UNIVERSITY OF GRANADA
FACULTY OF MEDICINE
Laboratory of Physical Anthropology – Morphological Sciences

EVOLUTION, COINCIDENCE AND CHARACTERIZATION OF CILIATED AND TUBAL METAPLASIA OF THE HUMAN ENDOMETRIUM

PhD Thesis, Doctor Europaeus

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EVOLUCIÓN, COINCIDENCIA Y CARACTERIZACIÓN DE LA METAPLASIA CILIADA Y TUBÁRICA DEL ENDOMETRIO HUMANO

Tesis Doctoral, Doctor Europaeus

Presentada por:

Alina Nicolae

Bajo la dirección de:

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Dr. Jose Palacios Calvo

Granada, 2011

D. Francisco Nogales Fernández, catedratico del Departamento de Anatomia Patologica y D. Miguel C Botella López, catedratico del Laboratorio de Antropologia Física de la Facultad de Medicina de la Universidad de Granada, así como D. José Palacios Calvo, jefe de Servicio de Anatomía Patológica de Hospital Universitario Virgen del Rocío, Sevilla

CERTIFICAN

Que Dña ALINA NICOLAE, licenciada en Medicina, ha realizado bajo nuestra dirección el trabajo de Tesis Doctoral:

"***Evolución, coincidencia y caracterización de la metaplasia ciliada y tubárica del endometrio humano***".

Ha sido revisada por los que suscriben y estimamos que está conforme para ser presentada y aspirar al grado de Doctor Europeo ante el Tribunal que en su día se designe.

Fdo.: Francisco Nogales Fernandez Fdo.: Miguel C Botella López Fdo.: José Palacios Calvo

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Index

Abbreviations

ACH	atypical complex hyperplasia
ADCa	Invasive adenocarcinoma
AIS	adenocarcinoma in situ endocervical
APC	adenomatous polyposis coli
ASC-H	atypical squamous cells, cannot exclude high-grade squamous intraepithelial lesion
ASC-US	atypical squamous cells of undetermined significance
ATM	atypical tubal metaplasia
CDK	cyclin-dependent-kinases
CEA	carcinoembrionic antigen
CGIN	cervical glandular intraepithelial neoplasia
CIN	cervical intraepithelial neoplasia
CH	complex hyperplasia without atypia
CHT	complex hyperplasia tubal type
CTMI	complex tubal metaplasia in isolated glands
EGD	endocervical glandular dysplasia
EIC	endometrial intraepithelial carcinoma
EIN	edometrial intraepithelial neoplasia
EGFR	epidermal growth factor receptor
EMCs	endometrial metaplasia and changes
EmGD	endometrial glandular displasia
Endoc ep	endocervical epithelium
ER	oestrogen receptor
ESC	endometrial serous carcinoma
FISH	fluorescence in situ hybridization
HNPCC	hereditary non-polyposis colorectal cancer
HR-HPV	high risk human papillomavirus
HRT	hormone replacement therapy
ISH	in situ hybridization
LR-HPV	low risk human papillomavirus
MDA	minimal deviation adenocarcinoma
MM	mucinous metaplasia
MMR	mismatch repair proteins
MSI	microsatellite instability
Occ	occasionally

PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
POD	postovulatory day
PR	progesterone receptor
PTEN	phosphatase and tensin homolog
SH	simple hyperplasia
SPSC	surface papillary syncitial changes
Sq. C	Squamous carcinoma
STM	simple tubal metaplasia
TEM	tubo-endometrial metaplasia
TM	tubal metaplasia
TZ	transitional zone
UNKs	uterine natural killer's cells

Summary

Endometrial biopsies and curettings account for one fifth of routine gynecological specimens. The evaluation of endometrial pathology is a challenge, even for the expert, due to the wide range of morphological patterns found in both normal and pathological endometrial. Before pronouncing (classifying, diagnosing adjudicating) an endometrial biopsy abnormal, the pathologist should be familiar with the many faces of functional endometrium: its changes during the menstrual cycle and throughout a woman's life.

The remodeling of the endometrium under hormonal stimuli is detailed in the first chapter. A brief systematical approach outlining the expected findings in the endometrial biopsy according to the patient's age is presented. Until recently, the endometrial biopsy was the gold standard in the management of infertility; however, histological dating (Noyes') is now considered too subjective and has been surpassed by other techniques. Currently endometrial biopsies are carried to examine the response of the endometrium to exogenous hormones and to search for pathological lesions.

In the second chapter, the alterative changes of endometrium are described, some of which are very frequently seen in routine practice but often overlooked and misdiagnosed. They are the subject of the recently published review: **"Endometrial metaplasias and reactive changes: a spectrum of altered differentiation"** in ***Journal Clinical of Pathology***. In this comprehensive article we introduced new concepts of metaplasia, proposed a new classification and a practical morphological approach for their diagnosis, together with clinical guidelines for their treatment. Furthermore, recently we have also written two related articles: "**p16INK4A positivity identifies endometrial surface papillary syncitial change as a regressive feature associated with desquamation"** accepted for publication in ***Histopathology*** and **"Endometrial intestinal metaplasia: a report of two**

cases, including one associated with cervical intestinal and pyloric metaplasia" accepted for publication in ***International Journal of Clinical pathology*.**

The second part of the chapter is dedicated to cervical ciliated tubal metaplasia, the second most common metaplasia after squamous metaplasia. Its prevalence, pathogenesis and morphological aspects, with emphasis on the features that lead to misdiagnosis with preneoplastic endocervical lesions, are described and the theories related to its behaviour are discussed.

The incidence of cervical and endometrial adenocarcinomas continues to increase in many countries. Consequently, there is a need for improved early diagnosis and one of the most efficient methods of achieving this is with accurate diagnosis of cancer precursors. Taking into account that morphological diagnosis of these lesions is highly subjective, new ancillary techniques have been developed in the last few years for diagnostic purposes, particularly the immunophenotypic pattern of various antibodies. The commonly found tubal metaplasia (TM) is the subject of our extensive study, the largest to date. In the chapter "*Aims, background and justification*" the main pathways of endometrial and endocervical carcinogenesis are described and the possible role of TM as a precursor lesion is discussed. In this chapter, a fresh look is taken at the interpretation of its structure and eventual potential using the identification of its immunophenotype and genetics.

For this, 164 cases have been studied, 100 of which are endometrial, 40 cervical ciliated, tubal metaplasia and the remaining 24 cases comprise the control group, which included normal functional endometria, endometrial and endocervical adenocarcinoma and normal Fallopian tubes. The morphology of all the cases was evaluated by two independent observers, the immunohistochemistry was studied using a wide range of antibodies and a genetic assessment of PTEN by FISH and of K-ras by PCR was carried out in selected cases. The techniques are presented in the chapter "*Material and methods*".

The *results* are subdivided in two sections for TM in endometrium and cervix. We studied their clinical profile, morphological findings: topography, architectural pattern and coexistence with other types of metaplasia. This was followed by a systematical analysis of the antibodies immunoexpression in normal endometrium, TM, other types of associated metaplasia and preneoplastic and neoplastic epithelia as well as in the normal tubal epithelium. The genetic results of the selected cases are shown. A comparative study

between various morphological entities, their correspondent immunophenotype and genetic changes was performed.

