. Pellets obtained after centrifugation during 5 minutes at 500 rcf were used to extract total protein. Cell lysates were obtained using RIPA buffer (Tris 20 mM pH 8.8, NaCl 150 mM, EDTA 5 mM, Triton X-100 1%, 1:100 protease inhibitors) and PhosphoStop 1x (Roche, Basel, Switzerland), incubated at 4ºC for 1h and passed through a syringe for cell disruption. After 15 minutes of centrifugation at 15,000 rcf, supernatants containing proteins were quantified by BioRad DCTM Protein Assay (Reagent A, Reagent B and Reagent S, Bio-Rad Laboratories, Hercules, CA, USA) and then boiled with Laemmli Buffer (100 mM Tris-HCl pH 6.8, 4% SDS and 20% glycerol) during 5 minutes at 95ºC. After centrifugation, the protein fractions were stored at -20ºC until use.

For western blot, samples were run on a 10% SDS-PAGE and transferred to a PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked in 5% non-fat milk solution (TBS-0.1% Tween) for 1 hour at room temperature and incubated with indicated primary antibody diluted in 5% non-fat milk solution overnight at 4°C. The membranes were washed 3 times for 10 minutes in TBS-0.1% Tween at room temperature and incubated for 1 hour with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibody. Proteins were detected by the Enhanced Chemiluminescence System (Amersham Pharmacia Biotech, Little Chalfont, UK) as described by the manufacturer's instructions.

Incubation with primary antibodies was performed using 1:500 mAb ALCAM (MOG/07, Abcam, Cambridge, UK); 1:500 mAb LAMC2 (CL2980, Abcam, Cambridge, USA); 1:2000 rAb α-Tubulin, (11H10, Cell Signalling, Beverly, MA, USA); 1:2000 rAb TXNRD1, (HPA001395, Atlas, Bromma, Sweden); 1:1000 rAb FLNB, (HPA004747, Atlas); 1:2000 rAb ERK1/2 (9102, Cell Signalling, Beverly); 1:1000 rAb P-ERK1/2 (9101, Cell Signalling).

3.2.3 Enzyme-linked immunosorbent assay (ELISA)

The ALCAM Duoset ELISA kit (R&D Systems, Minneapolis, MN, USA) was used to detect ALCAM levels in the supernatant of the uterine aspirates. All samples were diluted 1:100 in BSA 1%. All steps were performed at room temperature.

For the plate preparation, the 96-well microplates were coated with 100 µl/well of capture antibody at 2 µg/ml and incubated overnight. Each well was washed three times with 400 µl of 0.05% Tween 20 in PBS 1x. Then, plates were blocked by adding 300 µl of BSA 1% to each well, incubated for 1 h and afterwards washed.

For the assay procedure, 100 µl of each sample and standards were added per well and incubated 2 h. After washing, 100 µl/well of the detection antibody at 100 ng/ml were added and incubated 2 h. Subsequently to another washing step, 100 µl/well of Streptavidin-HRP (1:200) were added and incubated for 20 min. After washing, 100 µl/well of substrate solution (3,3',5,5'-tetramethylbenzidine (TMB), hydrogen peroxide (H2O2), and proprietary catalysing and stabilising agents) were added and incubated for 20 min in the dark. The reaction was stopped by the addition of 50 µl of 2N H2SO4 to each well. The optical density of each well was immediately determined, by using a microplate reader set to 450 nm and 570 nm for correction.

3.2.4 Immunofluorescence

Immunofluorescence (IF) uses the specificity of antibodies to their antigen to target fluorescent dyes to specific biomolecules, allowing the visualization of their expression and distribution in the cell.

IF was used to localize ALCAM-cherry in the transiently transfected Hec1A-ETV5 cells. Cells seeded onto glass coverslips were fixed with 4% paraformaldehyde (PFA) for 15 min, treated with 50mM NH4Cl for 30 min to prevent autofluorescence, blocked in PBS BSA 4% and incubated with DAPI for 15 min at room temperature in the dark. Coverslips were mounted using

the Aqua/Poly Mount medium (Polysciences Europe GmbH, Eppelheim, Germany). Fluorescence images were captured with Spectral Confocal Microscope FV1000 (Olympus, Hamburg, Germany).

3.3 Zymography

Zymography is an electrophoretic method for measuring proteolytic activity. It is based on a sodium dodecyl sulphate gel impregnated with a protein substrate, which is degraded by the proteases resolved during the incubation period. Coomassie blue staining of the gel reveals sites of proteolysis as white bands on a dark blue background.

Proteins with metalloprotease activity (MMP-2 and -9) were identified by zymography. Equal quantities of protein, from the uterine aspirates, were size fractionated under non-reducing conditions on SDS-PAGE gels impregnated with gelatin (7.5%; 1.5 mg/ml), at 20 mA/gel, 4ºC. Gels were washed with 2.5% TritonX-100 (2x15 min); 50 mM Tris-HCl pH 7.5/2.5 Triton X-100 (2x15 min); 50mM Tris-HCl pH 7.5 (2x15 min) to remove SDS, before incubation in assay buffer (50 mM Tris-HCl 7.5/150 mM NaCl/10 mM CaCl2; 1% Triton X-100; NaN3 0.02%; 24h, 37ºC, gentle shaking). Enzyme activity was visualized by staining the gels with 0.25% Coomassie Blue, and appeared as clear bands within the stained gel. Activity was semiquantitatively determined by densitometry. One control sample was loaded on each gel to normalize band intensities between gels.

3.4 Cell lines, constructs and cell lines generation

3.4.1 Human cell lines

Hec1A (ATCC®HTB-122^{TM}) and Ishikawa (#99040201, Sigma-Aldrich, St Louis, MO, USA) endometrial cancer cell lines were grown in McCoy's and DMEM/F-12 culture media (BioWest, Nuaillé, France), respectively, supplemented with 10% FBS and 1% penicillin-streptomycin in a 5% CO2

humidified atmosphere at 37ºC. Both cell lines were established from an endometrial adenocarcinoma. The Hek 293T human embryonic kidney cell line was maintained in DMEM (Gibco, Grand Island, NY, US) supplemented with 10% FBS and 1% penicillin-streptomycin in a 5% CO2 humidified atmosphere at 37ºC.

3.4.2 Lentiviral stable generation cell lines for ALCAM knockdown

Hec1A (ATCC®HTB-122^{TM}) and Ishikawa (#99040201, Sigma-Aldrich, St Louis, MO, USA) endometrial cancer cells were used for infection. To generate downregulated stably transfected cells, we used the lentiviral pGIPZ vectors: shALCAM1 (V3LHS-3600072), shALCAM2 (V3LHS-3600074) and the empty control vector, shControl (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA).

To generate downregulated stably transfected cells, we used lentiviral vectors carrying a CMV-driven Lac Z gene packaged with attenuated HIV-derived constructs and pseudotyped with VSV-G envelope, prepared by transient transfection of Hek 293T cells, together with pGIPZ against ALCAM. The 293T cells were split into 100 mm culture dishes at a density of 4x10^6 cells per plate using 10 ml of DMEM media supplemented with 10% FBS and 1% penicillin-streptomycin. One hour prior to transfection, the medium was changed for free DMEM without FBS or antibiotics. The viral, packaging and envelope vectors (ratio 3:2:1, respectively) were put in 0.5 ml of H2O and 62.5 µL of CaCl2 2M. The mixture was added dropwise in 0.5 ml of buffer HBS 2x at pH 7.1 (0.28 M NaCl, 0.05 M HEPES and 1.5 mM Na2HPO4; optimal pH range, 7.00–7.28) under continuous bubbling. The day after transfection, the medium was replaced by new completed DMEM. Lentiviral supernatants were collected 48 h after transfection, centrifuged at 2,500 rpm to precipitate any 293T cells and filtered through 0.45-µm-pore-size filters. Cleaned lentiviral medium was used to infect cells in the presence of polybrene (8 µg/ml; Millipore, Billerica, MA, USA). Fresh medium was added to the 293T cells and the collection and infection was repeated the following day. Puromycin (1

µg/ml, Sigma, St. Louis, MO, USA) was used to select and maintain a mixed population of the shRNA expressing cells.

3.4.3 ETV5 and ALCAM overexpression in endometrial cancer cell lines

The modified Hec1A cell line for the overexpression of ETV5 was previously generated and characterized by Monge M. et al. [191] and maintained in selection with Geneticin (500 µg/ml; Invitrogen, Carlsbad, CA, USA).

For ALCAM overexpression, we amplified ALCAM full-length and ALCAM without the cytoplasmic tail (ALCAMcytoless) from an EC tissue, using the forward primer 5'-GCAACTCGAGATGGAATCCAAGGGGGCCA-3', and 2 reverse primers 5'-GCTTGAATTCCGGCTTCAGTTTTGTGATTGTT-3' and 5'-GCTTGAATTCGCAGCCAGTAGACGACACCAG-3', respectively. Both amplified regions were inserted in pmCherry-N1 vector (Clontech Laboratories, Mountain View, CA, USA) with *Xho*I and *Ecor*I. Transfection was performed using Lipofectamine 2000 (Invitrogen, Life technologies, Carlsbad, CA, USA) following manufacturers' instructions.

Full-length ALCAM was stably overexpressed in Hec1A. To generate stable cultures, cells were selected and maintained with Geneticine (500 µg/ml; Invitrogen, Carlsbad, CA, USA) and the Cherry positive mixed population was selected by flow cytometry. The two constructs of ALCAM overexpression were transiently transfected in Hec1A-ETV5.

3.4.4 Luciferase expression in Hec1A and Hec1A-ETV5 cell lines

To monitor non-invasively tumour grafts of Hec1A and Hec1A-ETV5 cells, they were infected with lentivirus bearing pLenti CMV V5-LUC Blast (w567-1) (Addgene, Cambridge, MA, USA) to constitutively express the luciferase reporter gene. Lentivirus transfection and stable cells expressing luciferase generation were performed by L. Alonso-Alconada and M. Abal in the University Hospital of Santiago de Compostela (CHUS). To produce lentiviral particles, 293T cells were co-transfected with polyethyleneimine (PEI method)

with the virion packaging elements (VSV-G and D8.9) and the FSV on 293T human embryonic kidney. Supernatants were collected after 3 days, concentrated by centrifugation through a filter column of 100 kDa (VWR International LLC, West Chester, PE, USA) for 1 h at 4,000 rpm. Cells were incubated overnight in the presence of medium containing lentiviral particles. After this period, medium was replaced for fresh medium and cells were incubated for at least 72 h to allow endogenous protein knockdown. Stable infected cells expressing luciferase were selected with Blasticidine S HCl (3 µg/ml; Invitrogen, Carlsbad, CA, USA).

A summary of the generated cells with each specific modification is illustrated in Table 16.

Cell line	Modification
Hec1A shControl	Hec1A + empty shControl vector
Hec1A shALCAM1	Hec1A + shALCAM1 vector
Hec1A shALCAM2	Hec1A + shALCAM2 vector
Hec1A NCherry	Hec1A + empty pmCherry-N1 vector
Hec1A ALCAM Cherry	Hec1A + ALCAM full-length pmCherry-N1 vector
Hec1A-ETV5 NCherry	Hec1A + GFP-ETV5 vector + empty pmCherry-N1 vector
Hec1A-ETV5 ALCAM Cherry	Hec1A + GFP-ETV5 vector + ALCAM full-length pmCherry-N1 vector
Hec1A-ETV5 ALCAMcytoless Cherry	Hec1A + GFP-ETV5 vector + ALCAM without the cytoplasmic tail pmCherry-N1 vector
Hec 1A Luciferase	Hec1A + pLenti CMV V5-LUC
Hec1A-ETV5 Luciferase	Hec1A + GFP-ETV5 vector + pLenti CMV V5-LUC

Table 16. Summary of generated cells.

3.5 RNA extraction and reverse transcription

3.5.1 Total RNA extraction and quantification

Total RNA was collected and purified using the RNeasy kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Before extraction, cultured cells were washed with PBS 1x and then scraped. Cell suspensions were centrifuged at 1,500 rpm and cleaned again with PBS 1x. Cell pellets obtained after centrifugation were used to extract RNA. To quantify the isolated RNA two different techniques were used:

1) Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA). This microvolume spectrophotomer allows the determination of the absorbance at 260 nm (RNA) and 280 nm (protein) in small volume samples. Sample purity value is given as a ratio of 260/280 nm.
2) Bioanalyzer Assay (Agilent, Santa Clara, CA, USA). This microfluidic-based platform is used for sizing, quantification and quality control of DNA, RNA, proteins and cells. In addition to measuring total RNA, the Bioanalyzer allows the determination of RNA integrity (RNA integrity number, RIN).

3.5.2 RNA reverse transcription

Reverse transcription (RT) is the procedure of converting RNA into DNA. A reverse transcriptase enzyme (DNA polymerase) with other reagents (dNTPs and primers) converts mRNA into complementary DNA (cDNA). By definition, cDNA is double-stranded DNA that originates from the mRNA. RT is needed prior to the determination of gene expression levels inside the cells using RT-qPCR.

One microgram of total purified RNA was used for reverse transcriptase reaction using 1 µl of Random Primers (50 µM, Invitrogen, Carlsbad, CA, USA), 1 µl of dNTPs mixture (10 mM, Promega Madison, WI, USA) and sterile distilled water to reach 13 µl of total volume for each reaction. The

thermocycler program used for the RT is described as follows: 5 min at 65ºC, then an incubation of 1 minute on ice; addition of 4 µl of 5X First-Strand Buffer, 1 µl of DTT (0.1 M, and 1 µl of SuperScript III (Invitrogen, Carlsbad, CA, USA); and incubation for 5 min at 25ºC, 60 min at 50ºC, 10 min at 70ºC, and a final step at 4ºC.

3.6 Gene expression analysis

3.6.1 Quantitative real-time PCR (RT-qPCR)

The polymerase chain reaction (PCR) is a technique used to amplify DNA across several orders of magnitude, generating thousands to millions of copies of a specific DNA sequence. The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Almost all PCR applications employ a heat-stable DNA polymerase, which assembles a new DNA strand by using single-stranded DNA as a template and DNA oligonucleotides, under specific thermal cycling conditions.

Real-time quantitative PCR (RT-qPCR) is a PCR technique used to measure the quantity of a PCR product in real-time. It quantitatively measures starting amounts of DNA, cDNA, or RNA. This technique is commonly used to determine whether a DNA sequence is present and the number of copies in the sample.

RT-qPCR was performed following the manufacturer's protocol for SYBR® Green (Roche, Basel, Switzerland). SYBR® Green is a commonly used fluorescent dye that binds double-stranded DNA molecules by intercalating between the DNA bases. It is used in quantitative PCR because the fluorescence can be measured at the end of each amplification cycle to determine, relatively or absolutely, how much DNA has been amplified. The master mix formulation contains a blend of dTTP and dUTP, which ensures optimal PCR results and compatibility with AmpErase® UNG treatment. In addition, the master mix includes AmpliTaq Gold® DNA Polymerase, LD (Low DNA). The enzyme is provided in an inactive state to automate the Hot Start

PCR technique and allow flexibility in the reaction setup, including pre-mixing of PCR reagents at room temperature. SYBR® Green designed primers are listed in Table 17. Fold-change (FC) expression values were calculated with the ddCT-method. Data were normalized to *GAPDH*.

SYMBOL	Gene ID	Left Primer	Right Primer
IGFBP6	*Homo sapiens insulin-like growth factor binding protein 6, mRNA.*	CGAGGGGCTCAAACACTCTA	ACAGCTCCATGTTCCTTCCA
NDRG1	*Homo sapiens N-myc downstream regulated gene 1, mRNA.*	GTGAATGACATGAACCCCGG	AGTGGTCTTTGTTGGGTCCA
CYFIP2	*Homo sapiens cytoplasmic FMR1 interacting protein 2, mRNA.*	CAACGAGACAAACCTGCCAA	CACAGCGATGCCCTGATAAC
FHL2	*Homo sapiens four and a half LIM domains 2, mRNA.*	GCAAGAAGTACATCCTGCGG	CCACCAGTGAGTTTCTGCAC
LAMB3	*Homo sapiens laminin, beta 3, mRNA.*	CAGACCGGACCTATGGAGAC	TAGGTCTGGAAGCAAGGGTG
LAMC2	*Homo sapiens laminin, gamma 2, mRNA.*	CTGGTAATGGATTCCGCTGC	CATCCGTGAGCATGTGGAAG
CD44	*Homo sapiens CD44 molecule (Indian blood group), mRNA.*	TCCTCACATCCAACACCTCC	GCTGCTCACGTCATCATCAG
KLK6	*Homo sapiens kallikrein-related peptidase 6, mRNA.*	TTGTGTGCTGGGGATGAGAA	TGCAGACGTTGGTGTAGACT
LGALS1	*Homo sapiens lectin galactoside-binding soluble, 1, mRNA.*	AGGCTGTCTTTCCCTTCCAG	CGTCAGCTGCCATGTAGTTG
TXNRD1	*Homo sapiens thioredoxin reductase 1, mRNA.*	GGTCTCGGAGGAACATGTGT	ACTCGGTAGCCCCAATTCAA
MTSS1	*Homo sapiens metastasis suppressor 1, mRNA.*	GTGAGGAGATGGAGGCTTGT	ACTTGTCGAACTCCTGCTGA
FLNB	*Homo sapiens filamin B, beta (actin binding protein 278), mRNA.*	AGTTAACCAGCCAGCATCCT	TGACATCGATGGTGTGGACA
ITGB4	*Homo sapiens integrin, beta 4, mRNA.*	GAGGATGACGACTGCACCTA	AGGCACAGTACTTCCAGCAT
LMTK3	*PREDICTED: Homo sapiens lemur tyrosine kinase 3, mRNA.*	GATTGGGAGTGGCTGGTTTG	TAATCAGCAGAAACGGCAGC
CAPN1	*Homo sapiens calpain 1, (mu/I) large subunit, mRNA.*	CCGGCATCTTCCATTTCCAG	GCCTCGTAGCTGCCATTTAC
S100A14	*Homo sapiens S100 calcium binding protein A14, mRNA.*	TTCAGTGATGTGGAGAGGGC	TCAGCTCCCAGAAACTCCTG
S100A10	*Homo sapiens S100 calcium binding protein A10, mRNA.*	GGACCACACCAAAATGCCAT	GCCATCTCTACACTGGTCCA
PLAU	*Homo sapiens plasminogen activator, urokinase, mRNA.*	TTCACCACCATCGAGAACCA	TTGCGTGTTGGAGTTAAGCC

PLAUR	*Homo sapiens plasminogen activator, urokinase receptor, mRNA.*	ATGCAGTGTAAGACCAACGG	TGATCTTCAAGCCAGTCCGA
ITGB1	*Homo sapiens integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12), mRNA.*	TCTGGGCTTTACGGAGGAAG	TCTACCAACACGCCCTTCAT
EPHA2	*Homo sapiens EPH receptor A2, mRNA.*	GGCTTCTTTATCCACCGCAG	CGAGGATGTCTTCAGCATGC
AHNAK	*Homo sapiens AHNAK nucleoprotein, mRNA.*	GTCTTTGTGCAGGAGGTGAC	GAACCACTTCAGAGCTGCAG
ALCAM	*Homo sapiens activated leukocyte cell adhesion molecule (ALCAM), mRNA.*	TCCTGCCGTCTGCTCTTCT	TTCTGAGGTACGTCAAGTCGG

Table 17. Primers designed for SYBR® Green RT-qPCR.

3.6.2 Microarray analysis

A DNA microarray (also DNA chip or biochip) consists of a collection of microscopic DNA spots attached to a solid surface. Each spot contains a specific DNA sequence, known as a probe or reporter. Gene expression arrays provide a comprehensive view of gene activity in biological samples. Common uses of gene expression microarrays include genome-wide differential expression studies, disease classification, pathway analysis, expression-based quantitative trait loci mapping, among others.

3.6.2.1 Microarray analysis Illumina HumanHT-12 v4

Triplicates of Hec1A shControl and Hec1A shALCAM1 were used to carry out this study. The concentration and the quality of isolated RNA were first measured by Bioanalyzer Assay (Agilent, Santa Clara, CA, USA). Microarray analysis was performed by the Genome Analysis Platform in the CIC Biogune (Derio, Bizkaia, Spain). The gene expression profile was studied with the HumanHT-12 v4 Expression BeadChip (Illumina Inc, San Diego, CA, USA), which targets 31,325 annotated genes with >47,000 probes. RNA was subjected to reverse transcription to produce first and second strand cDNA and biotin-labelled cRNA fragments were generated following the manufacturer's instructions. The labelled cRNA was hybridized overnight to

the arrays. The beadchips were washed, stained with dye-labelled streptavidin, and scanned with an Illumina IScan to measure the intensity. The raw data images were analysed with Illumina Genome Studio software, which generated the average probe intensity for each sample. Raw data for the analysis were extracted with Illumina's GenomeStudio data analysis software, in the form of GenomeStudio's Final Report (sample probe profile). Gene expression data was analysed using the R/Bioconductor statistical computing environment (www.r-project.org, www.bioconductor.org). Using the lumi Bioconductor package, raw expression data were background corrected, log2 transformed and normalized. Probes not detected in at least one sample were excluded for subsequent analyses as they are considered to represent transcripts that are not expressed.

For the detection of differentially expressed genes, a linear model was fitted to the corrected, transformed and normalized data and empirical Bayes moderated t-statistics were calculated using the limma package from Bioconductor. Adjustment of p-values was done by the determination of false discovery rates (FDR) using the Benjami-Hochberg procedure. Microarray data is available on the GEO database (GSE86543).

3.7 Gene ontology analysis

For the gene ontology analysis, deregulated genes from the microarray with a fold-change >1.2 and an adjusted p-value <0.05 were introduced in the PANTHER database [309] and in the QIAGEN'S Ingenuity® Pathway Analysis (IPA® QIAGEN Redwood City, CA, USA) to identify the most significant canonical routes, biological processes and gene interaction networks in which they are involved.

3.8 Migration 2D and 3D assays

Cell migration is a key process in the development and maintenance of multicellular organisms. Tissue formation during embryonic development, wound healing and immune responses, require the orchestrated movement

of cells. Errors during this process have serious consequences, including tumour formation and metastasis.

3.8.1 Wound healing assay

The wound healing assay is a method that allows the study of directional cell migration *in vitro*. It mimics cell migration during wound healing *in vivo*. The assay consists in making a "wound" in a confluent cell monolayer and then capturing images at time 0h and at different time points until the wound closes. The images are used to quantify the migration rate of the different cell lines.

To perform the wound healing migration assay, we used Hec1A and Ishikawa shControl and shALCAM cell lines. A total of 5-8×10^5 cells were seeded into 24-well plates and cultured until confluence. A wound was made by scraping the cell monolayer with a 10µl pipette tip. Images were captured at 0, 24 and 48 h using an inverted microscope FSX100 (Olympus, Hamburg, Germany) to measure the wound closure rate.

3.8.2 Aggregate model

We used a 3D spheroid model to quantify the spreading of the aggregates with ALCAM-depletion on a fibronectin-coated pattern (Hec1A shControl and Hec1A shALCAM). The aggregate model was also used to test the effect on migration in Hec1A-ETV5 cells after ALCAM-recovery. Moreover, this specific model allows the study of competition between cell–cell and cell–substratum adhesion on tissue spreading.

