Expression of PAX2 and PTEN Correlates to Therapy Response in Endometrial Hyperplasia

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Abstract. *Aim: To investigate if a levonorgestrel-impregnated intrauterine system (LNG-IUS) was more efficient compared to oral progestin in the clearance of the paired box 2 gene (PAX2) - and phosphatase and tensin homolog (PTEN)-null endometrial glands and assess the significance of PAX2- and PTEN-null glands as markers for therapy response in endometrial hyperplasia. Patients and Methods: Immunohistochemical staining using antibodies against PAX2 and PTEN was performed in 141 pre- and post-treatment endometrial biopsies comparing the effect of LNG-IUS, 10 mg medroxyprogesterone acetate (MPA) taken continuously, or 10 mg MPA taken 10 days per cycle for six months. PAX2- and PTEN-null glands were investigated by light microscopy in pre-and post-treatment biopsies. Results: Clearance of PAX2- and PTEN-null glands was significantly more efficient by LNG-IUS compared to oral MPA ($p<0.000$ and $p=0.008$, respectively) and significantly related to therapy response ($p<0.000$ and $p=0.002$, respectively).*

Endometrial cancer, considered to develop through preliminary stages of endometrial hyperplasia, is presently the most frequent gynaecological malignancy in the Western world, and its incidence is still increasing (1). Nearly 80% of all endometrial cancer cases are preceded by endometrial hyperplasia as the initial stage (2). Thus, optimal and individualized therapy for endometrial hyperplastic lesions is of high importance to combat this trend. During the past decades, progestin has been introduced and accepted as a conservative therapy for low- and medium-risk endometrial hyperplasia (3-6). In observational studies and meta-analyses, the levonorgestrel-impregnated intrauterine system (LNG-IUS, Mirena®; Bayer) has exhibited greater efficiency compared to oral progestin (7-10). A recently published randomised multicentre controlled trial (RCT) showed that all the included women had therapy response after LNG-IUS, whereas only 69% were cured after oral low-dose progestin (11).

The pathogenesis of malignant endometrial proliferation is not completely understood but is generally accepted as an interaction between oestrogen-driven changes and underlying genetic defects preceding progression to endometrial cancer (12-16). Commonly occurring inactivation of the *PTEN* tumor-suppressor gene by mutation or deletion in endometrial carcinoma and clonal loss of *PTEN* has been demonstrated to occur in up to 83% of endometrioid endometrial adenocarcinomas and 63% of atypical hyperplasias (12, 16). In women with Cowden's syndrome, characterized by *PTEN* germline mutations, the lifetime risk of endometrial cancer is 29% compared to 0.6% in the general population (17). The mechanism of PAX2 (a gene required for embryonic uterine development) protein loss in the pathogenesis of endometrial hyperplasia and endometrial cancer is not as thoroughly characterized (14, 15). Recent immunohistochemical studies have demonstrated that occurrence of PAX2 loss in endometrial hyperplasia increases with malignant progression and PAX2 loss was demonstrated in up to 77% of women with endometrial adenocarcinomas and 71% of endometrial atypical hyperplasias (14, 15). A pattern of synchronous PAX2 and PTEN inactivation has also been demonstrated by immunohistochemistry in endometrial hyperplasia (14, 15).

Zheng and collaborators compared the occurrence of PTEN-null glands (lack of expression of PTEN by immunohistochemical staining) in women with endometrial hyperplasia after progestin therapy with occurrence in women with normal cycling endometrium (18). These researchers' study demonstrated that PTEN-null glands disappeared in 90% of women with endometrial hyperplasia after progestin therapy compared to 17% in normal cycling women (18). In

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another study of peri- and postmenopausal women with endometrial hyperplasia who underwent progestin therapy with either LNG-IUS or low-dose oral progestin or surveillance only, the LNG-IUS group demonstrated the highest clearance rate of PTEN-null glands (11).

Whereas the LNG-IUS group had a 62% pre-therapy and 5% post-therapy rate of PTEN-null glands, there was no significant change in the expression of PTEN-null glands after oral low-dose therapy or for the surveillance group (11). The mechanism of PTEN-null gland clearance is not completely understood, and to date, no study has investigated the effect of LNG-IUS therapy on clearance of PAX2-null glands.

The main intention of the current study was, firstly, to investigate if LNG-IUS was more efficient compared to oral progestin in the clearing of immunohistochemically detected PAX2- and PTEN-null endometrial glands and if the clearance rate of PAX2-null glands after progestin therapy was comparable to the level recently described for PTEN (11). The endometrial biopsy material used in the present study was obtained in the first national multicentre RCT ever, demonstrating that the LNG-IUS was safe and efficient therapy for endometrial hyperplasia (19).