Partial results were published as abstracts: "**Tubal (tubo-endometrial) metaplasia of the endometrium (TEM). A frequent source of misdiagnosis of malignancy. A study of 64 cases**" and **Tubal (tubo-endometrial) metaplasia in cervical pathology. A source of error with endocervical adenocarcinoma"** in ***Virchows Archiv***.

The *discussion* was divided into two sections, corresponding to endometrial and cervical TM. Their clinical context, architecture and associations are analyzed. The comparative pattern of expression of each antibody in normal, architectural subtypes of TM, hyperplastic and neoplastic epithelia is discussed. These results were compared with a partial molecular analysis. It was found that the most important features for the consideration of TM as a premalignant lesion in the endometrium are architectural alteration and extension. For endocervical TM, our main concern was to establish a practical diagnostic approach with emphasis on its differential diagnosis with malignant lesions.

The *conclusions* are exposed progressively with a final purpose of delineating a practical, reproducible histological and immunopathological approach that will be helpful in routine diagnosis.

Finally, almost 500 recent *bibliographical references* are included.

Resumen

La biopsia endometrial representa una quinta parte de los especímenes de la rutina ginecológica. La evaluación de la patología endometrial representa un desafío; incluso para patólogos expertos, debido al amplio espectro de patrones morfológicos encontrados en las muestras normales y patológicas. Antes de diagnosticar anomalías en una muestra endometrial (basado en su clasificación y diagnóstico), el patólogo debe de familiarizarse con las fases del endometrio funcional: los cambios durante el ciclo menstrual y sus variantes durante la vida de la mujer.

En el primer capítulo se detalla el remodelamiento endometrial bajo el estimulo hormonal. Se presenta un breve y sistemático abordaje de los hallazgos esperados en la biopsia endometrial acordes con la edad del paciente. Hasta hace poco, la biopsia endometrial era la norma estándar en el manejo de la infertilidad; sin embargo, el fichaje histológico (Noyes) se considera muy subjetivo y ha sido superado por otras técnicas. Hoy en día se orienta la biopsia endometrial para examinar la respuesta hormonal exógena y para la búsqueda de lesiones patológicas.

En el segundo capítulo, se describen los cambios alternos del endometrio, algunos de los cuales se presentan frecuentemente en la práctica rutinaria; estos en ocasiones son pasados por alto o mal diagnosticados. Estos fueron sujeto de revisión en la reciente publicación: **“Endometrial metaplasias and reactive changes: a spectrum of altered differentiation”** en el ***Journal Clinical of Pathology***. En este exhaustivo artículo introducimos nuevos conceptos de metaplasia, se propuso una nueva clasificación y un práctico abordaje morfológico para su adecuado diagnóstico, en conjunto con las guías de tratamiento. Además, se publicó recientemente otros dos artículos más relacionados: “**p16INK4A positivity identifies endometrial surface papillary syncitial change as a regressive feature associated with desquamation”** aceptado en la revista ***Histopathology*** y

"Endometrial intestinal metaplasia: a report of two cases, including one associated with cervical intestinal and pyloric metaplasia" aceptado en la revista ***International Journal of Clinical pathology*.**

La segunda parte de este capítulo está dedicada a la metaplasia tubárica cervical; la segunda metaplasia más frecuente después de la escamosa. Se describió su prevalencia, patogénesis y aspectos morfológicos, enfocados a las características que conllevan al diagnóstico equivoco con lesiones preneoplásicas endocervicales, discutiendo las teorías relacionadas con su comportamiento.

La incidencia del adenocarcinoma cervical y endometrial continúa en ascenso en muchos países. Consecuentemente, existe la necesidad de mejorar los métodos de detección temprana, diagnosticando efectivamente las lesiones preneoplásicas. En vista que el diagnóstico morfológico de estas lesiones es altamente subjetivo, se han desarrollado nuevas técnicas de apoyo durante los últimos años, particularmente la determinación de los perfiles inmunohistoquímicos. El hallazgo frecuente de la metaplasia tubárica y ciliada fue el sujeto de nuestro exhaustivo estudio; sin duda, el más extenso realizado hasta la fecha. En el capítulo "*Objetivos, antecedentes y justificación*" se describieron los principales mecanismos de la carcinogénensis endometrial y endocervical, también se discutió el posible papel que desempeña la metaplasia tubarica como una lesión precursora. En este capítulo, hemos dado una interpretación cauta a su estructura y al potencial uso de su eventual identificación inmunofenotípica y genética.

Para esto, se estudiaron 164 casos, 100 correspondían a endometrio, 40 a metaplasia tubárica cervical y los restantes 24 casos pertenecían al grupo control en el cual se incluyó el endometrio funcional normal, adenocarcinoma endocervical y endometrial y por último trompa uterina normal. Dos observadores reprodujeron independientemente la morfología de todos los casos, la inmunohistoquímica se evaluó usando una amplia gama de anticuerpos y el estudio genético de PTEN por FISH y de K-ras por PCR se llevó a cabo en casos seleccionados. La técnica empleada se detalla en el capítulo de "*Materiales y metodos*".

Los *resultados* se dividieron en dos secciones: para la metaplasia tubárica del endometrio y del cuello uterino. Estudiamos su perfil clínico, los hallazgos morfológicos: la topografía, el patrón arquitectónico y la coexistencia con otros tipos de metaplasia.

Posteriormente se hizo un análisis sistemático de los anticuerpos expresados en el endometrio normal, metaplasia tubárica, otros tipos de metaplasias asociadas y epitelios preneoplásicos y neoplásicos, así como en el epitelio normal de las trompas uterinas. Los resultados genéticos de los casos seleccionados también se incluyeron. Se realizó un estudio comparativo entre las distintas entidades morfológicas, su inmunofenotipo correspondiente y sus cambios genéticos.

Los resultados parciales fueron publicados como resumenes: "**Tubal (tubo-endometrial) metaplasia of the endometrium (TEM). A frequent source of misdiagnosis of malignancy. A study of 64 cases**" y **Tubal (tubo-endometrial) metaplasia in cervical pathology. A source of error with endocervical adenocarcinoma"** en ***Virchows Archiv***.

La *discusión* fue dividida en dos secciones, correspondiendo a la metaplasia tubárica endometrial y cervical. Se analizó su contexto clínico, arquitectural y asociaciones. Se discutió comparativamente el patrón de expresión de los anticuerpos entre el epitelio normal, hiperplásico y neoplásico abarcando todos los subtipos estructurales de la metaplasia tubárica. Los resultados fueron comparados con un análisis parcial molecular. La característica más importante encontrada ante la presencia de metaplasia tubárica y ciliada, es el hallazgo y extensión de la complejidad arquitectural; considerándola como una lesión premaligna endometrial. En el endocérvix, nuestra principal inquietud era establecer un enfoque práctico al diagnóstico, con énfasis en el diagnóstico diferencial de las lesiones malignas.

Las *conclusiones* se exponen progresivamente, con el propósito de delinear un enfoque práctico y reproducible tanto histológico como inmunopatológico, a fin de ser útil en el diagnóstico rutinario.

Finalmente se incluyen aproximadamente 500 *referencias bibliográficas* recientes.

Chapter 1

Endometrium - morphology and pathophysiology

I. Endometrium - morphology and pathophysiology

Endometrial biopsies account for 20% of routine gynecological specimens sent for pathological consultation[1]. Evaluation of the endometrium is a challenge, especially because of the wide range of morphological patterns resulting from normal and abnormal cyclic changes, exogenous hormones, infections and tumours. The success in resolving these challenges resides in understanding the clinical questions in the systematic approach used by both gynaecologists and pathologists.

