# Tailoring therapy in endometrial and cervical cancer

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# CHAPTER 3

## Exploring morphologic and molecular aspects of endometrial cancer under progesterone treatment in the context of fertility preservation

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## CHAPTER 3

**Exploring morphologic and molecular aspects of endometrial cancer under progesterone treatment in the context of fertility preservation**

## 3.1 ABSTRACT

**Objective**

The standard treatment of early stage (FIGO I) endometrioid endometrial cancer (EEC) is a hysterectomy with bilateral salpingo-oophorectomy. An alternative approach for younger women with low-grade EEC who wish to preserve fertility may be hormonal treatment. Previous studies have suggested that progesterone may elicit its anti-tumour effect in EEC by interacting with the Wnt and/or PI3K/Akt pathways. Therefore, we explored whether common activating genetic alterations in Wnt and PI3K/Akt signaling correlated with non-responsiveness to progesterone therapy for low-grade EEC. Additionally, we investigated whether benign morphology under progesterone treatment is accompanied by absence of genetic changes.

**Materials and Methods**

We analysed molecular alterations in the Wnt and PI3K/Akt signaling in 84 serial endometrial samples from 11 premenopausal patients with progesterone receptor (PR) positive, low-grade EEC conservatively treated with progesterone and correlated these with histological and clinical follow-up.

**Results**

There were six responders and five non-responders to progesterone treatment. The response rate to progesterone treatment was 55% and the relapse rate 83%. All responders had alterations in both the Wnt and PI3K/Akt pathway prior to treatment. In the non-responder group, tumours inconsistently showed alterations in none, one or both pathways. Normalisation of the endometrium morphology under progesterone treatment is accompanied by absence of the genetic changes found in the specimen prior to treatment.

**Conclusions**

We found that activating molecular alterations in either Wnt or PI3K/Akt signaling pathways did not predict resistance to progesterone treatment. It seems that morphological response goes along with disappearance of the established mutations. This exploratory study suggests that Wnt or PI3K/Akt status is unable to predict response to progesterone treatment in patients with EEC.

## 3.2 INTRODUCTION

Endometrial cancer is the most common cancer of the female genital tract in most Western countries, with an incidence of 20 in 100.000 and is the fourth most common cancer in women after breast, lung, and colorectal cancer. [1, 2] A minority (4%) of the patients is younger than 40 years of age at diagnosis. [3] Standard treatment in early stage endometrial cancer is total hysterectomy with bilateral salpingo-oophorectomy (BSO). Hormonal treatment with progesterone may also be an option in women with well-differentiated endometrioid endometrial cancer (EEC) who wish to preserve their fertility. The initial response rate to hormonal treatment is generally good (76.2%). However, there is a high risk of relapse. [4]

The premise for progesterone treatment is the concept that unopposed estrogen stimulation is the driver for both initiation and progression of EEC. [5] In advanced endometrial cancer, progesterone receptor (PR) expression has a strong predictive value for response to progesterone therapy. [6, 7] In these patients it is possible to identify progesterone resistant tumours with an accuracy of about 90%, by determining the PR expression. Therefore, pre-treatment PR expression analysis is commonly used in the selection of patients prior to initiating progesterone therapy. [6-8] Whether PR expression is as accurate in predicting responsiveness to progesterone in low-grade EEC is unknown.

The mechanism by which progesterone induces involution of EEC is not fully understood. There is data suggesting involvement of the Wingless (Wnt) and Phosphatidylinositol 3-kinase (PI3K)-Akt signal transduction pathways. Both Wnt and PI3K/Akt signaling are frequently altered during endometrial tumourigenesis and act additively and synergistically in inducing cell proliferation. [9, 10] Constitutive activation of the Wnt-signaling can be the result of a mutation in the *CTNNB1* gene, alterations in the *APC* gene or functionally, under influence of (endogenous) estrogen. [9, 11] Nuclear β-catenin staining, indicative of active Wnt signaling, has been demonstrated in 30-85% of EECs. [12, 13] Furthermore, endogenous activation of the Wnt and PI3K/Akt pathways occurs throughout the normal menstrual cycle following changes of estrogen and progesterone levels and induces proliferation of the endometrium. [9, 14] High levels of progesterone have been shown to result in inhibition of Wnt signaling *in vitro* through up-regulation of FOXO1 and DKK1. [9, 11], thereby providing a possible mechanism for the anti-tumour effect of progesterone.