Patients and Methods

Patients. The biopsy material was obtained from 153 women with low- or medium-risk endometrial hyperplasia in a national multicentre randomized study who had been treated with either LNG-IUS (Mirena®, Bayer), or 10 mg medroxyprogesterone acetate (MPA) 10 days per cycle, or 10 mg MPA daily for six months (ClinicalTrials.gov, NCT01074892) (19). The study inclusion period was from the 1st of January 2005 till the 1st of November 2011. The treatment period was completed on the 1st of May 2012. From each participant, one pre- and one post-treatment (after six months) biopsy had been obtained by Pipelle® (Endometrial suction curette) (19). For the present study, adequate paired biopsy material from 141 women was available for immunohistochemical analyses; 48 of these women had been treated by LNG-IUS, 44 by continuous MPA and 49 by cyclic MPA. Insufficient biopsy material in the paraffin blocks was the reason for excluding eight of the original 153 women. Patient characteristics, such as age, menopausal status, parity, and WHO diagnosis, were registered and related to clearance of PAX2- and PTEN-null glands.

Endometrial biopsies. The endometrial biopsy material was sent to the Department of Pathology at the University Hospital of North Norway for routine assessment. The specimens were fixed in buffered formaldehyde, embedded in paraffin and further processed in the laboratory before standard histological sections were made. Diagnostic assessment of WHO classification by light microscopy was performed by a trained gynaecology pathologist (AO) and one additional routine pathologist, both of whom were blinded to each other's diagnosis. Agreement after discordant results was always obtained after discussion at a two-headed microscope. The index biopsies were classified into one of three groups: simple hyperplasia, complex hyperplasia, or atypical hyperplasia according to the WHO classification, which was considered the gold standard for evaluation of endometrial hyperplasia at the time the study was performed (20, 21). Normalized histology in the control biopsies after therapy was defined as ordinary proliferative endometrium or endometrium with progestin effect (20, 21). All information from the WHO classification of the index and control biopsies was registered and maintained in a separate database and subsequently supplemented by information from hospital records.

PAX2 immunohistochemistry. Histological material (with a thickness of 4-5 μm) was routinely cut from paraffin blocks and placed on Superfrost+ glass slides (D-38116; Thermo Scientific, Braunschweig, Germany) followed by incubation overnight at 60˚C, necessary for fixation. For the PAX2 staining procedure, de-paraffinisation, pre-treatment in Tris-based, slightly alkaline reagent for 60 minutes (CC1 standard) at 100˚C, antibody incubation (rabbit monoclonal anti-PAX2, clone: Z-RX2; (Invitrogen Corporation, Camarillo, CA, USA), and staining were automatically performed in a Benchmark XT module (Ventana Medical Systems, Inc. Tucson, AZ, USA). The initial immunoglobulin concentration was 0.25 mg/ml, and the applied dilution was 1/200 in Antibody Diluent (Ventana Medical Systems, Inc.). The slides were treated with a 0.05% solution of glutaraldehyde in normal saline to strengthen the antibody–antigen binding. To increase the intensity of the specific bound antibody, the slides were also treated with an Amplification Kit (Ventana Medical Systems, Inc.). After addition of the primary antibody, slides were incubated for 60 min at 37˚C and automatic 3,3'-diaminobenzidine (DAB) staining in several steps was performed by the use of an UltraView DAB IHC Detection kit from Ventana Medical Systems, Inc. Dehydration and counterstaining with haematoxylin was performed manually. The slides were mounted in Tissue-Tek Film (Sakura Finetek Europe B.V. Alphen an Den Rijn, the Netherlands). The negative control used was 100 μl of Antibody Diluent (Ventana Medical Systems, Inc.). As positive controls endometrial biopsies in proliferative phase were used.

PTEN immunohistochemistry. The incubation procedure was similar to that described for PAX2. For PTEN (PTEN antibody, clone: 6H2; Dako Agilent Technologies Company, Glostrup, Denmark), tissue specimens were de-paraffinised in xylene and hydrated in a graded series of ethanol solutions. Antigen retrieval was performed in a pressure cooker with tris/EDTA buffer, at pH 9 for 10 min, followed by cooling and washing. The initial immunoglobulin concentration was 292.5 mg/l, and the applied dilution was 1/300 in Dako Antibody Diluent. The slides were incubated in a humidity chamber overnight in a refrigerator at 4˚C. A further step was performed manually according to manufacturer's instructions for Dako REAL EnVision System Peroxidase/DAB, Rabbit/Mouse kit (Dako Agilent Technologies Company, Glostrup, Denmark). After washing in water, the sections were counterstained with haematoxylin and bluing reagent, dehydrated in a series of ethanol solutions and cleared with xylene. The slides were mounted in Tissue-Tek Film (Sakura Finetek Europe B.V. Alphen an Den Rijn). The negative control used was 100 μl of Dako Antibody Diluent. Endometrial biopsies in proliferative phase were used as positive controls.

Evaluation of PAX2 and PTEN immunohistochemistry. The PAX2- and PTEN-stained slides were investigated with a light microscope by a trained gynaecological pathologist (AO) and a trained technician (MA). A slide was defined as "PTEN-null" or "PAX2-null" if one or more endometrial glands devoid of PAX2 or PTEN

Table I. *Patterns of change in the paired endometrial biopsies comparing pre- and post-treatment paired box 2 gene (PAX2) protein loss after different progestin therapy regimens.*

Therapy regimen	Absence, n	Persistence, n	Regression, n	Total, n
Cyclic oral MPA	18	19	12	49
Continuous oral MPA	11	8	25	44
LNG-IUS	11	4	33	48
Total	40	31	70	141

MPA: Medroxyprogesterone; LNG-IUS: levonorgestrel-impregnated intrauterine system. Pearson chi square p=0.00014. Absence: No PAX2-null glands before or after therapy, Persistence: PAX2-null glands present before and persisting after therapy, Regression: PAX2-null glands present before but not after therapy.