3.8.2.1 Spheroid formation and coated surface preparation

Aggregates were obtained from 5 ml of cell suspension in CO2-equilibrated culture medium at a concentration of 4x10^5 cells/ml in 25 ml Erlenmeyer flasks and placed in a gyratory orbital shaker at 75 rpm at 37°C for 22 h. The flasks were pretreated with 2% dimethylchlorosilane in chloroform to prevent adhesion of cells to the glass surface.

For the preparation of coated glass substrates: twenty-five mm circular glass coverslips were sonicated in ethanol for 5 min, dried at ambient temperature, and exposed to deep UV for 5 min. Fibronectin (Sigma-Aldrich, St Louis, MO, USA) coating was performed using a 0.1 mg/ml solution of fibronectin in PBS (pH 7.4) for 1 h. A Mixed coating of fibronectin and PEG-PLL (PLL(20)-g[3.5]-PEG(2), Surface Solution) was made by mixing at various rates 0.1 mg/ml fibronectin in PBS and 0.1 mg/ml PEG-PLL in Hepes solution (pH 7.3) for 1 h. Coverslips were then rinsed with PBS (pH 7.4).

3.8.2.2 Aggregate spreading

Cell aggregates were deposited randomly on fibronectin-coated coverslips, which were placed in a magnetic imaging chamber (Chamlide CMB, CM-B25-1) filled with CO2-equilibrated culture medium. Spreading was observed using a NIKON confocal microscope equipped with an x10 air objective and a 37°C heating cube system. Bright field images were recorded with a CCD camera (Luca-R, Andor) using NIS-Elements software every 15 min for 36-48 h. The area of each aggregate was measured with Image J software at time 0h and at the end of the assay (National Institutes of Health, Bethesda, MA, USA).

For Hec1A-ETV5 ALCAM-recovery, cell aggregates were deposited on 200 µm striped fibronectin-coated surface. The increased spreading was measured at different time points with Image J software (National Institutes of Health, Bethesda), which allowed calculating the speed of migration for each cell line.

3.9 Invasion

The movement of cancer cells into surrounding tissue and the vasculature is the first step in the spread of metastatic cancers. Cell invasion is related to, and encompasses, cell migration, but in addition invasive cells move through the extracellular matrix into neighbouring tissues in a process that involves ECM degradation and proteolysis.

3.9.1 Transwell invasion assay

A total of $5x10^5$ cells were seeded on 8 μm pore size transwell filters of the CytoSelect 24-well cell invasion assay kit according to manufacturers' instruction (Cell Biolabs, San Diego, CA, USA), and allowed to invade for 36-48 h. The upper faces of the inserts were coated with a basement membrane matrix solution. Only the cells able to degrade the matrix proteins in the layer and travel through the pores of the polycarbonate membrane are considered invasive cells. Invading cells were stained with DAPI and Phalloidin and counted with Image J (National Institutes of Health, Bethesda, MA, USA).

3.10 Cell-cell adhesion

Tumour cells often show aberrant cell-cell and/or cell-matrix adhesion. This cell-cell adhesion change has been correlated with tumour invasion and metastasis. Cell adhesion molecules are responsible for cell adhesion and they can also function as ligand-activated cell surface receptors, activating signals involved in the regulation of cell shape, migration, proliferation, differentiation, and survival. These two functions show considerable interdependence with the regulatory processes exercising feedback control over cell adhesion, often through inside-out signalling.

3.10.1 Dual-pipette adhesion force assay

The dual pipette assay used here, provides an overall quantification in terms of mechanical force of the adhesive properties of the cells conferred by ALCAM modulation during the development of adhesion.

To preserve intact the cell surface proteins, cells were dissociated with Cell dissociation enzyme-free buffer (Gibco, NY, USA), rinsed and then transferred in working medium (CO2-independent medium, Invitrogen, CA, USA) and used immediately. Two isolated cells were brought gently into contact and held for a predetermined period of time (1, 4, 10 and 20 min). The cells were manipulated in suspension, to eliminate matrix-mediated signalling, by diminishing the contribution of generalized membrane events, and avoiding

the initiation of intercellular adhesion through lamellipodial and filopodial activities. Isolated cells were manipulated at 37ºC with two micropipettes, each held by one micromanipulator connected to a combined hydraulic/pneumatic system and a pressure sensor making it possible to control and measure the aspiration applied to the cells. The cell doublet was cyclically brought back into contact with the left pipette and then withdrawn to the right, each time a stepwise increase in the strength of aspiration by the left pipette was applied, until the cell doublet was disrupted. A pressure sensor measured the aspiration applied to the left pipette. Aspiration was monitored continuously during the separation process, and the values recorded for each of the last two cycles in the series (Pn-1 and Pn) were used to calculate the separation force (SF) for each doublet using the equation: $SF = \pi (d/2)^2$ (Pn-1+Pn)/2 where d is the inside diameter of left pipette. SF was considered to be zero for pairs of cells that did not form adherent doublets in this assay. Results for 15–40 measurements were used to obtain the mean force of separation for a specific contact time.

3.11 Cell proliferation

Cell proliferation and differentiation is crucial from embryogenesis to development of whole organism and also in maintenance of adult tissue homoeostasis. Abnormal cell proliferation has been also associated with various human diseases like cancer. Consequently, cell proliferation assays become critical to examine the rate of cell proliferation *in vitro* and *in vivo*. There exist several assays to determine cell proliferation rate like amount of deoxyribonucleic acid synthesis, metabolic activity of cells, different antigens associated with cell proliferation and adenosine triphosphate concentration. One of the simplest methods uses crystal violet staining.

During cell death, adherent cells detach from cell culture plates. This characteristic is used for the indirect quantification of cell death and finally to determine the difference in the proliferation rate between cells. Crystal violet dye binds to proteins and DNA. Cells that undergo cell death lose their

adherence and are subsequently lost from the cell population, decreasing the amount of crystal violet staining.

3.11.1 Crystal violet

A total of 4,000 cells per well were seeded into 96-well plates. Cell viability was measured after 0, 24, 48, 72, 96 and 120 h. Cells were fixed with 50 µl/well of Formaldehyde 4% and incubated for 20 min. After washing, cells were stained with 100 µl/well crystal violet 0.5% in H2O (Sigma, St Louis, MO, USA) and incubated at room temperature for 20 min. Crystal violet was washed by immersing and shaking plates in water and leaving to dry for 5 min. Then, crystal violet was dissolved in 100 µl/well of 15% acetic acid for 10 min with shaking, and finally the supernatant was read at 595 nm.

3.12 Cell cycle assay for flow cytometry

Cancer is a disease accompanied by uncontrolled cell division. Its development and progression are usually linked to a series of changes in the activity of cell cycle regulators. The DNA of mammalian, yeast, plant or bacterial cells can be stained by a variety of DNA binding dyes. The principle of these dyes is that they are stoichiometric and as a consequence bind in proportion to the amount of DNA present in the cell. Cells that are in S phase will have more DNA than cells in G1. They will take up proportionally higher quantity of dye and will fluoresce more brightly until they have doubled their DNA content. The cells in G2 will be approximately twice as bright as cells in G1.

3.12.1 Propidium iodide DNA staining

Propidium iodide (PI) is an intercalating agent and a fluorescent molecule that can be used to stain cells. After 48 h, a total of $1x10^6$ cells were trypsinized, resuspended in 300 µl of PBS, and fixed with 700 µl of pre-cooled absolute EtOH for 2 h on ice. EtOH was slowly added while mixing to avoid aggregates. Cells were pelleted (5,000 rpm), resuspended in working solution

(940 µl of PBS, 30 µl of solution A and 30 µl of solution B) and incubated for 30 min at room temperature. Finally, cells were analysed by flow cytometry.
Solution A: Sodium Citrate (38 mM) and propidium Iodide (500 µg/ml).
Solution B: RNAse A (10 mg/ml in PBS).

3.13 Mouse model

The use of clinically relevant mouse models, which mimic the tumour growth, progression, invasion and metastases steps, is an essential requirement to better understand the endometrial cancer molecular mechanisms. Previously in the laboratory, we developed an orthotopic murine model that represents a realistic approach towards the process of dissemination of endometrial cancer. Tumour cells are directly injected into the uterus of the mice and so they are localized in the same microenvironment as the original tumour, replicating the processes of tumour growth and myometrial infiltration under endometrial stimuli.

3.13.1 Orthotopic murine model of endometrial cancer

All the procedures were performed according to the guidelines of the Spanish Council for Animal Care and the institutional guidelines for animal welfare (CEEA 23/16).

For objective (i), we tested the ability of Hec1A ALCAM-depleted cells in tumour growth and dissemination. For this, a total of 24 six-week old female athymic nude mice (Charles River Laboratories, Inc, Wilmington, MA, USA) were inoculated by transmyometrial injection of stably transfected Hec1A shControl and Hec1A shALCAM2 cells (n=12 per group). Mice were anesthetized with 2% isofluorane (ABBOT Laboratories, Madrid, Spain), and the lower abdomen was swabbed with Betadine®. A longitudinal incision (medial laparotomy) was performed and the murine uterus was exposed. EC cells were inoculated into the uterine body and the uterine horns. A 27G insulin syringe (Myjector® 1 ml, Terumo, Somerset, NJ, USA) was used for the injection. The strain on the endometrial cavity and the expulsion of a small

quantity of fluid through the vagina ensured the correct localization of the injection. Mice were sacrificed seven weeks after the injection. Tumour growth and dissemination were evaluated macroscopically and histologically *ex vivo*. For histological analysis, all extracted tissues were formalin-fixed, stained with haematoxylin and eosin (H&E), and evaluated by a pathologist. Ki67 expression was scored by using the ACIS® III Instrument automated imaging system (DAKO, Glostrup, Denmark).

For objective (ii), Hec1A control and Hec1A-ETV5 luciferase cells were injected into the uterus of 5 and 4 mice, respectively and following the aforementioned protocol. The *in vivo* model was performed by L. Alonso-Alconada and M. Abal in the CHUS (Santiago, Spain). Mice were followed weekly after cell injection and before sacrifice by using IVIS system (Xenogen Corporation) coupled to Living Imaging software 4.2 (Xenogen Corporation) to detect tumour growth by bioluminescent imaging. For non-invasive bioluminescence tumour imaging, luciferin (Firefly Luciferin, Caliper Lifescience Corp, Hopkinton, MA, USA) was used as the substrate for the luciferase expressing tumour cells and injected intraperitoneally at a concentration of 150 mg/kg in PBS. Mice were sacrificed 3 weeks after injection and before metastases could be observed.

3.14 Statistical analysis

The statistical approaches used for objective (i) are explained as follows:

- **For categorical variables**

The relationship between ALCAM expression and clinicopathological parameters was tested in univariate analysis by chi-square and Fisher's exact test, two-sided p-values. Stratified analysis was performed to study the relation between recurrence and ALCAM status. Follow-up studies were analysed by Kaplan-Meier and Cox’s proportional hazards test. Recurrence-free survival was evaluated in the present study, but not overall survival as the number of deaths from disease did not allow a reliable statistical analysis.

- **For continuous variables**

The normal distribution of data was verified (Shapiro-Wilk or Kolmogorov-Smirnov) and accordingly, parametric (t-test, ANOVA, and multiple post-hoc comparisons (Scheffe, Dunnet) and non-parametric (Mann-Whitney) tests were performed for each comparison.

The statistical approaches used for objective (ii) are explained as follows:

- **For continuous variables**

Univariate regression analyses were performed to evaluate the association between ALCAM and different epithelial and mesenchymal markers at the superficial and the invasive front. Variables that were statistically significant in univariate linear regression analysis were included in the multiple linear regression models. For each cohort, a stepwise method was used to select the explanatory variables based on analysis of variance, checking the normality (Shapiro-Wilk or Kolmogorov-Smirnov) and the independence of residuals (Durbin-Watson).

Receiver operating characteristic (ROC) curve analysis was conducted on individual marker soluble ALCAM (sALCAM), detected in uterine aspirates at diagnosis, to discriminate patients presenting myometrial infiltration. To explore its relationship with the levels of the different forms of MMP-9 present in the uterine aspirates, we performed a factor analysis with extraction of principal component (PCA) for MMP-9 expression and then a regression analysis with sALCAM levels. The normal distribution of data was verified (Shapiro-Wilk) and levels were Ln-transformed for linear analysis.

Non-parametric tests (Mann Whitney) were used for comparisons of data sets whose distributions deviate from normal.

- **For categorical variables**

The relationships between ALCAM expression, at the superficial and the invasive front, and clinicopathological parameters were tested in univariate analysis by Chisquare and Fisher's exact test. Stratified analysis was performed to study the molecular features ALCAM/MMP-9/ETV5 and the correlation between ALCAM/MMP-9 and clinicopathological parameters. The

odds ratios (OR) were calculated across the strata, with their 95 % confidence interval (IC 95 %) obtained through bootstrapping methods. In addition, logistic regression multivariate analysis was done for the clinicopathological parameters and status biomarkers to determine independent predictors for myometrial infiltration. Categorical variables are presented as an absolute number and percentage.

For both objectives, measurements were made in triplicate in three independent experiments and presented with the Mean±SD (except for the separation force experiments, which were presented by Mean±SEM). All statistical analyses were performed using the IBM SPSS Statistics 21. All two-sided pvalues<0.05 were considered statistically significant.

Chapter 4. Results

The results section is organized into two blocks, where we provide the results obtained when addressing the two main objectives of this thesis, previously described in Chapter 2.

4.1 ALCAM as a marker of recurrence and as a promoter of tumour progression in EEC

4.1.1 Framework

As stated in Chapter 1, 1.1.4.2 section, abnormal vaginal bleeding is an early symptom of endometrial cancer and as consequence allows the diagnosis of around 75% of patients at an early stage of the tumour. Recurrence risk groups are basically defined based on the assessment of clinical prognostic factors like age, FIGO staging, grade, and histology, to guide the management of patients. Despite those early-diagnosed patients are classified and treated according to their established risk of recurrence, a number of them relapse or even die of the disease.

For this reason, in order to improve the proper classification of these patients, the main objective of this section was to evaluate the immunohistochemical

determination of ALCAM in 174 EEC primary tumours as a reliable marker of recurrence. In addition, we used *in vitro* and *in vivo* orthotopic murine models as well as microarray technology, to unveil the functions regulated by ALCAM to promote EEC dissemination and metastasis.

4.1.2 ALCAM-positive expression is a marker of recurrence in early stage moderately-poorly differentiated tumours

Among the 174 EEC patients analysed for ALCAM expression, we found 131 (76.20%) to be ALCAM-positive (Table 18).

Variable, No. (%)	Σ	ALCAM Positive*	ALCAM Negative†	P-value
Age	172	131 (76.2)	41 (23.8)	0.589
≤64 years	76	56 (73.7)	20 (26.3)	
>64 years	96	75 (78.1)	21 (21.9)	
FIGO	172	132 (76.7)	40 (23.3)	0.125
Stage IA	85	70 (82.4)	15 (17.6)	
Stage IB	49	38 (77.6)	11 (22.4)	
Stage II	25	16 (64.0)	9 (36.0)	
Stage III	13	8 (61.5)	5 (38.5)	
Grade	172	130 (75.6)	42 (24.4)	0.049
Grade 1	99	81 (81.8)	18 (18.2)	
Grade 2	46	29 (63.0)	17 (37.0)	
Grade 3	27	20 (74.1)	7 (25.9)	

*ALCAM positive ≥ 30
†ALCAM negative < 30

Table 18. Clinicopathologic parameters according to ALCAM expression (N=174).

Expression of ALCAM was homogeneous across the section, and predominantly localized at the membrane and the cytoplasm (Figure 26-D). Of the included cancers, 77.9% were early stage EEC (stage IA and IB according to the FIGO classification), containing 88 well-differentiated (G1) and 45 moderately-poorly differentiated tumours (G2-G3); whereas 22.1% of remaining cases were diagnosed at advanced stages.

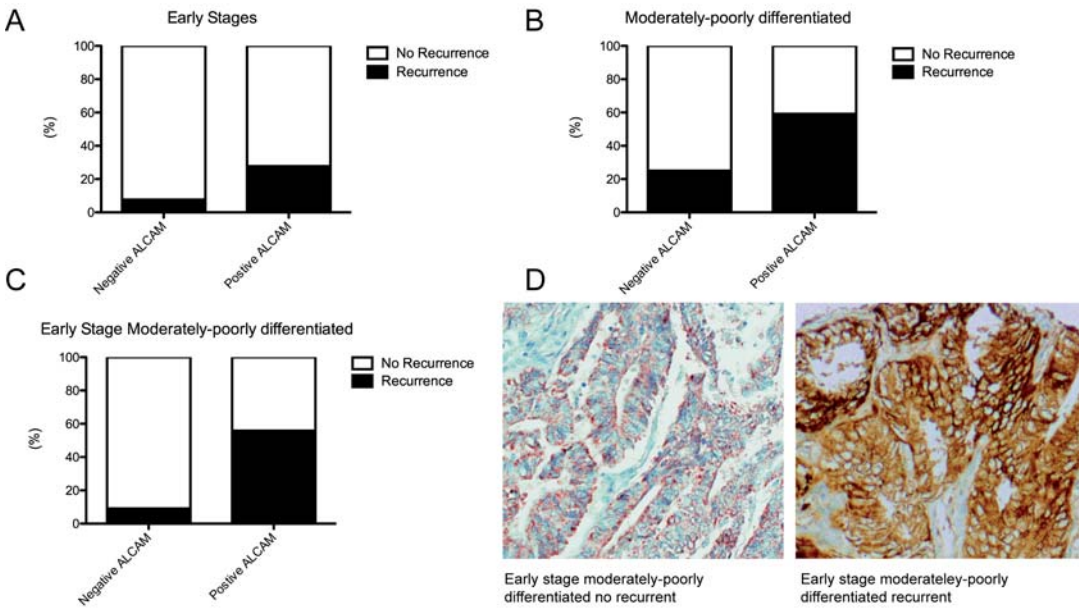


Figure 26. ALCAM-positivity is a marker of recurrence. (A) Patients diagnosed with tumours in early stage (rate of recurrence in ALCAM positive tumours is 27.8% vs. ALCAM negative tumours is 7.7%, p=0.039); (B) patients diagnosed with moderately-poorly differentiated tumours (59.2% versus 25%, p=0.007); and (C) patients diagnosed with early stage moderately-poorly differentiated tumours (55.9% versus 9.1%, p=0.012). (D) Representative images of ALCAM expression in a recurrent (left) and non-recurrent (right) early stage moderately-poorly differentiated tumour.

The univariate analysis between ALCAM expression and the most common prognostic factors only showed a minor correlation with tumour grade when studied in the whole population (Table 18). Interestingly, in a stratified analysis, ALCAM expression showed a significant correlation with the recurrence for patients diagnosed in early stage (N=134) (p=0.039), with moderately-poorly differentiated tumours (N=73) (p=0.007), and with tumours combining these two clinical parameters (N=45) (p=0.012) (Table 19). Specifically, in the subset of patients diagnosed at early stage, the recurrence rate was significantly higher for ALCAM-positive patients compared to ALCAM-negative patients (27.8% vs 7.7%). The results were similar for patients with moderately-poorly differentiated tumours (59.2% vs. 25.0%); and noteworthy, for the early stage moderately-poorly differentiated cohort in which the percentage of ALCAM-positivity in recurrent patients increased up to a 55.9% compared to a 9.1% for ALCAM-negative patients (Figure 26A-C).

Variable, No. (%)	Σ	No recurrence	Recurrence	P-value
Staging				
Early Stage I	134	102 (76.1)	32 (23.9)	0.039
ALCAM Positive*	108	78 (72.2)	30 (27.8)	
ALCAM Negative†	26	24 (92.3)	2 (7.7)	
Advanced Stages II-III	38	19 (50.0)	19 (50.0)	0.737
ALCAM Positive*	24	11 (45.8)	13 (54.2)	
ALCAM Negative†	14	8 (57.1)	6 (42.9)	
Grade				
Differentiated tumors (G1)	99	84 (84.8)	15 (15.2)	1.00
ALCAM Positive*	81	69 (85.2)	12 (14.8)	
ALCAM Negative†	18	15 (83.3)	3 (16.7)	
Moderately- Poorly differentiated tumors (G2-3)	73	38 (52.1)	35 (47.9)	0.007
ALCAM Positive*	49	20 (40.8)	29 (59.2)	
ALCAM Negative†	24	18 (75.0)	6 (25.0)	
Early Stage I & Moderately-Poorly differentiated tumors	45	25 (55.6)	20 (44.4)	0.012
ALCAM Positive*	34	15 (44.1)	19 (55.9)	
ALCAM Negative†	11	10 (90.9)	1 (9.1)	

* ALCAM positive ≥ 30

† ALCAM negative < 30

Table 19. ALCAM expression signature of EEC recurrence (N=174).

These results were supported by univariate Cox regression and Kaplan Meier analyses. Recurrence-free survival was lower in patients with ALCAM-positive tumours than in patients with ALCAM-negative expression. For early stage EEC patients, median recurrence-free survival in ALCAM-negative patients was 111.44 months vs. 95.47 months for ALCAM-positive cancers (p=0.031) with a hazard ration (HR) of 4.237 (p=0.048) (Figure 27-A). Similarly, for patients with moderately-poorly differentiated tumours, recurrence-free survival was 93.243 vs. 64.762 months (p=0.011) with a HR 2.966 (p=0.016) (Figure 27-B). Again, the highest difference was observed in patients with early stage moderately-poorly differentiated tumours, in which ALCAM reached a HR 9.259 (p=0.034). In this subcohort, recurrence-free survival was significantly longer in

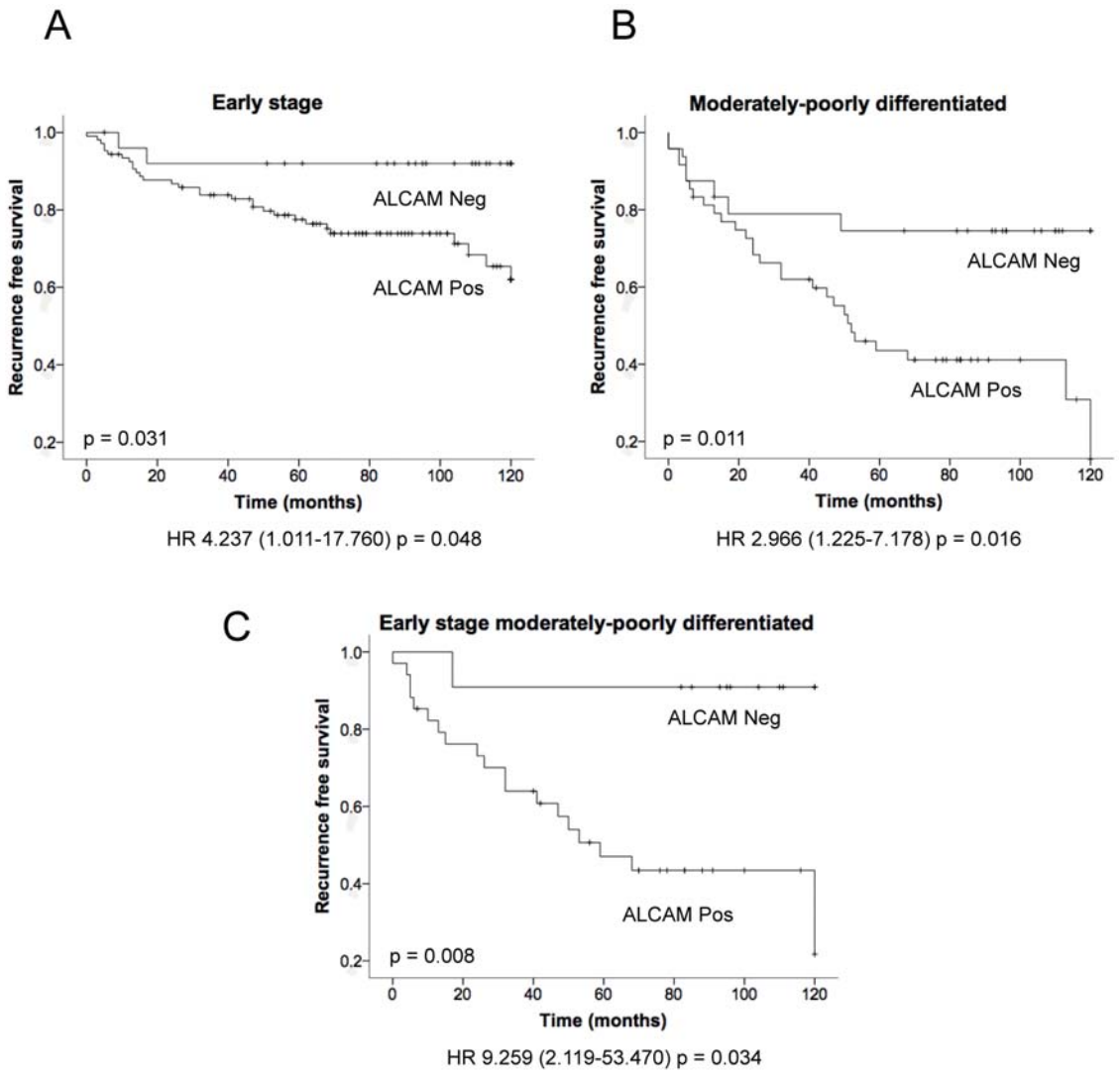


Figure 27. Univariate survival analyses according to ALCAM expression in 174 EEC patients, (A) early stage, (B) moderately-poorly differentiated, and (C) early stage moderately-poorly differentiated tumours. Differences in survival between ALCAM positive (ALCAM pos) and ALCAM negative (ALCAM neg) groups were assessed by the two-sided log-rank test. The HR (95% CI) for each comparison is given below the graphs.