Activation of the PI3K/Akt pathway in EECs is most frequently due to inactivation of the tumour suppressor gene *PTEN* or by activating hotspot mutations in *PIK3CA* and/or *KRAS*. In EEC, loss of PTEN has been reported in 50 to 80% of cases. [15, 16] *PIK3CA* and *KRAS* have been shown to be mutated in up to 52% and 17% of EECs, respectively. [17, 18] One study of serial endometrial samples of patients with complex atypical hyperplasia (CAH), which is considered the precursor of EEC, shows significant decrease in phospho-Akt during

progesterone treatment. [19] This suggests that anti-tumour effect of progesterone may be mediated by inhibiting the PI3K/Akt signaling through dephosphorylation of Akt.
The aim of our study was to evaluate the morphological and molecular changes in sequential biopsies obtained prior to, during and after progesterone treatment in 11 women with EEC. We focussed on the Wnt and PI3K/Akt signaling pathways and explored whether activation of these pathways was related to response to therapy and recurrence.

## 3.3 MATERIALS AND METHODS

### Patients

Between 2002 and 2012, pre-treatment specimens of eleven patients treated with progesterone for EEC were sent in for central pathological review at our institution. We retrospectively collected the clinical data of these patients and obtained all available consecutive endometrial specimens for histopathological review and molecular analysis. Patient data is administered according to the principles of the Declaration of Helsinki on the ethics of research with humans. Clinical, pathological and follow-up data are summarised in table 1. All patients were diagnosed as having well-differentiated (grade 1) EEC according to the criteria stated by the WHO Classification of Tumours of the Female Genital Tract. [15] All tumours showed PR expression by immunohistochemistry (IHC) in their pre-treatment samples. Myometrial invasion and extra-uterine disease were evaluated by transvaginal ultrasound and contrast enhanced magnetic resonance imaging, revealing <50% myometrium invasion and no extra-uterine disease in all cases. All patients were treated with high-dose progesterone followed by three-monthly hysteroscopy with curetting and/or biopsy. Ten patients received medroxyprogesterone (MPA) with a median dosage of 250 mg/day (range 100-600 mg) and 1 patient was treated with megestrol acetate 200 mg/day. In addition to MPA, two patients received a levonorgestrel-releasing intrauterine system (LNG-IUS). During follow up, if responders had completed family or revised their wish for offspring, hysterectomy with BSO was advised.

### Pathology evaluation

We examined 84 curettings or biopsies and 5 hysterectomy specimens. Samples were handled in a coded fashion and all procedures were performed according to the ethical guidelines as outlined in the "Code for Proper Secondary Use of Human Tissue in The Netherlands" (Dutch Federation of Medical Scientific Societies). Formalin fixed paraffin embedded (FFPE) blocks of at least 6 consecutive samples were available for each patient (range 6 - 10, median 8). All slides and immunohistochemical stains were evaluated by one senior pathology resident (A.N.) and two gynaecopathologists (T.B. en V.S.) independently. Evaluation of the specimen was performed while unaware of

patient characteristics and clinical outcome. Discordant findings were reviewed until consensus was reached. The following parameters were evaluated on haematoxylin and eosin slides: architectural abnormalities, cytological atypia and presence of progesterone induced changes (atrophy of glands, secretory changes of the epithelium and pseudodecidualisation of stroma). Complete pathological response to progesterone therapy was defined as proliferative, secretory, inactive or atrophic endometrium without hyperplasia or atypia, as previously described [20] and according to the criteria stated by the WHO Classification of Tumours of the Female Genital Tract. [15] Absence of response was defined as persistence of well-differentiated EEC or CAH/endometrial intraepithelial neoplasia (EIN).

**Endometrial sampling**

63% of the pre-hysterectomy samples were taken by conventional dilatation and curetting or micro-curetting, 13% by biopsy/hysteroscopic resection, and 17% by both. The sampling method was unknown in 7% of the samples.