Table II. *Patterns of change in the paired endometrial biopsies comparing pre- and post-treatment phosphatase and tensin homolog (PTEN) protein loss after different progestin therapy regimens.*

Therapy regimen	Absence n	Persistence, n	Regression, n	Total,
Cyclic oral MPA	17	15	17	49
Continuous oral MPA	15	6	23	44
LNG-IUS	18	2	28	48
Total	50	23	68	141

MPA: Medroxyprogesterone; LNG-IUS: levonorgestrel-impregnated intrauterine system. Pearson chi square p=0.0080. Absence: No PTEN-null glands before or after therapy, Persistence: PTEN-null glands present before and persisting after therapy, Regression: PTEN-null glands present before but not after therapy.

Table III. *Patterns of change in the paired endometrial biopsies comparing pre- and post-treatment paired box 2 gene (PAX2) protein loss in relation to therapy response.*

Response to therapy	Absence, n	Persistence, n	Regression, n	Total, n
Response	32	17	63	112
No response	8	14	7	29
Total	40	31	70	141

Pearson Chi square p=0.0003. Absence: No PAX 2-null glands before or after therapy, Persistence: PAX2-null glands present before and persisting after therapy, Regression: PAX2-null glands present before but not after therapy.

Table IV. *Patterns of change in the paired endometrial biopsies comparing pre- and post-treatment expression of phosphatase and tensin homolog (PTEN) protein loss in relation to therapy response.*

Response to therapy	Absence, n	Persistence, n	Regression, n	Total, n
Response	43	12	57	112
No response	7	11	11	29
Total	50	23	68	141

Pearson chi square p=0.0019. Absence: No PTEN-null glands before or after therapy, Persistence: PTEN-null glands present before and persisting after therapy, Regression: PTEN-null glands present before but not after therapy.

Table V. *Patterns of change in the paired endometrial biopsies comparing pre- and post-treatment paired box 2 gene (PAX2) protein loss after different progestin therapy regimens when only women with therapy response were assessed.*

Therapy regimen	Absence, n	Persistence, n	Regression, n	Total, n
Cyclic oral MPA	11	8	8	27
Continuous oral MPA	10	5	22	37
LNG-IUS	11	4	33	48
Total	32	17	63	112

MPA: Medroxyprogesterone; LNG-IUS: levonorgestrel-impregnated intrauterine system. Pearson chi square p=0.017. Absence: No PAX2-null glands before or after therapy, Persistence: PAX2-null glands present before and persisting after therapy, Regression: PAX2-null glands present before but not after therapy.

Table VI. *Patterns of change in the paired endometrial biopsies comparing pre-treatment and post-treatment expression of phosphatase and tensin homolog (PTEN) protein loss after different therapy regimens with progestin therapy when only women with therapy response were assessed.*

Therapy regimen	Absence, n	Persistence, n	Regression, n	Total, n
Cyclic oral MPA	11	7	9	27
Continuous oral MPA	14	3	20	37
LNG-IUS	18	2	28	48
Total	43	12	57	112

MPA: Medroxyprogesterone; LNG-IUS: levonorgestrel-impregnated intrauterine system. Pearson chi square p=0.036. Absence: No PTEN-null glands before or after therapy, Persistence: PTEN-null glands present before and persisting after therapy, Regression: PTEN-null glands present before but not after therapy.

protein were observed. When all endometrial glands visualized expressed PAX2 or PTEN protein, the specimen was defined as normal. Null glands were generally devoid of PAX2 or PTEN protein in all cells of the gland. Cases with "null glands" were grouped as either two or fewer, or more than two. Depending on PAX2 and PTEN staining in pre-treatment and post-treatment specimens, each case was assigned to one of the following groups: "Absence" when no null glands were observed in either specimen, "persistence" when null glands were observed in both specimens, and "regression" when null glands were present only in the index specimen but not in the post-treatment biopsy. The presence or absence of staining was always obtained after consensus between the two investigators using a two-headed microscope. This