1.1. Embryological aspects

Embryologically, the human endometrium is of mesodermal origin, and constitutes the mucosal lining of the fused Müllerian ducts of the uterus. When the ducts fail to fuse or fuse only partially (double uterus, septate uterus etc), the endometrium lines each of the ductal units. When the structural abnormalities are minor, the endometrial lining is usually of normal appearance. In some women, there may be a decrease in the sensitivity to hormonal stimulation in the septal endometrium [2]. When the structural abnormalities are severe and accompanied by a degree of hypoplasia, the endometrium may be unresponsive to the hormonal stimulation and retain a prepuberal appearance. In extreme cases of Müllerian duct hypoplasia, there may be no canalization and hence no endometrial lining.

In the next pages we will discuss the histological components of the endometrium with a further discussion of the physiologically dynamic endometrial functionalis layer.

1.2. Normal histology

Endometrium is one of the body tissues with the highest morphological variation. It presents not only a temporal but also a spatial/regional variation closely related to stimulation by ovarian hormones. A different hormonal response occurs in the isthmus compared with the mucosal lining the corpus. In the endometrium itself there are differences in hormonal response in thickness with variations taking place on the surface and the deep, basal portions. It is not completely clear if these differences in hormonal response are due to dissimilar vascular supply or to its receptor content, or both. The transition with endocervical type mucosa and with Fallopian tube mucosa in the uterine cornua may be abrupt but is most frequently gradual.

The isthmic part is generally thinner than the corporeal mucosa and it tends to respond only sluggishly to the ovarian stimulus. Its development often lags behind the rest of the endometrium [3]. The hybrid appearance of the isthmus helps the pathologist to identify it in endometrial curettage. Towards the endocervix the stroma becomes more fibrous and less cellular, with endocervical type epithelium present in the surface of otherwise endometrial glands.

The geographical variation of hormone sensitivity correlates with different biological functions:

The functionalis is the part which exhibits the most variable changes. Based on the morphological features during the late secretory phase it has also been divided into *compactum and spongiosum*. The upper functional layer serves as the implantation site, providing an appropriate metabolic and physical environment for the implanted blastocyst.

After incomplete shedding of the functionalis during menstruation or after curettage, the basalis considered the “reserve cell layer” will play the crucial role in regeneration of the endometrium. Its importance is clearly demonstrated when its damage or loss results in Asherman’s syndrome or intrauterine synechiae. The remaining part of the functionalis [4] and also parts of the lower uterine segment surface epithelium participate in this process [5]. Regeneration is linked to the presence of endometrial stem cells in various locations, mainly in the basalis. This latter layer has an inactive, undifferentiated appearance with weakly proliferative glands and a dense spindle cell stroma due to its minimal hormone response.

An exception to its inactive appearance is encountered in the later half of pregnancy, when it exhibits secretory glandular changes and stromal decidualisation.

There is no sharp line of demarcation between these functional layers as there is not between basal endometrium and myometrium. These features are important in the evaluation of myometrial involvement by an endometrial carcinoma or in the diagnosis of adenomyosis.

1.2.1. Histological components of the endometrium

Morphologically, the endometrium is represented by both epithelial (tubular glands and surface epithelium) and mesenchymal elements, (stroma and blood vessels) with a distinct distribution and morphology in the upper functionalis and a lower basalis layer. During reproductive years, the endometrium follows a precisely programmed series of morphologic and physiologic events. Epithelium, stroma and vessels proliferate synchronously and also differentiate and degenerate accordingly.

1.2.1.1. Epithelial elements:

The glands and the surface epithelium are composed of four cellular types; two of them representing functional variants of the same cell [6].

Proliferative type cell of the functionalis and basalis are morphologically similar. These cells have a basophilic appearance with scanty, dark cytoplasm with cylindrical nuclei and common mitotic figures.

Secretory cells can be of vacuolated and non-vacuolated types. The vacuolated ones have non-mucin secretory vacuoles and proliferative-like nuclei while the non-vacuolated cells are characterized by round, large nuclei with visible nucleoli in a dense eosinophilic cytoplasm. A second type of secretory cells, similar to the Fallopian tube secretory cells, is easily identified in the surface epithelium. Some of these may represent “exhausted” ciliated cells. Their cytoplasm prolongs in the lumina forming a bleb; their elongated nuclei presenting coarse chromatin.

Ciliated cells are a constant occurrence in the normal endometrium, especially in the isthmus mucosa and during the proliferative phase [7 8]. The origin of these cells is not fully understood. Some authors [9] sustain their origin from the basalis type epithelium, as a distinctive line of differentiation. Interestingly, these cells change their

shape and position during ciliogenesis. Their round nuclei with smooth membrane and finely dispersed chromatin are a constant feature. Initially the cells have a basal position and present a pyramidal shape with clear cytoplasm. The intracellular cilia can only be visible only in EM. Afterwards they become closer to the lumen and the cell presents a fusiform shape. When these cells become the predominant population of the glands the term "ciliary metaplasia" has been used.

1.2.1.2. Morphology of the glands

The glands and their cells undergo continous remodeling during the menstrual cycle. The glands vary from tubular, straight with narrow lumens (early proliferative phase) to coiling, branching (late proliferative, early secretory phase) and culminate in a serrated appearance in the late and menstrual endometrium. Also, the cells are changing the shape, position, characteristics of the nuclei and cytoplasm.

The surface epithelium presents a relatively constant appearance during the cycle, being represented predominantly by apocrine-like secretory and ciliated cells.

1.2.1.3. Mesenchymal elements

They are represented by cellular components (stromal and hematolymphoid cells), reticulin network and vascular elements.

Stroma of the basal endometrium appears more densely cellular, with spindle shaped darkly staining nuclei, scanty cytoplasm and ill defined cell borders. The cells remain constant in the appearance throughout the normal cycle. In the fuctionalis, the morphological aspects of the stromal cells are dependent on the hormonal stimulus (see below in the functional endometrium). Interestingly, both the stromal and smooth muscle cells express vimentine, SMA (smooth muscle actin), bcl-2, however, CD10 is positive only in the stromal cells.

Lymphocytes, lymphocytic aggregates and lymphoid follicles may be found in the normal endometrium. Their profile changes also with the menstrual phase, being CD8+ T cells and B cells during the proliferative phase [10] and granulated lymphocytes with characteristics of natural killer cells CD56+, CD16- (the so-called endometrial stromal granulocytes) in the late secretory phase [11 12]. The function of these latter cells has been the subject of discussion for a long time. Some workers showed that UNKs (uterine natural killer

cells) play a role in pregnancy, controlling trophoblast invasion and spiral artery remodeling. Alternatively, death of UNKs might be an early event in the onset of endometrium breakdown [13]. Other hematolympoid, menstrual cycle dependent cells include: neutrophils and eosinophils (not easily identified until the premenstrual phase), histiocytes and mast cells [14]. Traditionally, it has been emphasized that plasma cells are not present in normal endometria and their presence is associated with pathological conditions such as subclinical endometritis or endometrial carcinoma. In fact, using flow-citometry, small number of plasma cells can be detected in the endometrial samples [15]. Some studies have nevertheless raised questions about the clinical relevance of scattered plasma cells [16].

The sterile nature of the endometrium is also sustained by negligible synthesis of immunoproteins by the epithelial cells [17], by very scarce Langerhans cells and finally by the absence or rare IgG containing plasma cells.