**Table 1.** Clinicopathological findings and follow-up of 11 patients with low-grade EEC treated with progesterone

| Case no. | Age at Diagnosis, y | BMI | Menstrual cycle Prediagnosis | Indication Analysis | MRI | Prediagnosis GxPx |
|---|---|---|---|---|---|---|
| 1 | 27 | 31.1 | Oligomenorrhea | Polyp | normal | G1P1 |
| 2 | 30 | 38 | Irregular | Fertility analysis | - | G0 |
| 3 | 35 | 20.8 | Regular | Fertility analysis | normal | G0 |
| 4 | 37 | 30 | Regular | Fertility analysis | normal | G0 |
| 5 | 35 | 20 | Oligomenorrhea | Fertility analysis | - | G0 |
| 6 | 36 | 33.2 | Irregular | Thickened endometrium | - | G0 |
| 7 | 38 | 22.8 | Regular | Fertility analysis/ polyp | < 50% myometrium | G0 |
| 8 | 27 | 27 | - | Fertility analysis/ polyp | normal | G0 |
| 9 | 31 | 23.9 | Irregular | Metrorrhagia | normal | G0 |
| 10 | 33 | 28.7 | Irregular | Fertility analysis/ polyp | - | G1P0A1 |
| 11 | 28 | 39 | Irregular | Irregular bleeding | normal | G0 |

MPA. Medroxyprogesterone; mo. months; G, gravida; P, para; A, abortion; LNG-IUS, Levenogestrel-releasing intrauterine sysem; * But relapsed and responded again. † Complete family. ‡ After relapse.

**Immunohistochemistry**

Immunohistochemistry for PTEN and β-catenin was performed as described previously. [21] PTEN was scored positive if it showed cytoplasmic staining in the entire tumour or the majority of the tumour cells, and negative if it showed no cytoplasmic staining in the tumour cells. [22] Adjacent stromal cells or normal endometrial glands served as positive internal controls. Activated Wnt signaling was defined as nuclear beta-catenin staining in > 1% of the tumour cells, in accordance with previous studies. [12, 23] PR staining was performed using clone PGR 636, DAKO, 1: 100 dilution and ER staining using clone 1D5, DAKO, 1:100 on each sample. Nuclear PR and ER positivity in tumour cells was scored using a continuous percentage scale.

**DNA isolation**

From FFPE blocks containing sufficient tissue (curettings), two whole sections of 10 µm were used for DNA isolation with the Tissue Preparation System (Siemens Healthcare Diagnostics). From samples with a small amount of tissue (micro-curettings or biopsies), 5 whole sections of 10 µm were used. Endometrium from hysterectomy specimens was micro dissected from five whole sections of 10 um, to avoid contamination with normal myometrium.



| Treatment, mg/d | Duration of Treatment, mo | No. Curettages | Response | Hysterectomy | Follow-up, mo | GxPx at End of Follow-up |
|---|---|---|---|---|---|---|
| MPA 200 | 17.5 | 10 | Yes* | No | 47 | G3P1A2 |
| MPA 600 (10 mo) + MPA 300 (4 mo) +LNG-IUS post tx | 17 | 8 | Yes* | Yes† | 128 | G3P2A1 |
| MPA 200 | 18 | 9 | Yes* | No | 38 | G1P0A1 |
| MPA 200 | 19 | 6 | No | Yes | 32 | G0 |
| Megace 200 | 17 | 7 | No | No | 25 | G0 |
| MPA 100 + LNG-IUS (4 mo) + MPA 500 | 10 | 8 | No | Yes | 42 | G0 |
| MPA 400 | 9 | 6 | No | Yes | 22 | G0 |
| MPA 200 | 15 | 8 | Yes | No | 22 | G0 |
| MPA 200 | 8.5 | 7 | Yes | Yes‡ | 23 | G0 |
| MPA 300 (3 mo) + MPA 200 (3 mo) | 6 | 7 | Yes | No | 75 | G2P1A1 |
| MPA 500 + LNG-IUS | 12 | 6 | No | No | 19 | G0 |

**Allele specific PCR (KRAS and PIK3CA)**

Allele specific PCR was performed to identify the seven *KRAS* and three *PIK3CA* hot spot mutations most frequently encountered in EEC, using a custom-made panel of hydrolysis probe assays as described previously. [21, 24]

**Sanger sequencing**

*CTNNB1* exon 3 was amplified using the following primers: forward: GATTTGATGGAGTTGGACATGG, reverse: TGTTCTTGAGTGAAGGACTGA. Sanger sequencing was performed on purified PCR products (Macrogen, Amsterdam, the Netherlands). Sequences of the forward and reverse strands were analysed with Mutation Surveyor software (Softgenetics, State College, PA, USA).

**Genetic susceptibility exclusion**

According to the clinical data there was no suspicion of Lynch syndrome. MLH1 immunohistochemistry was performed in all patients and showed no loss of expression, thereby excluding the possibility of sporadic EEC with microsatellite instability.