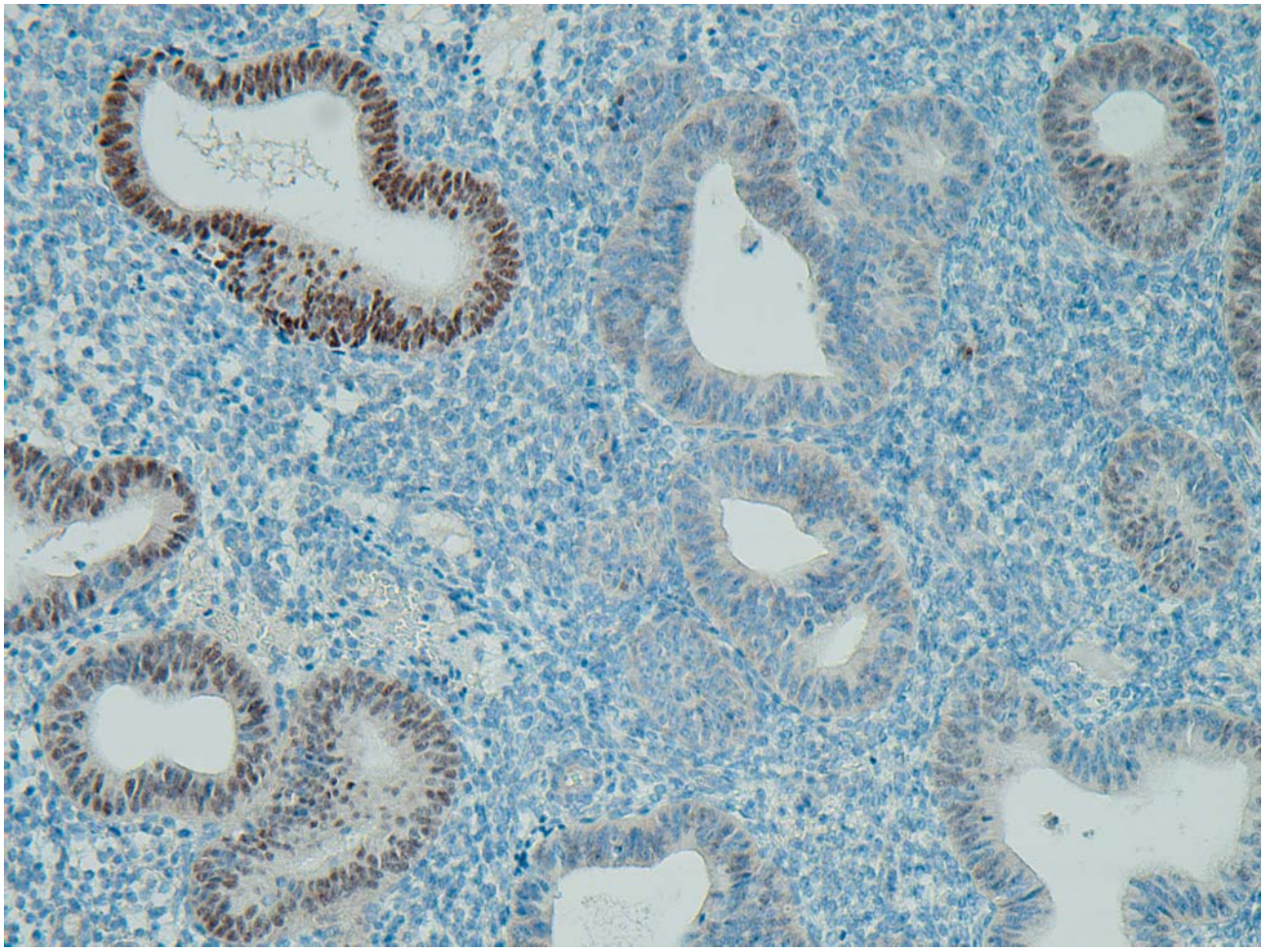

Figure 1. *Before levonorgestrel-impregated intrauterine device therapy paired box 2 gene (PAX2)-null glands were found to be unstained in contrast to the surrounding normal brown-stained glands (×20). Stain used: PAX2, clone: Z-RX2; Invitrogen Corporation, Camarillo, CA, USA.*

procedure was always performed twice. Loss of PAX2 and PTEN immunostaining was mostly characterized by an overlapping pattern occurring in the same foci in the histological specimens.

Ethical approval. The study was approved by the Regional Committee for Medical and Health Research Ethics (approval number PREKNORD 25/2004), the Norwegian Council of Medical Advice, and the Norwegian Medicines Agency.

Statistical analysis. All statistical analyses are presented in simple cross tables and *p*-values for Pearson chi square tests are reported.

Results

Patients. Among the 153 women originally included in the study, all those treated by LNG-IUS had complete response (normal proliferative endometrium or endometrium with atrophic glands and pseudodecidualised stroma) after six months of treatment. Only 69% of women were cured by cyclic use of low-dose oral MPA (10 mg, 10 days per cycle), whereas 96% had response by using oral MPA (10 mg) daily (19). In the present study, biopsies from 141 out of the 153 women were available for immunohistochemical analyses. Among these women, 123 were responders and 18 were non-responders. All 48 women treated with LNG-IUS were responders, whereas 33 out of the 49 treated with cyclic MPA and 42 out of the 44 treated with continuous MPA were responders. The mean age of the responding and non-responding women was 47.6 years and 48.4 years, respectively.