ALCAM-negative cancers than in patients with ALCAM-positive expression (110.636 vs. 68.279 months, respectively; p=0.008) (Figure 27-C).

Furthermore, in the early stage patients, ALCAM-positivity was identified as an independent prognostic factor of recurrence with a HR 6.027 (p=0.015) together with the tumour grade with a HR 5.634 (p<0.001) in a multivariate Cox regression analysis (Table 20).

	Recurrence-free survival		
Variable	**HR**	**95% Interval confidence**	**P-value**
ALCAM			
Negative	1		
Positive	6.027	(1.411-25.746)	0.015
Age			
≤64 years	1		
>64 years	0.935	(0.450-1.943)	0.857
Grade			
Differentiated tumours (G1)	1		
Moderateley-poorly differentiated tumours (G2-3)	5.634	(2.623-12.101)	<0.001

Table 20. Multivariate Cox regression model for patients with early stage tumours (N=134).

4.1.3 Inhibition of ALCAM in EEC cell lines decreased cell migration, invasion and cell-cell adhesion

To understand the role of ALCAM in endometrial tumour progression and dissemination, we studied the effect of ALCAM inhibition in the Hec1A EEC cells. We generated two ALCAM-depleted Hec1A cell lines by using two specific shRNAs obtaining >70% of inhibition (Figure 28A-B).

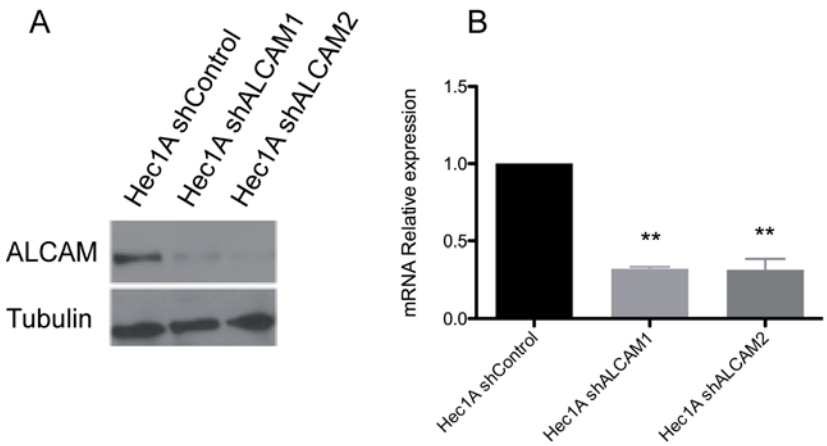


Figure 28. Inhibition of ALCAM in Hec1A cell line. (A) Stable downregulation of ALCAM in Hec1A cell line was analysed by western blot and (B) by RT-qPCR, normalized with *GAPDH* as housekeeping (**p<0.01).

We used *in vitro* 2D and 3D models to evaluate the role of ALCAM in EEC motility and a dual-micropipette assay to determine quantitatively ALCAM function in cell-cell adhesion. ALCAM-depletion decreased the wound closure rate of Hec1A cells in a 2D wound healing assay (Figure 29-A). To mimic the tumour progression in patients, we used a 3D model to quantitatively study the spreading of cell aggregates with ALCAM-depletion on a fibronectin-coated surface. Three-dimensional models fill the gap between 2D cell cultures and animal systems as they mimic characteristics of the *in vivo* environment [310]. Specifically, this model allows the study of the competition between cell–cell and cell–substratum adhesion on tissue spreading [311]. We found that, in agreement with 2D studies, ALCAM-depleted cells presented a decreased motility in the 3D model (Figure 29-B). In addition, ALCAM-depleted cells showed a significant reduced invasive capability on a matrigel transwell assay (Figure 29-C).

Finally, we evaluated how ALCAM affects the formation and strength of cell-cell adhesion. Two isolated cells maintained in suspension, to avoid cell-matrix interactions, were put into contact to initiate adhesion by using two micropipettes. After a defined period of contact, we measured the forces required to separate them. The mean separation force (SF) is used as readout of the strength of adhesion for a specific contact time. In both control and ALCAM-depleted cells the SF required to disrupt the cell doublet increased with the time of contact. However, at all time points, the intercellular cell adhesion was weaker in the ALCAM-depleted cells (Figure 30). Cell-cell adhesion and aggregate spheroid assays pointed to ALCAM as an important player in the overall balance of adhesion, which is crucial in cancer processes. Those effects were independent of cell proliferation, as no significant differences were shown in cell viability or progression through the cell cycle (Figure 31).

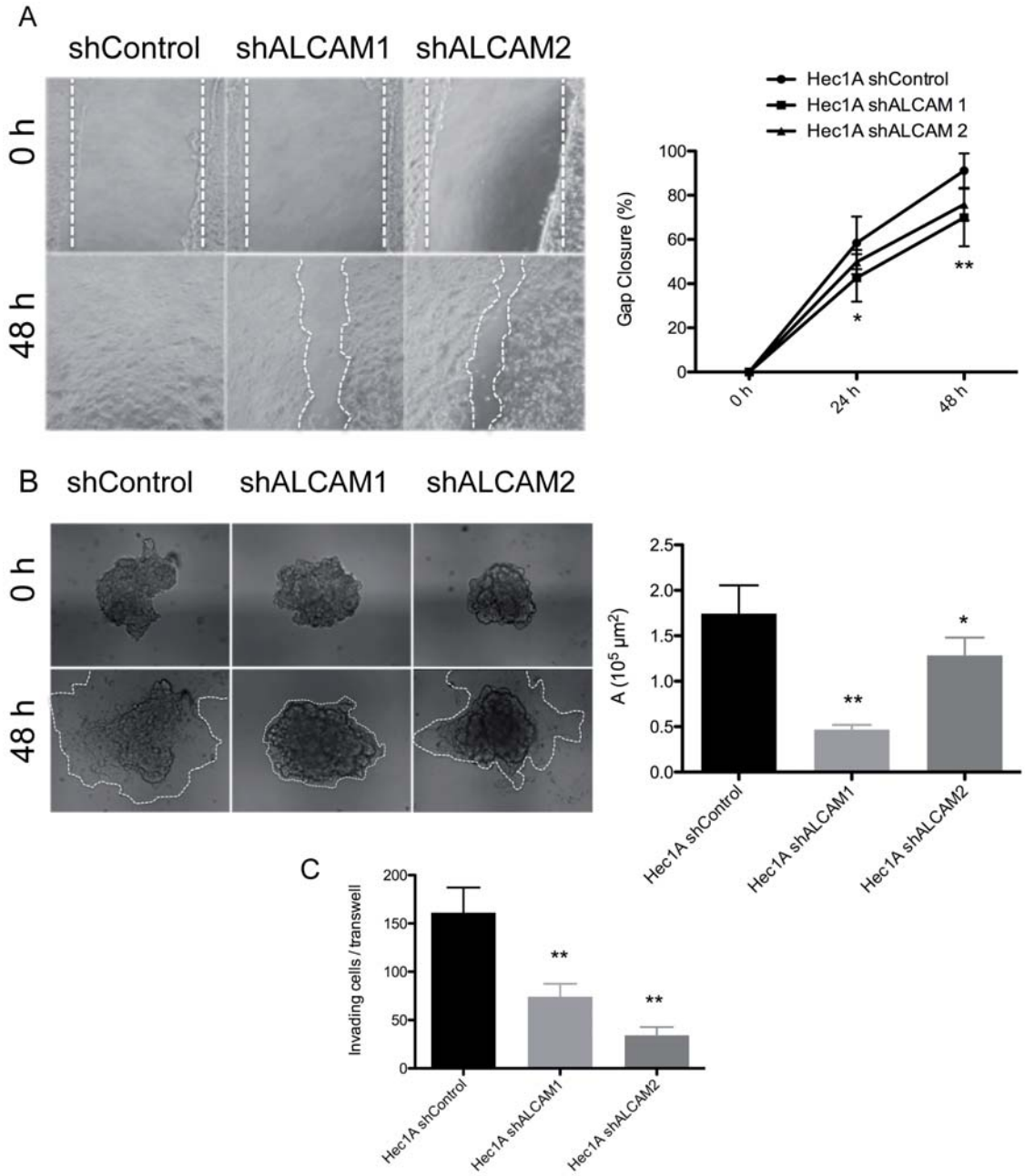


Figure 29. ALCAM inhibition decreased migration and invasion in Hec1A cell line. (A) ALCAM inhibition decreased wound closure rate at 24 and 48 h. Up, wound is delimited with a dotted line at 0 h and 48 h for each cell line. Bottom, the gap closure is quantified at 24 and 48 h. (B) The effect of ALCAM silencing in cell motility was assessed by a 3D spheroid-spreading model on fibronectin-coated surface. The spreading area is outlined in the upper image and measured at 48 h in the bar graph. (C) ALCAM depletion decreased the invasive abilities of Hec1A cells in a matrigel transwell assay after 36 h. (For all, **p<0.01, *p<0.05).

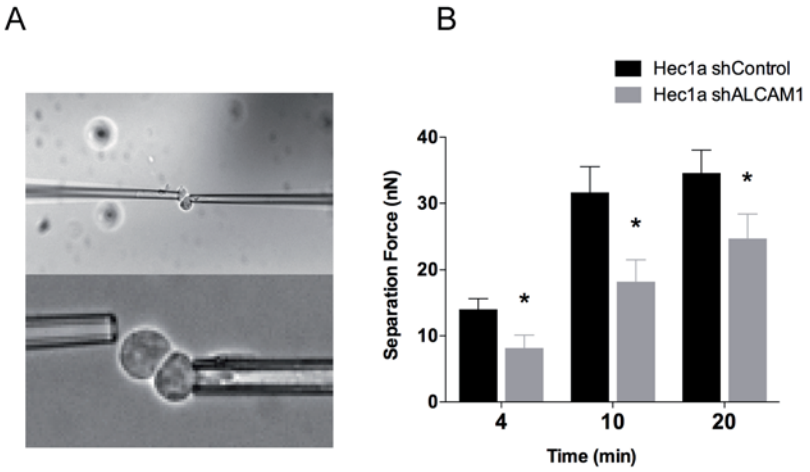


Figure 30. ALCAM inhibition decreased cell-cell adhesion in Hec1A cell line. (A) Two cells were put into contact to form adhesion. (B) After 4, 10 and 20 min ALCAM inhibition decreased the cell-cell adhesion in Hec1A cells as shown by the quantification of the separation force for each time point. (*p<0.05, Mean ± SEM).

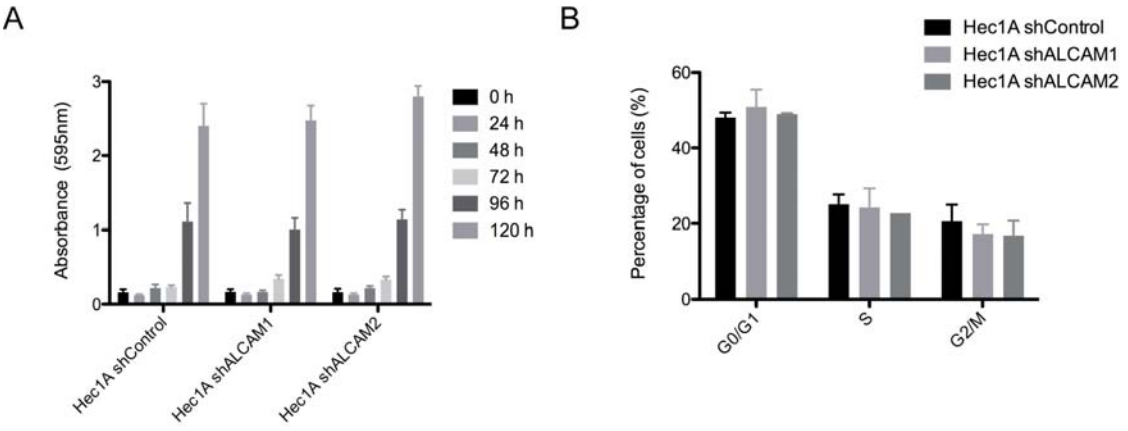


Figure 31. ALCAM inhibition had no effect on cell proliferation or progression throughout the cell cycle. (A) A crystal violet assay was performed at 0, 24, 48, 72, 96 and 120 h. ALCAM inhibition had no effect on cell proliferation in Hec1A cell line. (B) The percentages of cells in G1, S and G2 were quantified by using propidium iodide DNA staining and flow citometry. ALCAM inhibition in Hec1A had no effect on cell cycle progression.

The same results were observed with the ALCAM-depleted Ishikawa EEC cell line (Figure 32). Specifically, Ishikawa shALCAM cells presented lower rate of wound closure, decreased invasive ability, and no differences in cell proliferation were observed (Figure 33). Taken together, these results supported the role of ALCAM in key processes for tumour dissemination, leading to less aggressive phenotypes when supressed.

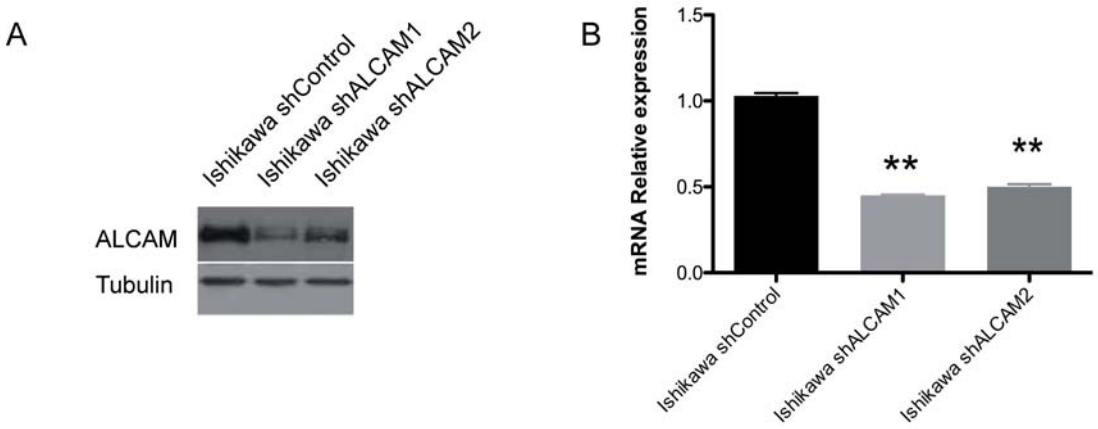


Figure 32. Inhibition of ALCAM in Ishikawa cell line. Stable downregulation of ALCAM by two shRNAs in Ishikawa cell line was analysed by (A) western blot and (B) by RT-qPCR and normalized to *GAPDH* as a reference transcript (**p<0.01).

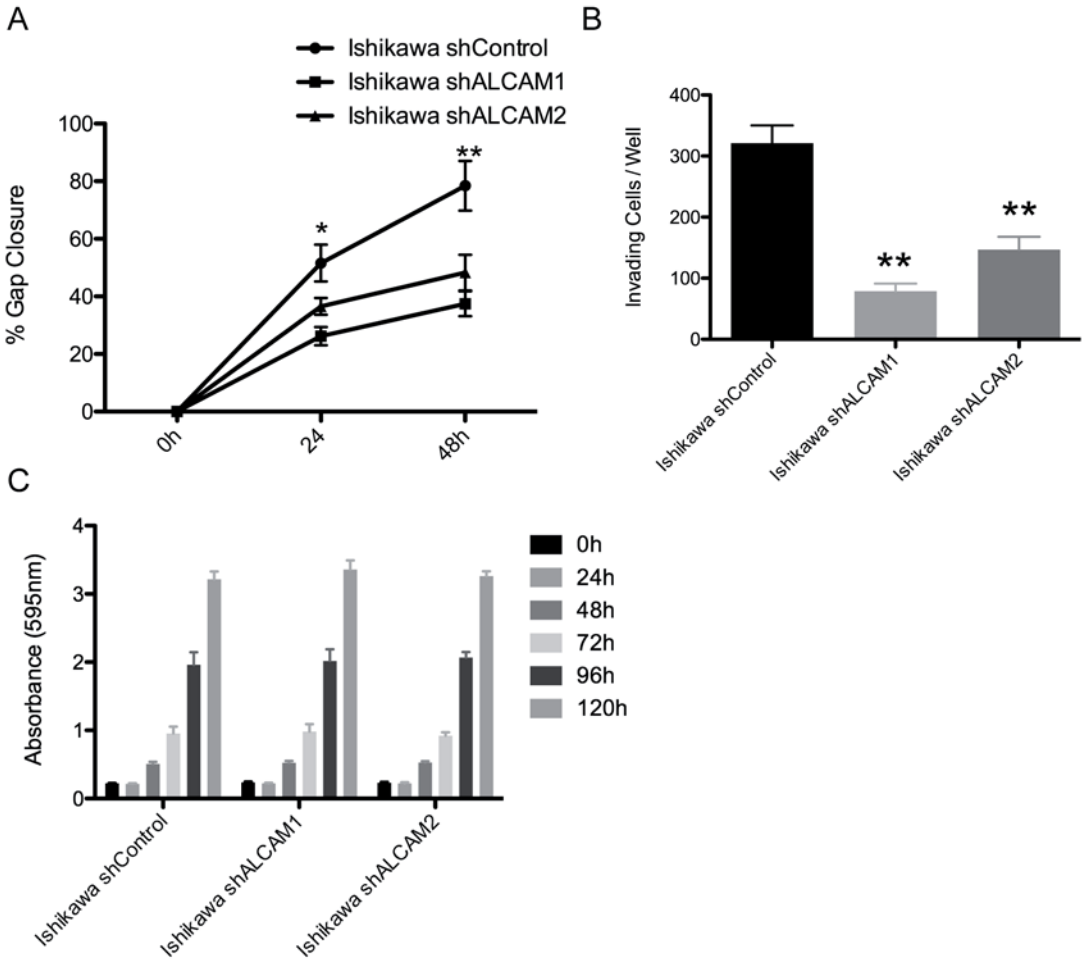


Figure 33. ALCAM inhibition in Ishikawa cell line decreased migration and invasion. (A) ALCAM inhibition decreased the wound closure rate in a 2D wound healing assay at 24 h and 48 h (24 h *p<0.05; 48 h **p<0.01). (B) ALCAM depletion decreased the invasive abilities of Ishikawa cells in a Matrigel transwell assay after 36 h (**p<0.01). (C) A crystal violet assay was performed at 0, 24, 48, 72, 96 and 120 h. ALCAM inhibition had no effect on cell proliferation in the Ishikawa cell line.

4.1.4 Depletion of ALCAM reduced primary tumour size and inhibited metastasis in an orthotopic murine model of EEC

To assess the effect of ALCAM on tumour development and metastasis we used a clinically relevant EEC orthotopic murine model. We inoculated stably transfected control or ALCAM-depleted cells into the uterus of mice and after 7 weeks both conditions developed solid tumours (Figure 34-A). Hec1A shControl tumours were larger than those generated by shALCAM cells (Figure 34B-C).

However, no differences were observed in the H&E or Ki67 staining (Figure 34-A; p=0.3), indicating that the dissimilarity in tumour size was not due to cell proliferation. These results might indicate that ALCAM interferes with the ability of tumour cells to communicate with the surrounding microenvironment. Reinforcing this hypothesis, mice with ALCAM-depleted cells developed fewer metastases and more significantly, fewer local metastases than the control (Figure 35A-B). Local metastases were differentially found in the peritoneum, pelvic fat, kidney, spleen, pancreas, colon, liver and diaphragm (Figure 35-C). Accordingly, we observed that a larger proportion of control mice presented macro metastases (>5 mm) (Figure 35D-E). Taken together, we evidenced that ALCAM-depletion profoundly affected the ability of tumour cells in developing metastasis.