## 3.4 RESULTS

**Clinical aspects**

Eleven patients diagnosed with grade 1 EEC were included. The mean BMI was 25.8 (median 27.9, range 20-39). None of the patients suffered from diabetes. The duration of treatment varied between 6 and 19 months, with a median of 15 months. The median follow up was 32 months (range 19-128 months). Based on the aforementioned definition of complete response (CR) there were 5 non-responders (45%) and 6 responders (55%), of whom 5 patients (83%) relapsed during the follow-up of our study. All responders showed CR within the first 12 months of treatment, with a median time to response of 6 months (table 3). Four of the responders became pregnant with a total of 7 pregnancies (4 miscarriages, 3 term deliveries of healthy neonates). All pregnancies were achieved by assisted fertility treatment through intrauterine insemination (IUI) (n = 4) or in vitro fertilisation (IVF) (n = 3). Five responders had recurrent disease after discontinuation of progesterone and 4 of them responded again after restarting hormonal treatment. Two of the responders underwent hysterectomy after completion of their pregnancies (table 1). The non-responders continued treatment up to a follow up of respectively 15, 18, 24, 24 and 27 months. Of these, three have undergone hysterectomies and two are awaiting hysterectomy. In the 5 non-responders, there was persistence of residual disease in every curetting and in the hysterectomy specimen. Histological analysis of none of the available hysterectomy specimens showed upgrading or upstaging of the presumed stage of disease. Nine patients are alive without evidence of disease (table 1). Two are awaiting hysterectomy.

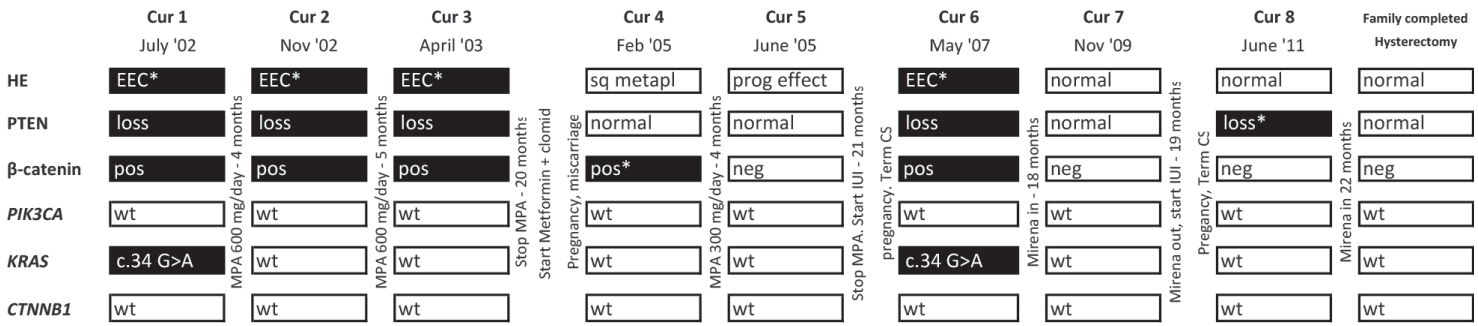



**Figure 1.** Histological changes and molecular findings in sequential endometrium samples under progesterone treatment, patient 2: response, recurrent disease, response.

Legend: Sq metapl, squamous metaplasia; prog effect, progesterone effect; wt, wild type; * focally present

**Histology**

During treatment, the tumours showed prominent architectural as well as cellular changes. Decrease of the gland-to-stroma ratio was observed. All patients showed decreased glandular cellularity, decreased mitotic activity of glandular epithelium and decreased cellular atypia during treatment. Atrophy of pre-existing glandular epithelium and pseudodecidualisation of stroma were present in the first curetting after initiation of hormonal treatment in most patients (82%). Figure 1 displays the HE and molecular findings of an initial responder, who relapsed and responded again. In samples with residual disease under progesterone treatment, glandular confluence with a cribriform growth pattern persisted at least focally (supplement 1).

**Hormone receptor status**

All patients had PR and ER expression in 90-100% of tumour cells in the initial curetting and maintained positivity during treatment.

**Molecular changes**

All 6 responders had both pathways activated. In the non-responder group, tumours inconsistently showed alterations in none, one or both pathways. An overview of alterations in the PI3K/Akt and Wnt signaling pathways in the pre-treatment (diagnostic) samples in relation to the clinical response is listed in table 2. The genetic aberrations in subsequent curettings are summarised in table 3.