The stromal intercellular space is occupied by *reticulin network* and is rich in high molecular weight mucopolisacharides. This network becomes progressively denser as the endometrium develops during the menstrual cycle. In the late secretory phase, each cell is enmeshed in reticulin which undergoes dissolution during menstruation.

Vascular elements are derived from the myometrial arcuate system. The radial arteries give rise to the basal branches and then continue as endometrial spiral arteries. The basal arteries are unresponsive to the steroid hormones, whereas those of the functionalis are eliminated and must re-grow within the endometrium in each cycle. Angiogenesis is the central player, not only for the menstrual cycle, but also for the implantation phenomenon and subsequent gestation.

The spiral arteries in the early proliferative phase are straight and thin; they become elongated and coiled in the secretory phase and differentiate muscle cells in their walls under the influence of progesterone. All these changes are checked by activators and inhibitors [18] and it appears that the initial growth of the vessels is independent from oestrogen and progesterone control [19].

1.2.2. Temporal variations of the endometrium

The renewal capacity of the endometrium can be compared to the gastro-intestinal mucosa, but if the latter presents a constant appearance throughout the life, the endometrium undergoes dramatic temporal morphologic changes. There is a biphasic

temporal variation: either during the lifetime of an individual woman or during each menstrual cycle.

During lifetime, the endometrium presents a bimodal curve, with its active remodeling in the first two weeks of life (due to high levels of circulating maternal and placental steroids) and in the reproductive years. Prior to the menarche and after the menopause, the endometrium is a shallow dormant mucosa.

1.2.2.1. Functional and morphological changes of the functionalis during the reproductive years

The endometrium is a sensitive biological reflection of ovarian steroidogenesis, with its cyclic structural changes mirroring those in the cells metabolism. The endometrial growth, modeling and breakdown are controlled by a series of balanced but opposed events. There are factors that promote growth, such as oestrogen, and factors that initiate breakdown and apoptosis, inducing tissue dissolution. There is evidence of disturbance of this process in women with infertility, dysfunctional bleeding [20] and endometriosis [21]. It should also be emphasized that glandular and stromal compartments have distinct biological profiles which can explain the differences in their responsiveness to circulating hormones.

Morphologically, the endometrium is one of the most dynamic target tissues in women. Its assessment represents a challenge for the pathologist due to the wide spectrum of changes occurring in conjunction with normal and abnormal cycle, hormone therapy, infectious or neoplastic pathology. Pathologist should be familiar with these many faces of the cycling endometrium and should try to correlate the histological findings with clinical data in order to avoid a misdiagnosis.

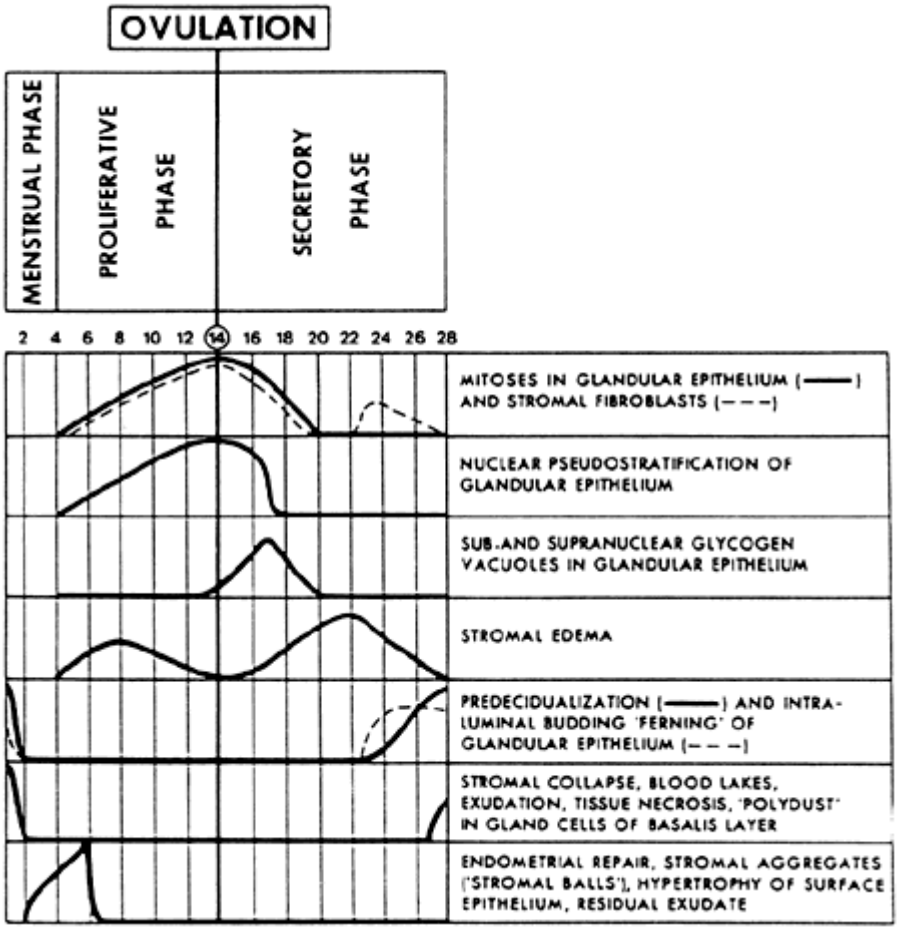
Endometrial cycle length is often described as an idealized 28 days in duration, but it can vary from 24 to 38 days between individuals and also during the lifetime of the same woman.

The phases of menstrual cycle could be defined as proliferative, oestrogenic or follicular prior to ovulation and secretory, progestational or luteal phase after ovulation. Initially, it was believed that the physiological variation in the length of menstrual cycle was related to the variation of the proliferative phase with a constant secretory phase and this constancy provided the base for endometrial dating. Numerous later studies proved that also luteal phase vary in duration [22 23].

In 1950, Noyes, Hertig and Rock published the first comprehensive treatise on evaluation the endometrial [24]. Major advances in understanding the menstrual cycle have occurred since then, and excellent bibliography is available for the interpretation of benign endometrium [25 26].

Cyclic endometrium can be subdivided into six phases based on morphological appearance: 1) menstrual phase, 2) late menstrual/early proliferative endometrium, 3) proliferative phase, 4) early secretory phase, 5) mid secretory phase and 6) late secretory phase. The major morphological features of the endometrium throughout the cycle are shown in the figure 1.

Figure 1. Endometrial morphological alterations during the menstrual cycle (reproduced from original drawings in the Noyes study)



1.2.2.1.1. Mentrual phase (cycle days 1-4)

It is defined as the spontaneous shedding of secretory-type functionalis. The endometrium appears thick, red and soft. Initially, a cord like aggregation of predecidual cells with collapse of the stroma takes place. There is an interstitial hemorrhage, oedema

and exudate of acute inflammatory cells. Subsequently, the process becomes generalized with a plane of cleavage apparent throughout the spongy layer leading to detachment of the superficial endometrium from the basalis. As the stroma disintegrates, the large irregular endometrial glands, more resistant to dissolution, become closely packed and may appear artifactually complex. This, together with degenerative atypia of the glandular cells and necrotic background, may suggest a diagnosis of malignancy. The ruptured, collapsed, exhausted glands and predecidual stromal aggregates typify ovulatory breakdown and the diagnosis for these changes is *menstrual (ovulatory) endometrium*. Molecular events of endometrial breakdown are complex, involving a number of factors from which the proteolityc activity of lysosomal enzymes within epithelial, stromal and endothelial cells is the most important. Vascular luminal injury promotes thrombosis with appearance of multiple minute foci of ischaemic tissue necrosis [27]. Other factors implicated in the breakdown include: apoptosis, disturbance of the cell adhesion molecules, loss of filamentous actin from cell borders, various integrins [28], matrix metaloproteinases [29] and granulated lymphocytes which mediate apoptosis by their content in perforin and granzyme B.

