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## Histopathologic diagnosis of endometrial precancers: updates and future directions

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### Abstract

Early detection of endometrial cancer, especially its precancers, remains a critical and evolving issue in patient management and the quest to decrease mortality due to endometrial cancer. Due to many factors such as specimen fragmentation, the confounding influence of endogenous or exogenous hormones, and variable or overlapping histologic features, identification of *bona fide* endometrial precancers and their reliable discrimination from benign mimics remains one of the most challenging areas in diagnostic pathology. At the same time, the diagnosis of endometrial precancer, or the presence of suspicious but subdiagnostic features in an endometrial biopsy, can lead to long clinical follow-up with multiple patient visits and serial endometrial sampling, emphasizing the need for accurate diagnosis. Our understanding of endometrial precancers and their diagnosis has improved due to systematic investigations into morphologic criteria, the molecular genetics of endometrial cancer and their precursors, the validation of novel biomarkers and their use in panels, and more recent methods such digital image analysis. Although precancers for both endometrioid and non-endometrioid carcinomas will be reviewed, emphasis will be placed on the former. We review these advances and their relevance to the histopathologic diagnosis of endometrial precancers, and the recently updated 2020 World Health Organization (WHO) Classification of Female Genital Tumors.

### Keywords

endometrial cancer; endometrial precancer; endometrial atypical hyperplasia (AH); endometrioid intraepithelial neoplasia (EIN); Pax2; Pten;  $\beta$ -catenin

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#### Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Introduction

Endometrial cancer (EC) is the most common gynecological cancer and the third most common cancer in women<sup>1, 2</sup>. The incidence of EC has been on the rise in the past decade and poses a major threat to public health<sup>3, 4</sup>. Earlier and more accurate diagnosis of EC, and particular its histologic precursors, represents an outstanding opportunity for prevention and improved patient management of this commonly-encountered malignancy. The diagnosis of endometrial atypical hyperplasia/endometrioid intraepithelial neoplasia (AH/EIN), the accepted precancerous lesion for endometrioid adenocarcinomas, remains challenging and subjective, with variable histologic criteria and differences of opinion among gynecologic pathologists, potentially leading to under- or overtreatment, thereby delaying diagnosis or adding to healthcare costs<sup>5-8</sup>. In this review, and in accordance to prior literature, we draw a distinction between “precancer” and “precursor”. A precancer is a precursor with a high risk for progression to a malignancy (typically necessitating clinical intervention), whereas a precursor is an entity believed to precede the eventual malignancy but not necessarily with a high risk to do so. Thus, “precursor” encompasses a wider range of lesions than “precancer”. We consider Pten or Pax2-deficient clones in otherwise normal endometria (discussed below) to be “precursors” whereas AH/EIN are “precancers”. These definitions are somewhat fluid and subject to refinements as more is learned. Serous endometrial intraepithelial carcinoma (SEIC) and its relationship to endometrial serous carcinoma will also be briefly reviewed.

The practical challenges in reliable diagnosis are well-known to pathologists and include 1) specimen fragmentation/limited tissue; 2) variations in glandular architecture, density, and cytology during normal cycling; 3) treatment with hormonal agents that obscure architectural and cytologic features; 4) presence of fragments of endometrial polyps, which can normally exhibit considerable gland crowding—but conversely often harbor precancers, and 5) gradual variations in gland architecture can make it difficult to identify or clearly demarcate definitive regions of neoplasia. Naturally, there has been sustained interest in the refinement of histologic criteria for the diagnosis of endometrial precancer, and more recently, in the use of other approaches such as specific immunohistochemical markers as adjuncts in AH/EIN diagnosis.

The molecular pathways and genetic driver lesions underlying endometrioid adenocarcinoma are now well established. Building upon past studies, the EC genome atlas project (TCGA, National Cancer Institute)<sup>9</sup> revealed the pathways most frequently misregulated *via* mutations in genes such as *PTEN*, *CTNNB1* ( $\beta$ -catenin), or *ARID1A*. These refined views of molecular genetics of EC combined with numerous studies of early endometrial neoplasia provide a rational basis for the systematic exploration and validation of biomarkers that could be useful in the diagnosis of AH/EIN. In this review, we summarize the gradual evolution of WHO criteria. We then present several individual biomarkers of potential diagnostic use, and discuss efforts to define combinations of these biomarkers that work well together in limited immunohistochemistry panels. Finally, we close with a brief discussion of other methods that merit further investigation as promising future approaches to the diagnosis of endometrial precancers.

## Changes in the diagnostic system for the precancer of endometrioid adenocarcinoma from WHO 1994 to 2020 classifications

**WHO 1994 4-tier classification system.**—In WHO 1994, hyperplasia was classified into 4 diagnostic categories based on 1) the degree of architectural complexity (simple vs. complex) and 2) nuclear atypia of the lesional glands (presence vs. absence). In this system, all presumptive lesions were divided into 2 binary categories leading to 4 entities: hyperplasia without atypia (simple or complex) and hyperplasia with atypia a.k.a. atypical hyperplasia (simple or complex) based on a seminal 1985 study showing that the 4 categories were predictive of risk of progression to invasive adenocarcinoma<sup>10</sup>. This diagnostic scheme was a major advance, as it was a more systematic and unified nomenclature and classification system than the pre-existing and non-uniform terminology.

**Development of 2-tier endometrioid intraepithelial neoplasia (EIN) scheme.**—On the other hand, over time, some limitations of the WHO 1994 became apparent. One challenge was the lack of specific, reliable, and reproducible cutoffs for architectural complexity and nuclear atypia, or at least, difficulties in their application in day-to-day cases. Another limitation was the creation of 4 diagnostic categories that did not clearly align with clinical management options (e.g. treatment vs. continued follow-up) including one category that remains controversial or rarely seen (simple atypical hyperplasia). These perceived limitations led to the advent of a distinct 2-tier scheme with different histologic criteria and terminology: endometrioid intraepithelial neoplasia/EIN<sup>11, 12</sup>. While early literature used the term “endometrial intraepithelial neoplasia”<sup>13</sup>, this was later modified to endometrioid intraepithelial neoplasia to emphasize the distinct endometrioid pathway of carcinogenesis (e.g. vs. serous). This 2-tier EIN scheme dropped atypia and subgrading of glandular complexity as formal criteria. Another point stressed by the proponents of EIN was that the term “neoplasia” is preferable to “hyperplasia” when referring to a *bona fide* cancer precancer, because hyperplasias have generally been defined as physiologic i.e. non-clonal, non-neoplastic processes. In the EIN classification system, lesions were subclassified into 2 categories: “hyperplasia without atypia” (i.e. non-neoplastic) and “EIN”. The diagnostic criteria for EIN are 1) glandular crowding (gland/stroma ratio >1); 2) cytology differs between architecturally crowded focus and background; 3) size of lesion >1 mm; and 4) exclusion of carcinoma or mimics such as endometrial polyp or artifactual crowding. The EIN system emphasizes “cytologic distinctiveness” (nuclear and/or cytoplasmic features) relative to entrapped or adjacent normal glands that serve as internal morphologic controls for assessing putatively neoplastic foci. Inherent to the EIN scheme was the argument that nuclear atypia is subjective, and hence not highly reproducible, whereas comparison of normal vs. putative neoplastic glands provides a more reliable and readily applied diagnostic criterion. The adoption of this 2-tier system has led to better reproducibility among pathologists, according to some studies<sup>14, 15</sup>.