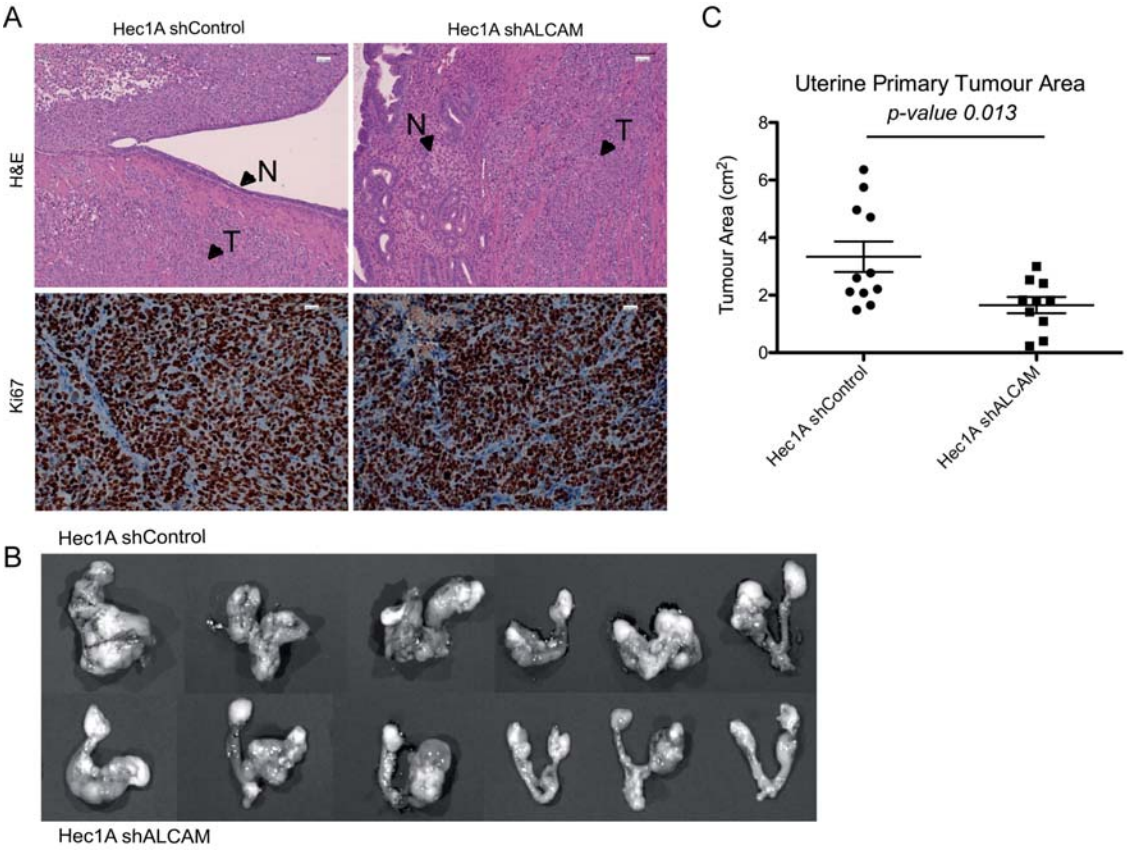


Figure 34. ALCAM-depletion decreased primary tumour size in an orthotopic mice model of EEC. (A) Up, representative H&E sections of the mice uterus, both cell lines produced solid primary tumours (T arrow) within the normal mice uterus (N arrow). Below, Ki67 representative images of the primary tumours showed no differences in cell proliferation. (B) Image of 6 uteri containing representative primary tumours in Hec1A shControl and shALCAM (the 3 largest and smallest tumours for each cell line). (C) ALCAM-depletion inhibited significantly the size of the primary tumour ($p<0.05$).

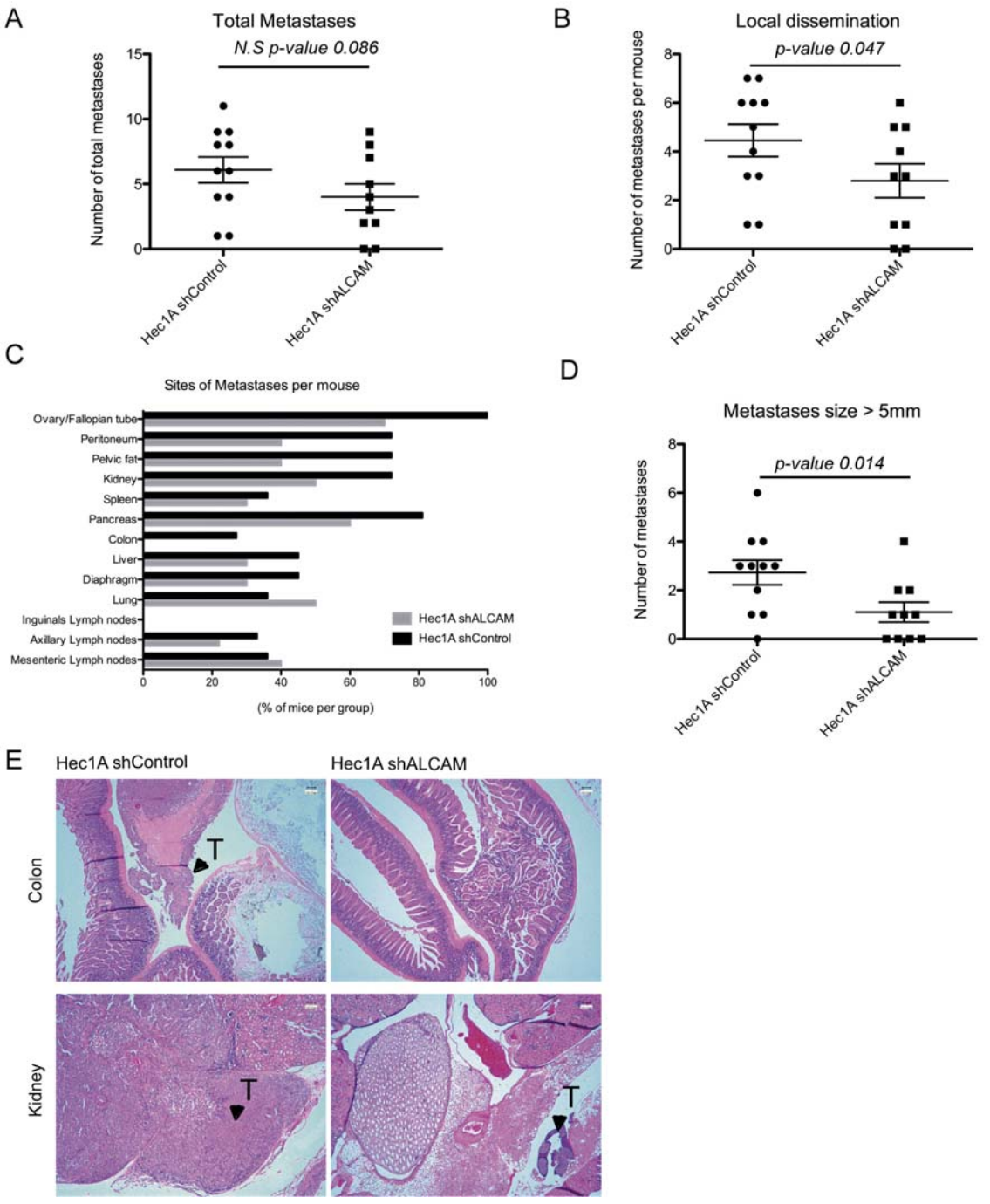


Figure 35. ALCAM-depletion reduced metastasis in an orthotopic mice model of EEC. (A) ALCAM-depletion reduced the total number of metastases per mouse and (B) reduced significantly the number of metastases produced by local invasion ($p<0.05$). (C) Graph representing the percentage of mice affected by metastases in the shControl and the shALCAM groups per organ. (D) The number of metastases >5 mm was significantly higher in the control group ($p<0.05$). (E) Representative images of colon and kidney metastases in Hec1A shControl and shALCAM mice (tumours are indicated by arrows).

4.1.5 Microarray analysis of ALCAM-dependent gene expression in Hec1A cell line

To elucidate the molecular mechanisms governed by ALCAM to promote EC dissemination and metastasis, we performed a cDNA microarray to compare the gene expression profile of Hec1A shControl and shALCAM cells. A total of 315 genes were found to be deregulated with FC >1.2 and adjusted p-value <0.05. Among those, a heatmap of the 50 most deregulated genes is represented in Figure 36.

All the differentially expressed genes were introduced in IPA and PANTHER databases for gene ontology analysis. We found that the deregulated genes were related to commonly altered pathways in cancer (Figure 37-A) and among those, the integrin signalling pathway was the most significantly altered (Figure 37-B). The IPA analysis revealed that the most enriched functions corresponded to cell movement, cell migration and cell invasion. We also observed that ALCAM inhibition had an effect in cellular assembly and organization through the regulation of genes involved in formation of cellular protrusions, microtubule dynamics, organization of cytoplasm and cytoskeleton among others; as well as functions related to cell-to-cell adhesion (Figure 37-C). All of them presented a negative z-score and thus are predicted to be inhibited in ALCAM-depleted cells, in agreement with our previous observations.

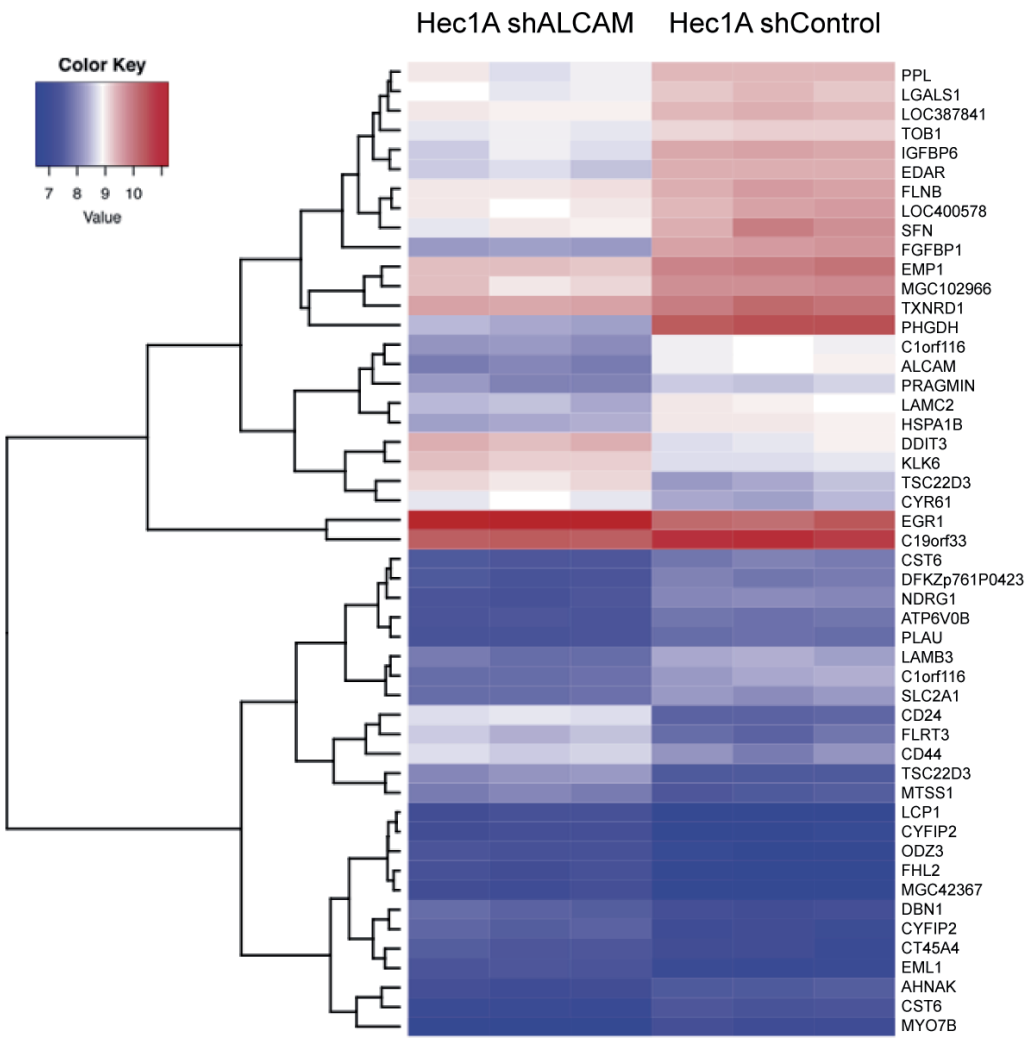


Figure 36. Gene expression analysis of ALCAM-depleted cell lines. Heatmap of the most significant deregulated genes of microarray data for three replicates of Hec1A shALCAM and Hec1A shControl cell lines.

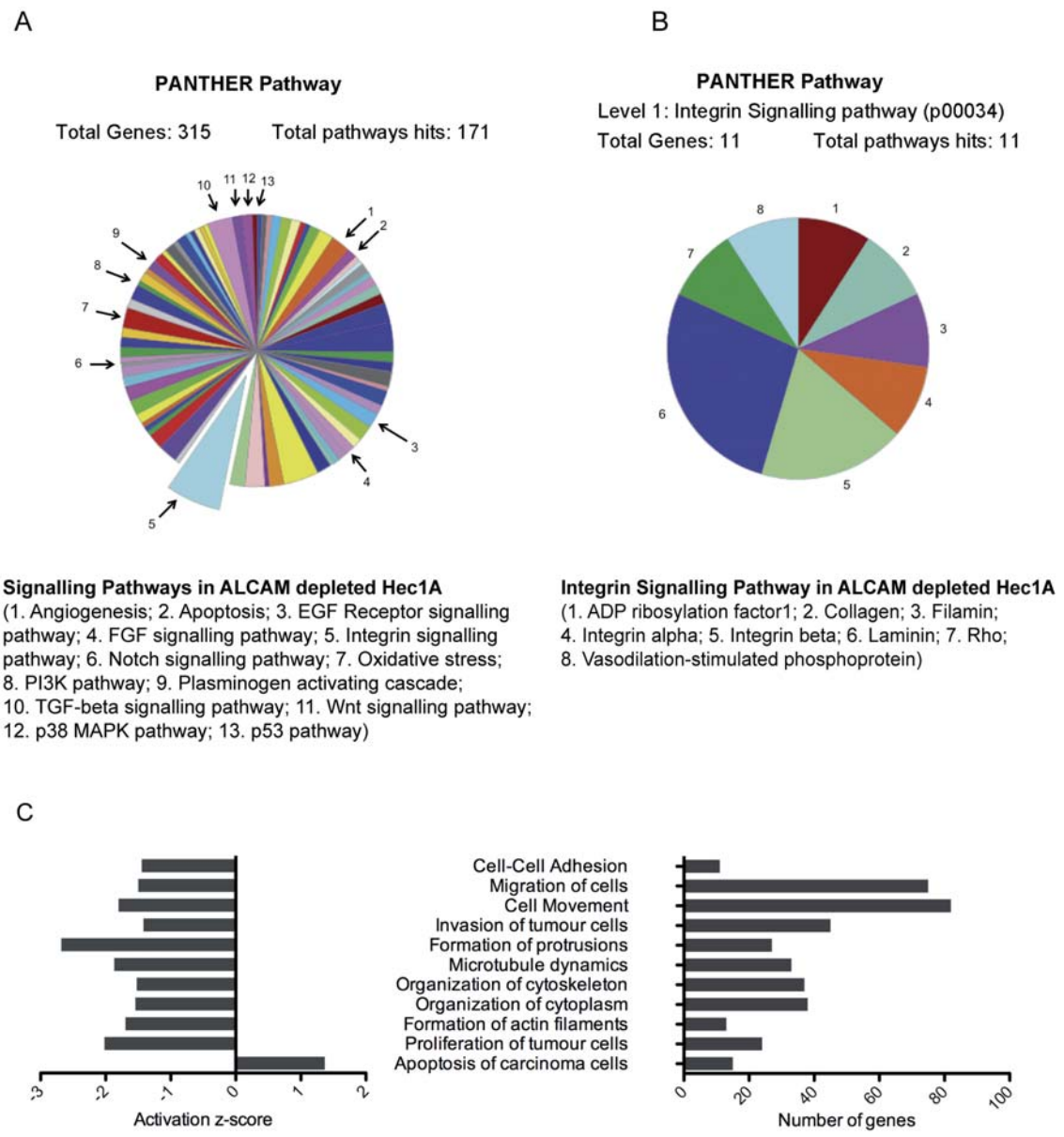


Figure 37. Gene ontology analyses of deregulated ALCAM-depleted cell lines. (A) Signalling pathways of deregulated genes in ALCAM-depleted cells by Panther analysis. The legend describes the most important affected pathways. (B) The integrin signalling pathway was the most significant represented pathway ($p<0.05$) and the specific genes are represented in the graph and described in the legend. (C) Functional categories of deregulated genes in ALCAM inhibited cells by Ingenuity pathway analysis. The activation z-score was generated by the software using the information about the direction of gene regulation. The representation of the number of genes deregulated for each functional category evidenced enrichment in cell motility and invasion.

4.1.6 ALCAM regulates LAMC2, FLNB and TXNRD1 during EEC dissemination

To characterize the downstream effectors of ALCAM-mediated functions, we selected from the microarray data the most deregulated genes (FC >1.4, adjusted p-value <0.05) related to cell motility, cell movement and cell invasion functions. Additionally, genes from the plasminogen activating cascade, which participated in the same functions, were included (Table 21). The levels of 21 genes were validated by RT-qPCR, using both knockdown and Hec1A-ALCAM overexpressing cells. Among those, six genes -*LAMC2*, *TXNRD1*, *FLNB*, *EPHA2*, *PLAU* and *KLK6*-, presented a coherent behaviour in ALCAM-depleted and ALCAM-overexpressing cells (Figure 38A-B). LAMC2, TXNRD1 and FLNB also showed decreased protein levels in ALCAM-depleted cells (Figure 39A-B). This finding supported their role as downstream effectors of the ALCAM-related functions. The genes that were not fully consistent by RT-qPCR are presented in Figure 40A-B.

SYMBOL	Gene ID	Acc_No	Fold-Change	Adj. P-Value
CD24	*Homo sapiens CD24 molecule, mRNA.*	NM_013230.2	2.191	8.37E-08
IGFBP6	*Homo sapiens insulin-like growth factor binding protein 6, mRNA.*	NM_002178.2	-1.7949	1.70E-05
NDRG1	*Homo sapiens N-myc downstream regulated gene 1, mRNA.*	NM_006096.2	-1.6342	5.35E-05
CYFIP2	*Homo sapiens cytoplasmic FMR1 interacting protein 2, mRNA.*	NM_014376.2	1.4927	1.54E-04
FHL2	*Homo sapiens four and a half LIM domains 2, mRNA.*	NM_201555.1	1.4673	2.76E-04
LAMB3	*Homo sapiens laminin, beta 3, mRNA.*	NM_000228.2	-1.4721	3.25E-04
LAMC2	*Homo sapiens laminin, gamma 2, mRNA.*	NM_005562.1	-1.4504	4.42E-04
CD44	*Homo sapiens CD44 molecule (Indian blood group), mRNA.*	NM_001001391.1	1.506	4.42E-04
KLK6	*Homo sapiens kallikrein-related peptidase 6, mRNA.*	NM_001012964.1	1.4234	4.53E-04
LGALS1	*Homo sapiens lectin, galactoside-binding, soluble, 1, mRNA.*	NM_002305.3	-1.4032	5.26E-04

TXNRD1	*Homo sapiens thioredoxin reductase 1, mRNA.*	NM_001093771.1	-1.4451	6.48E-04
MTSS1	*Homo sapiens metastasis suppressor 1, mRNA.*	NM_014751.2	1.397	6.83E-04
FLNB	*Homo sapiens filamin B, beta (actin binding protein 278), mRNA.*	NM_001457.1	-1.4373	7.14E-04
ITGB4	*Homo sapiens integrin, beta 4, mRNA.*	NM_001005619.1	-1.4425	1.92E-03
LMTK3	*PREDICTED: Homo sapiens lemur tyrosine kinase 3, mRNA.*	XM_936372.2	-1.4319	1.92E-03
CAPN1	*Homo sapiens calpain 1, (mu/I) large subunit, mRNA.*	NM_005186.2	-1.4049	1.92E-03
S100A14	*Homo sapiens S100 calcium binding protein A14, mRNA.*	NM_020672.1	-1.4442	2.39E-03
S100A10	*Homo sapiens S100 calcium binding protein A10, mRNA.*	NM_002966.2	-1.4119	5.39E-03
PLAU	*Homo sapiens plasminogen activator, urokinase, mRNA.*	NM_002658.2	-1.3676	7.47E-04
PLAUR	*Homo sapiens plasminogen activator, urokinase receptor, mRNA.*	NM_001005376.1	-1.245	1.80E-02
ITGB1	*Homo sapiens integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12), mRNA.*	NM_033668.1	-1.2938	2.35E-03
EPHA2	*Homo sapiens EPH receptor A2, mRNA.*	NM_004431.2	-1.2124	3.89E-02
AHNAK	*Homo sapiens AHNAK nucleoprotein, mRNA.*	NM_001620.1	-1.3103	1.92E-03

Table 21. List of the selected genes deregulated in the gene expression analysis of Hec1A shALCAM cells relative to Hec1A shControl.

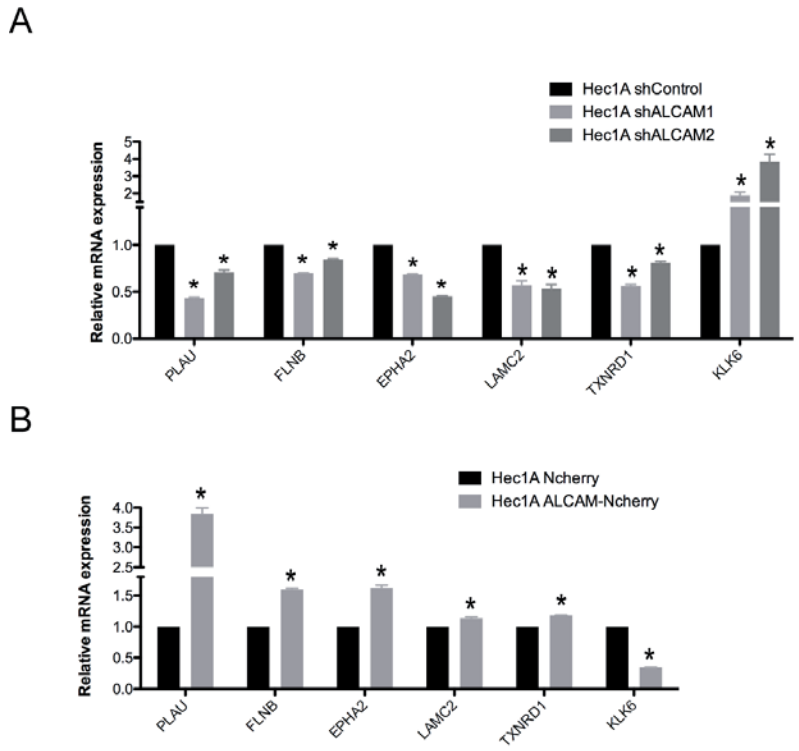


Figure 38. *LAMC2, TXNRD1* and *FLNB* were decreased at mRNA level in ALCAM-depleted cells. The expressions of *PLAU*, *FLNB*, *EPHA2*, *LAMC2*, *TXNRD1* and *KLK6* were analysed by RT-qPCR in Hec1A cells with (A) ALCAM-depletion (B) and overexpression versus control (*p<0.01).