Clearance of PAX2- and PTEN-null glands related to therapy regimen. The LNG-IUS was more effective in obtaining clearance of PAX2-null glands compared to the oral cyclic treatment. Among the women treated with LNG-IUS, 69% had regression and only 8% had persistence of PAX2-null

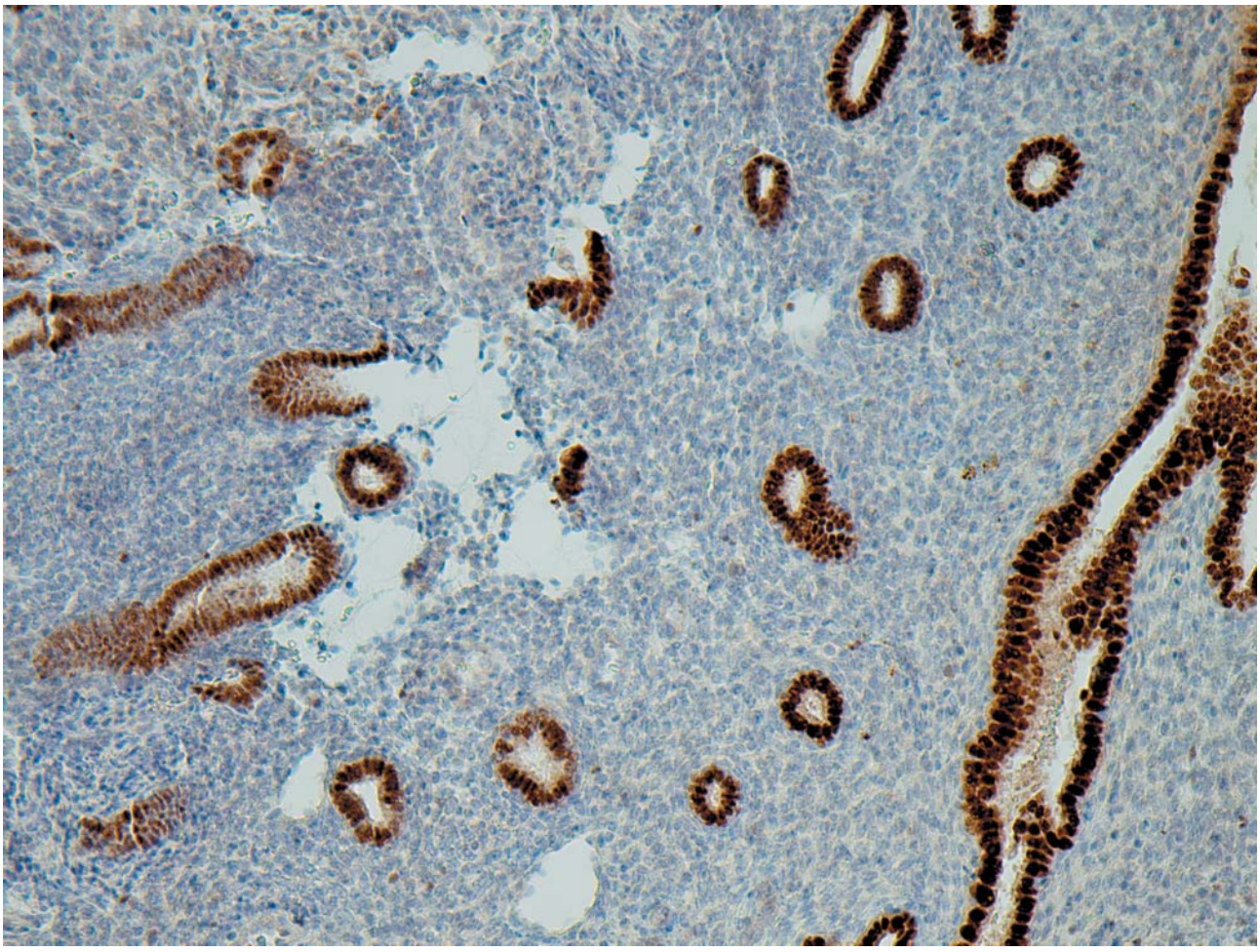

Figure 2. *After levonorgestrel-impregated intrauterine device therapy all glands appeared atrophic and no paired box 2 gene (PAX2)-null glands were detected (×20). Stain used: PAX2, clone: Z-RX2; Invitrogen Corporation, Camarillo, CA, USA.*

glands after therapy. PAX2 staining before and after LNG-IUS therapy is shown in Figure 1 and 2, respectively. After cyclic oral treatment, 24% had regression, and 38% had persistence of PAX2-null glands. For the group treated with continuous MPA, the persistence and regression rates were 16% and 57%, respectively (p=0.00014) (Table I).

The LNG-IUS was also more effective in obtaining clearance of PTEN-null glands compared to the oral cyclic treatment. Regression and persistence of PTEN-null glands after LNG-IUS was 58% and 4%, respectively. PTEN staining before and after LNG-IUS therapy is shown in Figure 3 and 4, respectively. Out of those on oral cyclic therapy, 34% had regression and 30% persistence of PTEN-null glands and the corresponding rates for those on continuous therapy we 13% and 52% (p=0.008) (Table II). The number of PAX2- and PTEN-null glands (1-2 or >2 negative glands) counted in the specimens taken prior to therapy was not significantly different in the treatment groups after therapy (data not shown).