**WHO 2014 and 2020: Incorporation of EIN system, continued refinement of the diagnostic criteria for atypical hyperplasia/EIN, and use of biomarkers.**—In the 2000s and 2010s, the differences and advantages of the atypical hyperplasia (AH) vs. EIN schema were debated, and some pathology departments began using the EIN nomenclature. The existence of 2 competing schema with different criteria may have been

somewhat disconcerting, a less than ideal state of affairs requiring some resolution. To a large extent, WHO 2014 was conciliatory and largely (if not explicitly) merged the AH and EIN systems into one 2-tiered system<sup>16</sup> where AH and EIN were considered equal: “AH/EIN”. Per WHO 2014, “cytologic atypia superimposed on endometrial hyperplasia defines AH/EIN”. The influence of the EIN conceptual framework is clearly evident in the collapse to 2 categories, while at the same time the concept of atypia intrinsic to the AH system was maintained. While this could seem counterintuitive, it may make sense in light of the fact that pathologists using the AH system were likely aware of normal glands and their cytologic distinctiveness, even if this had not been emphasized as a specific diagnostic criterion<sup>10</sup>. The WHO 2020 classification continued with the combined AH/EIN terminology, specifying as essential diagnostic criteria crowded glandular architecture with altered epithelial cytology distinct from non-neoplastic glands. The initial 1 mm size cutoff in the WHO 2014 system, based on morphometric studies (10) to exclude artifact crowding, was modified to “sufficient size that artifact can be excluded”. Therefore, some small lesions that were subdiagnostic in WHO 2014 could be classified as AH/EIN in the WHO 2020 system. Notably, WHO 2020 also specified loss of immunoreactivity for Pten, Pax2, or mismatch repair proteins as “desirable” diagnostic criteria. This was a significant development, as it was the first time that incorporation of biomarkers was recommended in the diagnostic workflows for endometrial precancers. The use of biomarkers in the diagnosis of AH/EIN is discussed at length below. The evolving criteria in the WHO systems are summarized in Table 1.

### **Disordered proliferative endometrium (DPE) and hyperplasia without atypia**

The WHO diagnostic criteria for “non-atypical” hyperplasia has not explicitly changed over the years. In the current WHO 2-tiered system, hyperplasia without atypia is considered a “benign” hyperplasia resulting from a physiological polyclonal proliferation typically caused by prolonged unopposed estrogen, which is common in the perimenopause. The range of histopathological features [so-called benign hyperplasia sequence<sup>17</sup>] is likely dependent on the quality and duration of unopposed estrogen in the perimenopause or other anovulatory conditions such as polycystic ovarian syndrome.

Changes at the lower end of the histological spectrum are referred to as “disordered proliferative endometrium” (DPE), which describes a proliferative endometrium (PE) lacking the usual regularity of gland size and spacing. Instead, DPE is characterized by irregularly shaped, cystically dilated glands producing a disordered arrangement. True gland cribriforming is not seen and the epithelium remains as a single layer without stratification. Metaplastic changes are common, including tubal or eosinophilic syncytial metaplasia<sup>18</sup>. Other changes associated with endometrial stromal breakdown are common. Most importantly, the endometrium retains a relatively normal gland to stroma ratio. When the gland:stroma area exceeds 50% (1:1), the term “hyperplasia without atypia” may be used, although this threshold is somewhat arbitrary and does not necessarily signify a clear-cut biological or clinical distinction. Of note, the changes associated with hyperplasia without atypia involve the entire endometrium (global changes), best appreciated at low magnification. In contrast, local admixtures of irregularly shaped glands have variable appearances among different fields at medium magnification. This combination of low

magnification uniformity and medium magnification variability has been described as “regularly irregular”<sup>17</sup>. A feature of hyperplasia without atypia is that cytological features of crowded glands are unchanged from field to field. This global change serves as a helpful criterion for the distinction between hyperplasia without atypia (presumed non-clonal) and AH/EIN (clonal).

DPE and hyperplasia without atypia represent a spectrum of continuous histological change caused by excess estrogen exposure and are not considered precancers. Because there are no well-established criteria for distinguishing the 2 entities (the use of 1:1 ratio as a threshold notwithstanding), the separation of these 2 diagnoses is essentially subjective<sup>19</sup>, highly variable among individual pathologists, and thus of questionable value. The diagnosis of “hyperplasia without atypia” might imply an entity more worrisome than “DPE”, or one more likely to represent a *bona fide* endometrial precancer requiring management. This is particularly the case because the term hyperplasia persists in the AH/EIN nomenclature, potentially causing confusion among clinicians and possibly leading to overly aggressive clinical management in some cases. On the other hand, some lesions diagnosed as simple hyperplasias, perhaps at the higher end of the spectrum, might be more significant precursor lesions<sup>20</sup>, a possibility that warrants investigation and is further discussed below. Similarly, there is no generally accepted criterion to separate PE from DPE. In our practice, the presence of 10% dilated or architecturally irregular glands is sufficient for the diagnosis of DPE.

### Molecular genetics of AH/EIN

The integrated genomic analysis of EC by The Cancer Genome Atlas (TCGA) has provided a more complete understanding view of the genetic EC landscape<sup>21</sup>. Per this analysis, ECs can be divided into 4 categories based on patterns of genomic instability: 1) POLE/ultramutated 2) microsatellite unstable and mismatch repair deficient/hypermethylated 3) copy-number low, and 4) copy-number high, largely corresponding to *TP53*-mutant serous and high-grade endometrioid carcinomas. In addition to the original publication in 2013, there have been several outstanding reviews<sup>22–24</sup> about these discoveries and their implications for patient management. Frequent tumor driver genes include *PTEN*, *CTNNB1*, *PIK3CA*, *ARID1A* and *KRAS*. Genetic changes lead to frequent disruption of diverse pathways including the PI3K pathway (*PI3K–PTEN–AKT–mTOR*, *RAS–MEK–ERK*), WNT- $\beta$ -catenin) and the SWI/SNF chromatin remodeling complex, which includes *ARID1A*. Immunohistochemical loss of PAX2 is also frequently observed in EC and AH/EIN, suggesting that PAX2 loss is a very early event, although interestingly, this does not appear to be due to mutational inactivation<sup>21, 25–35</sup>.

Recent genome-wide mutation profiling<sup>34, 36–38</sup> in paired AH/EIN and EC cast new light on the clonal evolution of endometrioid carcinoma. While stepwise acquisition of cancer driver mutations and progressive accumulation of tumor mutation burden have been observed in some cases, private mutations (i.e., present in only one sample) are not uncommon. Thus, beside an idealized linear pathway which involves stepwise accumulation of molecular events, more complex pathways in which AH/EIN and/or carcinoma diverge early or

develop independently—leading to considerable genetic heterogeneity—may also exist (see example case described below).

### Potential immunohistochemical biomarkers in the diagnosis of AH/EIN

Several factors that participate in early endometrial neoplasia are aberrantly expressed in some AH/EIN, stimulating interest in their use as practical biomarkers in the diagnosis of AH/EIN. Below, we briefly present each of these protein markers, followed by a discussion of their potential use together as a group (i.e. in panels). Photomicrographs showing normal and aberrant patterns of expression are presented in Figs. 1 and 2.

**Pax2**—Pax2 is a DNA-binding transcription factor localized exclusively within nuclei. Loss of nuclear expression of PAX2 is observed in endocervical adenocarcinoma<sup>39</sup>, EC, and AH/EIN<sup>35</sup>. Frequent immunohistochemical loss of Pax2 in AH/EIN was first reported by George Mutter in 2010<sup>35</sup>. Pax2 is usually strongly expressed in all endometrial epithelial nuclei. Loss of expression, when it occurs, appears complete (i.e. “all or none” within individual cells) in AH/EIN. Such complete Pax2 loss relative to the strong and uniform expression in control glands makes Pax2 an easily-scored and attractive AH/EIN marker, with several investigations confirming its utility and the robustness of Pax2 IHC<sup>11, 35, 40–43</sup>. Widespread loss of nuclear expression of Pax2 in AH/EIN is specific and demonstrates high sensitivity (~80% of AH/EIN exhibit significant Pax2 loss, Figs. 3, 4) as also confirmed in a recently published meta-analysis<sup>44</sup>. Interestingly, as noted above, the mechanism(s) for Pax2 loss are not fully understood, although some studies have implicated epigenetic misregulation, perhaps due to hypermethylation of the *PAX2* promoter<sup>45</sup>.