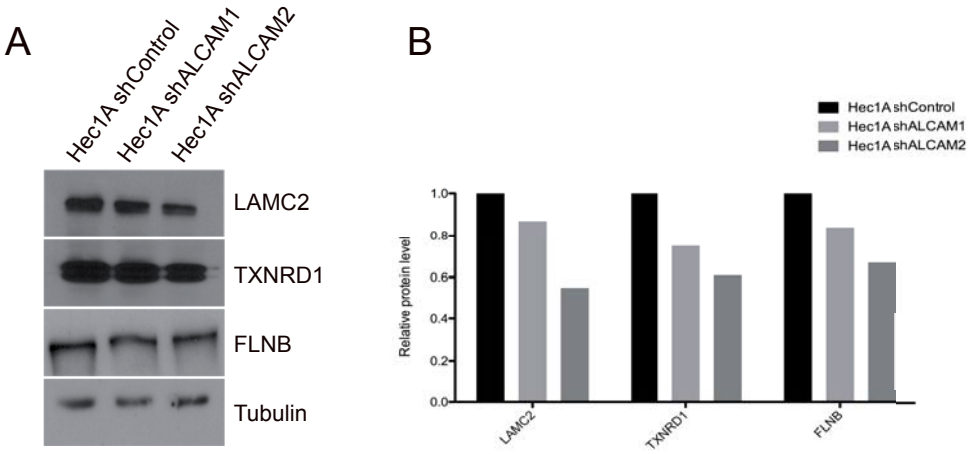
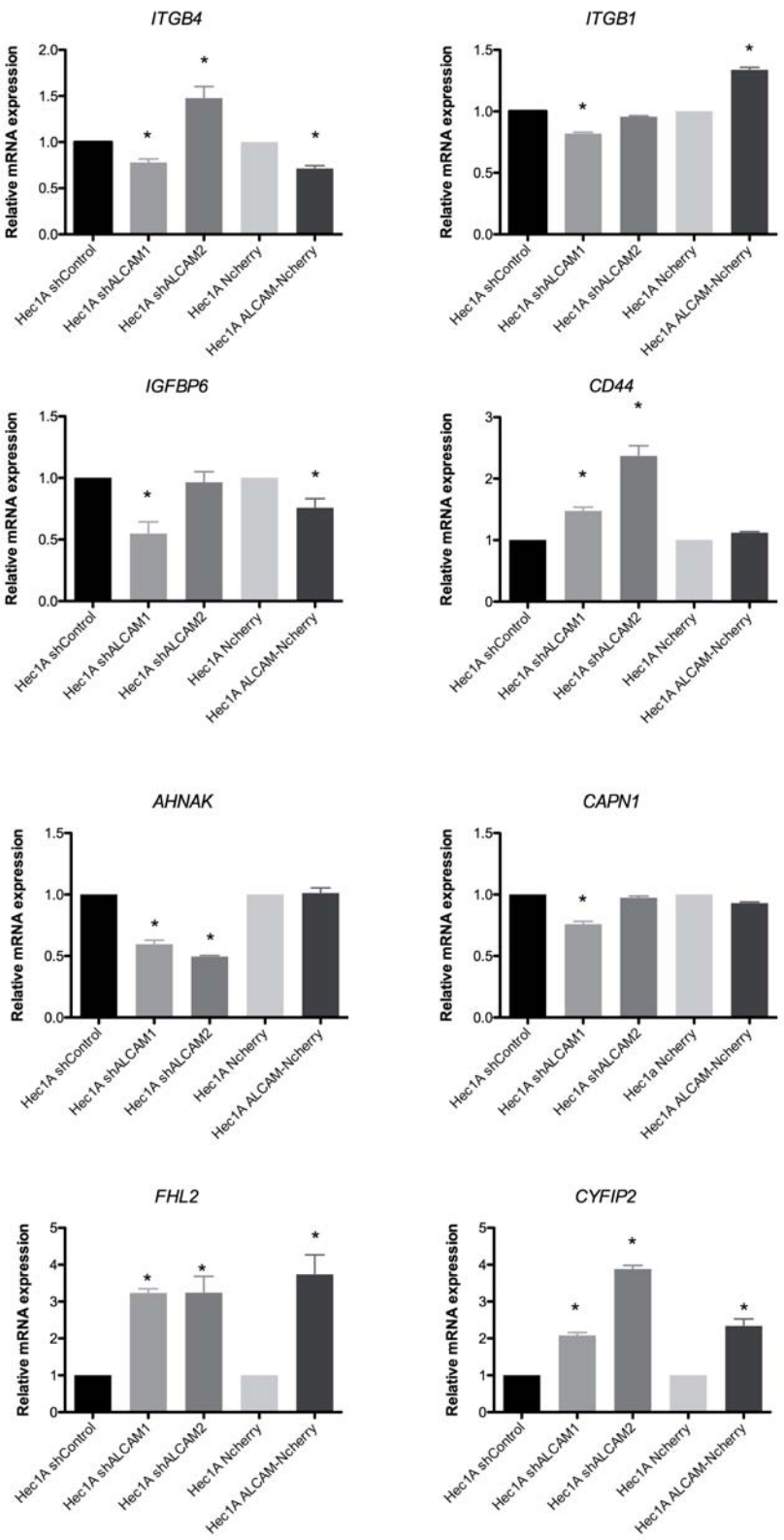


Figure 39. LAMC2, TXNRD1 and FLNB were decreased at protein level in ALCAM-depleted cells. (A) Western blot analysis confirmed the down-regulation at protein level of LAMC2, TXNRD1 and FLNB in ALCAM inhibited cells. (B) Western blot was quantified using Image J software.

A


ITGB4
ITGB1
IGFBP6
CD44
AHNAK
CAPN1
FHL2
CYFIP2
Relative mRNA expression
Hec1A shControl
Hec1A shALCAM1
Hec1A shALCAM2
Hec1A Ncherry
Hec1a Ncherry
Hec1A ALCAM-Ncherry

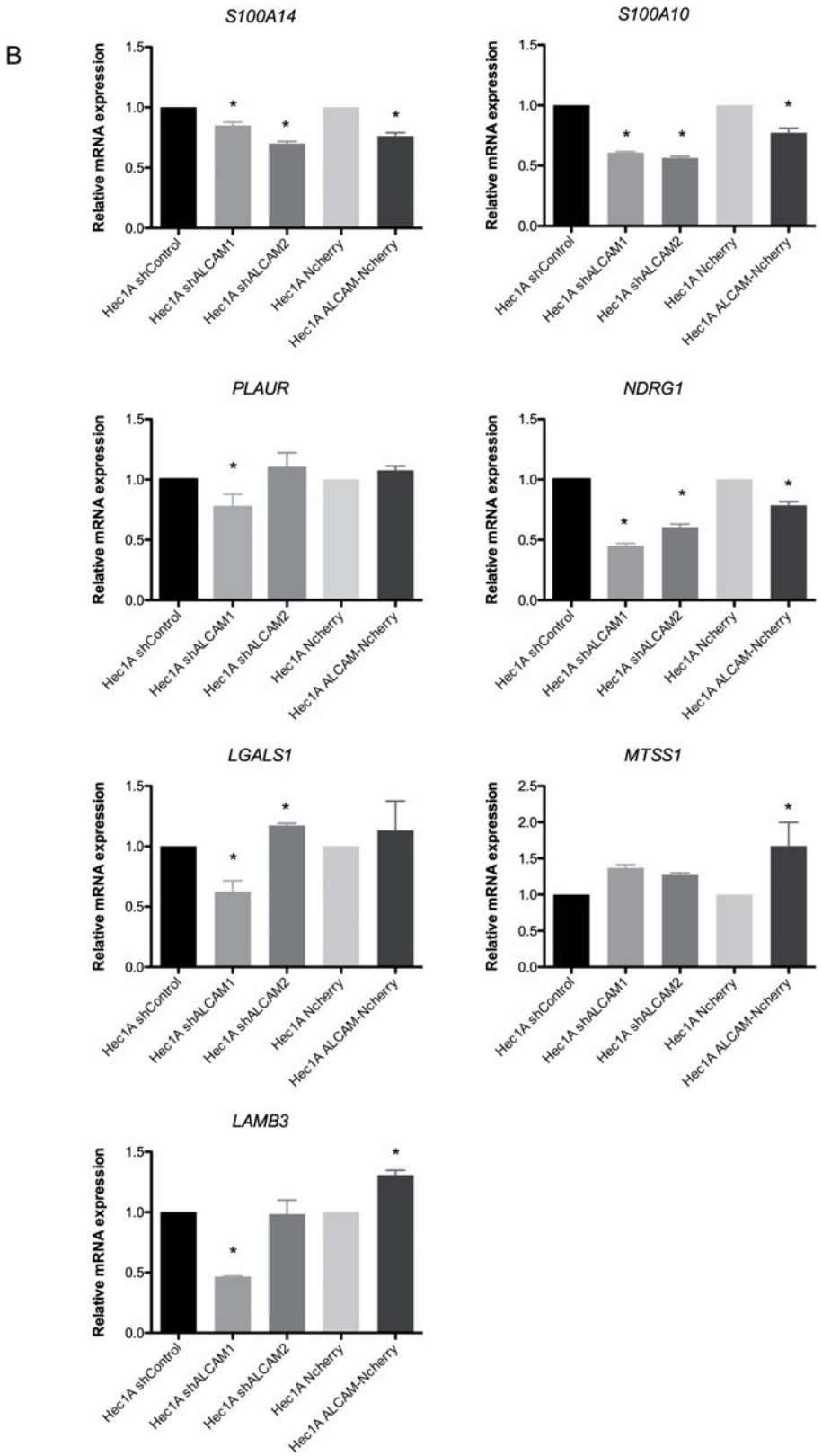


Figure 40. RT-qPCR performed on deregulated genes from the microarray study. Genes with FC >1.4 (except for PLAUR, FC >1.2) and adjusted p-value<0.05, all associated with the functional category of cell migration and invasion. (A, B) Genes included in this figure are not fully consistent to ALCAM expression, as they either present contradictory results or are not validated in both ALCAM-overexpressing and depleted cells (*p<0.01).

4.2 Characterization of the molecular mechanisms associated with ALCAM in EEC and its relation to myometrial invasion

4.2.1 Framework

The first step of myometrial invasion is characterized by the dissociation of tumour cells from the epithelial layer of the endometrial glands, and their infiltration through the basement membrane into the adjacent layer, the myometrium [312]. For that to happen, several authors suggested that epithelial tumour cells undergo an EMT, either transiently or stably. Specifically, it is known that in invading tumours this process could take place at the invasive front of the tumour [169,313,314]. As explained in Chapter 1 section 1.1.10.2, multiple factors have been described as being responsible for producing EMT in EC, such as ETV5, oestrogen and progesterone receptors, and TGF-β, among others [312].

ALCAM expression in the cell surface can be regulated by endocytosis or protein cleavage by ADAM 17 metalloproteinase [268,315,270], generating a soluble 96kDa and a truncated membrane-bound. Interestingly, in addition to its clear association with the tumorigenesis of many cancers in tissues [289,290,296,299,302,303,316,317], ALCAM shedding has also been reported in the serum of breast, ovarian, thyroid and pancreatic cancer patients and in all cases has been related to poor clinical outcome [303,305,318,319].

We have previously demonstrated that ALCAM is an important player in EEC dissemination [320]. For this reason, in this results section, we go further in our understanding of ALCAM in EEC tumorigenesis by evaluating its full-form expression (non-cleaved protein) in different areas of the tumour as well as its relation with key molecules associated to the epithelial (E-cadherin, β-catenin) and mesenchymal (ETV5, COX-2, MMP-2 and -9) context in EC. Moreover, we evaluated the levels of shed ALCAM (sALCAM) in uterine aspirates as a potential prognostic tool to predict myometrial invasion at the time of diagnosis.

4.2.2 ALCAM partners differed in the superficial compared to the invasive area of the tumour

We studied the immunohistochemical expression of ALCAM extracellular domain (non-cleaved protein) and a representative set of epithelial (E-cadherin/β-catenin adhesion complex) and mesenchymal molecules involved in EEC dissemination (ETV5, COX-2, MMP-2 and -9), in both the superficial and the invasive areas of the tumour. As our previous results indicated the differential behaviour of ALCAM regarding tumour differentiation, analyses were performed in the whole population and in the well-differentiated (G1) and poorly-differentiated (G2-3) subcohorts [320] .

Univariate regression analyses were performed as a first approach to evaluate the association between ALCAM and epithelial or mesenchymal markers. Variables significantly correlated with ALCAM (Table 22) were included in multivariate linear regression models.

Multivariate linear regression analyses evidenced a significantly positive correlation between ALCAM and the adhesion complex only at the superficial area of the tumour. Whilst β-catenin was retained in the whole population and the well-differentiated cohort (R=0.416 and 0.391, respectively), E-cadherin was retained in the moderately-poorly differentiated tumours (R=0.445) (Table 23, Figure 41). By contrast, at the invasive front of the tumour, the model performed in all patients retained COX-2 and MMP-9. Specifically, COX-2 is presented as a predictor that correlated positively with ALCAM, whilst MMP-9 was negatively correlated. However, the best regression adjustment was reached in the moderately-poorly differentiated subcohort of patients, in which the model retained ETV5 and MMP-9 with a R=0.559. Both molecules showed a negative correlation with ALCAM (Table 24, Figure 41). Altogether, the results suggest a different functional profile of the molecule that correlated strongly with the epithelial markers at the superficial tumour; and by contrast, evidenced a correlation with mesenchymal markers at the invasive front.

	All cases		Grade 1		Grade 2-3	
	β	**p-value**	**β**	**p-value**	**β**	**p-value**
Superficial area of the tumour						
β-catenin	0.419	< 0.001***	0.394	< 0.001***	0.410	0.013*
E-cadherin	0.346	< 0.001***	0.275	0.014*	0.462	0.004**
ETV5	0.026	0.791	0.033	0.780	0.027	0.878
COX-2	0.109	0.248	0.153	0.180	0.013	0.938
MMP-9	-0.176	0.060	-0.014	0.906	-0.395	0.015*
MMP-2	0.247	0.008**	0.251	0.026*	0.246	0.143
Invasive front of the tumour						
β-catenin	0.144	0.174	0.181	0.157	0.017	0.933
E-cadherin	0.160	0.125	0.173	0.182	0.111	0.546
ETV5	-0.034	0.748	0.118	0.365	-0.408	0.025*
COX-2	0.210	0.038*	0.221	0.073	0.188	0.310
MMP-9	-0.242	0.018*	-0.137	0.280	-0.438	0.014*
MMP-2	0.035	0.723	0.029	0.808	-	-

Table 22. Patterns of ALCAM in the tumour: univariate linear regression analysis.

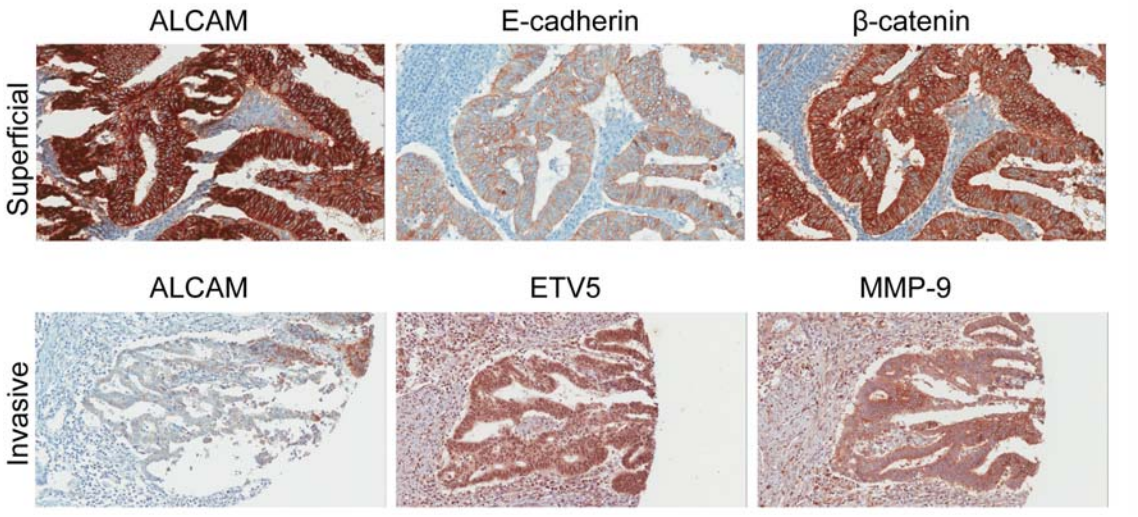


Figure 41. ALCAM patterns at the tumour. Upper: full-length ALCAM was positively correlated positively with the E-cadherin/β-catenin complex at the superficial area of the tumour and its mainly localized at the cell membrane. Down: At the invasive front, full-length ALCAM was significantly inversely correlated with MMP-9 and ETV5, in the moderately-poorly differentiated EEC.

Superficial area of the tumour							
	Summary model			**Coefficients**			
	R	R^2 adj.	p-value	B	CI95%	β	p-value
All cases	0.416	0.166	<0.001***				
Predictors							
β-catenin				1.690	(0.990; 2.391)	0.416	< 0.001***
E-cadherin						0.172	0.081
MMP-9						-0.148	0.090
MMP-2						0.120	0.194
Grade Well-differentiated	0.391	0.141	0.001**				
Predictors							
β-catenin				1.585	(0.715; 2.455)	0.391	0.001**
E-cadherin						0.092	0.460
MMP-9						-0.079	0.470
MMP-2						0.129	0.261
Moderately-poorly differentiated	0.445	0.174	0.007**				
Predictors							
E-cadherin				0.673	(0.201; 1.146)	0.445	0.007**
β-catenin						0.272	0.109
MMP-9						-0.264	0.108

Table 23. ALCAM patterns at the superficial area of the tumour: multivariate linear regression analysis.

Invasive front of the tumour							
	Summary model			Coefficients			
	R	R^2 adj.	p-value	B	CI95%	β	p-value
All cases	0.382	0.126	0.001**				
Predictors							
COX-2				0.630	(0.202; 1.058)	0.296	0.004**
MMP-9				-1.493	(-2.607; -0.379)	-0.270	0.009**
ETV5						-0.101	0.363
Grade Well-differentiated	0.344	0.102	0.008**				
Predictors							
COX-2				0.754	(0.203; 1.305)	0.344	0.008**
ETV5						0.008	0.952
MMP-9						-0.215	0.091
Moderately-poorly differentiated	0.559	0.259	0.008**				
Predictors							
ETV5				-1.175	(-2.283; -0.067)	-0.363	0.039*
MMP-9				-1.987	(-3.917; -0.057)	-0.353	0.044*
COX-2						0.279	0.120

Table 24. ALCAM patterns at the invasive front of the tumour: multivariate linear regression analysis.

4.2.3 ALCAM-negativity at the invasive front of the tumour is a marker of myometrial invasion

In order to further investigate in the role of ALCAM at the invasive front, as well as in its relationship with ETV5 and MMP-9, categorized analyses were performed. In the superficial area, uncleaved ALCAM expression was unrelated to the clinical parameters of myometrial invasion, tumour grade, and tumour progression. Interestingly, it acts as a marker of tumour progression and invasion when analysed at the invasive front. In that case, ALCAM was shown to significantly decrease throughout the tumour stages and in tumours with myometrial invasion >50% (Table 25, Figure 42A-B). In fact, the potential of ALCAM-negativity as an independent prognostic factor of myometrial invasion

was confirmed by multivariate logistic regression analysis with an OR 3.273, together with the tumour grade (OR 3.484) (Table 26).

Variable, No. (%)	Σ	ALCAM Positive*	ALCAM Negative†	P-value
Superficial area of the tumour				
FIGO	116	82 (70.7)	34 (29.3)	0.208
IA	56	42 (75.0)	14 (25.0)	
IB	41	28 (68.3)	13 (31.7)	
II	15	11 (73.3)	4 (26.7)	
III	4	1 (25.0)	3 (75.0)	
Grade	116	82 (70.7)	34 (29.3)	0.131
Grade 1	79	60 (75.9)	19 (24.1)	
Grade 2	24	13 (54.2)	11 (45.8)	
Grade 3	13	9 (69.2)	4 (30.8)	
Myometrial infiltration	116	82 (70.7)	34 (29.3)	0.541
< 50%	58	43 (74.1)	15 (25.9)	
≥ 50%	58	39 (67.2)	19 (32.8)	
Invasive front of the tumour				
FIGO	106	71 (67.0)	35 (33.0)	0.004**
IA	53	42 (79.2)	11 (20.8)	
IB	36	21 (58.3)	15 (41.7)	
II	13	8 (61.5)	5 (38.5)	
III	4	0 (0.0)	4 (100.0)	
Grade	106	71 (67.0)	35 (33.0)	0.213
Grade 1	72	52 (72.2)	20 (27.8)	
Grade 2	22	13 (59.1)	9 (40.9)	
Grade 3	12	6 (50.0)	6 (50.0)	
Myometrial infiltration	106	71 (67.0)	35 (33.0)	0.014*
< 50%	55	43 (78.2)	12 (21.8)	
≥ 50%	51	28 (54.9)	23 (45.1)	

* Positive ≥ 65

† Negative < 65

Table 25. ALCAM expression and clinical parameters.

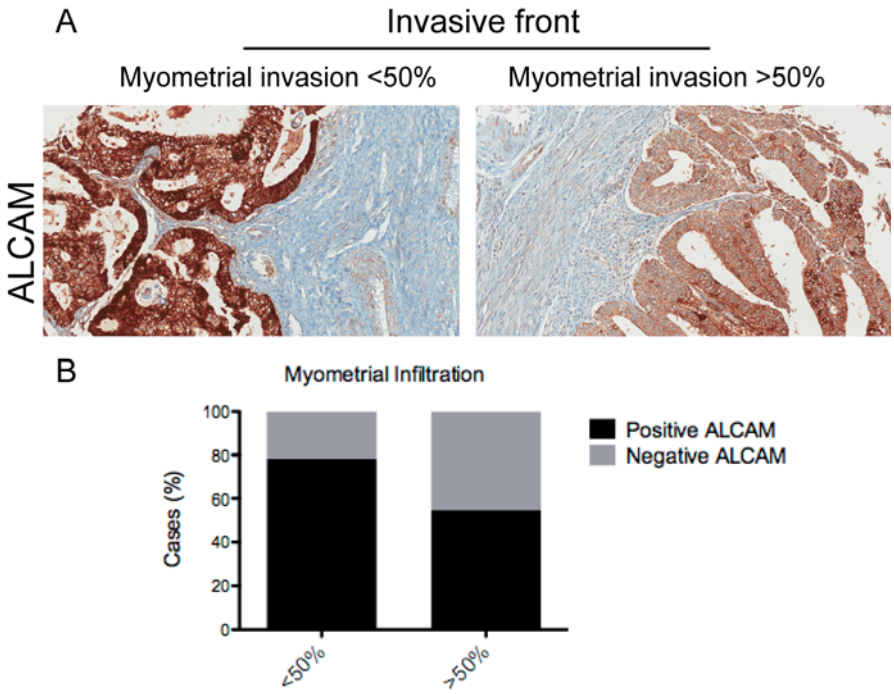


Figure 42. ALCAM expression was decreased at the invasive front of patients with myometrial invasion. (A) ALCAM-negativity, at the invasive front, is a marker of myometrial invasion. (B) At the invasive front, ALCAM-negative tumours increased from a 21.8% in patients without myometrial invasion to a 45.1% in patients presenting myometrial infiltration (p= 0.014).

Variable	OR	95% Interval confidence	p-value
Grade			
Differentiated tumours (Grade 1)	1		
Moderateley-poorly differentiated tumours (Grade 2-3)	3.484	(1.284-9.524)	0.014*
ALCAM			
Positive	1		
Negative	3.273	(1.200-8.932)	0.021*
MMP-9			
Positive	1		
Negative	1.629	(0.602-4.409)	0.337
ETV5			
Positive	1		
Negative	0.556	(0.194-1.592)	0.274

Table 26. Multivariate logistic regression model, at the invasive front, related to myometrial invasion >50% (N=89).

ALCAM maintained a close and inverse relation with MMP-9 in the invasive front of the tumours (p=0.004; OR 0.26) (Table 27).

This relation was only maintained in poor prognosis tumours when the cohort was divided according to clinical parameters, i.e. either in tumours with moderately-poorly differentiated EEC histology (p=0.02; OR 0.08) or in patients presenting >50% of myometrial infiltration (p=0.016; OR 0.19). Interestingly, when we studied this relationship in terms of ETV5-expression, we observed that ALCAM and MMP-9 correlation was only significant in the ETV5-positive tumours (p=0.009). Altogether, our results demonstrated that ALCAM-negativity is a marker for myometrial invasion. Moreover, its close and inverse relation with MMP-9 led us speculate that ALCAM-negativity might be due to an increased shedding of the protein by the metalloproteinase at the invasive front of poor prognostic tumours. This dialog could be orchestrated by the mesenchymal transcription factor ETV5, whose up-regulation has been strongly associated with the myometrial infiltration event.