Shedding is more prominent in the first two days with approximately 50% of menstrual detritus [4 30] expelled in the first 24 hours. This is composed by heavy PMN exudate, red blood cells and proteolytic enzyme [25], from which blood plasmine is an important fibrinolytic agent preventing clotting of menstrual blood and facilitating expulsion of the degenerated functionalis. Uterine homeostasis is assured by vasoconstriction of ruptured basal arteries, radial and arcuated arteries of the myometrium. Due to the lack of elastin in their walls, the arteries of functionalis fail to participate.

Menstrual endometrium involves various diagnostic pitfalls, the most dangerous ones being the misinterpretation of stromal breakdown with epithelial proliferation with adenocarcinoma [1].

1.2.2.1.2. Late menstrual/early proliferative endometrium (3-5 days of menses)

The early repair phase begins during the 2nd or 3rd day and completed on the 4th or 5th day and is considered to be oestrogen independent [19 31]. Also, the initial regrowth of the vessels appears to be influenced rather by VEGF secretion, dependent from the ischaemic conditions, than by the oestrogen stimulation [32 33].

The endometrium is repaired by migration and proliferation of intact surface cornual and isthmic mucosa, as well as by epithelial stumps of amputated glands. This process is believed to be mediated by Tenascin, an extracellular matrix protein [34]. Recently, it has been proved that migration, proliferation and differentiation of endometrial stem cells are the main mechanisms involved in endometrial regeneration [35]. Also tumour suppressor, PTEN-positive endometrial glands, which appear to persist in the basalis during menstruation may also contribute to post-menstrual endometrial regeneration [36].

This phase is characterized by the persistence of some features of menstruation, continued breakdown of functionalis (the lower part) together with the process of reepithelization and abortive features of proliferation (slight stratified nuclei and occasional mitotic figures). The morphological hallmark of this period is represented by the so-called "stromal balls", which represent residual stromal breakdown. They are composed by clumps of specialized fibroblastic cells surrounded by regenerative surfacing epithelial cells. The latter can present enlarged polyploid nuclei with conspicuous nucleoli, features consistent with repair process.

It is not possible to confirm ovulation in the late stage of endometrial shedding due to the absence of the characteristic signs such as predecidualized stroma and exhausted secretory cells. Also, the "stromal balls" are not pathognomonic for postovulatory menstrual regeneration, being seen in the endometrium after anovulation, oestrogen or progesterone breakthrough bleeding, or withdrawal of exogeneous oestrogen and progesterone.

1.2.2.1.3. Proliferative phase endometrium

The ideal duration of this phase has been considered to be 14 days, but a variable length of 10-20 days is commonly accepted. Its daily morphological changes are not sufficiently characteristic to permit an accurate dating and are not important for diagnosis or management.

The functional or morphological changes are subordinated to a raised oestrogen level. It is well known that oestrogen induces, through nuclear receptors, a proliferative activity of both glandular and stromal cells. The active, synchronous growth of the glands, stroma and vessels is responsible for a 10 fold thickening of functionalis. The morphological patterns of these elements are not stable during the proliferative phase, as they constantly remodel. In the first third of this period there is a coordinated growth rate of glands and

stroma. The glands are straight, tubular and round in cross section, the stroma is immature in aspect and the vessels are non-coiled. In mid and late proliferative phase the growth of glands and vessels outstrips that of the stroma and in consequence, these structures become more convoluted. Despite the variation of shape and size, the glands remain parallel, regardless of the orientation of the section. In this permanent process of change, each glandular and stromal cell is involved and a variation of the morphology and mitotic count is observed. Initially, the glandular epithelium presents a minor degree of pseudostratification of the cubo-columnar basophilic cells. Scarce mitoses are present, more easily identified at the functionalis-basalis junction. The delicate, translucent appearance of immature functional stroma is in sharp contrast with compact, darkly staining basalis. The former presents ill-defined cells with scant cytoplasm and round to fusiform, mitotically active nuclei.

As the cycle progresses the stratification of the cells becomes more obvious and the vessels more conspicuous. In a stroma with transient oedema, lymphoid aggregates can be identified. The proliferative index reaches the maximum between the 8^{th} and 10^{th} day. This corresponds to the peak of plasma oestradiol levels and the maximum concentration of its receptors. The mitotic figures decline in number and disappear after the third postovulatory day (POD3). The surface and glandular cells acquire numerous cilia and microvilli, particularly around glandular openings [37]. Their presumed role is an involvement in mobilization and distribution of endometrial secretion during the postovulatory phase [38].

Endometrial growth is revealed by the variable distribution of Ki-67 proliferation marker [39] and at molecular level is explained by a tight balance between promoting proliferation factors (oestrogen, EGFR [40], Insulin-like growth factors) and initiators and promoters of apoptosis [41]. Oestrogen binding to its nuclear receptors involves translation of hormonal stimulus with subsequent protein synthesis and increase of RNA in the glandular epithelium.

Misinterpretation of intraluminal material as secretion and telescoping phenomenon of the glands, a technical artifact, taken as criteria for endometrial hyperplasia are the most frequent pitfalls in assessment of proliferative phase.

1.2.2.1.4. Secretory phase

Glandular secretion initially takes place as a response to the increased levels of progesterone produced by postovulatory corpus luteum and simultaneously stromal luteinization.

Traditionally, the secretory phase was divided in four periods based on the consistent sequence of histological changes which permitted endometrial "dating": 1) 16 day endometrium (interval phase or POD 2), 2) vacuolar phase (early secretory, 17-19 days, POD 4-6), 3) exhausted phase (mid secretory, 20-22 days, POD 6-8), 4) predecidual phase (late secretory, 23-28 days, POD 9-14).

Functional progesterone inhibits the proliferative activity of the endometrium and induces a complex secretory activity with glycogen secretion. There are many mechanisms by which the progesterone antagonizes the growth process, such as: down-regulation of oestrogen receptor; conversion of oestradiol into a less active form, oestrone under the action of 17β-hydroxydehydrogenase, a progesterone-specific enzyme [34]; decreased level of bcl-2, an anti-apoptotic protein and tumour suppressor gene p16. PTEN takes also part at the regulation of apoptosis [42] as does Fas [43]. Apoptotic bodies in the glandular epithelium increase in number from the mid secretory phase [44] to the 2nd day of menstruation, when they reach the peak. In stromal cells, this happens two days later and to a lesser degree.

Preceding endometrial breakdown there is an increase of Ki-67 index in the stroma due to mitotic activity of the stromal cells, and also augmentation of the number of granulated lymphocytes [44].

a. Sixteen-day endometrium (POD 2)

Cylindrical subnuclear vacuoles appear in an endometrium with proliferative features (tall cell, psudostratified nuclei, mitosis). These are irregular in distribution in the same gland and throughout the functionalis glands, being conspicuous in the mid-zone and least obvious in the upper part. The glands appear slightly larger and their basophilia decreases at low power magnification.