**Pten**—Pten is a ubiquitously-expressed lipid phosphatase present throughout the cell in the nucleus, cytoplasm, and cell membrane<sup>46</sup>. *PTEN* is among the most frequently mutated genes in EC, usually as an early driver event<sup>9, 22</sup>. The first studies documenting Pten loss in AH/EIN were, as for Pax2, performed by Mutter’s group<sup>35, 47, 48</sup>. Pten was not immediately adopted as an AH/EIN biomarker, with some relatively recent practice consensus publications arguing against its routine use<sup>49</sup>. Resistance to its acceptance may stem from the existence of diverse commercially monoclonal antibodies, some of which may be suboptimal for formalin-fixed paraffin-embedded tissue. However, optimal commercial monoclonal antibodies are reliable in the clinical pathology laboratory (Figs. 1, 2). Although the frequent loss (~50%) of Pten protein in AH/EIN (Figs. 3, 4) is consistent with the high frequency of *PTEN* point mutations in EC, the frequent immunohistochemical loss of Pten protein is not yet entirely explained at the molecular level. For example, to fully rationalize complete loss of the Pten protein, biallelic mutations would have to be identified (or a single mutation with documented loss-of-heterozygosity), and each mutation would have to be predicted or demonstrated to lead to protein loss (i.e. premature stop, frameshift, etc.). Molecular studies to date have not been systematically carried out at this level of resolution. Thus, it remains possible that diverse mechanisms contribute to Pten loss, such as nonsense-mediated decay, destabilization of the protein by point mutations, intragenic or larger deletions not readily detectable by standard (short-read) next-generation sequencing methods, or epigenetic/autoregulatory mechanisms operating at the transcriptional level.

**β-catenin.**—In endometrial neoplasia, mutations in the *CTNNB1* gene encoding β-catenin are frequent early events<sup>27, 50</sup> occurring in ~50% of ECs, most commonly in copy-number low endometrioid adenocarcinomas<sup>9, 51</sup>. Most *CTNNB1* mutations alter specific residues within exon 3 that form a β-catenin protein degradation motif. These mutations inhibit proteasomal degradation and lead to stabilization of β-catenin, resulting in increased protein levels and abnormal relocalization from the membrane/cytoplasm to the nucleus<sup>51–53</sup>. Strong nuclear β-catenin localization, usually associated with overall overexpression, is a reliable indicator of β-catenin activation in AH/EIN or endometrial adenocarcinoma and such aberrancy occurs in ~48% of cases (Figs. 3, 4)<sup>43, 53, 54</sup>. Studies of ECs showed that nuclear β-catenin staining has 90% specificity and sensitivity for *CTNNB1* mutations<sup>52, 53, 55</sup>.

Although abnormal nuclear localization of β-catenin in AH/EIN associated with *CTNNB1* mutations has been documented since 1999<sup>27, 56, 57</sup> only recently have studies begun to formally explore the utility of β-catenin as a practicable diagnostic AH/EIN marker<sup>58, 59</sup>. Unlike Pten or Pax2, where loss of expression is the feature indicating aberrance, relocalization of β-catenin to the nucleus is the principal immunohistochemical finding indicating an underlying molecular defect. The presence of strong, distinctively nuclear expression in glands observed in many AH/EIN cases, even when focally present, makes scoring such cases straightforward. Morular squamous metaplasia, which has been associated with underlying *CTNNB1* mutations, always exhibits nuclear β-catenin<sup>60</sup>, and β-catenin should be assessed in endometrial epithelium without obvious morules. When morules are present, adjacent epithelium usually exhibits distinctive nuclear localization in some non-morular epithelial cells. Characteristically, nuclear localization occurs in scattered cells within AH/EIN glands, and is not uniform among all cells in a gland.

**Mlh1 and other mismatch repair (MMR) factors.**—The use of Mlh1 as an AH/EIN marker is analogous to its use in the standard 4-marker panel for MMR deficiency and Lynch Syndrome screening in newly-diagnosed EC<sup>61–63</sup>. Mlh1 could be an attractive AH/EIN marker, at least in principle, in that 1) pathologists are experienced in its use 2) it is the most commonly aberrant MMR marker in AH/EIN and 3) MMR defects are early if not initiating defects in endometrioid carcinogenesis. AH/EINs with Mlh1 protein deficiency are easily scored, with the strong stromal nuclear staining normally present in endometrial stroma serving as an internal control (Figs. 1, 2)<sup>64</sup>.

However, studies investigating MMR expression in biopsies with AH/EIN have reported that only 5–10% of AH/EIN demonstrated loss of Mlh1 expression by immunohistochemistry (IHC)<sup>64, 65</sup> vs. the 29% incidence of Mlh1 loss in EC<sup>66</sup>. Thus, the prevalence of MMR deficiency in AH/EIN appears to be much lower than in EC, perhaps because MMR deficient AH/EIN have a shorter transition time to carcinoma relative to MMR proficient AH/EIN<sup>67</sup>. More rapid progression may be a general feature of hypermutant (MMR deficient) and ultramutant (POLE) ECs<sup>20, 50</sup>. In our previous study, most AH/EIN cases with loss expression of MMR proteins other than Mlh1 were patients with Lynch syndrome. However, the low prevalence of MMR deficiency in unselected AH/EIN is comparable to the reported 2–4% incidence of LS in unselected EC cases<sup>66, 68, 69</sup>. Therefore, MMR testing in AH/EIN appears to be highly useful in the early detection of LS, even if Mlh1 or other MMR factors were not found to be highly effectual markers in the diagnosis of AH/EIN.

**Arid1a.**—*ARID1A* (a.k.a. *BAF250A*), which encodes a component of the SWI/SNF nucleosome-remodeling complex, is one of the most frequently mutated genes in endometrioid adenocarcinomas, leading to complete immunohistochemical loss of the protein in some mutant cases<sup>70</sup>. Arid1a protein is, like Mlh1, nuclear and ubiquitously expressed. Although Arid1a IHC is not available in most pathology laboratories, robust IHC protocols with monoclonal antibodies are available<sup>71</sup>. Scoring of Arid1a loss in AH/EIN and identification of Arid1a-deficient cases is analogous to Mlh1<sup>71, 72</sup>, making it also a potentially useful marker (Figs. 1, 2). A recently published meta-analysis of *ARID1A* in AH/EIN also showed that *ARID1A* loss is highly specific as a diagnostic marker for AH/EIN<sup>73</sup>, with loss occurring in 5–10% of cases. Arid1a protein loss is clearly associated with *ARID1A* mutations, although as with *PTEN*, it is not always obvious why some mutations lead to protein loss while others do not, or if mutations must be biallelic for protein loss to occur.

**Foxo1 and phospho-Akt.**—Pten loss leads to hyperphosphorylation of the kinase Akt, which can be immunohistochemically detected by phospho-specific AKT antibodies. Phosphorylated Akt in turn phosphorylates the forkhead transcription factor Foxo1, leading to its export from the nucleus to the cytoplasm and hence its functional inactivation. Foxo1 and phospho-Akt are aberrant in some AH/EIN, but both markers appear less suitable as practicable biomarkers due to challenges in detecting phospho-proteins in paraffin-embedded tissues, or the influence of steroid hormone variation during normal cycling that impact levels of Foxo1 and or phospho-Akt. Nonetheless, these markers may merit further investigation<sup>59</sup>.

**p53.**—The use of p53 as a marker of endometrial serous cancers is well-known, and pathologists are familiar with identification of p53 mutant patterns<sup>74</sup>. Although p53 is typically considered as a marker of serous EC precancers, it is also mutated in some endometrioid adenocarcinomas, particularly those that are high grade. It is mentioned here for completeness, but the fact that p53 mutations occur very late in disease progression (i.e. in the progression from well- to poorly-differentiated invasive adenocarcinoma) makes it unlikely to be a useful AH/EIN marker. However, some studies have identified aberrant p53 expression in at least rare AH/EIN cases<sup>75</sup>.

### **Systematic evaluation of potential biomarkers for AH/EIN and selection of an optimal panel**

In recognition of the challenges in the reliable diagnosis of AH/EIN, and the promise of at least some of the above biomarkers, the 2020 WHO Classification of Female Genital Tumors stated that “loss of immunoreactivity for Pten, Pax2, or MMR proteins is desirable” in the diagnosis of AH/EIN<sup>76</sup>. This statement implies that a panel of immunostains is desirable in the diagnosis of AH/EIN. However, this raised critical questions. How many markers should be employed? And which ones? To address these unresolved issues, we recently performed a systematic analysis of the performance characteristics of 6 immunohistochemical markers (Pax2, Pten,  $\beta$ -catenin, Arid1a, Mlh1, and p53) both individually and in combinations in AH/EIN and normal controls, and the findings are reviewed below.