4.2.4 ALCAM shedding in uterine aspirates is a marker of myometrial invasion and is closely related to MMP-9 expression

In order to evaluate the possible association between ALCAM shedding and myometrial invasion, we analysed the expression of soluble ALCAM (sALCAM) in 40 uterine aspirates (UA) from moderately-poorly differentiated EEC patients presenting different grades of myometrial infiltration. The UA is a body fluid that is collected from inside of the uterine cavity, and is formed mainly by the secretion of the tumour cells and other cells from the endometrium, enabling the detection of cleaved ALCAM by ELISA. We confirmed that sALCAM was significantly increased in patients presenting myometrial invasion >50% (Figure 43-A). Moreover, ROC analysis showed that sALCAM in UAs is a significant predictor of myometrial infiltration (AUC 0.80; p=0.001) (Figure 43-B). In fact, when ALCAM is set at a cut-off of 9.375 ng/mg, the model presents a sensitivity of 87% and a specificity of 70.6%.

Variable, No. (%)	Σ	ALCAM Positive*	ALCAM Negative†	P-value	OR (Positive/ Negative)
All	95	64 (67.4)	31 (32.6)	0.004**	0.26 (0.10-0.66)
MMP-9 Positive	47	25 (53.2)	22 (46.8)		
MMP-9 Negative	48	39 (81.3)	9 (18.8)		
Clinical parameters					
Grade Differentiated tumours	64	46 (71.9)	18 (28.1)	0.162	N.S
MMP-9 Positive	26	16 (61.5)	10 (38.5)		
MMP-9 Negative	38	30 (78.9)	8 (21.1)		
Moderately- Poorly differentiated tumours	31	18 (58.1)	13 (41.9)	0.020*	0.08 (0.03-0.63)
MMP-9 Positive	21	9 (42.9)	12 (57.1)		
MMP-9 Negative	10	9 (90.0)	1 (10.00)		
Myometrial infiltration <50%	53	41 (77.4)	12 (22.6)	0.190	N.S
MMP-9 Positive	25	17 (68.0)	8 (32.0)		
MMP-9 Negative	28	24 (85.7)	4 (14.3)		
Myometrial infiltration ≥50%	42	23 (54.8)	19 (45.2)	0.016*	0.19 (0.03-0.65)
MMP-9 Positive	22	8 (36.4)	14 (63.6)		
MMP-9 Negative	20	15 (75.0)	5 (25.0)		
Molecular features					
ETV5 Positive	64	40 (62.5)	24 (37.5)	0.009**	0.22 (0.07-0.69)
MMP-9 Positive	37	18 (48.6)	19 (51.4)		
MMP-9 Negative	27	22 (81.5)	5 (18.5)		
ETV5 Negative	25	19 (76.0)	6 (24.0)	1.000	N.S
MMP-9 Positive	7	5 (71.4)	2 (28.6)		
MMP-9 Negative	18	14 (77.8)	4 (22.2)		

* Positive ≥ 65

† Negative < 65

Table 27. ALCAM and MMP-9 at the invasive front of the tumour.

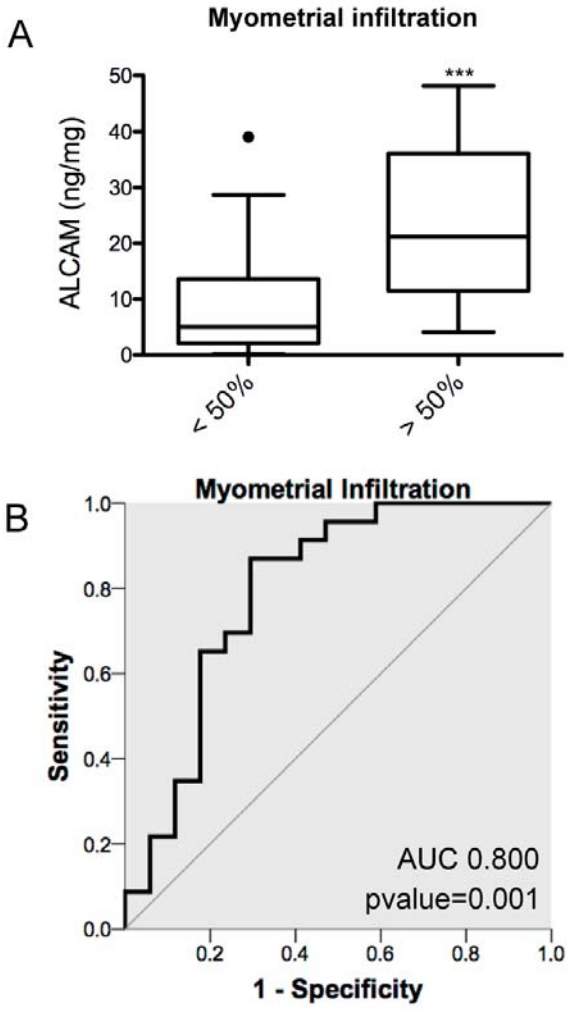


Figure 43. Soluble ALCAM detected in uterine aspirates is a marker of myometrial invasion. (A) Soluble ALCAM detected by ELISA in uterine aspirates was significantly increased in patients presenting myometrial invasion (***p<0.001). ALCAM values were normalized to total protein amount from the uterine aspirates. (B) ROC curve of sALCAM individual marker demonstrated its prognostic value to discriminate patients with myometrial invasion (AUC=0.800; p=0.001).

Then, the relation between sALCAM and MMP-9 was assessed in UA by performing a gelatine zymography of the same patients (Figure 44-A). Univariate linear regression analyses (Table 28) evidenced a significant positive correlation between sALCAM with all MMP-9 detected forms, with the exception of the more labile active form. No correlation was found with the MMP-2 forms (data not shown). In order to avoid the problems derived from the simultaneous use of several measurements that reflect expressions of molecular forms of the same enzyme (Table 29), PCA was addressed to explore the close relationship

among the four MMP-9 forms and reduce their number before performing regression analysis. Two main components C1, which explains 75.18% of the variability of the original dataset, and C2 which only explains 13.86%, were extracted (Figure 44-B). Regression analyses showed a strong and positive correlation between sALCAM and C1 at the global level and specifically in patients presenting myometrial invasion >50% (R=0.575; p=0.004) (Table 28, Figure 44C-D). These results suggested that at the invasive front of the tumour ALCAM extracellular shedding by MMP-9 could be an important player in myometrial invasion.

	Univariate linear regression					
	All cases		**Myometrial infiltration <50%**		**Myometrial infiltration >50%**	
	R	**p-value**	**R**	**p-value**	**R**	**p-value**
MMP-9 Dimeric	0.355	0.025*	0.298	0.246	0.484	0.019*
MMP-9 NGAL	0.407	0.009**	0.313	0.221	0.446	0.033*
MMP-9 Latent	0.385	0.014*	0.282	0.272	0.326	0.129
MMP-9 Active	0.100	0.541	0.197	0.449	0.095	0.666
C1 Regression Factor Score	0.447	0.004**	0.322	0.207	0.575	0.004**

Table 28. ALCAM correlation with MMP-9 in uterine aspirates.

	MMP-9 forms (*)			
	MMP-9 Dimeric	MMP-9 NGAL	MMP-9 Latent	MMP-9 Active
MMP-9 Dimeric		0.815***	0.826***	0.552***
MMP-9 NGAL	0,815***		0,699***	0,545***
MMP-9 Latent	0.826***	0.699***		0.543***
MMP-9 Active	0.552***	0.545***	0,543***	

(*ln-transformed)

Table 29. Matrix correlation MMP-9 forms.

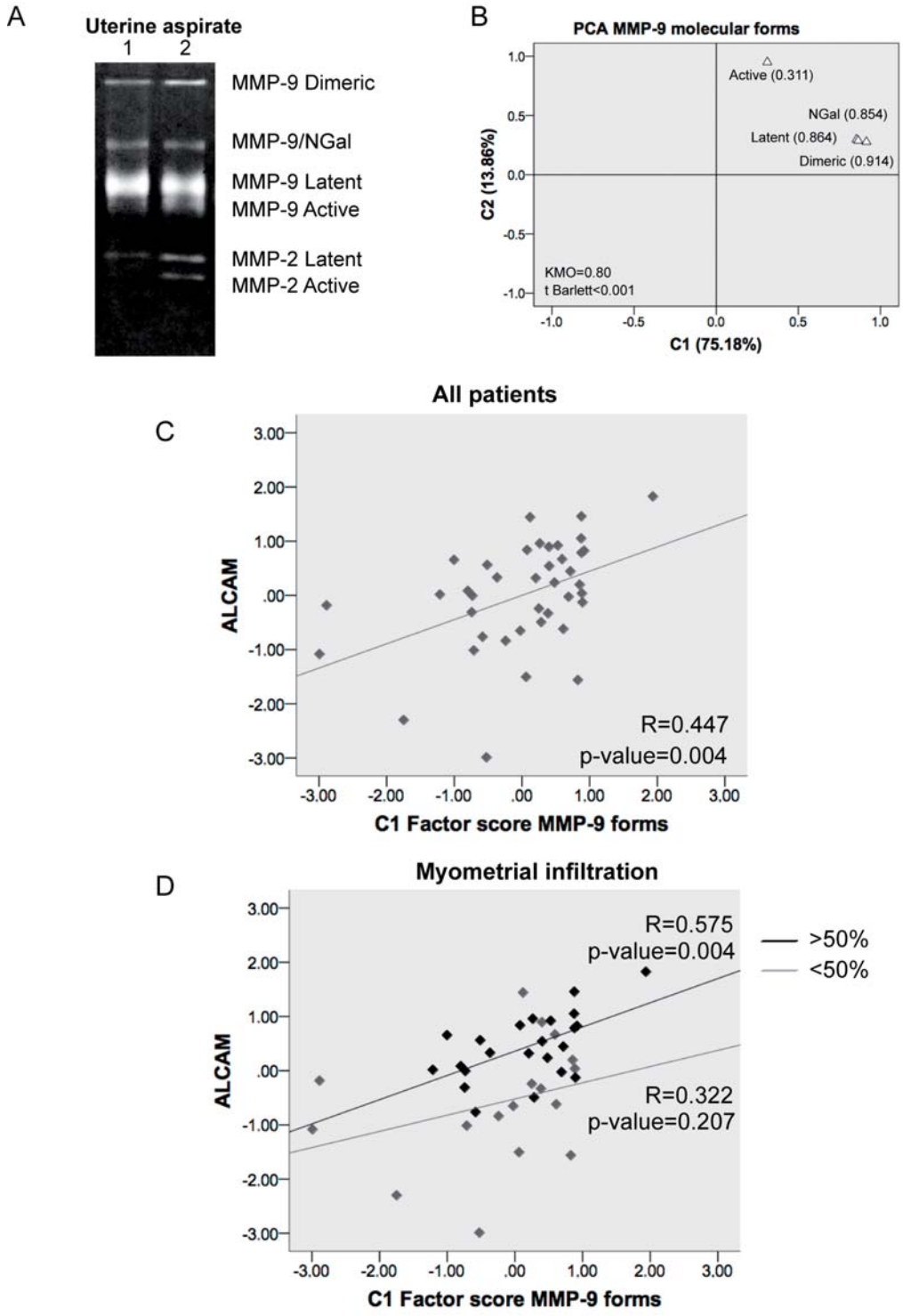


Figure 44. Soluble ALCAM and MMP-9 expressions were correlated in uterine aspirates. (A) Two different examples of gelatine zymography from uterine aspirates and detection of the different forms of MMP-9 and MMP-2. (B) Principal component analysis from the MMP-9 detected molecular forms evidenced two principal components: C1 (75.18% of the variability) and C2 (13.86% of the variability). The factor loadings, correlation between the original variables and the main components of each form can be observed in the graph. The KMO obtained was 0.80 and the t Barlett <0.001. (C) The principal component C1 and the sALCAM were significantly correlated in all patients (R=0.447; p=0.004). (D) The correlation between the C1 component and sALCAM was significant in patients with myometrial invasion >50% (R=0.575; p=0.004).

4.2.5 ALCAM and MMP-9 are important actors at the invasive front of an in vivo model of EEC dissemination

The ETV5 overexpression in Hec1A cell line has been extensively used as a model that mimics the step of tumour invasion in EEC [190,191,321], as ETV5 overexpression is known to induce EMT in EEC. Furthermore, the parental Hec1A cell line is representative of moderately-poorly differentiated EEC superficial tumours. In order to study ALCAM in a controlled environment, which represents a realistic approach towards the process of dissemination of EC, we generated orthotopic murine models by injecting Hec1A (n=5) or its ETV5 stable overexpressing cells (n=4) directly in the mice uterus. In these models, tumour cells are localized in the same microenvironment of the original tumour, resembling the processes of tumour growth and myometrial infiltration under endometrial stimuli.

As expected, the tumours generated by the ETV5-overexpressing cells presented larger myometrial invasion and tumour burden as seen in the H&E and in the quantified luminescence from the IVIS images (Figure 45, Figure 47). While in the ETV5-overexpressing tumours, we observed disseminated cells in finger-like strands or single-cells projecting into the stroma at the invasive front; in the Hec1A tumours, cells invade forming clusters from the primary tumour (see arrows, Figure 45). The immunohistochemical staining of uncleaved ALCAM, MMP-9 and ETV5 in the mice's primary tumours unveiled that ALCAM expression is reduced in the invasive front of ETV5-overexpressing compared to Hec1A tumours ($p<0.05$; Figure 46), and moreover, the pattern of expression is modified from a very membranous staining in Hec1A tumours to a diffuse cytoplasmic staining in ETV5 overexpressing cells (Figure 45, 48). This could be promoted by the shedding of ALCAM in ETV5 overexpressing tumours.

In relation to this, we observed that the intensity of ALCAM and MMP-9 markers showed no variation between the superficial or the invasive area in the Hec1A tumours. But interestingly, their intensity was graded in the ETV5 overexpressing tumours. In those, MMP-9 increased was concomitant with a

decrease in ALCAM, when comparing the superficial to the invasive front of the tumour (Figure 46-B, Figure 47).

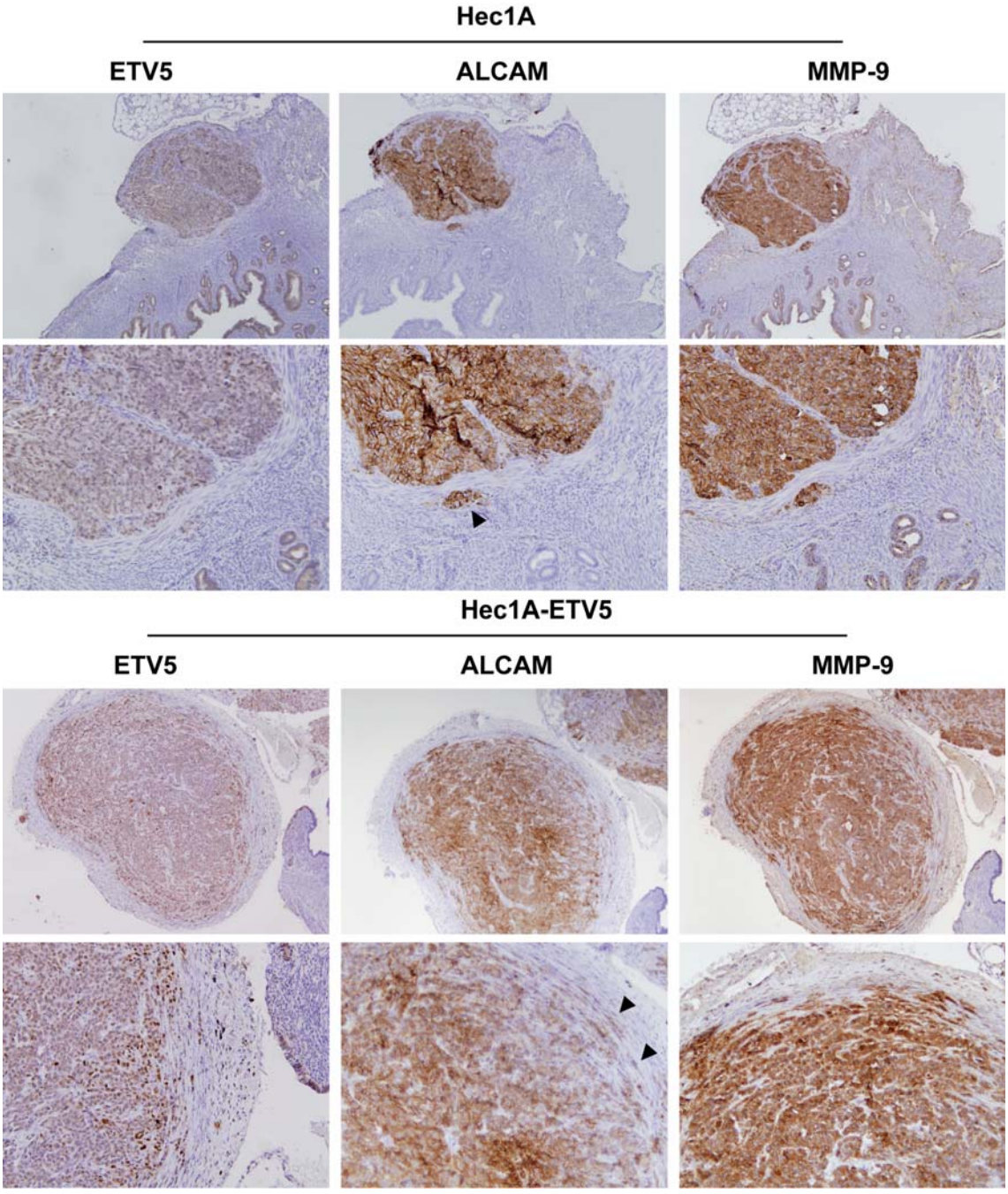


Figure 45. ALCAM was decreased at the invasive front of the primary tumours in a controlled model of EEC dissemination. Upper: Immunohistochemistry of ETV5, ALCAM and MMP-9 in Hec1A control mice. Both ALCAM and MMP-9 presented a homogeneous staining between the superficial and invasive front or disseminated cells. Black arrow signals a cluster of disseminated cells released from the primary tumour. Down: Immunohistochemistry of ETV5, ALCAM and MMP-9 in Hec1A-ETV5 mice. ALCAM expression was decreased at the invasive front of the tumour, concomitant with an increase of MMP-9 expression. Black arrows evidenced disseminated cells in finger-strand or individual cells, released from the ETV5-overexpressed primary tumour.

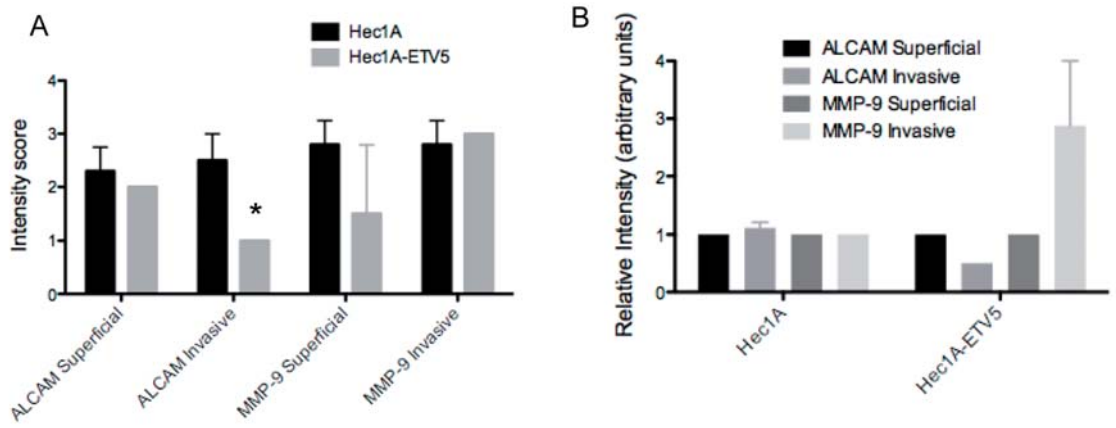


Figure 46. ALCAM staining was significantly decreased at the invasive front of the primary tumours in a controlled model of EEC dissemination. (A) Representation of the intensity of staining of the two molecules. ALCAM expression was only significantly decreased at the invasive front of the ETV5-overexpressing mice, compared to control (*p<0.05). (B) Relative intensity of the two markers at the invasive front compared to the superficial tumour. While in the control mice, ALCAM and MMP-9 where homogeneous across the section, in the ETV5-overexpressing mice we observed a decrease in ALCAM expression concomitant with an increase in MMP-9 expression in the invasive front of the primary tumours.

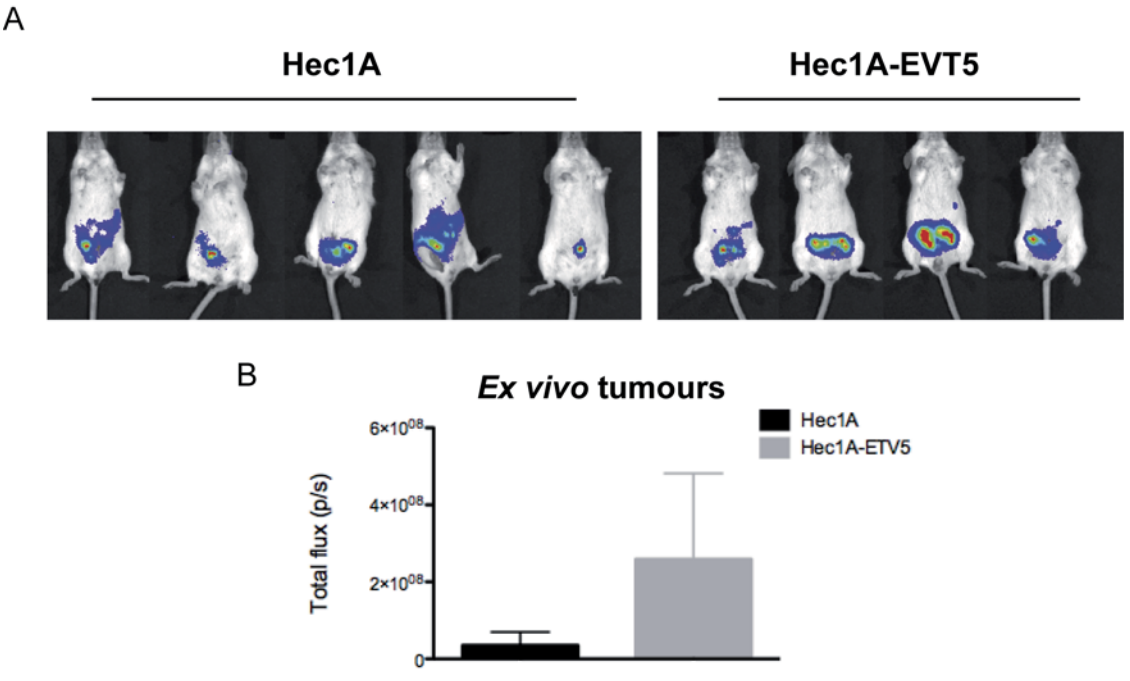


Figure 47. Hec1A and Hec1A-ETV5 orthotopic murine models followed by IVIS. (A) IVIS images of the injected mice evidenced a larger tumour growth in the ETV5-overexpressing cells. (B) Quantification of the luminescence of the primary tumours *ex vivo* evidenced a higher intensity in the ETV5-overexpressing mice (p=0.032, one-sided test).