This morphologically indeterminate endometrium, termed "interval" does not represent the hallmark of the ovulation. The clinical context is important for assessing an endometrium with these changes, since similar modifications can be produced by oestrogen. Furthermore the discriminatory features of POD 2 (pseudostratified nuclei with scarce mitotic figures) present high interoberver variability [45 46].

b. Vacuole phase of secretory endomerium (early secretory phase, 17-19 days, POD 4-6)

The first unequivocal histological alteration considered specific for ovulation is seen on the 17th day (POD 3) of the cycle [47]. The hallmark features of this period are uniform, glycogen containing subnuclear vacuoles which involve more that 50% of the endometrial glands. Their lining epithelium presents palisading nuclei with virtually no mitotic activity.

The proliferative profile of the glands changes around the day 18, when the vacuoles reach their peak and are pushed towards the lumen. The glands appear larger, pale pink and present uniform, non-stratified nuclei with a central position within the individual cell.

In the day 19th the nuclei have nearly migrated back to the base of the cells. Scattered, residual subnuclear vacuoles can be identified as early signs of secretory exhaustion.

The ovulation process can be demonstrated immunohistochemically using a mucin-like glycoprotein that is exclusively found in postovulatory secretory cells (TAG-72 or B72.3) or by electron microscopy which shows two ultrastructural features unique for ovulation: giant mitochondriae and the so-called nucleolar channel system. The first one represents a cellular response to the high energy demand for the glycogen metabolism and the second one has no known significance but is presumably produced by the infolding of the nuclear membranes under progesterone stimulation [25 48].

The irregular enlargement of the glands and their false crowding could create confusion and misinterpreted as “secretory hyperplasia”.

c. Exhausted/mid secretory endometrium (20-22 days, POD 6-8)

Mid secretory endometrium typically presents somewhat distended, angular shape glands. As a continuity, the supranuclear cytoplasmic protrusions containing glycoproteins are expelled into the glandular lumen by apocrine type secretion [48]. The peak of intraglandular secretion coincides with the time of implantation of the free blastocyst, if fertilization takes place, on the 21st day of the cycle (POD 7). Stromal oedema is also important in preparing the endometrium for an eventual pregnancy. It reaches the maximum on the day 22 (POD 8), being marked in the mid-zone of the spongiosa. PgE2 (prostaglandine E2) and is considered to be the main trigger of stromal oedema due to its active change on capillary permeability via histamine [37]. Luminal secretion and stromal oedema were described by Noyes as a part of the main criteria for endometrial dating. In fact, it was demonstrated that these changes do not have a discriminatory power for dating

purposes. Also, serrated glands with linear/basal nuclei considered the best descriptive feature for this period could not be used for dating due to their temporal persistence [45].

The first hint of perivascular cuffing appears on the day 22, but predecidual changes are lacking by this time. The stromal, rather than glandular changes are evaluated in dating the endometrium from cycle day 21 onwards.

d. Predecidual/late secretory phase (23-28 days, POD 9-14)

The distinctive feature of the late secretory endometrium is represented by stromal predecidualization. The term "psudodecidualization" is not appropriate and should not be used.

Predecidual cells represent precursor forms of gestational decidual cells (decidua vera) with some phagocytic properties, capable of removing collagen fibrils and therefore with a role in menstrual breakdown. They appear not to be implicated in the implantation process because their development occurs once implantation has taken place.

Histologically, decidual epithelioid cells present distinct borders and an "out of focus" appearance due to decreased nuclear/cytoplasmic contrast (paler tetraploid nuclei in abundant darker cytoplasm). The immunoreactivity of predecidual cells is consistent with their stromal derivation, being vimentin and desmin positive and cytokeratins and EMA negative. They express oestradiol receptors and appear to be relatively independent from progesterone influence [49]. This morphologic transformation appears to be mediated by PGF2α and other peptides [34].

The predecidual changes involve initially the stromal cells around the spiral arteries, where they form a cuff (days 23-24). Perivascular aggregates involving single vessels correspond to day 23, while aggregates bridging multiple vessels characterize the day 24. Subsequently, the process expands as a spiderweb, initially in foci (day 25), then into the subsurface stroma (day 26) and eventually extends downwards between the folds of the compacta, along the spiral arteries (day 27).

Predecidual changes are not strict criteria to define this interval. They can appear earlier than POD 9 and their interpretation is affected by a high interobserver variation [45].

Mitoses reappear in the stromal cells by day 26-27 as a reflection of minor recrudescence of the oestrogenic effect.

The adjacent glands may display a transition from dilated glands with simple contour to those having a "saw-toothed" configuration. An increase of secretory cells apoptosis with

accumulation of tingible bodies within macrophage can be observed along the progressed late secretory phase.

Granulated lymphocytes start to appear within the stroma by the day 26 and their intensity is closely correlated with the menstrual onset. “Leukocytic infiltration” was also used as useful criteria in the comprehensive scheme of Noyes et al, but again, the interobserver variability was unacceptable high [45].

The potential pitfalls of this phase are represented by confusion of oedematous predecidua with day 21 endometrium but here, the space between the cells is occupied by cytoplasm and not by oedema fluid. Also, the stromal granulocytes may lead to a diagnosis of chronic endometritis; however here plasma cells are not present.

1.2.2.2. Morphology of gestational endometrium

The implantation of the blastocyst (POD 6-8) is associated with resurgence of glandular secretion and persistence of stromal oedema. The predecidua is gradually converted to decidua after POD 14, being complete by the end of the first month of gestation. The endometrial stroma will become a pavement-like sheet of epithelioid cells with well defined borders and central vesicular nuclei. Decidua cells may exhibit a striking nuclear pleomorphism and cytological atypia, particularly in the region of the implantation site. In zona compacta, the glands are compressed and lined by endothelial-like epithelium. The glands are tooth-shaped and lined by vacuolated cells with dysmorphic nuclei. Exaggeration of these features produces the so-called Arias Stella phenomenon [50], which consists of packed glands whose extreme coiling and collapse throw the lining epithelium into papillary folds. The cells present marked nuclear pleomorphism, with a smudged appearance and sometimes with large biotin inclusions [51]. The cytoplasm may be clear, hypervacuolated or eosinophilic. The confusion with clear cell adenocarcinoma may be avoided by the secretory features and clinical history.

The diagnosis of intrauterine pregnancy should be made in the presence of chorionic villi or on the basis of implantation site reaction, but not on decidual reaction. The latter is seen in the presence of ectopic pregnancy and in various progestational changes.

Cuffs of hyalinized decidua with hyperchromatic, degenerated nuclei of intermediate trophoblast, around ectatic, hyalinized, spiral arteries, strongly suggest a previous intrauterine pregnancy or a postpartum endometrium.

1.2.2.3. Morphology of postmenopausal endometrium

After age 50, the endometrium rarely exhibits proliferative or secretory features. However, postmenopausal endometrium fits in the usual patterns of: 1) inactive and atrophic, 2) hormonal replacement therapy (HRT) and 3) tamoxifen

In the postmenopause the precise morphology of the endometrial variations have been studied extensively [52] The basic picture of post-menopausal endometrium is that of atrophy. Initially, it appears inactive, devoid of morphological features of either active proliferation or secretion and ends up as a thin atrophic layer.