As single markers, Pax2, Pten, or  $\beta$ -catenin were aberrant in a high percentage of AH/EIN cases (Pax2, 81.1%; Pten, 50.5%;  $\beta$ -catenin, 47.7%) (Fig. 3). Arid1a, Mlh1, or p53 were aberrant in a significant, but much smaller percentage of cases (7.2%, 4.5%, and 2.7% respectively). With a hypothetical panel consisting of 6 markers, at least 1 of the markers was aberrant in 92.8% of AH/EIN (Fig. 4A). The 5 non-Pax2 markers identified 83.0% of cases, while Pten and  $\beta$ -catenin combined identified 78.4% of cases. The additive effects of each marker in order of aberrancy in AH/EIN provides a useful way to assess the value of including additional markers. A panel consisting of only Pax2, Pten, and  $\beta$ -catenin identified 92.8% of cases. Inclusion of Arid1a or Mlh1 did not increase the diagnostic yield further because all of the cases detected by Arid1a/Mlh1 were already scored as aberrant by Pax2, Pten, or  $\beta$ -catenin. p53 did not prove useful, because of the rarity of p53 mutant clones in AH/EIN, and the occurrence of rare p53-overexpressing clones in normal control endometria. Also of note, most AH/EIN cases were aberrant for 2 or 3 markers, further increasing diagnostic confidence (Fig. 4B).

One challenge in the use of Pten or Pax2 as practical AH/EIN biomarkers is the surprisingly common occurrence of sporadic Pax2 or Pten negative glands in normal endometria, also first described by Mutter. Pten-null glands were considered to represent extremely early “latent precancers” (i.e. precursors) for ECs, although their high incidence in biopsies (>40% of histologically normal PE) clearly indicates that most do not progress to EC, and do not represent a critical rate-limiting step in endometrial carcinogenesis<sup>35</sup>. That such Pten-null glands are indeed very early precursors to AH/EIN and cancer is an appealing idea, and a recent serial genomic analysis of early EC progression provided additional support for this notion. In some patients the same point mutations were identified in the pre-precancerous lesions as in the eventual endometrioid adenocarcinomas, many years apart<sup>20</sup>. The possibility that Pax2-null glands in normal endometria represent precursors also seems likely, although interestingly, Pax2 and Pten loss do not generally occur in overlapping patterns in normal endometria<sup>35</sup>. While the presence of rare Pax2 or Pten null glands is an important fact to be aware of, it does not greatly limit the utility of either marker in the diagnosis in AH/EIN. This is because the sporadic loss in normal endometria occurs in very rare and isolated glands, usually just 1 or a few in an entire section (<1% of the sample), whereas AH/EIN are typified by loss over large areas comprising >>5% of the sample. Thus the patterns of Pax2 and Pten loss in normal endometria and AH/EIN are quite different and usually resolvable. However, loss in the 1–5% range should be interpreted with caution and in the context of other histologic features and sampling (Fig. 4C, D).

In summary, in addition to refining specific criteria for scoring these markers, our findings demonstrated that a panel of only 3 markers (Pax2, Pten, or  $\beta$ -catenin) had optimal performance characteristics and is practical, feasible, efficient, and of considerable utility in the diagnosis of AH/EIN<sup>77</sup>. Also, the approach serves as a useful template for assessing any additional markers that may be discovered or further considered in the future<sup>78</sup>.

### **Other benefits to the general use of the Pax2/Pten/ $\beta$ -catenin panel**

Most cases of AH/EIN can be confidently diagnosed without the use of immunostains. Nonetheless, there should be considerable benefit to the routine use of the 3-marker AH/EIN

biomarker panel. First, routine use of the panel will help pathologists refine their diagnostic accuracy and skills<sup>48</sup>. Second, and more importantly, many women with AH/EIN undergo conservative management with long-term progestin administration. This necessitates routine surveillance with repeat endometrial samplings, and yet, progestin profoundly masks the histologic features of AH/EIN, making surveillance difficult in practice<sup>79, 80</sup>. A large recent longitudinal investigation of Pax2 and Pten expression patterns in serial biopsies from women treated with progestin found that expression patterns in pretreatment AH/EIN were consistently recapitulated by AH/EIN present following treatment<sup>78</sup>.  $\beta$ -catenin patterns are also likely to be recapitulated following treatment<sup>20</sup>. Thus, in addition to facilitating the initial diagnosis of AH/EIN, establishment of baseline expression patterns should be useful diagnostically in follow-up biopsies in the setting of progestin treatment. Additional investigations will be needed to determine the incidence and patterns of marker aberrance in mimics of AH/EIN, including endometrial polyps, DPE, or non-atypical hyperplasia. The utility of the IHC panel in the diagnosis of AH/EIN in progestin-treated endometrial specimens is discussed in another article in this issue (see Chang *et al*).

In addition, the diagnosis of AH/EIN is challenging in other situations, such as minute lesions of questionable significance, secretory AH/EIN, AH/EIN within endometrial polyps or in the background of polyps (including atypical polypoid adenomyoma). Pathologists often find it difficult to diagnose AH/EIN when secretory change is present because 1) glandular crowding criteria are difficult to apply since glandular crowding is a feature of secretory endometrium; and 2) under the influence of high circulating levels of progesterone/progestins, nuclear atypia is less prominent or absent<sup>81</sup>, especially when the background endometrium is also secretory type. The diagnosis of AH/EIN within a polyp or polyp background remains difficult in that both glandular crowding and metaplasia are common in benign endometrial polyps. Thus, in practice it is often difficult to exclude endometrial polyp, or reliably identify AH/EIN in a polyp. In these diverse and challenging situations, the 3-marker panel is particularly useful (Figs. 5, 6).

In closing, we present a case (Fig. 7) that—though unusual—illustrates the power of the 3-marker panel in providing more refined views of AH/EIN. In this initial biopsy, the 3 marker panel yielded unexpected results, revealing 2 distinct EINs. In the first, Pax2 and Pten were lost, but  $\beta$ -catenin was wild-type (membrane-associated). In the second, Pax2 and Pten were retained but  $\beta$ -catenin was diffusely mutant with strong overexpression/nuclear localization. Intriguingly, the 2 EINs were histologically distinct, with the second exhibiting less impressive architectural features. These distinct EINs cannot be readily explained as sharing a common precursor, suggesting that the 2 EINs are likely to be clonally independent.

**Strengths and limitations of AH vs. EIN schema: a reassessment.**—Although there has been some conciliation of the AH and EIN systems, at least in the recent WHO classifications, they do have different conceptual underpinnings and debate will continue as to their relative merits. On one hand, the EIN approach is pragmatic in that it seeks to provide a defined cutoff to distinguish benign from precancerous/neoplastic in a manner aligned to clinical decision making (i.e. no follow-up needed vs. extensive follow-up and/or treatment). Indeed, the EIN system is geared towards providing an optimal definition of

this cutoff based on a precise percentage, currently set at >1:1 gland:stroma ratio. Even if the 50% (1:1) cutoff is the most practical from a diagnostic and clinical perspective, what comprises an EC precancer must be more nuanced. For most cancer types, high-grade *in situ* precancers (i.e. ductal carcinoma of the breast, prostatic intraepithelial neoplasia, pancreatic intraepithelial neoplasia, etc.) already harbor most of the mutations evident in their frankly invasive malignant counterparts<sup>82–84</sup>. This is also the case in AH/EIN, which harbor a similar spectrum of mutations as EC<sup>36, 37, 85, 86</sup>. Thus, AH/EINs must progress from “pre-precancer” precursors, which could include Pten or Pax2 null but morphologically normal glands, or other intermediates, pointing to practical limits of purely histologic evaluation. Also, we often encounter cases that are subdiagnostic for EIN, but raise considerable suspicion for a significant lesion. In some cases, there are diffuse architectural changes that may not fit well into the conception of a focal or clonal EIN, resulting in considerable diagnostic ambiguity.