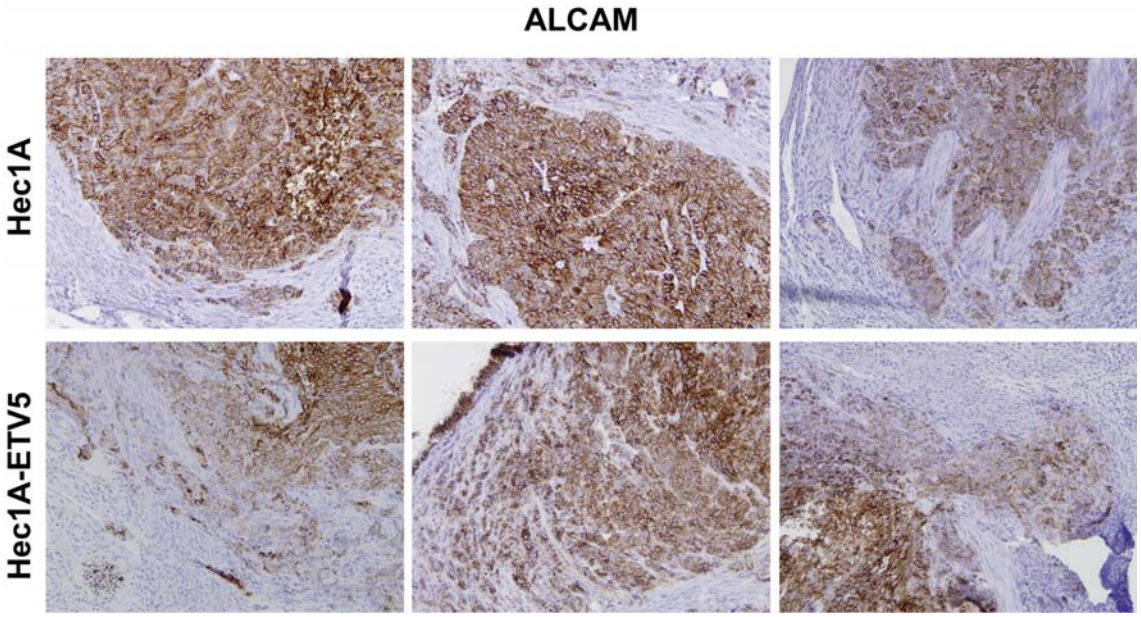


Figure 48. The expression of the full ALCAM protein was decreased at the invasive front of Hec1A-ETV5 cells. Upper: Images of ALCAM expression in Hec1A control mice, presenting less invasion and scattered clusters of cells. Down: Images of ALCAM in Hec1A-ETV5 mice, with higher rate of invasion and disseminated cells, with a finger-strand pattern. ALCAM staining was more homogeneous in the control model with lower invasion and more collective invasion.

As a result of the *in vivo* model, we evidenced that the cell-cell contacts of the Hec1A invading cells seem to be preserved, as shown by the highly collective migration and a homogeneous ALCAM expression. However, the ETV5-overexpressing invading cells were more prone to present switching between thin cords and single-cells, both presented decreased or more transient contacts and higher rate of cleaved ALCAM expression. Moreover, we finally confirmed that in an invasive scenario, ALCAM and MMP-9 are important actors at the invasive front of the tumour. Whilst ALCAM diminishes its expression as an uncleaved protein, MMP-9 increases its expression, probably promoting ALCAM shedding.

4.2.6 Full ALCAM recovery impaired migration and decreased cell-cell adhesion of invasive EEC cells

In order to understand the role of ALCAM in the invasive Hec1A-ETV5 model, we constructed two ALCAM overexpression vectors: a full-length ALCAM and an ALCAM containing only the extracellular and transmembrane region

(ALCAMcytoless) in pmCherry-N1 vectors (Figure 49-A). We transiently transfected the Hec1A-ETV5 cells with the Cherry empty-vector as a control, and both ALCAM constructs. As expected, ALCAM overexpression was localized primarily in the plasma membrane and in the cytoplasm.
(Figure 49-B).

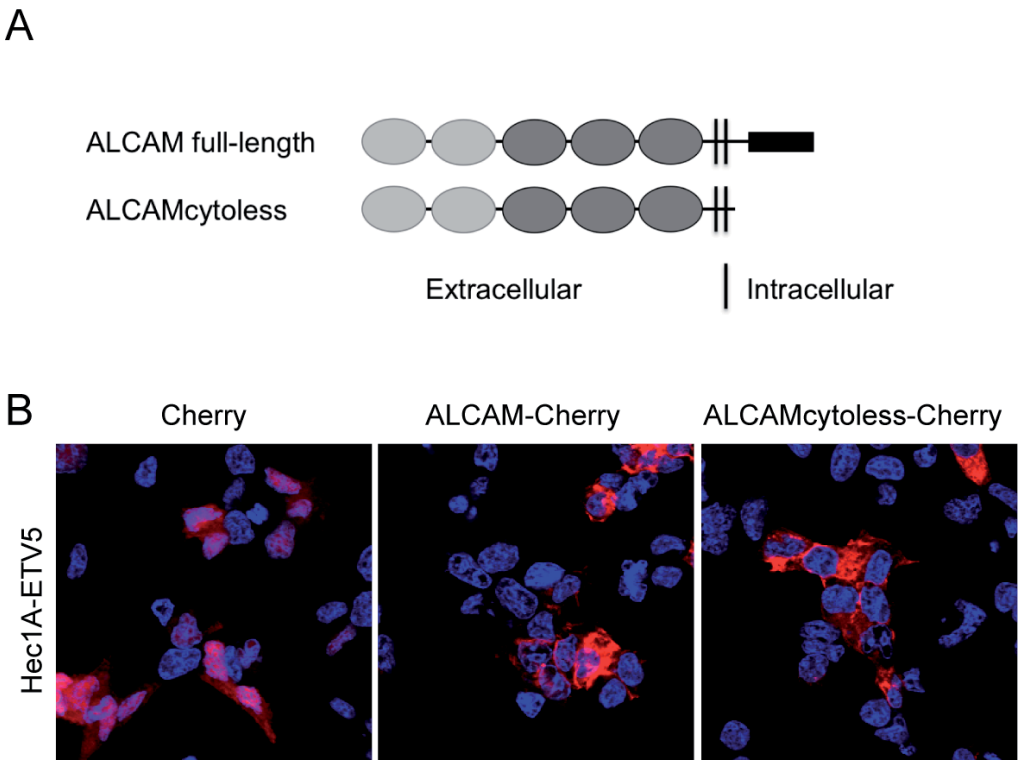


Figure 49. ALCAM recovery in mesenchymal Hec1A-ETV5 cells. (A) Representation of the two vectors constructed to overexpress ALCAM: full-length and ALCAMcytoless inserted in the pmCherry-N1 construct. (B) Images of the Hec1A-ETV5 cell line transfected with the pmCherry vector as a control, and both ALCAM-overexpression constructs.

To evaluate the effects of ALCAM-recovery in ETV5-overexpressing cells, we used 3D *in vitro* approaches that closely mimic the *in vivo* settings. We used a spheroid model to quantitatively study the spreading of cell aggregates on a striped fibronectin-coated surface. In addition to reproducing characteristics of the *in vivo* environment, as stated in objective (i), this model also allows us to analyse the effects of the competition between cell–cell and cell–substratum adhesion on tissue spreading [311]. In both conditions (ALCAM full-length and ALCAMcytoless) the speed was significantly decreased compared to the control cells (Figure 50-A). However, the larger difference was found in the transfected cells expressing full-length ALCAM. In fact, we observed that the mean time of

disaggregation of the Cherry-control spheroids was around 5 h, while ALCAM full-length and ALCAMcytoless expressing aggregates needed approximately 21 h and 18 h, respectively (Figure 50-B). Moreover, the pattern of spreading in control cells presented a more spindle-shaped phenotype, with larger presence of individual cells and protrusions, and highly dynamic transient cell-cell contacts, migration in a sheet fashion and presentation of chemotactic abilities. In contrast, full-ALCAM and ALCAMcytoless cells presented an increased intercellular adhesion phenotype and a more collective pattern of spreading (Figure 50-B). Consequently, ALCAM recovery clearly impaired the migratory abilities of ETV5 overexpressing cell lines.

To verify these effects we evaluated how ALCAM-rescue affects the formation and strength of cell-cell adhesion. We used two micropipettes to put into contact two isolated cells maintained in suspension, to avoid cell-matrix interactions, and to initiate adhesion. Single cells were chosen under a fluorescence microscope to verify the Cherry-transfection. The mean separation force (SF) is used as a read-out of the strength of adhesion for a precise contact time. In all the cases, the SF required to disrupt the cell doublet increased with the contact time. We observed that at 4 min both ALCAM transfected cells presented significantly larger adhesion than the control line (Figure 51A-B). Cells expressing full-length ALCAM displayed stronger intercellular adhesion, as shown by the two-fold increase in their SF compared to control cells, and were more adhesive than cells expressing ALCAMcytoless. In contrast, at 10 min only the full-length ALCAM-transfected cells presented significantly stronger adhesion than the control. Thus, we showed that ALCAM shedding was important to also reduce ETV5-adhesive properties. In this particular context, the cytoplasmic tail was an important player in cell-cell adhesion formation or maturation.

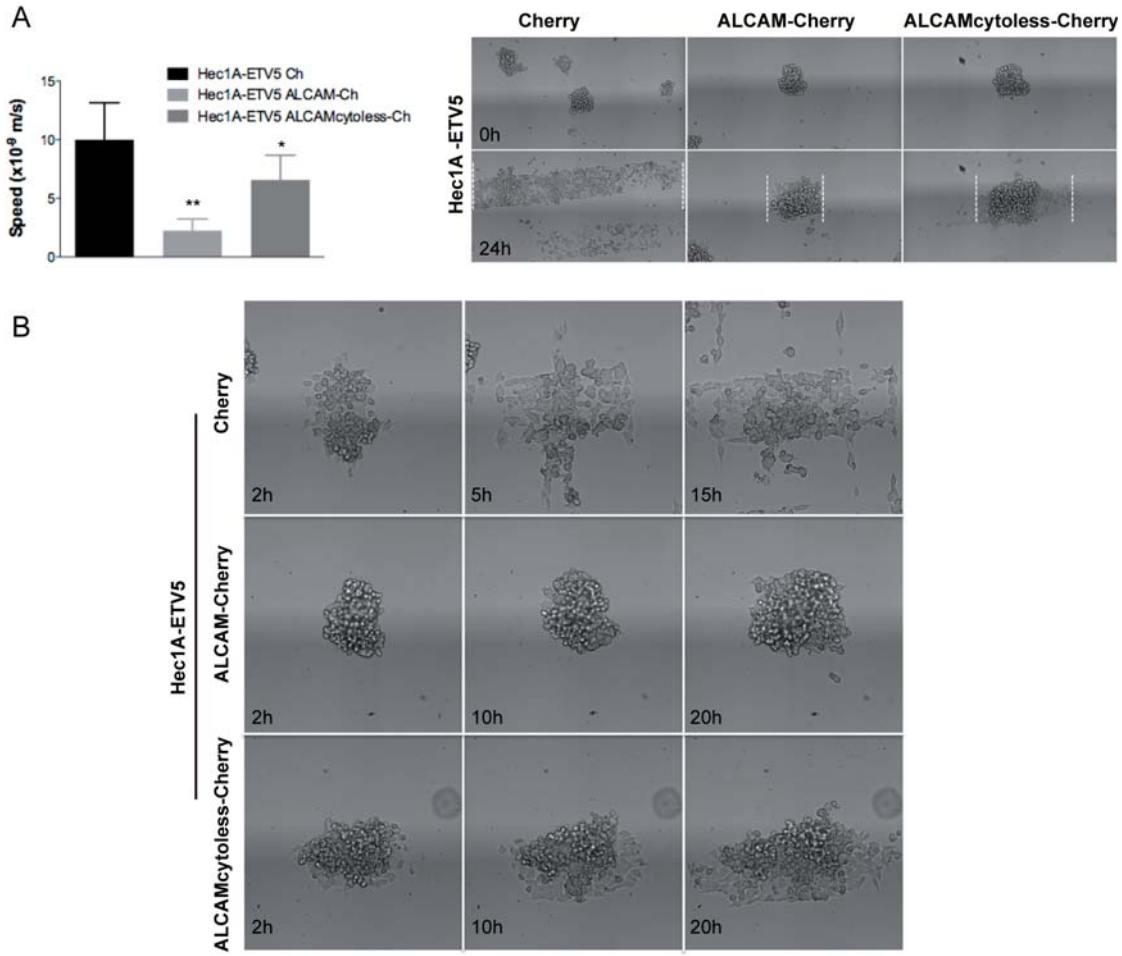


Figure 50. ALCAM overexpression in mesenchymal Hec1A-ETV5 cells decreased cell migration. (A) Effect of ALCAM overexpression on migration was assessed by a 3D spheroid-spreading model on 200 µm fibronectin-coated stripes. On the left, box plot of the speed of migration for each cell line. On the right, representative images at time 0h and at 24h are illustrated and the spreading reached at 24h is outlined in the images. Migration decreased in cells transfected with full-length ALCAM (**p<0.01) and ALCAMcytoless (*p<0.05) compared to control. (B) Images of the 3D spheroid-spreading model are illustrated at different time points. In the control cells, the spheroids were almost disaggregated after 5 h, whilst the mean time for disaggregation for the ALCAMcytoless cells was around 18 h, and more than 21 h for the full-length ALCAM cells.

The molecular pathway by which ETV5 promotes its invasive functions in EEC has been recently postulated by Alonso-Alconada et al. [322]. The authors described how ETV5 works through the BDNF–TrkB–ERK1/2 axis by inducing ERK1/2 phosphorylation, leading to the promotion of migration and invasion. In this work, we observed that ALCAM-overexpression was concomitant with a decrease in p-ERK levels, without reverting to an epithelial phenotype (Figure 52), but impairing the migratory and adhesive properties promoted by ETV5. Although overexpression of the extracellular-ALCAM is sufficient to reduce the

levels of ERK phosphorylation and to decrease spheroid spreading, full-length ALCAM is necessary to increase cell-cell adhesion with time.

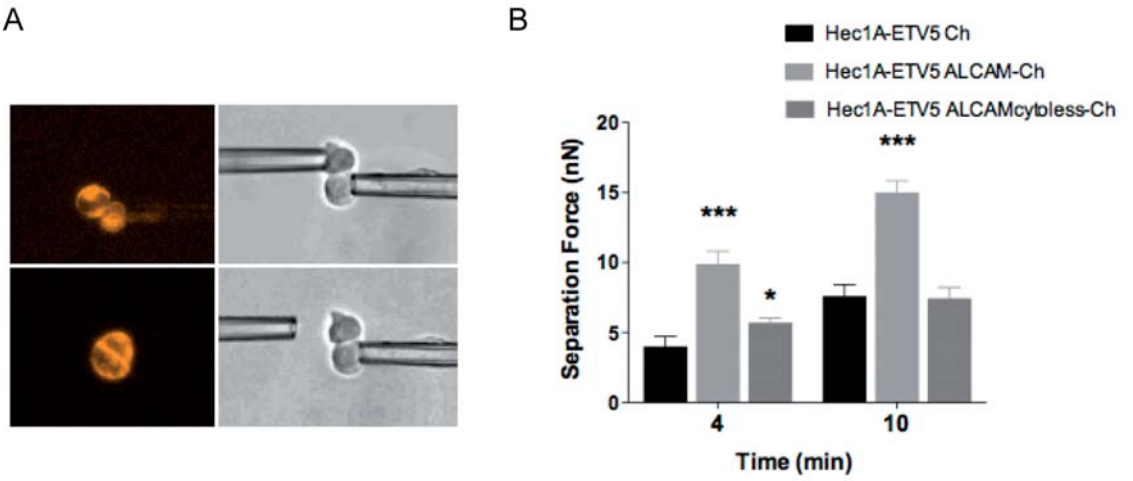


Figure 51. ALCAM recovery in mesenchymal Hec1A-ETV5 cells increased cell-cell adhesion. (A) Two Cherry transiently transfected cells were put into contact and allowed to form adhesion. After 4 and 10 min, we applied an increasing measurable force (nN) up to the disruption of the formed cell doublet. (B) Full-length ALCAM-overexpression increased cell-cell adhesion at all points, as shown by quantification of the separation force (***p<0.001, Mean±SEM). However, ALCAMcytoless only increased cell-cell adhesion at 4 min and showed no differences with control line at 10 min.

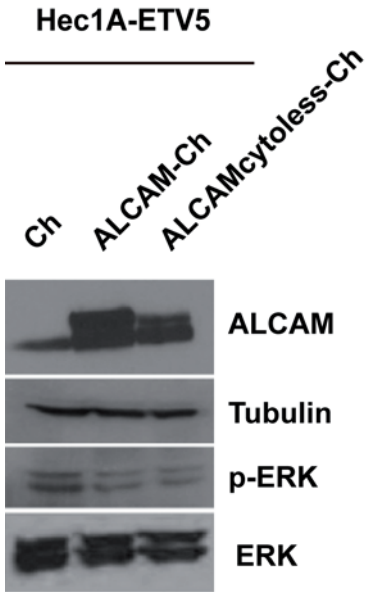


Figure 52. ALCAM recovery in mesenchymal Hec1A-ETV5 cells and p-ERK. Western blot analysis of ALCAM-overexpression in Hec1A-ETV5 cells demonstrated a significant decrease in the levels of ERK-phosphorylation.

Chapter 5. Discussion

Endometrial cancer (EC) is the most common gynaecological cancer in the western countries and the second leading cause of gynaecological malignancy worldwide [1]. Endometrial cancers are classified into two broad categories, endometrioid and non-endometrioid carcinoma, which are associated with different pathogeneses and prognoses [323]. Endometrioid endometrial cancers (EEC) comprise ≈80% of the tumours, present a better outcome, and are manifest basically of low-grade endometrioid histology whereas non-endometrioid endometrial carcinomas (NEEC) are usually represented by serous carcinomas. While EEC are associated with a premalignant hyperplastic lesion and are related to hyperoestrogenism, NEEC are associated with atrophic endometrium and hormone-independent carcinomas [324]. The symptomatology allows an early stage diagnosis, when the disease is confined in the uterus. These patients are classified in FIGO stage I, and present a favourable prognosis. The FIGO stage, assessed after pathological examination of the hysterectomy sample, is the most compelling independent prognostic factor.

The main goal of risk stratification systems in EC is to identify those patients who will benefit from a specific adjuvant treatment. A general consensus was recently reached between the European Society for Medical Oncology (ESMO), the European Society for Radiotherapy & Oncology (ESTRO) and

the European Society of Gynaecological Oncology (ESGO) to define recurrence risk groups based on the assessment of prognostic factors: age, FIGO stage, grade and histology [46]. Following this classification, EEC patients at low- and intermediate-risk of recurrence achieve an overall 5-year survival rate of ≈90%. Nonetheless, a significant number of patients stratified according to these clinicopathological parameters experience recurrence and die from the disease. This statement underlines an important unmet clinical need in the definition of more accurate risk stratification systems

In the first part of this thesis, we demonstrated that ALCAM-positivity expression was an independent prognostic factor of recurrence in the early stage EEC patients in a multicentre retrospective study, reaching a HR 9.293 (95% IC 2.119-53.470) in the subset of early stage moderately-poorly differentiated tumours. Moreover, in these cohorts ALCAM-positive expression was correlated with a decrease in the recurrence-free survival. Thus, ALCAM-positivity demonstrated to be a reliable biomarker to discriminate patients currently identified at an intermediate-risk of recurrence. Recent studies have also identified other markers, such as L1CAM [325] and ANXA2 [326], which improved risk prediction in early stage EEC. Actually, L1CAM also belongs to the IgSF, adding weight to the role of CAMs in EC dissemination and metastasis. The authors evidenced that L1CAM is a reliable prognostic factor of recurrence in FIGO stage I, type I EC. They also proved that L1CAM positive expression indicated the need for adjuvant treatment. Further validations are needed in order to foster the implementation of ALCAM and other markers in new risk prediction models that may favourably impact on the survival of EC patients.

Cell adhesion molecules (CAMs) are essential for cell attachment, tissue organization and development, and they also play a major role in transmembrane signalling and cell motility. Interaction of cadherins, integrins, selectins and IgSF members result in signal transduction into the cell [327]. In fact, they translate external mechanical forces through the cytoskeleton to activate biochemical signals within the cell [328]. ALCAM homotypic adhesion is a tightly regulated process, and it has been associated with cell motility and metastasis in cancer [329]. ALCAM has been described to interact with the actin

cytoskeleton, since low concentrations of cytochalasin D allowed a partial disruption of the cytoskeleton, which is necessary for the transient release of ALCAM from the actin cortex and for the stable homotypic ALCAM-mediated adhesion [252]. Actually, ALCAM is composed of 5 extracellular domains, a transmembrane region and a short cytoplasmic tail that can interact, via adaptor proteins, with the actin cytoskeleton [253]. The short cytoplasmic tail includes a positive-charge-rich domain (PCRD) as well as a PDZ-binding motif KTEA placed at the C-terminus [254]. FRET-FLIM and single-cell force spectroscopy measurements demonstrated the existence of a supramolecular complex, where ALCAM binds to syntenin-1 and ezrin, these binds allow ALCAM to interact with the actin [256]. In addition, PKCα also plays a role in the cytoskeleton-dependent avidity modulation of ALCAM [252]. However, the cytoplasmic domain of ALCAM lacks both of PKC phosphorylation motif and the ability to be phosphorylated upon PKC activation. PKCα regulates the supramolecular complex formed by ALCAM and Ezrin [254]. Tetraspanin CD9 was also described to work as a scaffold interacting with the ALCAM extracellular Ig-like domain [330].

In our study, less force was required to separate ALCAM-depleted cells, demonstrating its role in cell-cell adhesion. As such, several studies have evidenced crosstalk between cell-cell and cell-matrix adhesion in regulating cell migration [331,332]. ALCAM modulation may have impacted the balance between cell-cell and cell-matrix with an effect on cell polarization and migration. We also demonstrated that ALCAM acted as a positive regulator of migration and invasion in Hec1A and Ishikawa cells. Moreover, we tested the tumorigenic and metastatic abilities of ALCAM-depleted cells *in vivo* in an EC orthotopic murine model. We found that ALCAM inhibition decreased both the size of primary tumour and the number of metastases. Specifically, ALCAM inhibition impaired the ability of tumour cells to disseminate at the locoregional level. These results were independent of an increase in cell proliferation and lead to speculation that ALCAM-depletion interferes with the ability of tumour cells to communicate with the surrounding microenvironment. Similarly, ALCAM knockdown in malignant mesothelioma cells was related to supressed

migration and invasion [333]. Moreover, Wilger et al. used a fully human scFv antibody recognising ALCAM on cancer cells that reduced cell invasion [334].

Likewise, microarray analysis evidenced enrichment in deregulated genes involved in cell migration and invasion. Microarray validation by RT-qPCR and western blot demonstrated that FLNB, TXNRD1 and LAMC2 are dependent upon ALCAM regulation, so we hypothesize that those proteins may be downstream effectors of ALCAM-mediated functions. Filamins are a type of acting-binding proteins that play an important role in cross-linking cortical actin filament in 3D structures. They have been described to interact directly with many functional proteins [335,336], including channels, IgSF members integrins, kinases and transcription factors. Specifically, FLNB interacts with ICAM-1, this allowing its recruitment and ICAM-1 induced transmigration [336]. FLNB has been described to promote cancer cells invasive capabilities and a spindle-morphology [337]. Iguchi et al. proposed a model whereby FLNB enhances contractile forces generated by actomyosin, via regulation of diphosphorylation of the myosin regulatory light chain. They also state that FLNB may be responsible for promoting invasion through FAK-mediated regulation of focal adhesion turnover. Under this scenario, it is tempting to hypothesize that FLNB could bind to the cytoplasmic tail of ALCAM and serve as a scaffold for ALCAM-mediated functions into the cell.