Some clinical reports have suggested the existence of an endometrial ageing [53 54], similar to age-dependent morphological alterations of the endometrium in several mammalian species [55 56]. Noci *et al.* showed that human endometrium does not age, at least while cyclical hormonal stimulation and menstruation are present [57]. Even if the ovarian hormonal stimuli decrease to levels unable to induce endometrial proliferation, the oestrogen receptors (ER) are maintained, as it also is nuclear DNA synthesis. These explain the "revitalizing" of the endometrium under endogenous stimulus, exogenous oestrogen and [58] tamoxifen administration, which also leads to the acquisition of progesterone receptors.

With the absence of the ovarian cycle, a deprivation of progestational stimulus is evident; however, some degree of oestrogen stimulation persists due to the peripheral conversion of androgens [59]. As a result, the number of glands and stromal volume decrease. The uterus appears lined by shallow basalis that measures half or less of the thickness of the basal layer of cyclic endometrium (less than 5 mm). Endo-myometrial junction is more irregular than in the reproductive years. If initially the stroma resembles the proliferative endometrium, after several years it becomes rich in collagen fibres resembling the isthmic stroma.

The glands are usually narrow, tubular, oriented parallel rather than perpendicular to the surface epithelium. The lining epithelium appears cubo-columnar, of indeterminate type, with mitotically inactive unistratified nuclei, scanty cytoplasm and with cilia especially in the surface epithelial cells.

The number of lymphocytes or lymphoid aggregates may increase.

If an endogeneous oestrogen production persists (obesity, ovarian cortical stromal hyperplasia) the endometrium presents a proliferative pattern with glandular dilatation

similar to anovulation. In some postmenopausal cases, the endometrium develops a senile cystic atrophy. As a result of blockage of the gland neck by fibrous stroma, many glands become cystically dilated. They are lined either by cuboidal inactive epithelium or flat, indeterminate one.

50% of post-menopausal endometrial uterine bleeding is related to endometrial vascular alterations [60] in the absence of other uterine pathology.

Postmenopausal endometrium under HRT or tamoxifen is described in the section covering hormonal therapy.

1.3. Ultrasound pattern of the normal endometrium

The ultrasound appearance of the endometrium differs during a female's life and varies with the stage of menstrual cycle.

At birth it appears as a thin, echogenic line and approximately 25% of neonates will present fluid collection within the endometrial cavity [61].

During the adult life, the endometrial thickness is better appreciated by transvaginal scan. The thickness of endometrium is evaluated on a sagital midline, which crosses the endometrium cavity and has the echogenic borders at its ends.

The ultrasonographic pattern of reproductive type endometrium changes during the menstrual cycle. It appears as a thin, echogenic line of 1-4mm thickness throughout menstruation [62]. During the proliferative phase, the endometrium appears hypoechogenic in comparison with adjacent myometrium and its thickness continues to increase from 5-7mm up to 11mm around ovulation. Periovulatory endometrium shows a characteristic multilayer appearance. Three hyperechogenic lines represented by basal layers and luminal interface in the middle separate two hypoechogenic inner functionalis. This pattern frequently disappears 48 hours after ovulation [63]. The echogenicity and endometrial thickness continues to increase during the secretory phase [62]. Endometrium measures between 7-16mm, being the thickest during the mid secretory phase. The hyperechogenicity results from the summary of stromal oedema and large glands filled with mucus and glycogen. The ultrasonographic pattern of the endometrium has to be interpreted together with the clinical data. Due to the overlap of features, it is sometimes difficult to make a distinction between a normal secretory endometrium and a pathological hyperplastic one.

The literature offers contradictory findings related to the utility of endometrial ultrasonography in the screening of infertile women. Some studies consider that this tool does not show substantial differences between fertile and infertile endometrial [64] whilst others maintain that a triple layer pattern assessment could be useful, being present in 99% of conception cycles and only in 44% of the non-conception ones [65].

The postmenopausal endometrium appears ultrasonograpically as a thin, homogeneous, echogenic layer. This pattern of atrophic endomerium is not a static feature and could be modified by HRT [66] or any condition associated with abnormal uterine bleeding. While some authors support the idea that endometrial thickness decreases with age [67], others believe that there are not significant changes unless there are HRT stimuli or peripheral oestrogen production [68]. It is accepted as a consensus that a ≤5mm double layer homogeneous thickness generally excludes significant pathology (however with many exceptions) [69] [70]. Because of thickness variation during HRT administration it is advisable to perform a measurement of endometrial thickness at the beginning and the end of the therapy, when the endometrium is thin and pathological changes can be easily documented. A biopsy should be considered in the cases with an endometrial thickness of >8mm [66]. Also in the cases of postmenopausal uterine bleeding it is better to evaluate the endometrium when the bleeding has stopped as the endometrium is thinner, allowing a good evaluation.

In MRI imaging the endometrium is well visualized on T2-weighted images and appears as a uniform, high intensity signal in comparison with the low intensity signal of the inner myometrium. [71]

1.4. Expected findings in the endometrium according to the age

The wide range of morphological patterns resulting from both normal and abnormal cyclical changes seen in the endometrium during reproductive and menopausal years poses a challenge for many gynaecologists.

A practical approach would consist in subdividing women with endometrial alterations into three general categories: 1) women in their second-fourth decade undergoing evaluation for infertility; 2) women in their fifth decade with abnormal uterine bleeding and 3) women in their sixth decade and beyond with postmenopausal bleeding [1].

The clinical pattern and morphological features of each group are unique and are summarized in the table 1.1.

Table 1.1. Expected findings as a function of age

Age	Most likely clinical scenario	Diagnostic concerns
20-40	Infertility	Luteal phase defect Response to hormones (assisted reproduction)
35-50	Dysfunctional uterine bleeding	Anovulation Submucosal leimyoma Chronic endometritis Endometrial polyp Mixed patterns Hyperplasia
>50	Hormonal replacement	Excess oestrogen Complex metaplasias Atypical hyperplasia Adenocarcinoma

1.4.1. Luteal phase defect and implications of endometrial biopsy in the screening of infertile women

For many decades the daily changes of the secretory endometrium were considered significant enough to permit an accurate evaluation of the endometrial cycle. It was proved that various histological changes are less temporarily distinct or occur in more variable combinations than previously believed thereby compromising their discriminatory power [45]. Also, their variation between individuals, between the cycles of the same individual, their inconsistent interpretation by different pathologists [72 73] or by the same pathologist at different times alter the possibility to define a specific luteal day or even a narrow interval of days [45]. With the exception of stromal breakdown which announces the menstrual time, no single feature or combination of changes can reliably define a postovulatory day of the cycle [45 46].

Histological dating of the secretory endometrium remains substantially subjective. Even in the expert's hands it is not a highly reproducible judgment, describing continuity of development.

Endometrial biopsy frequently underestimates the cycle day. Even applying refined scoring parameters using 32 criteria and not only the classical 8 criteria of Noyes, the

accuracy of endometrial dating did not improve [45]. Interobserver concordance in evaluation of maturation of endometrium along secretory phase varied in different studies from 29% (for exact histological date) to 62% (+/- 1 day) [74] and 77% [73]. Also the same pathologist may be inconsistent in reading the same biopsy at different time in as many as 27% of cases [75]. The percentage of agreement is dependent if it is considered as an exact appreciation or within a spectrum of one or two days.