Such pre-precancerous lesions might be found among DPEs and hyperplasias without atypia, and there is already considerable evidence for this. First, the work of Mutter identified “latent precancers” that are entirely normal histologically yet harbor mutations in *PTEN*, leading to its apparently complete genetic inactivation. We recently performed a retrospective analysis of women who developed EC but for whom preceding biopsies were available, including at least one prior to the histologic diagnosis of AH/EIN. In most cases,

1 mutation present in the invasive cancer could be identified in biopsies preceding AH/EIN, and in most such cases, at least 1 mutation could be confirmed by IHC to Pten, Arid1a,  $\beta$ -catenin or Mlh1<sup>34</sup>. Also, “focal gland crowding” that is diagnostically ambiguous and/or falls short of diagnostic criteria for AH/EIN has been identified as an endometrial biopsy finding associated with a significant risk of AH/EIN or carcinoma in subsequent biopsies (23% and 4% respectively with 1.5 year average follow-up)<sup>7</sup>. This finding further supports the idea that there is a currently ill-defined subset of endometrial biopsies that fall short of AH/EIN but nonetheless carry a significant risk of subsequent neoplasia.

Thus, the AH system remains appealing in that it embraces the notion that there is in fact a continuum of cancer progression risk based on histologic and/or molecular features. The diagnosis of EIN on a biopsy signifies a 45-fold increase in the risk of EC >1 year after the diagnosis of EIN (excluding cases likely to have concurrent EC and EIN)<sup>11</sup>. However, 45x may be too stringent a criterion that risks not identifying patients with lower, but still substantial risk of EC. It would be desirable to be able to perform better risk stratification in the evaluation of endometrial biopsies and identify women with a lower but still significant risk of EC (e.g. 10–30x).

**Early forms of endometrial serous and clear cell carcinoma.**—Serous endometrial intraepithelial carcinoma (SEIC) is a recognized entity related to endometrial serous carcinoma (ESC). SEIC epithelium shows cytological features typical of serous carcinoma (marked nucleomegaly and pleomorphism) and p53 mutant patterns by IHC characteristic of ESC. SEIC may represent an early variant of ESC, as SEIC lesions are typically relatively small with less extensive involvement of the endometrium than ESC. However, SEIC is strongly associated with extra-uterine spread and should be considered as a malignancy even when invasion is not identified<sup>87–90</sup>. Thus, in the WHO 2020 classification, SEIC is

considered as early stage serous carcinoma. Zheng *et al* proposed that ESC is preceded by a sequence similar to that of tubo-ovarian high-grade serous carcinoma [endometrial p53 signature lesion→endometrial glandular dysplasia (EmGD)→SEIC→ESC]<sup>89, 91, 92</sup>. In this sequence, EmGD is most likely an earlier precancer of SEIC/ESC<sup>93–98</sup>. EmGD is usually found adjacent to ESC and SEIC, and only rarely associated with endometrioid adenocarcinoma<sup>99</sup>. It is defined by endometrial glands with nuclear atypia falling short of SEIC, but distinct compared to background endometrium. In addition, it has an IHC profile bridging benign resting endometrium and SEIC (i.e. p53 and p16 diffusely strongly positive, increased IMP3 expression, and reduced ER/PR expression).

Similarly, a spectrum of atypical endometrial glandular and surface changes is often found adjacent to clear cell carcinoma. The atypical glands and surface epithelia are cytologically distinct from the background benign endometrium and the adjacent endometrial clear cell carcinoma. Lining epithelia show clear cytoplasm, moderate nuclear atypia (less than clear cell carcinoma) and occasional hobnailing. Fadare *et al* have proposed the term “clear cell EmGD” as the precancer of endometrial clear cell carcinoma<sup>100</sup>. More research is needed to better define the histologic and molecular features of early precancers for non-endometrioid ECs including clear cell, serous, and carcinosarcomas.

**Future direction 1: Use of next-generation sequencing (NGS) for refining the diagnosis of endometrial precancer.**—NGS assays have become standard-of-care, including circulating tumor DNA assays that highlight the extraordinary sensitivity of such assays and their ability to detect rare mutations. NGS on selected endometrial biopsies might someday be useful in clinical practice, e.g. to 1) establish a lesion as a *bona fide* precancer signifying an increased cancer risk, 2) identify class-defining mutations such as *POLE* or in MMR factors earlier in clinical progression, which could further guide management, and 3) diagnose hereditary cancer syndromes earlier, which would trigger earlier surveillance and further enhance clinical management. One major challenge would be the need to establish thresholds capable of distinguishing age-related mutations i.e. in *PTEN*, which clearly occur in normal aging endometria<sup>101</sup>. Other and arguably even more ambitious approaches have included NGS analysis of lavage specimens during hysteroscopy, or pap smears<sup>102, 103</sup>. Much more research is needed to establish the utility and practicability of such approaches with respect to patient management and assess their cost-effectiveness<sup>8, 104</sup>.

**Future direction 2: Image analysis-aided assessment of endometrial biopsies.**—There is a long history of investigations of quantitative or computerized morphometric image analysis in the assessment of normal, hyperplastic, and malignant glands in the endometrium<sup>105, 106</sup>. Indeed, quantitative image analysis of diverse potential classifiers led to the identification of volume percentage stroma as the best predictor of progression to adenocarcinoma<sup>107</sup>. Efforts to refine such image analysis methods continue<sup>108</sup> and are likely to accelerate with the advent of deep learning/artificial intelligence methodologies for pathology image analysis<sup>109</sup>. The key questions are if 1) such algorithms can be efficiently used by pathologists or 2) if there are limits of even refined computational analysis, such that other methods like biomarker panels or NGS that add layers of non-morphologic information will ultimately prove more valuable.

**Future direction 3: Refinement of 3-marker panel.**—While the 3-marker panel (Pax2, Pten,  $\beta$ -catenin) is of demonstrated utility in the diagnosis of AH/EIN<sup>77</sup>, future refinements can be envisioned. The 3-marker panel detects >90% of AH/EIN but raising the sensitivity even further is desirable, so long as the panel remains “compact” and specificity is not sacrificed. There may be markers yet to be discovered that operate on new principles, or detect aberrancy of other signaling pathways involved in EC genesis. Alternatively, future markers may be identified that more reliably detect aberrations in their respective pathways than e.g. Pten or  $\beta$ -catenin.

## Conclusion

The accurate diagnosis of AH/EIN remains a common challenge frequently encountered by pathologists in their daily clinical practice. Reflecting these challenges, diagnostic schema based on histologic features have evolved considerably in the last 25 years. We believe that the recent adoption of select biomarkers, as introduced in WHO 2020 and as reinforced by studies showing the utility of the 3-marker panel (Pax2, Pten,  $\beta$ -catenin), also represent a significant advance. Our understanding of the molecular pathways and aberrations characterizing early endometrial cancers/precancers/precursors is rapidly moving forward, and these future insights should be continually applied to refine diagnostic approaches and improve risk stratification for this common premalignancy of women.

## Abbreviations

<b>AH</b>	atypical hyperplasia
<b>DPE</b>	disordered proliferative endometrium
<b>EC</b>	endometrial cancer
<b>EmGD</b>	endometrial glandular dysplasia
<b>ESC</b>	endometrial serous cancer
<b>EIN</b>	endometrioid intraepithelial neoplasia
<b>IHC</b>	immunohistochemistry
<b>MMR</b>	mismatch repair
<b>NGS</b>	next generation sequencing
<b>PE</b>	proliferative endometrium
<b>SEIC</b>	serous endometrial intraepithelial carcinoma
<b>WHO</b>	World Health Organization