**Wnt signaling**

Nuclear staining of β-catenin by IHC, consistent with Wnt activation, was seen in 9 patients in the pre-treatment samples, all 6 responders and 3 non-responders (table 3). Residual foci of EEC in subsequent samples showed the same β-catenin staining as the initial tumour. In pre-treatment samples of four of these 9 patients (44%) a *CTNNB-1* exon 3 mutation could be demonstrated; 3 of them were missense mutations (p.D32V, p.S33C and p.S37C) and one was an in-frame deletion (p.D32del4(DSGI)). In two non-responders the same *CTNNB-1* mutation was found in subsequent samples with residual disease, indicating persistence of the malignant clone. Moreover, in one patient with relapse after complete response, the same *CTNNB-1* mutation as demonstrated in the initial specimen was found in the relapse specimen (case 9, table 3).

**Table 3.** Pathway alterations in the pretreatment endometrial specimen (EEC), responders versus nonresponders

| | Responders | Nonresponders |
|---|---|---|
| Wnt activation only | 0 | 1 |
| PI3K/Akt activation only | 0 | 1 |
| Wnt + PI3K/Akt activation | 6 | 2 |
| No detected pathway alterations | 0 | 1 |

*Wnt activation* was defined as nuclear β-catenin by immuno-histochemistry (with or without *CTNNB-1* exon 3 mutation), PIK3/Akt activation as PTEN loss and/or PIK3CA or KRAS mutations.
All responders showed activation of both pathways. In the non-responders, the molecular alterations vary.

### PI3K/Akt signaling pathway

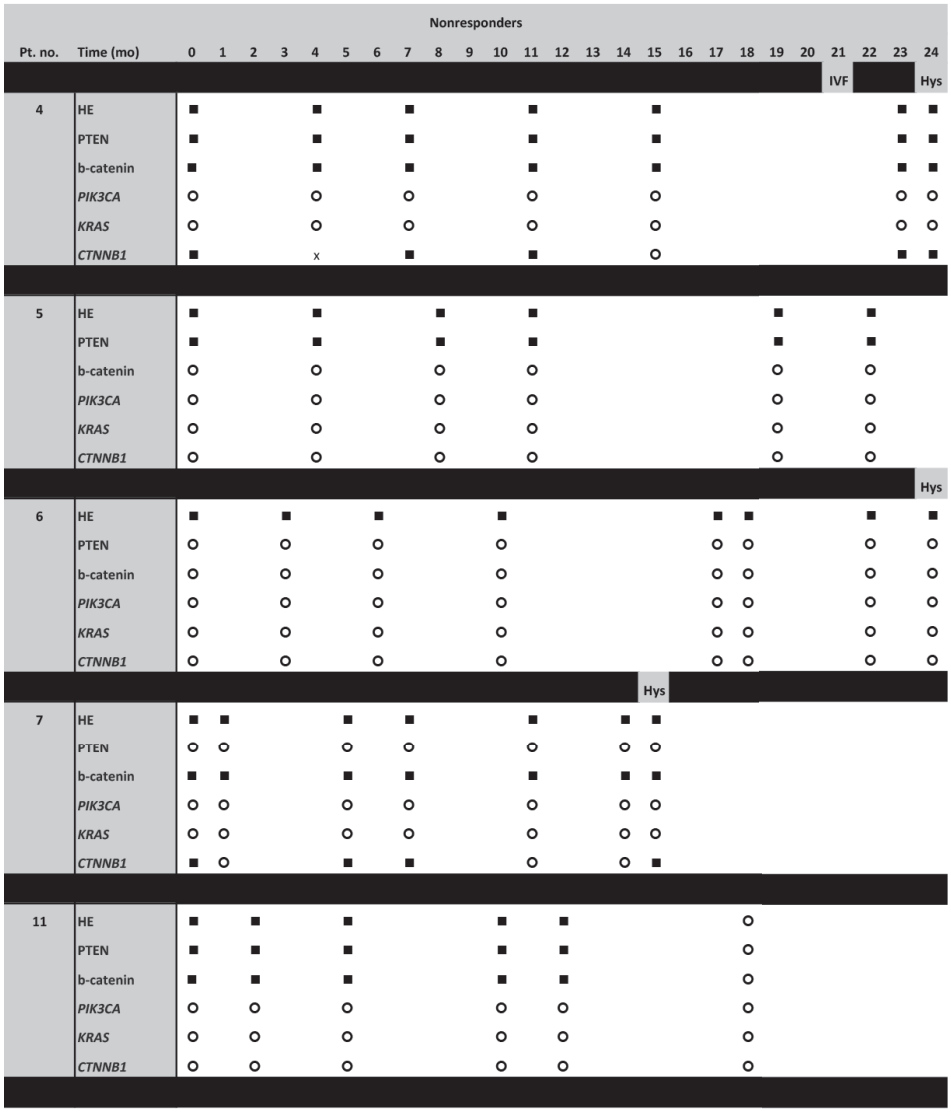
The PI3K/Akt pathway showed alterations in the pre-treatment samples in 8 patients (72%, table 2). Four of these had PTEN loss alone, one had simultaneous loss of PTEN and a *KRAS* exon 2 (p.G12S) mutation, and three had a *PIK3CA* mutation alone as activating event. Two of the *PIK3CA* mutations were located in exon 20 (H147R) and one in exon 9 (E542K). Residual foci of EEC in following samples had an identical PTEN-profile as the primary carcinoma. The same mutation (in *PIK3CA* and *KRAS*) was found in subsequent samples in two of the four patients, indicating persistence of the mutated clone. The same *KRAS* exon *2* mutation as in the initial specimen was found in one patient who relapsed after complete response followed by discontinuation of the treatment (table 3). None of the tumours harboured both *KRAS* and *PIK3CA* mutations simultaneously. In one of the non-responders no molecular alterations in Wnt or PI3K/Akt pathways were identified (table 2).