Clearance of PAX2- and PTEN-null glands related to therapy response. Tables III and IV show that clearance of PAX2- and PTEN-null glands after therapy was significantly correlated to therapy response. Among women having therapy response, 56% had regression of PAX2-null glands and 15% had persisting PAX2-null glands after therapy. In contrast, among the women lacking therapy response, only 25% had regression of PAX-null glands and 48% had persisting PAX2-null glands after therapy (p=0.0003) (Table III). Correspondingly, in women having therapy response, 51% showed regression and 10% persistence of PTEN-null glands after therapy. For women lacking therapy response, 38% had regression and 38% persistence of PTEN-null glands (p=0.0019) (Table IV). The number of PAX2- and

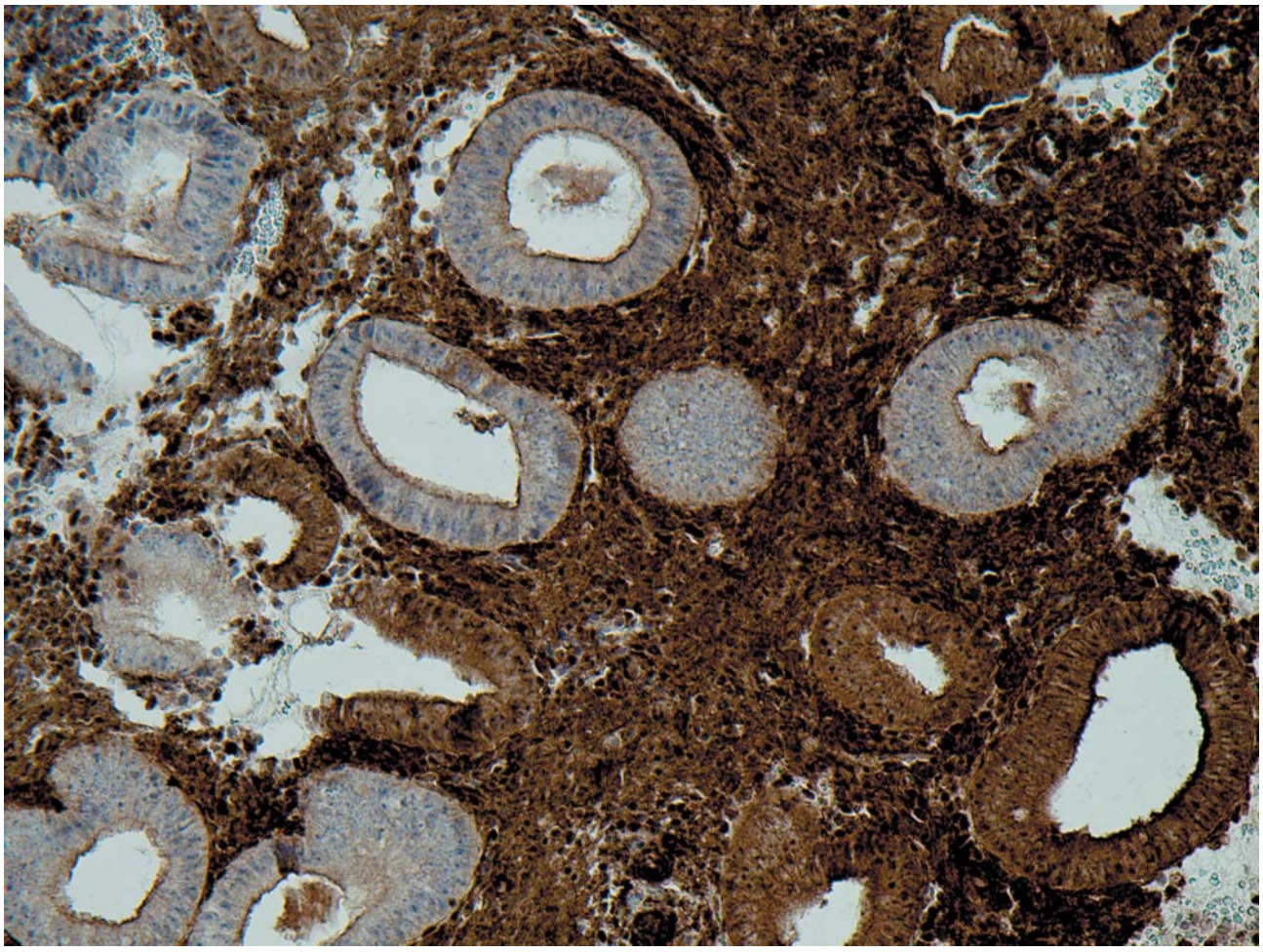

Figure 3. *Before levonorgestrel-impregated intrauterine device therapy phosphatase and tensin homolog (PTEN)-null glands were found to be unstained in contrast to the stained surrounding glands (×20). Stain used: PTEN antibody, clone: 6H2; Dako Agilent Technologies Company, Glostrup, Denmark.*

PTEN-null glands (1-2 or >2 negative glands) estimated prior to therapy was not significantly different in the treatment groups after therapy (data not shown).

Clearance of PAX2 and PTEN null glands in women with therapy response. When women with therapy response were assessed (n=112) separately, there were also significantly more women with regression of PAX2-null glands in the LNG-IUS group compared to the group treated with oral progestin therapy (p=0.017) (Table V). Similarly for PTEN, the LNG-IUS therapy was the most efficient therapy regimen compared to oral progestin (p= 0.036) (Table VI).