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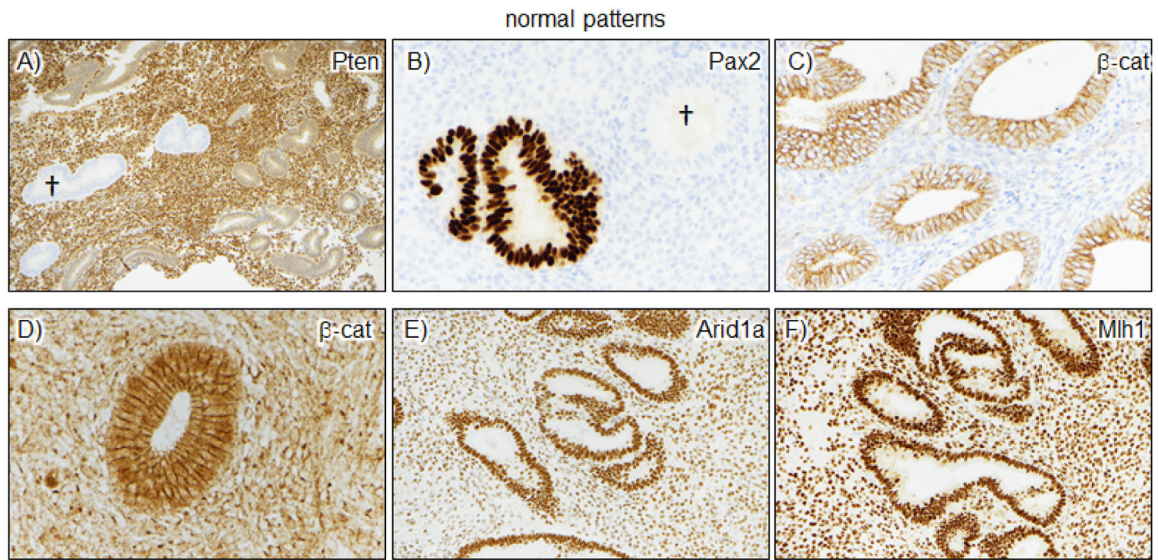
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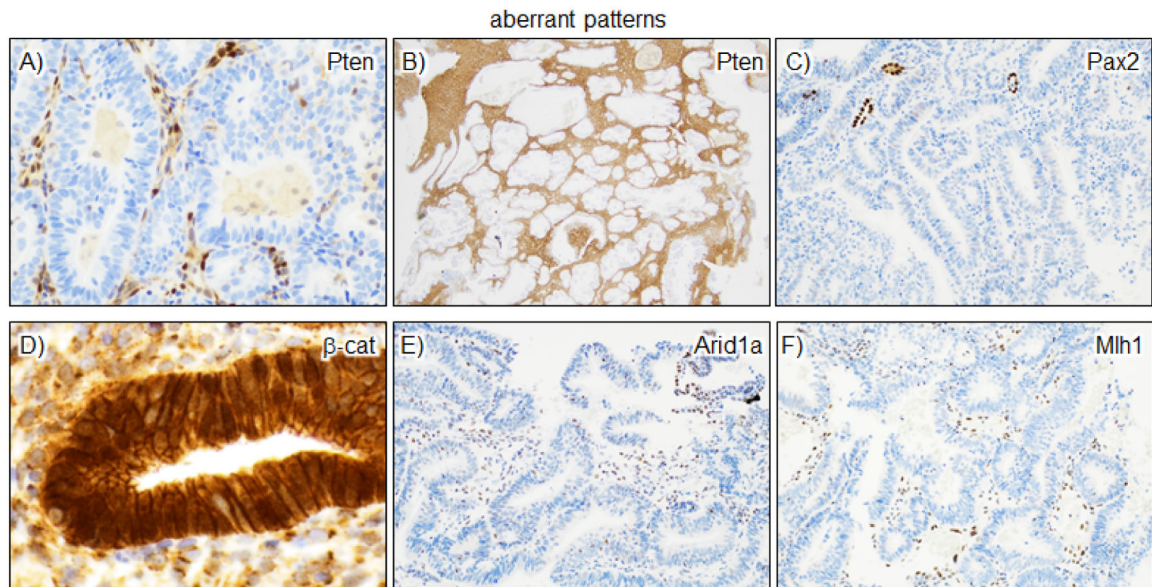
### Highlights

- Criteria for the diagnosis of endometrial precancers has evolved with each iteration of WHO guidelines, incorporating concepts and terminology from 4- and 2-tier systems
- Despite refined histologic criteria, diagnosis of atypical hyperplasia/ endometrioid intraepithelial neoplasia (AH/EIN) remains a common diagnostic dilemma faced by pathologists
- In WHO 2020, for the first time, use of biomarkers was specified as desirable
- A panel consisting of three biomarkers—Pax2, Pten, and  $\beta$ -catenin—has demonstrated utility in the diagnosis of AH/EIN
- Additional methods such as image or molecular analysis of endometrial biopsies represent future research directions in the refined diagnosis of AH/EIN with improved risk stratification



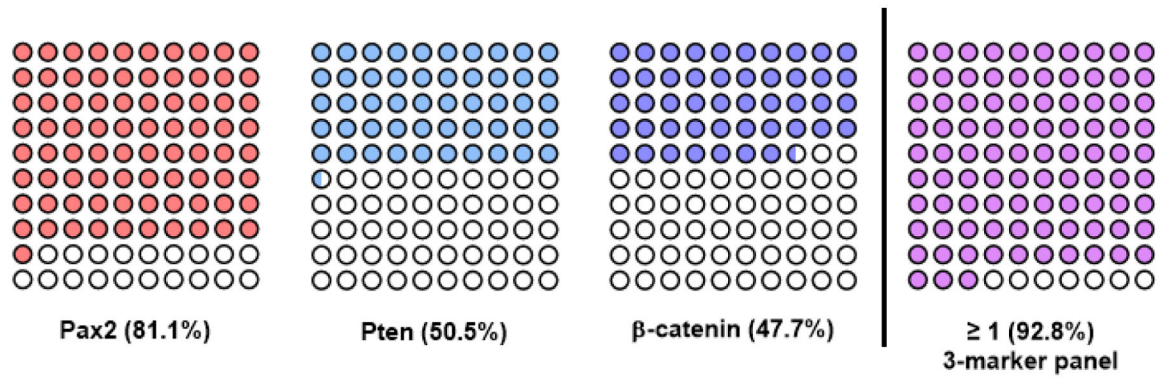
**Figure 1. Normal patterns of expression of biomarkers capable of identifying AH/EIN.**

A-B) Pten and Pax2 panels show examples of marker loss in scattered glands. The loss is striking and easily identified. Such loss is common in normal endometrial, but usually in <1% of the entire sample, and is thus usually readily distinguishable from the much more extensive loss seen in specimens with AH/EIN; †=glands with marker loss. C-D)  $\beta$ -catenin panels show the range of normal  $\beta$ -catenin expression, which can range from faint and entirely membranous to somewhat stronger expression with some nuclear localization. However, in normal endometria, the nuclear  $\beta$ -catenin expression is not stronger than the intervening cell membranes, a useful feature distinguishing it from mutant/aberrant patterns of  $\beta$ -catenin expression. E-F) Arid1a and Mlh1 are rarely lost in normal endometria; the two panels show the typical strong expression in glands and stroma, with the latter serving as a useful internal control.