### Samples without (pre)malignancy

Samples without histomorphological evidence of disease showed no *KRAS*, *PIK3CA or CTNNB-1* exon 3 mutations (N=32). In 6 of these samples, isolated benign looking glands showed nuclear beta-catenin positivity surrounded by strong pseudo-decidualized stroma. In 4 of the curettings without malignancy, sparse isolated PTEN negative glands with normal morphology were seen (PTEN null glands [25]).

**Table 2.** Histological and molecular findings in the first 24 months of follow-up, responders versus nonresponders

| | | Responders | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Pt. no. | Time (mo) | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 |
| | | | | | | | | | | | | | | | | | | | | | | | IVF | MC | | |
| 1 | HE | ■ | | | | | | ○ | | | ○ | | | | ○ | | | ■ | | | ○ | | | | | ○ |
| | PTEN | ○ | | | | | | ○ | | | ○ | | | | ○ | | | ○ | | | ○ | | | | | ○ |
| | b-catenin | ■ | | | | | | ■ | | | ■ | | | | ■ | | | ■ | | | ■ | | | | | ■ |
| | PIK3CA | ■ | | | | | | ○ | | | ○ | | | | ○ | | | ○ | | | ○ | | | | | ○ |
| | KRAS | ○ | | | | | | ○ | | | ○ | | | | ○ | | | ○ | | | ○ | | | | | ○ |
| | CTNNB1 | ○ | | | | | | ○ | | | ○ | | | | ○ | | | ○ | | | ○ | | | | | ○ |
| | | | | | | | | | | | | | | | | | | | | MC | | | | | | |
| 2 | HE | ■ | | | | ■ | | | | | ■ | | | | | | | | | | | | | | | |
| | PTEN | ■ | | | | ■ | | | | | ■ | | | | | | | | | | | | | | | |
| | b-catenin | ■ | | | | ■ | | | | | ■ | | | | | | | | | | | | | | | |
| | *PIK3CA* | ○ | | | | ○ | | | | | ○ | | | | | | | | | | | | | | | |
| | *KRAS* | ■ | | | | ○ | | | | | ○ | | | | | | | | | | | | | | | |
| | *CTNNB1* | ○ | | | | ○ | | | | | ○ | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | | | | IUI | MC | | |
| 3 | HE | ■ | | ■ | | | ■ | | | ○ | | | ○ | | | | | ○ | | | | | | | | |
| | PTEN | ○ | | ■ | | | ■ | | | ○ | | | ○ | | | | | ○ | | | | | | | | |
| | b-catenin | ■ | | ■ | | | ■ | | | ○ | | | ○ | | | | | ○ | | | | | | | | |
| | *PIK3CA* | ■ | | x | | | ○ | | | ○ | | | ○ | | | | | ○ | | | | | | | | |
| | *KRAS* | ○ | | x | | | ○ | | | ○ | | | ○ | | | | | ○ | | | | | | | | |
| | *CTNNB1* | ■ | | x | | | ○ | | | ○ | | | x | | | | | ○ | | | | | | | | |
| | | | | | | | | | | | | | | IUI | | | | | | | | | | | IUI | |
| 8 | HE | ■ | | | ○ | | | ○ | | | | | ○ | | | | | | ○ | | ■ | | | ○ | | |
| | PTEN | ■ | | | ○ | | | ○ | | | | | ○ | | | | | | ○ | | ■ | | | ○ | | |
| | b-catenin | ■ | | | ○ | | | ○ | | | | | ○ | | | | | | ○ | | ■ | | | ○ | | |
| | *PIK3CA* | ○ | | | ○ | | | ○ | | | | | ○ | | | | | | ○ | | xx | | | xx | | |
| | *KRAS* | ○ | | | ○ | | | ○ | | | | | ○ | | | | | | ○ | | xx | | | xx | | |
| | *CTNNB1* | ■ | | | ○ | | | ○ | | | | | ○ | | | | | | ○ | | xx | | | xx | | |
| | | | | | | | | | | | | | | | | | | | | | | | | | | Hys |
| 9 | HE | ■ | ○ | | | | ○ | | | ○ | | | ○ | | | | ■ | | | | | | | ■ | | ■ |