Additionally, TXNRD1 possesses glutaredoxin and thioredoxin-reductase activity. Moreover, specific functions of TXNRD1 splice variants have been described to regulate the cell cytoskeleton by induction of actin and tubulin polymerization, this leading to the formation of cell membrane protrusions identified as filopodia [338,339]. LAMC2 is a subunit of the heterotrimeric glycoprotein laminin-5, and it has been described as a fundamental constituent of the epithelial basement membrane [340]. LAMC2 expression has been correlated with poor clinical outcomes and metastatic potential in lung adenocarcinoma, oesophageal squamous cell cancer, bladder cancer and its expression in the serum of pancreatic patients was associated with higher aggressiveness [341–343]. The exact mechanisms leading to ALCAM regulation of TXNRD1 and LAMC2 remain unclear and require further study.

In summary, in our first objective we demonstrated the clinical utility of ALCAM expression as a biomarker of recurrence in early stage moderately-poorly differentiated EEC tumours. We have established the role of ALCAM as a positive modulator of cell adhesion, motility and invasiveness, *in vitro* and *in vivo*; and we have identified some of the downstream effector genes that are involved in ALCAM-mediated cell functions, specifically *FLNB*, *TXNRD1* and *LAMC2*. Altogether our results point to ALCAM as an important molecule in EEC carcinogenesis and metastasis development and suggest that identifying the intrinsic mechanisms in which it is involved may lead to the development of powerful clinical and therapeutic approaches.

At this stage, the important role of ALCAM in EEC carcinogenesis has been demonstrated. Next, in the second part of this thesis, we went further in the characterization of the involved mechanisms, and specifically, in its link to the myometrial invasion event as well as to some key epithelial and mesenchymal molecules in EC dissemination.

The principal components of adherens junctions in epithelial cells are E-cadherin and β-catenin. E-cadherin is a calcium-dependent transmembrane receptor that interacts homotypically to the E-cadherin of adjacent cells and heterotypically to cytoplasmic-binding proteins, including β-catenin, to transduce external signals within the cell and provide a physical link to the cytoskeleton [344]. Loss of epithelial polarity and decreased E-cadherin lead to EMT and have been described to promote invasion and are related to poor prognosis and high-grade EC tumours [345]. In addition, the upregulation of the ETV5 Ets transcription factor has been linked to EMT and associated with myometrial infiltration in EC [346]. Our previous *in vitro* and *in vivo* studies evidenced that ETV5 promotes EMT by modulation of ZEB1 expression and consequent E-cadherin repression, leading to a complete reorganization of cell-cell and cell-substrate contacts and in the acquisition of migratory and invasive capabilities [190,321]. Moreover, ETV5 has been shown to co-localize with MMP-2 and -9 at the invasive front of the tumour [346]. The high expression of both metalloproteinases and COX-2, another direct target of ETV5, has been correlated to poor prognosis and relapse in EC [347,348].

In this study, we demonstrated the role of ALCAM in the process of myometrial invasion. To do this, we used an extracellular ALCAM antibody to assess the uncleaved ALCAM protein expression by immunohistochemistry in two different tumour areas in 116 patients, at the superficial and the invasive front, and correlated to a set of epithelial (E-cadherin, β-catenin) and mesenchymal markers (ETV5, COX-2, MMP-2, and MMP-9).

While in the superficial tumour, uncleaved ALCAM was correlated with the major adhesion complex E-Cadherin/β-catenin; in the invasive tumour, where a more mesenchymal microenvironment takes place, this correlation is lost and replaced by the correlation between ALCAM and the mesenchymal markers COX-2, MMP-9 and ETV5. Our previous studies on ALCAM in EEC evidenced the important role of this molecule in recurrence and cancer progression in patients with moderately-poorly differentiated tumours [320]. Interestingly, the best regression models were obtained for the moderately-poorly differentiated tumours in this study. In this subcohort, only the E-cadherin was retained at the superficial level (R=0.445) versus ETV5 and MMP-9 (R=0.559) at the invasive front. While the correlation with E-cadherin was positive, both ETV5 and MMP-9 presented a significant negative correlation with extracellular ALCAM.

Moreover, multivariate logistic regression analysis evidenced that ALCAM-negativity is an independent prognostic marker of myometrial invasion together with the tumour grade (OR 3.273 and 3.484, respectively) when analysed at the invasive front of the tumour. Categorized analyses also demonstrated that extracellular ALCAM and MMP-9 present a negative significant correlation (p=0.004). However, this relationship was only maintained in the moderately-poorly differentiated tumours and in patients presenting >50% of myometrial invasion. Interestingly, the relationship was dependent on the ETV5-positive phenotype, suggesting that the mesenchymal transcription factor might promote ALCAM cleavage and release from the cell surface through MMP-9.

In the last few years, the interest in ALCAM ectodomain shedding and its use as prognostic biomarker for patient outcome has significantly increased. To demonstrate its cleavage in colorectal cancer, some authors used a dual-stain

system that detects both the extracellular and the intracellular domains in formalin-fixed tissue. ALCAM shedding was significantly elevated in patients with colon cancer and correlated with reduced survival. Moreover, they suggested that shedding could identify patients with early stage disease at risk of rapid progression for colon cancer [299]. In fact, soluble ALCAM has been detected in some biofluids and described as a prognostic marker of tumour progression in ovarian, oesophageal, pancreatic, breast, thyroid, colon and bladder cancers [298,301,302,304,349,350]. In this study, we found that soluble ALCAM, in uterine aspirates (UA) from patients with moderately-poorly differentiated EEC, is significantly increased when myometrial invasion is >50%. Moreover, ROC analysis corroborated sALCAM as a significant predictor of myometrial invasion in UA (AUC=0.80; p=0.001). These findings are particularly interesting as the uterine aspirates are the preferable biopsy used in the diagnostic process of EC and the assessment of sALCAM might open the possibility of developing a potential tool to determine pre-operatively myometrial infiltration (sensitivity 87.0%, specificity 70.6%) at the time of diagnosis even in earlier EC stages [351].

In addition, the use of UA allowed us to evidence that sALCAM and MMP-9 were significantly strongly correlated (R=0.575; p=0.004) only in patients presenting myometrial invasion >50%. Given that the disruption of ALCAM–ALCAM interactions between cells promotes tumour cell motility and metastasis, ALCAM shedding may predict malignant progression at a molecular level. In fact, ectopic expression of a mutant ΔN-ALCAM in melanoma cells has been described to induce loss of cellular aggregation, increased cell motility in skin reconstructs and larger spontaneous lung metastasis formation at the expense of primary tumour growth in mouse xenografts [257].

In our orthotopic *in vivo* model of EEC dissemination, Hec1A and the Hec1A-ETV5 luciferase overexpressing cells were injected directly into the mice uterus. ETV5-overexpressing mice presented larger tumour burden, invasion and disseminated cells with a more mesenchymal phenotype and a switch from finger-like strands to single-cells compared to the control mice. In the ETV5-overexpressing tumours, we observed a decrease of uncleaved

ALCAM expression at the invasive front concomitant with an increase in MMP-9 expression in comparison to a homogeneous staining in the less invasive control tumours. MMP-9 expression has been associated with increased metastatic potential in many cancer types including prostate, breast, melanoma, and brain cancer [352]. MMP-9 activity directly modulates the cell-cell and cell-matrix attachment and has been described to regulate the process of cell invasion and migration. Here, we could hypothesize that MMP-9 is responsible for ALCAM shedding, allowing cell invasion.

Moreover, we proved that ALCAM recovery in ETV5 overexpressing cells impaired the ETV5-related functions in the invasive process. Firstly, ALCAM-recovery in Hec1A-ETV5 cells decreased cell migration in a 3D spheroid model. In addition, ALCAM-transfected cells showed a pattern of spreading more prone to collective migration compared to the single-cell phenotype of ETV5-control, which are characterised by higher dynamic transient cell-cell contacts and the presence of protrusions. In fact, we evidenced that a larger adhesion force was necessary to separate the ALCAM-transfected cell doublet and especially, that after longer periods of adhesion, the only significant difference was found with the full-length transfected ALCAM indicating its capacity to promote intercellular adhesion strengthening. This fact, evidences the crucial role of its cytoplasmic tail in the maturations of cell-cell adhesion. In fact, as aforementioned, the cytoplasmic tail of ALCAM has been described to interact with the actin cytoskeleton, and could be essential in the translation of external mechanical forces into the cells to activate biochemical signals [257,328].

These differences in cell-cell adhesion, in the cell speed and the pattern of migration could be almost partially explained by the decrease of the active levels of p-ERK. The link between the role of BDNF in the modulation of EMT induced by ETV5 and the acquisition of cell migratory/invasive capabilities through p-ERK phosphorylation has been established [322]. The known role of the BDNF–TrkB–ERK1/2 axis on neuronal plasticity and organogenesis might be mediating pro-metastatic events in endometrial cancer and as consequence the decrease in the p-ERK levels would lead to a less aggressive phenotype [322]. Moreover, some authors evidenced that sustained

MAPK activation could lead to enhanced induction of proteolytic enzymes in the surrounding environment in tumours. Specifically, the inhibition of ERK activation by MEK inhibitor PD098059 has been described to partially block MMP-9 production and attenuated the *in vivo* invasiveness of head and neck squamous cancer cells [353].

Although further research is needed to define the exact mechanisms, our *in vitro* and *in vivo* models evidenced that ALCAM participates in cell-cell adhesion and collective cell migration, and suggest that to allow invasion in the frontier between tissue-restricted carcinoma and disseminated tumour cells, a dynamic and adaptive switch between ALCAM expression at the cell surface and cleaved ALCAM shed by MMP-9 might take place. In fact, cancer cell invasion is currently viewed as a heterogeneous and adaptive process [354] in which, the plasticity in cell adhesion, the dynamics of the cytoskeleton and the mechanotransduction of external stimuli are key processes. Taking into account that myometrial invasion is highly associated with poor prognosis and to a limited therapeutic response, our research opens an avenue for the use of ALCAM recovery as a therapeutic approach to control tumour dissemination.

Chapter 6. Conclusions

This manuscript gathers the main scientific contributions achieved through the current PhD thesis and framed in the field of endometrial cancer. These research achievements have led to the publication of a manuscript in The Journal of Pathology (paper A) and to the submission of a second one in another peer-review journal (paper B).

This thesis aimed to improve survival of endometrial cancer patients by enhancing the accuracy of current risk assessment methods and improving our knowledge in the specific mechanisms leading to invasion. As stated in Chapter II of this manuscript, this thesis has focused on two specific objectives: (*i*) The study of ALCAM as a marker of recurrence in endometrioid endometrial cancer and its function in tumour progression; and (*ii*) The characterization of the molecular mechanisms associated with ALCAM in the superficial and the invasive front of the tumour and its relation to myometrial infiltration.

In the first part of this thesis, we demonstrated the value of ALCAM as a marker of recurrence in endometrioid endometrial cancer by conducting a retrospective multicentre study in 174 primary tumours. ALCAM positive

expression was an independent prognostic factor of recurrence in the early stage EEC patients (HR 6.027, 95% CI 1.41-25.74). Moreover, in the subset of early stage moderately-poorly differentiated tumours, ALCAM-positivity was correlated with a significant decrease in recurrence-free survival and a high probability of relapse (HR 9.293, 95% IC 2.119-53.470; p=0.008). Then, we demonstrated *in vitro* a role for ALCAM in cell migration and invasion by using a loss-of-function model in Hec1A and Ishikawa endometrial cancer cell lines. Moreover, ALCAM depletion resulted in reduced primary tumour and metastases size, as well as in a reduction of the number of metastases formed by local spread in an orthotopic murine model. Microarray gene expression analysis of ALCAM-depleted cell lines supported that motility, invasiveness, cellular assembly and organization were the most deregulated functions associated with ALCAM. Finally, we evidenced some of the downstream effector genes that are involved in ALCAM mediated cell migration; specifically FLNB, TXNRD1 and LAMC2 were decreased at mRNA and at protein level when ALCAM expression was knockdown. In conclusion, our results highlighted the potential of ALCAM as a biomarker of recurrence in early stage endometrioid endometrial cancer and point to ALCAM as an important molecule in endometrial cancer dissemination by regulating cell migration, invasion and metastasis.

Once we demonstrated the role of ALCAM in EEC invasion, we went further in our understanding of its mechanisms to promote invasion and specifically regarding the myometrial infiltration event. Myometrial invasion in EC is an event of poor prognosis; so research to control this process is important in order to hamper tumour dissemination. It has already been described that ALCAM can be proteolytically cleaved at the cell surface causing the ectodomain to be shed, generating a soluble 96 kDa and a truncated membrane-bound. Although currently ALCAM shedding in endometrial cancer has not been studied, it has been reported in the serum of breast, ovarian, thyroid and pancreatic cancer patients and related to poor clinical outcome.

In the second part of this thesis, we used an antibody that specifically recognises the N-terminal domain of ALCAM extracellular domains. Thus, we were able to measure the non-cleaved protein at the cell surface. ALCAM

expression was assessed by immunohistochemistry in two tumour areas in 116 EC patients, at the superficial and the invasive front and correlated to a set of epithelial (E-cadherin, β-catenin) and mesenchymal markers (ETV5, COX-2, MMP-2 and MMP-9). Moreover, ALCAM shedding was analysed by ELISA in uterine aspirates from endometrial cancer patients diagnosed with moderately-poorly differentiated tumours, and related to clinical-parameters and to MMP-9 expression by using gelatin zymography. Finally, *in vivo* and *in vitro* assays were assessed to study ALCAM modulation effects in a controlled model of invasion. We described the heterogeneous functionality of ALCAM depending on its localization, i.e. superficial vs. invasive areas of the tumour. While in the superficial area of the tumour ALCAM correlated with the major adhesion complex, at the invasive front ALCAM correlated with the mesenchymal markers. Specifically, at the invasive front of moderately-poorly differentiated tumours, ALCAM correlated inversely with ETV5 and MMP-9. Only at the invasive front, was the extracellular ALCAM-negativity an independent marker of myometrial invasion (OR 3.273). The lack of ALCAM staining at the invasive front was due to an increase of ALCAM shedding, as proved by the increased soluble ALCAM seen in uterine aspirates from patients with moderately-poorly differentiated EC presenting myometrial infiltration (AUC=0.80). *In vivo* and *in vitro* ETV5-overexpressing models of EC were selected as models of EC invasion. We demonstrated that ALCAM shedding at the invasion front promotes cancer cell dissemination and that the recovery of the full-form of ALCAM reverts most of the ETV5-overepressing cells mesenchymal abilities, at least partially in a p-ERK dependent-manner. In conclusion, we demonstrated that ALCAM participates in cell-cell adhesion and collective cell migration, and our results suggest that a dynamic and adaptive switch between cell surface and cleaved ALCAM, in the frontier between tissue-restricted carcinoma and disseminated tumour cells, allows invasion to take place.

For the sake of clarity, the main conclusions achieved in this thesis are summarized in the following list:

1) A retrospective multicentre study in 174 EEC primary tumours evidenced that ALCAM-positivity was an independent prognostic factor of relapse in early stages of the disease (HR 6.027).

2) In early stage patients, recurrence-free survival was significantly lower in patients with ALCAM-positive compared to ALCAM-negative tumours. This difference was more relevant in patients with early stage moderately-poorly differentiated tumours, for whom the risk of relapse reached a HR 9.259.

3) ALCAM knockdown decreased cell migration and invasion in Hec1A and Ishikawa endometrial cancer cell lines and demonstrated its participation in cell-cell adhesion strengthening.

4) ALCAM knockdown resulted in reduced primary tumour and metastases size, as well as in a reduction of the number of metastases formed by local spread.

5) Gene expression analysis of Hec1A ALCAM-depleted cells confirmed that motility, invasiveness, and cellular assembly and organization were the most associated deregulated functions.

6) *FLNB*, *TXNRD1* and *LAMC2* were validated as downstream effector genes involved in ALCAM mediated functions. All these genes were decreased at mRNA and at protein level in ALCAM-depleted cells.

7) ALCAM presented a heterogeneous functionality depending on its localization within the tumour. At the superficial area, uncleaved ALCAM was correlated with the E-cadherin/β-catenin complex; whereas at the invasive front, this correlation was lost and COX-2, MMP-9 and ETV5 mesenchymal markers replaced the epithelial partners.

8) In the moderately-poorly differentiated tumours, uncleaved ALCAM correlated positively with E-cadherin at the superficial area, and negatively with ETV5 and MMP-9 at the invasive front.

9) Categorized analyses demonstrated that correlation between uncleaved ALCAM and MMP-9 was specifically significant in moderately-poorly differentiated tumours, tumours presenting more than 50% of myometrial invasion, and tumours with an ETV5-positive phenotype.

10) Lack of uncleaved ALCAM staining (or ALCAM-negativity) associated with protein shedding, at the invasive front of the tumour, was an independent marker of myometrial invasion.

11) In uterine aspirates from patients diagnosed with moderately-poorly differentiated tumours, soluble ALCAM was significantly increased when myometrial invasion was higher than 50%. ROC analysis corroborated sALCAM as a significant predictor of myometrial invasion in this body fluid (AUC=0.80) at time of diagnosis even in earlier EEC stages, and presented a sensitivity of 87.0% and a specificity of 70.6%.

12) MMP-9 and sALCAM levels were significantly correlated in uterine aspirates from patients diagnosed with moderately-poorly differentiated tumours presenting myometrial invasion higher than 50%.

13) The differential pattern of ALCAM expression seen in human tumours was corroborated in an *in vivo* model of EC dissemination. Whilst in the orthotopic murine model generated with the epithelial Hec1A cells, ALCAM and MMP-9 expressions were homogeneously distributed throughout the tumour; in the model generated with the invasive ETV5-overexpressing cells, they showed a differential staining in the superficial and the invasive front of the tumour. In this latter model, ALCAM expression decreased, whilst MMP-9 increased at the invasive front.

14) The recovery of the full-form of ALCAM in Hec1A-ETV5 cells impaired the ETV5-related functions associated with the invasive process. It decreased migration and showed a more collective migration pattern compared to the ETV5-control single-cell phenotype, which is characterised by higher dynamic

transient cell-cell contacts and presence of protrusions. Moreover, ALCAM recovery increased cell-cell adhesion.

15) ALCAM recovery in Hec1A-ETV5 cells was concomitant with a decrease in ERK phosphorylation level, which could explain the impairment of ETV5-related functions.

16) *In vitro* and *in vivo* models evidenced that ALCAM participates in cell-cell adhesion and collective cell migration, and suggest that a dynamic and adaptive switch between ALCAM expression at the cell surface and ALCAM cleavage by MMP-9 might take place in order to invade the frontier between tissue-restricted carcinoma and disseminated tumour cells.

Journal publications

This thesis has led to the publication (paper A) and submission (paper B) of the two following manuscripts:

[Paper A] L. Devis, C. P. Moiola, N. Masia, E. Martinez-Garcia, M. Santacana, T. V. Stirbat, F. Brochard-Wyart, Á. García, F. Alameda, S. Cabrera, J. Palacios, G. Moreno-Bueno, M. Abal, W. Thomas, S. Dufour, X. Matias-Guiu, A. Santamaria, J. Reventos, A. Gil-Moreno, and E. Colas, "Activated leukocyte cell adhesion molecule (ALCAM) is a marker of recurrence and promotes cell migration, invasion and metastasis in early stage endometrioid endometrial cancer," *J. Pathol.*, vol. 241, no. 4, pp. 475-487, 2017.

[Paper B] L. Devis, E. Martinez-Garcia, C. P. Moiola, M. T. Quiles, M. A. Arbos, T. V. Stirbat, F. Brochard-Wyart, Á. García, L. Alonso-Alconada, M. Abal, B. Diaz-Feijoo, W. Thomas, S. Dufour, G. Mancebo, F. Alameda, J. Reventos, A. Gil-Moreno, E. Colas. "ALCAM shedding at the invasive front of endometrioid endometrial cancer is a marker of cancer progression, promotes myometrial invasion and tumour dissemination," (submitted to *Gynecol. Oncol.*).

In the course of this thesis, collaborations with other projects focus on endometrial and ovarian cancer research have also be done. Although these works are not part of the thesis, they are listed in the following articles:

[Paper C] B. Majem, M. Rosso, L. Lapyckyj, **L. Devis**, M. F. Abascal, M. Llaurado, M. L. Matos, M. J. Besso, L. Lanau, J. CastellvI, J. L. Sanchez, A. Perez-Benavente, A. Gil-Moreno, J. Reventos, A. Santamaria-Margalef, M. Rigau, M. Vazquez-Levin. "Changes in E-cadherin expression levels are associated with ovarian cancer progression, dissemination and aggressiveness," (manuscript in preparation for *PLOS ONE*).

[Paper D] E. Martinez-Garcia, A. Lesur, **L. Devis**, S. Cabrera, X. Matias-Guiu, M. Hirshfeld, J. Asberger, J. van Oostrum, , M. A. Casares, A. Gómez-Tato, , J. Reventos, B. Domon, E. Colas, and A. Gil-Moreno, "Targeted proteomics identifies proteomic signatures in liquid-biopsies of the endometrium to diagnose endometrial cancer and assist in the prediction of the optimal surgical treatment," (submitted to *Clin. Cancer Res.*).

[Paper E] E. Martinez-Garcia, A. Lesur, **L. Devis**, A. R. Campos, S. Cabrera, J. van Oostrum, X. Matias-Guiu, A. Gil-Moreno, J. Reventos, E. Colas, and B. Domon, "Development of a sequential workflow based on LC-PRM for the verification of endometrial cancer protein biomarkers in uterine aspirate samples," *Oncotarget*, vol. 7, no. 33, pp. 53102–53115, 2016.

[Paper F] N. Pedrola, **L. Devis**, M. Llauradó, I. Campoy, E. Martinez-Garcia, M. Garcia, L. Muinelo-Romay, L. Alonso-Alconada, M. Abal, F. Alameda, G. Mancebo, R. Carreras, J. Castellví, S. Cabrera, A. Gil-Moreno, X. Matias-Guiu, J. L. Iovanna, E. Colas, J. Reventós, and A. Ruiz, "Nidogen 1 and Nuclear Protein 1: novel targets of ETV5 transcription factor involved in endometrial cancer invasion," *Clin. Exp. Metastasis*, vol. 32, no. 5, pp. 467–478, 2015.

[Paper G] E. Colas, L. Muinelo-Romay, L. Alonso-Alconada, M. Llaurado, M. Monge, J. Barbazan, M. Gonzalez, M. Schoumacher, N. Pedrola, T. Ertekin, **L. Devis**, A. Ruiz, J. Castellvi, A. Doll, A. Gil-Moreno, M. Vazquez-Levin, L. Lapyckyj, R. Lopez-Lopez, S. Robine, E. Friederich, M. Castro, J. Reventos, D. Vignjevic, and M. Abal, "ETV5 cooperates with LPP as a sensor of extracellular signals and promotes EMT in endometrial carcinomas," *Oncogene*, vol. 31, pp. 4778–4788, 2012.

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