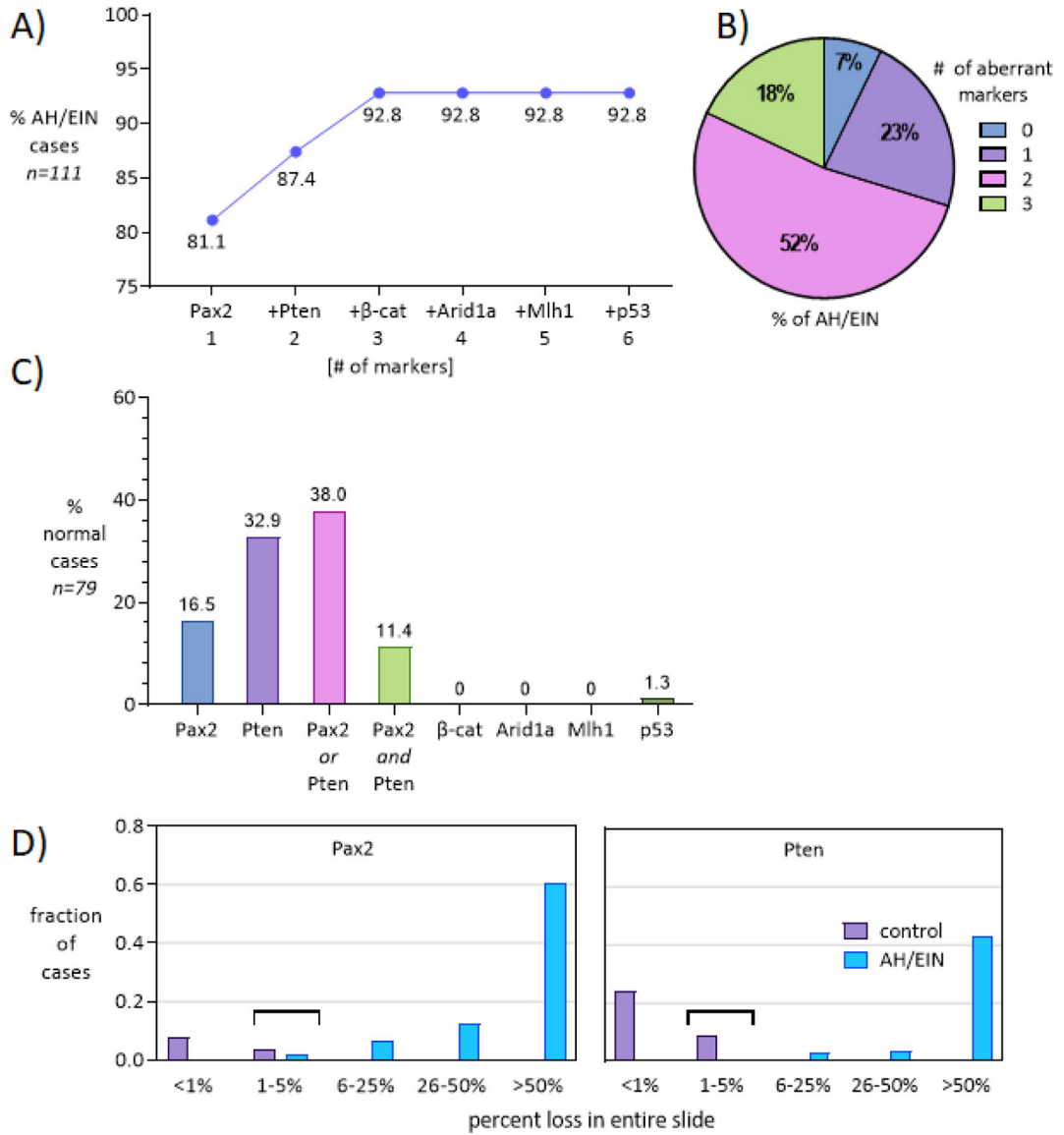
**Figure 2. Aberrant patterns of expression in AH/EIN.**

Pax2, Pten, and  $\beta$ -catenin are the most useful markers and comprise the most efficient panel for the detection of AH/EIN. A-B) For Pten, distinct loss produces a “punched-out” appearance of glands. Stroma and intraluminal macrophages/other leukocytes retain Pten. C) Pax2 loss is also usually easily scored, although the lack of expression in stroma or leukocytes means that other internal or external IHC controls are needed. Often scattered normal glands are present, providing an internal control, as in this example. D)  $\beta$ -catenin expression is readily scored by the presence of strong nuclear expression greater than that observed in the intervening cell membranes. E-F) Arid1a and Mlh1 loss in epithelium can be easily scored but are less useful in practice due to a lower incidence of biomarker aberrancy. In our study of n=111 AH/EIN, all cases were diagnosed with the 3-marker panel and inclusion of Arid1a and Mlh1 did not lead to the identification of any additional cases.



**Figure 3. Parts-of-whole representations for most useful AH/EIN biomarkers.**

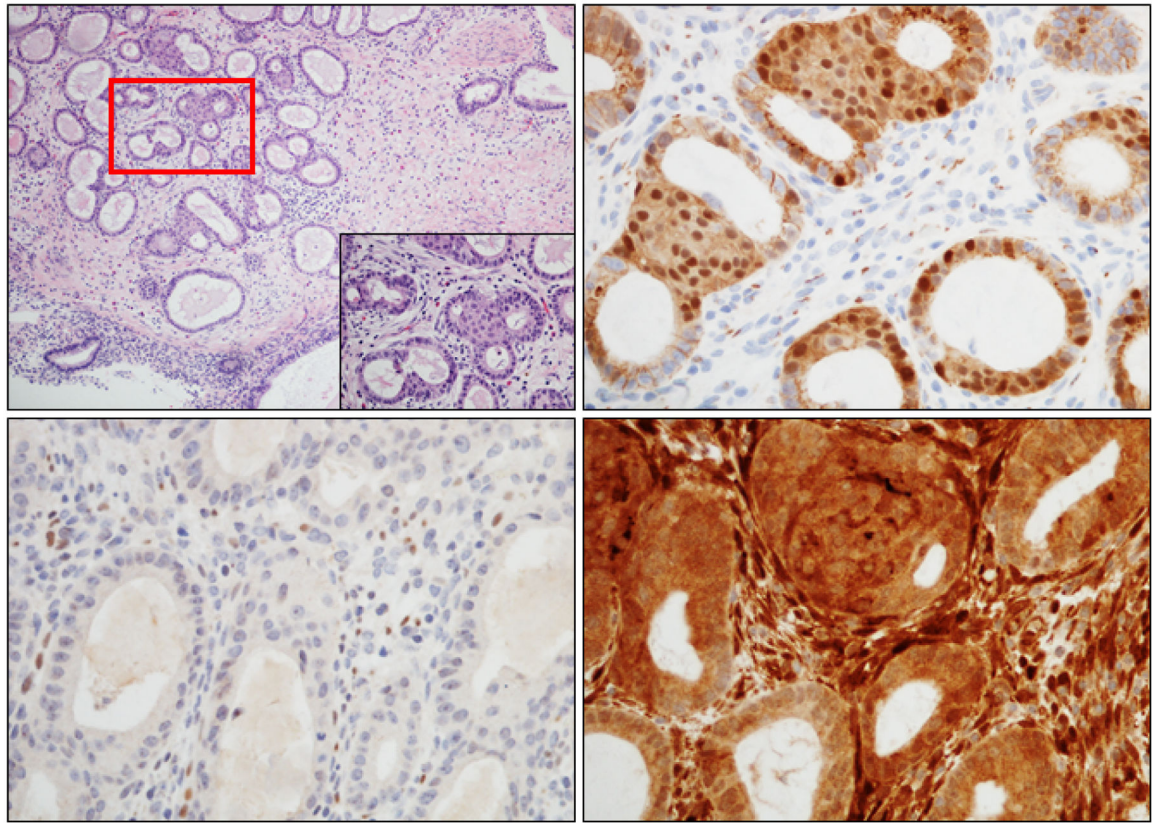
Graphs show individual cases (among idealized set of 100 patients) detected (filled circles) by each of the 3 markers individually and by 1 marker when all 3 markers are used. Data is based on a previous analysis of n=111 patients<sup>77</sup>.