| | PTEN | ○ | ○ | | | | ○ | | | ○ | | | ○ | | | | ○ | | | | | | | ○ | | ○ |
| | b-catenin | ■ | ○ | | | | ○ | | | ■ | | | ■ | | | | ■ | | | | | | | ■ | | ■ |
| | *PIK3CA* | ■ | ○ | | | | ○ | | | ○ | | | ○ | | | | xx | | | | | | | xx | | ■ |
| | *KRAS* | ○ | ○ | | | | ○ | | | ○ | | | ○ | | | | xx | | | | | | | xx | | ○ |
| | *CTNNB1* | ■ | ○ | | | | ○ | | | ○ | | | ○ | | | | xx | | | | | | | xx | | ■ |
| | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 10 | HE | ■ | | | | ■ | ■ | | | | | | ■ | | | | | | ○ | | | | | | | |
| | PTEN | ■ | | | | ○ | ■ | | | | | | ■ | | | | | | ■ | | | | | | | |
| | b-catenin | ■ | | | | ■ | ■ | | | | | | ■ | | | | | | ○ | | | | | | | |
| | *PIK3CA* | ○ | | | | ○ | ○ | | | | | | ○ | | | | | | ○ | | | | | | | |
| | *KRAS* | ○ | | | | ○ | ○ | | | | | | ○ | | | | | | ○ | | | | | | | |
| | *CTNNB1* | ○ | | | | ○ | ○ | | | | | | ○ | | | | | | ○ | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | | | | | | | |


Nonresponders
Pt. no.
Time (mo)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
IVF
Hys
4
5
6
7
11
HE
PTEN
b-catenin
PIK3CA
KRAS
CTNNB1
x
Hys
HE ■ Ca/CAH ○ normal
PTEN ■ loss ○ normal
b-catenin ■ positive ○ negative
PIK3CA ■ mutant ○ wildtype
KRAS ■ mutant ○ wildtype
CTNNB1 ■ mutant ○ wildtype
x Not enough tissue for analysis
xx Not available
MC Miscarriage
Hys Hysterectomy
IVF IVF attempt
IUI IUI attempt

## 3.5 DISCUSSION

This study is the first to explore whether alterations in the Wnt and PI3K/Akt signaling pathways in the pre-treatment endometrial sample are predictive for progesterone non-responsiveness in women with low-grade, FIGO I, PR positive EEC. Moreover we evaluated the alteration in these signaling pathways during treatment and follow-up. In contrast to most previous work concentrating on morphological changes alone [20, 26, 27], our study describes molecular genetic alterations in combination with the morphologic appearance in subsequent endometrium samples from progesterone treated patients with low-grade EEC. According to our results, molecular alterations in Wnt and/or PI3K/Akt pathways do not necessarily induce resistance to progesterone treatment.

Alterations in the Wnt or PI3K/Akt signaling pathways are the most frequent genetic abnormalities in low-grade EEC. [15] Previous studies have suggested that progesterone might rely on the Wnt or PI3K/Akt signaling pathways to elicit its therapeutic effect in EEC. [11, 19] We hypothesised that if somatic mutations activate one or both signaling pathways constitutively, that might lead to resistance to progesterone therapy. All six responders in our series had concomitant activation of both Wnt and PI3K/Akt signaling pathways suggesting that alterations in these important pathways in endometrial cancer may not be predictive of resistance to progesterone treatment.