Clearance of PAX2 and PTEN as related to patient characteristics. When the age of the participating women was divided into quartiles (≤44, 45-48, 49-52, and >52 years), there was no statistical correlation to regression or persistence of PAX2 or PTEN after therapy. These data were also not associated with response to therapy. Menopausal status (classified as not menopausal, perimenopausal or postmenopausal), defined according to serum levels of oestrogen and follicle-stimulating hormone (FSH), showed no significant correlation to regression or persistence of PAX2 or PTEN after therapy or to therapy response (19). Only 7% of the women were defined as postmenopausal and 26% as perimenopausal. When endometrial biopsies were investigated according to the modified WHO classification (simple hyperplasia, complex hyperplasia, and atypical hyperplasia) and correlated to regression or persistence of PAX2 or PTEN after therapy or to therapy response, no significant differences were found (19). Among the 141 included biopsies, 15% were simple, 74% were complex, and 11% were atypical hyperplasias.

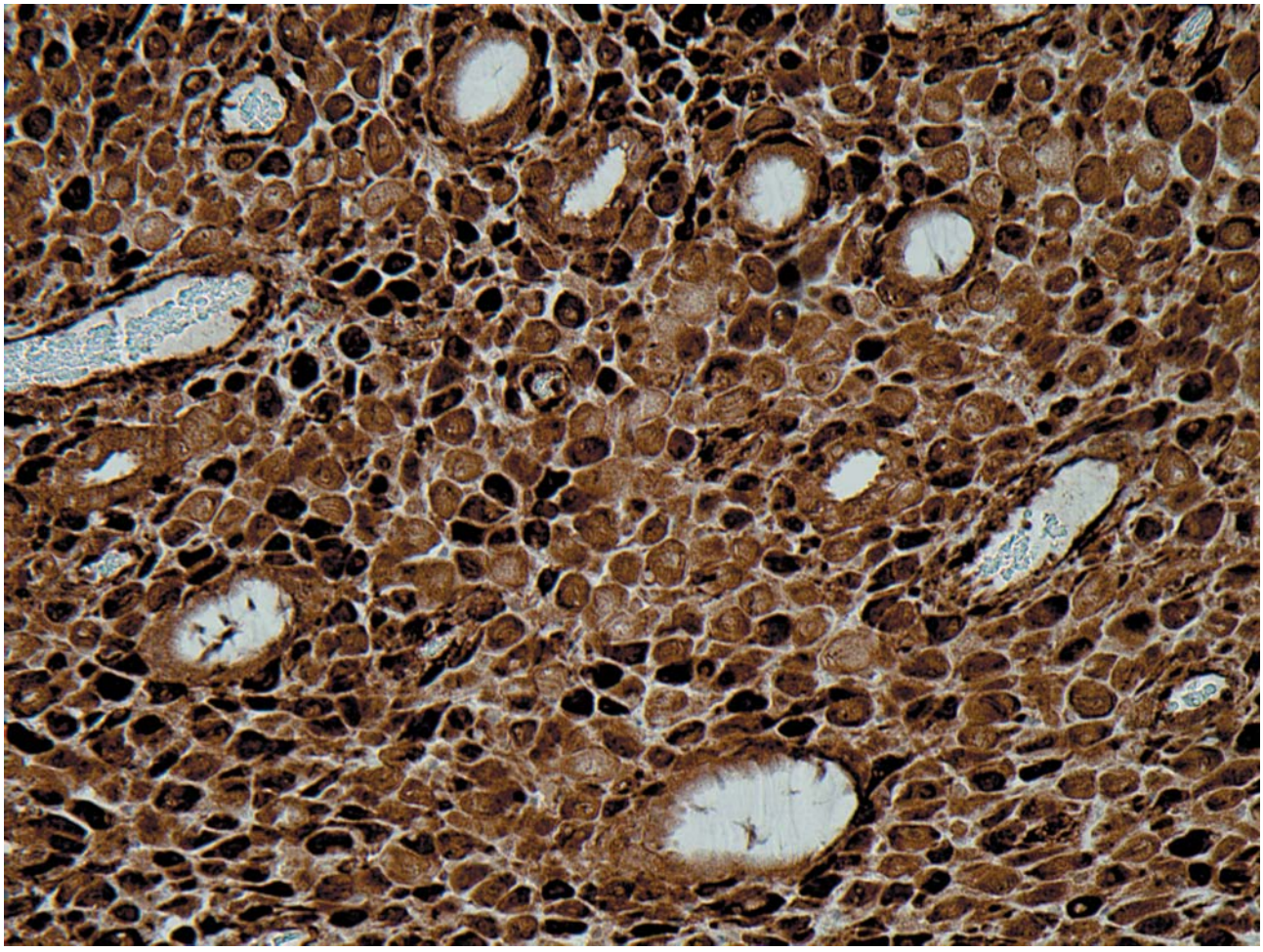

Figure 4. *After levonorgestrel-impregated intrauterine device therapy all glands were found to be atrophic and no phosphatase and tensin homolog (PTEN) -null glands were detected (×20). Stain used: PTEN antibody, clone: 6H2; Dako Agilent Technologies Company, Glostrup, Denmark.*