**Figure 4. Quantitative analyses of biomarker aberrance in AH/EIN including group performance.**

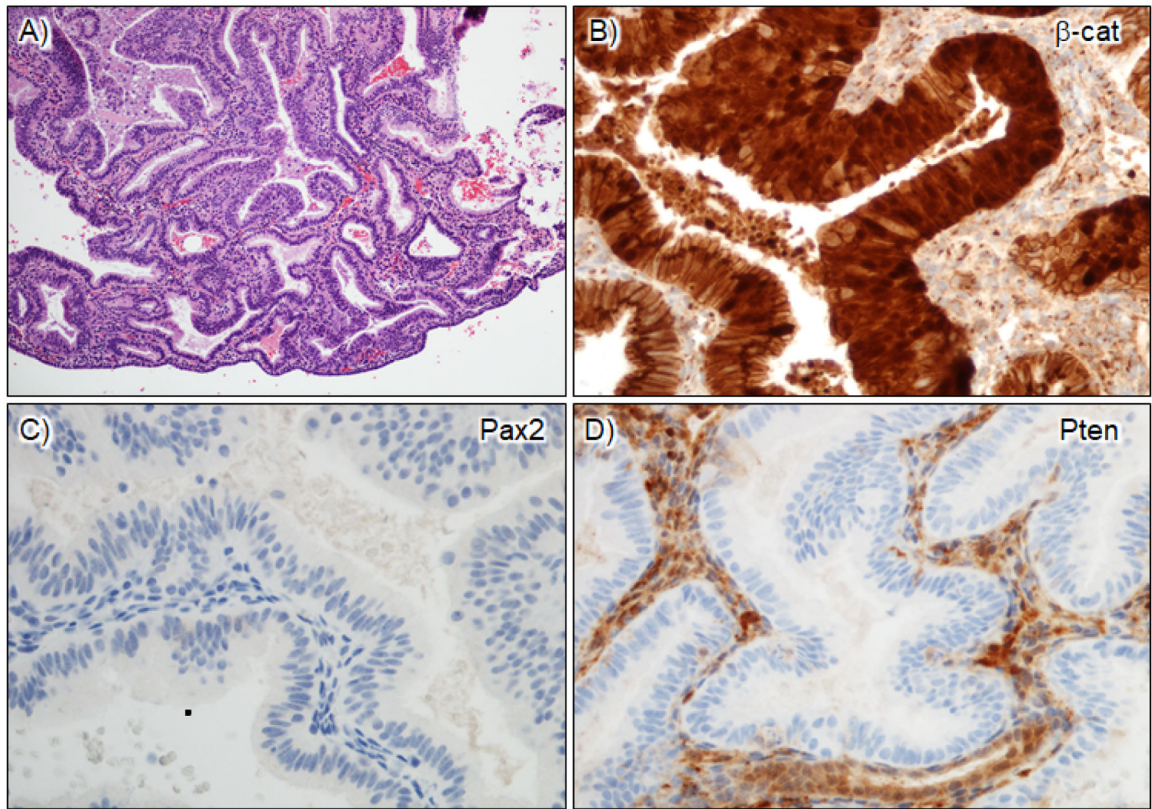
A) Diagnostic yields following addition of individual markers, in order of greatest to least likelihood of aberrancy. Arid1a, Mlh1, or p53 did not lead to the identification of any additional AH/EIN cases, pointing to Pax2/Pten/β-catenin as the most effective and compact panel. B) Pie chart showing the number of aberrant markers (0–3) among AH/EIN. Aberrance for 2 or more markers (which occurs in >50% of cases) can enhance diagnostic confidence. C) Percent of normal endometrial controls showing aberrancy for each marker. The numbers are not comparable to (A), because aberrancy within a single gland of normal endometrium is shown, whereas aberrancy within AH/EIN was characterized large areas involving many glands. Nonetheless, the findings indicate that evaluation of markers must always occur in the context of histologic features. D) Fraction (%) of control (purple) or AH/EIN (blue) cases exhibiting loss of Pax2 or Pten across different categories based on estimated overall loss on slide (<1%, 1–5%, 6–25%, 26–50%, or >50%).





**Figure 5. Aberrant patterns of expression in AH/EIN within an endometrial polyp.**

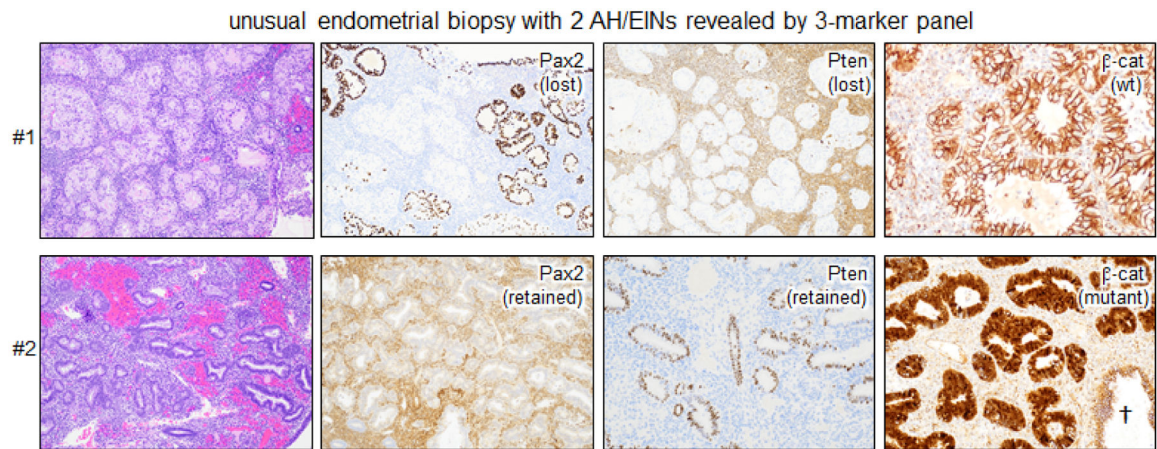
A) H&E, inset=higher magnification of boxed area; background glands are located at the periphery. B)  $\beta$ -catenin nuclear staining in both glandular and squamous morule components of AH/EIN. C) Loss of Pax2 expression. Intact expression in stromal cells serves as good internal control; D) Intact expression of Pten. Thus, the 3-marker panel supports the diagnosis of AH/EIN despite modest gland crowding (Pax2 and  $\beta$ -catenin aberrant) and the aberrancy of 2 markers enhances diagnostic confidence.



**Figure 6. Aberrant patterns of expression in secretory AH/EIN.**

A) H&E. B)  $\beta$ -catenin nuclear staining in AH/EIN; strong overexpression is also observed.

C) Loss of Pax2 expression; D) Loss of Pten expression. Intact expression in stromal cells serves as an internal control. This case is thus Pax2/Pten aberrant.



**Figure 7. An unusual endometrial biopsy harboring 2 separate AH/EIN lesions with entirely distinct biomarker profiles and different histologic features.**

†=normal entrapped gland with wild-type membranous pattern of  $\beta$ -catenin. EIN #1 is Pax2 and Pten aberrant, whereas EIN #2 is  $\beta$ -catenin aberrant.

**Table 1.**

The evolution of WHO criteria for the diagnosis of endometrial precancers.

Diagnostic criteria	WHO 1994	
Hyperplasia	<ul style="list-style-type: none"> <li>Gland to stroma ratio &gt;1</li> </ul>	
Nuclear atypia*	<ul style="list-style-type: none"> <li>Atypical</li> </ul>	<ul style="list-style-type: none"> <li>Non-atypical</li> </ul>
Architecture	<ul style="list-style-type: none"> <li>Simple atypical hyperplasia</li> </ul>	<ul style="list-style-type: none"> <li>Simple hyperplasia without atypia</li> </ul>
	<ul style="list-style-type: none"> <li>Complex atypical hyperplasia</li> </ul>	<ul style="list-style-type: none"> <li>Complex hyperplasia without atypia</li> </ul>
*Features of nuclear atypia: <ul style="list-style-type: none"> <li>Loss of polarity</li> <li>Irregular nuclear shape and contours</li> <li>Vesicular chromatin</li> <li>Hyperchromasia</li> <li>Prominent nucleoli</li> </ul>		
Diagnostic criteria	WHO 2014	WHO 2020
<b>Hyperplasia without atypia</b>		
Same in WHO 2014 and 2020. <ul style="list-style-type: none"> <li>Increased gland to stroma ratio</li> <li>Tubular, branching or cystically dilated glands</li> <li>Uniform distribution of cytological features across submitted tissue</li> </ul>		
<b>AH/EIN</b>		
Hyperplasia	Gland to stroma ratio >1	
Cytological atypia	<ul style="list-style-type: none"> <li>Difference in nuclear and or cytoplasmic features from background endometrium</li> </ul>	<ul style="list-style-type: none"> <li>Difference in nuclear and or cytoplasmic features (including various metaplasias) from background endometrium</li> <li>Difference in architectural pattern</li> </ul>
Size	<ul style="list-style-type: none"> <li>1mm</li> </ul>	<ul style="list-style-type: none"> <li>Subjective</li> </ul>
Exclusions	<ul style="list-style-type: none"> <li>Benign mimics and adenocarcinomas</li> </ul>	
Biomarkers*	N/A	
	<ul style="list-style-type: none"> <li>Essential: meeting the morphological criteria above</li> <li>Desirable: altered biomarker expression</li> </ul>	
*Biomarkers listed in WHO 2020 included Pten, Pax2 and mismatch repair proteins (MMR), with the latter not further specified		