Our findings indicate that the progesterone effect may at least partially be independent of alterations in the Wnt and/or PI3K/Akt signaling pathways. Other non-mutually exclusive hypotheses may explain how progesterone bypasses the Wnt and PI3K/Akt signaling pathways. First, progesterone may induce apoptosis through up regulation of Fas/FasL or decreasing Bcl-2 protein expression. [28-30] According to this hypothesis, progesterone induces apoptosis in all endometrial glands including those with Wnt and PI3K/Akt activation, followed by replacement with new glands without genetic defects. [19] Additionally, there may be a role for endometrial stroma in endometrial carcinogenesis and response to progesterone therapy through PR receptor or hypermethylation of the tumour suppressor *HAND2* in stromal cells. Epigenetic *HAND2* inactivation in stromal cells seems to induce resistance to progesterone in hyperplastic endometrium. [31] As *HAND2* hypermethylation was observed in 90% of EEC, it seems unlikely to be the only factor of progesterone resistance, knowing the response rate in grade 1 EEC reaches up to 75%. [4] Future studies on these alternative mechanisms will be required to evaluate their potential role in predicting progesterone responsiveness in EEC.

We also observed that normalisation of the endometrium morphology under progesterone treatment is accompanied by absence of the pre-treatment genetic changes. The absence of genetic alterations in most morphologically normal endometrium under progesterone treatment could support the hypothesis that progesterone acts through induction of apoptosis followed by replacement with wild-type glands. The significance

of sparse PTEN null glands and single glands with nuclear beta-catenin positivity in a few of the morphologically normal samples is not clear. The significance of sparse PTEN null glands and single glands with nuclear beta-catenin positivity in a few of the morphologically normal samples is not clear. It is unknown whether this implies residual disease, *de novo* (pre)malignant disease after complete response or functional increase of beta-catenin/ decrease of PTEN under hormonal influence. [9, 14]

Previous research identified persistent architectural abnormalities and/or cellular atypia after 7-9 months of treatment as predictive of treatment failure. [20] Another study found that presence of at least three out of five given unfavourable architectural features in the pre-treatment specimen, in combination with the Body Mass Index (BMI) could predict the likelihood of response. [26] The tumour PR status before initiating the treatment was assessed in only one of the previous studies that tried to identify predictive markers in conservative treated low-grade EEC before initiating treatment, but correlation with response was not stated. [19] Most low-grade EECs express PR [32], but only approximately 75% of these patients do indeed respond to fertility sparing progesterone therapy, indicating that other prognostic biomarkers are required in this subset of patients. [4, 33] Studies investigating the value of PR positivity in predicting response in conservatively treated, low-grade presumed FIGO IA EEC are lacking, most likely due to the low number of patients and the high prevalence of PR positivity of these tumours. [32, 34] In all our patients the tumours were PR positive.

This work has some limitations. Although we analysed a large collection of serial endometrial samples, the number of patients we were able to include was small. The sampling method varied among patients during follow-up, resulting in potential under sampling in selected cases, which might explain negative findings in some curettings (figure 1). This notion is supported by the fact that the same genetic alterations were found in recurrences after 'negative' curettings. Thus, 'field cancerisation' in which new clones would arise under unopposed estrogen stimulation is not sustained by our results. Furthermore, despite a national treatment advice, the patients of whom the endometrial samples were included in the study did not receive uniform treatment nor follow up regime.

In conclusion, we describe an in-depth morphologic and molecular analysis of a large collection of serial endometrium samples from EEC patients undergoing progesterone treatment for preservation of fertility. In contrast to the initial hypothesis, our findings indicate that activation of the Wnt and/or PI3K/Akt pathways does not result in lack of response to progesterone treatment. Studying a cohort with more homogenous data, taking bio-physical profiles into account, and performing large scale analyses using targeted next generation sequencing may allow selection of EEC patients who will benefit from progesterone treatment. Young women with low-grade early stage EEC and desire to conceive should be offered progesterone treatment while risks are explained exten-

sively and response is monitored according to a strict protocol. We suggest to include these patients in an international registration study and treat them according to the 2015 ESMO-ESGO-ESTRO guidelines presented at the 19th ESGO meeting.

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