Discussion

To the best of our knowledge, this study is the first ever to demonstrate that LNG-IUS was significantly more efficient compared to oral progestin therapy in obtaining clearance of PAX2-null glands in medium- and low-risk endometrial hyperplasia (p=0.00014). Loss of PAX2 protein expression in endometrial glands was recently shown to occur in hyperplastic and malignant endometrium, as well as proliferative and atrophic endometrium (14, 22). As neoplastic lesions progress from latent pre-cancer to carcinoma, occurrence of PAX2 loss increases, suggesting that PAX2 may contribute to development of endometrial cancer (23). In the present study comparison of three different progestin regimens as therapy for endometrial hyperplasia, the significant change in PAX2 expression in the paired samples taken before and after progestin therapy, gives some insight into the balance of regression and persistence of PAX2. The efficient clearance of PTEN-null glands obtained by the LNG-IUS, as demonstrated in the current study, is in accordance with the results of our previous report (11). The tissue concentration of levonorgestrel, due to the LNG-IUS in the uterine mucosa, has been shown to be more than 100-fold higher compared to the concentration found after oral treatment (24). However, clearance of PAX2- and PTEN-null glands after continuous use of daily oral MPA was demonstrated to be more efficient compared to oral cyclic therapy. Although the biological effects of different progestin derivatives, such as MPA and levonorgestrel, are not directly comparable, there is indication that the mechanism explaining clearance of PAX2- and PTEN-null glands might be directly dependent on the progestin dose delivered to the endometrial mucosa.

The present study also clearly demonstrates a significant correlation between therapy response and clearance of the PAX2- and PTEN-null glands (p=0.00014 and p=0.008, respectively). To the best of our knowledge, only one previous study exists in which PAX2 has been studied as a marker of resistance to progestin therapy (25). No correlation was found between therapy response and PAX2 and PTEN staining analyzed before therapy in complex atypical hyperplasia. However, all the patients had been treated with oral progestin and none had received high-dose therapy with LNG-IUS (25). Our study clearly demonstrates that many patients with histologically normal endometrium after therapy still had persistent PAX2- and PTEN-null glands (Tables V and VI). Thus, a higher intrauterine dose of progestin might be required for the clearance of deranged protein expression. The biopsies investigated in the current study were obtained from an RCT where dose and treatment time were consistent for all the included patients (19). This represents an important strength of this study compared to many other clinical studies of progestin therapy for endometrial proliferative lesions (19).

Whereas the exact mechanism for the clearance of PAX2-null glands by progestin is unknown, it has been suggested that the disappearance of PTEN-null glands is due to re-activation of a silenced gene. However, as the majority of *PTEN*-inactivating events are related to structurally damaged *PTEN* alleles, this theory has been considered less likely happen (13). Another possibility could be a direct influence of progestin on the downstream PTEN signalling pathway at the protein kinase B (AKT) level or below as such pathway is crucial for proliferation in endometrial cancer and precancer (26, 27). Decreased expression of AKT has been observed by immunohistochemistry in endometrial precancer after MPA therapy, suggesting that the antitumor action of MPA may be mediated by dephosphorylation of AKT and that immunohistochemical expression of phospho-AKT and PTEN may be able to predict the outcome of MPA therapy (28). In another study, progesterone was shown to inhibit the oestradiol-stimulated AKT/cyclinD1/pathway, blocking uterine epithelial proliferation (29). However, the specific histological normalization observed by microscopy after LNG-IUS in the present study demonstrates a characteristic glandular atrophy coexisting with the pseudo-decidualisation of stroma. Thus, clearance of PAX2- and PTEN-null glands can be explained by mechanical ablation due to the high-dose progestin delivered directly to the endometrial mucosa.

The real prognostic significance of PTEN and PAX2 loss after progestin is not fully understood and has not been investigated in follow-up studies. However, in a recent study by Akiyama-Abe and co-workers, clearance of PTEN loss proved to be an independent predictor of favourable survival in endometrial carcinomas (30).

In daily routine, immunostaining of PTEN has been proven to be technically challenging, and its pancellular distribution is often difficult and confusing to interpret (14). This makes staining a significant obstacle to routine use. In contrast, PAX2 has proven to be technically robust and its normally strong nuclear staining pattern is very easy to interpret (14). PAX2 and PTEN protein loss has been shown to occur independently in latent pre- cancer of normal premenopausal endometrium. In a recent study of Monte and collaborators, loss of function of both genes occurred in overlapping distribution in pre-malignant endometrial lesions (14). Overlapping of PAX2- and PTEN-null glands were also observed in most cases of the present study.

In conclusion, LNG-IUS was significantly more efficient compared to oral progestin therapy in obtaining clearance of PAX2- and PTEN-null glands in medium- and low-risk endometrial hyperplasia. More studies with long-term data are required to demonstrate that PAX2 and PTEN are efficient as prognostic markers for therapy response in daily routine as a supplement to the WHO classification system.

Acknowledgements

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