The reliability, validity and utility of traditional histological dating of the endometrium as an integral part in the evaluation of infertile couples remains a subject surrounded by controversy and discussion. Traditional criteria described by Noyes, Hertig and Rock [24] have been used for more than 50 years as a gold standard for the diagnosis of luteal pase defect, considered the main cause of infertility and recurrent abortion [76]. Frequently, this "accurate" morphologic evaluation of endometrial function and development was followed by "corrective" hormonal treatment [77]. LPD was first described by Jones et al 1969 as an alteration in the secretory maturation of the endometrium [78]. It was characterized by a morphological pattern which lags at least two days behind the estimated postovulatory day in two consecutive endometrial samplings [79]. A wide range of disorders were described in association with LPD and various diagnostic tests with different sensibility and specificity constituted the subject of numerous studies [80 81].

There are multiple factors which lead to the conclusion that traditional histological dating of the endometrium should no longer be considered a valid diagnostic tool in the guidance of the clinical management of infertile women. [82 83]. These include:

a. lack of precision and poor reproducibility in identifying the "true" day of ovulation for the diagnosis of luteal phase defect [72 73];
b. endometrial morphologic features that specifically indicate receptivity have not been identified;
c. the prevalence of "out of the phase" biopsies in fertile population ranges between 5-50% [45];
d. "out of the phase" biopsy does not clearly discriminate infertile women from fertile controls during the window of implantation or late luteal phase [82];
e. successful implantation in endometria with "out-of-phase" pattern [84];
f. lack of agreement regarding the optimal time of performing the procedure [79]

g. sensitivity and specificity of serum hormonal studies or transvaginal ultrasound for evaluation of the dominant follicle collapse [80 81].

Only if endometrial pathology is suspected (hyperplasia, endometritis, carcinoma) as a cause of infertility, the endometrial biopsy is a very useful diagnostic tool.

The persistent belief is that subtle periovulatory abnormalities in ovarian steroidogenesis or intrinsic molecular endometrial alterations are responsible for an important percentage of female infertility [82].

Given the prevalence of polycystic ovarian disease, dysfunctional uterine bleeding, iatrogenic hormonal use and clinicians' requests for endometrial dating, it remains important for the surgical pathologist to recognize when ovulation occurs and the normal response of the endometrium to various hormonal stimuli.

1.4.2. Dysfunctional uterine bleeding

In the fifth decade of life the reason to perform an endometrial biopsy is to evaluate the underlying cause of dysfunctional uterine bleeding, from which the most common are: 1) anovulatory cycles, 2) endometrial polyps, 3) chronic endometritis and 4) submucosal leiomyoma.

1.4.2.1. Anovulatory cycles

The morphological pattern related to changes in the normal curve of hormones due to anovulation, was defined under the terminology "disordered proliferative endometrium" [1]. This bridging nomenclature between proliferation and simple hyperplasia is however discouraged by WHO due to its involvement into the already poorly reproducible endometrial histopathology [85].

If anovulatory cycles are accompanied by persistent oestrogen production, the endometrium shows proliferative changes that are often misdiagnosed as simple non-atypical hyperplasia. These include: cystic glandular dilatation, patchy stromal breakdown associated with fibrin trombi, tubal metaplasia and surface repair. Also this condition is not readily distinguished from hormone withdrawal pattern of HRT endomerium. Sometimes, the oestradiol levels can decrease abruptly and the endometrium will show proliferative glands in a background of diffuse stromal breakdown.

1.4.2.2. Endometrial polyps

Endometrial polyps are the most common benign lesions of the endometrium and the second cause of dysfunctional uterine bleeding, with a prevalence ranging between 16-34% [86]. They are particularly common in patients treated with Tamoxifen. They can originate anywhere in the uterus, with different size and potential of recurrence after excision. The "gold standard" tools used for the diagnosis are hysteroscopy with subsequent polypectomy. Genetic studies proved that endometrial polyps are clonal lesions with genetical alterations restricted to the stromal component and subsequent induction of polyclonal glandular elements by undefined stromal-epithelial interactions. Four cytogenetic groups were identified based on specific rearrangements involving 7q22, 12p15, 6p21; the latter two result in dysregulation of HMG1-C and HMG1 [87 88].

Morphologically, a mixture of normal cyclic endometrium foci with fragments of different appearances is the clue for diagnosis at low power examination. Two out of three criteria are necessary for the histopathological diagnosis of endometrial polyps: 1) paucicellular, fibrous, dense stroma, 2) irregular architecture and distribution of glands with proliferative features, even in postmenopausal women, 3) thick walled blood vessels. A surface epithelium circumferentially lining the polyp is not a constant feature.

A diagnosis of simple hyperplasia within the polyp should not be made, since a glandular architecture reminiscent of simple hyperplasia frequently occurs within the polyp. The diagnosis of hyperplasia within the polyp can be difficult [89] and it should be reserved for the cases with considerable crowding and complex architecture of glands. As in the endometrium, atypical hyperplasia is a focal lesion with altered cytology. Various confusing terminologies have been used for this condition, such as: "hyperplastic polyp", "adenomatous polyp with or without atypia", "polyp with premalignant or malignant change", etc. The risk of hyperplasia or neoplasia within the polyp or in the underlying endometrium has been the subject of various studies[86 90 91] The frequency of hyperplasia without atypia within the polyp varied from 11.4% to 25.7%, and that of atypical hyperplasia was 3.3% and respectively 3.1% [90 91]. The underlying unremarkable endometrium showed 18% hyperplasia without atypia and 7.3% atypical hyperplasia [86]. Persistence of glands which do not desquamate is proposed as a possible hypothesis of malignant transformation of a polyp [89].

The risk of malignancy within the polyp or in the endometrium harboring a polyp is greater for postmenopausal women with polyps larger that 1.5cm and for these cases, a hysteroscopically directed polypectomy with biopsy of the adjoining endometrium is advised [86 90 91]. "Polyp cancer" may be endometrioid type, but serous carcinomas (invasive or intraepithelial endometrial carcinoma) have a peculiar propensity to arise in an endometrial polyp [92]. Endometrial polyps, especially when large and occurring in elderly patients, should be carefully scrutinized for small serous proliferation [93].

1.4.2.3. Chronic endometritis

It may be seen in various infections of the uterus, post-partum and post-menopause, in this latter condition without well defined circumstances.

Lymphocytes, including lymphoid aggregates, are the normal constituents of the endometrium, as are also the polymorphonuclear cells related to menstrual cycle. The hallmark of chronic endometritis is the presence of plasma cells, which frequently are intermixed with other inflammatory cells and often are located under the surface epithelium or on the periphery of lymphoid follicles. It can be associated with luminal exudates of inflammatory cells [94]. Stromal oedema, focal hormonally non-responsive areas and a characteristically spindle cell alteration of the stroma encircling the glands are often associated with the inflammatory infiltrates. Some degree of architectural complexity and also atypicality can be present as a reactive phenomenon [93].

In the routine practice, the main questions related to this process are: how many plasma cells are necessary for the diagnosis? Is the presence of a single plasma cell sufficient for diagnosis?. There are scattered plasma cells described in the late menstrual endometrium, within an endometrial polyp or in association with an endometrial malignancy. This topic was discussed in the section of "hematholymphatic cells of the endometrium".

In the presence of pyometra, a pathologist should discard the coexistence of adenocarcinoma and in the presence of granulomatous endometritis a tuberculous infection or sarcoidosis [1]. "Focal necrotizing endometritis" is a rare variant of endometritis in which the focal, patchy inflammatory population represented by neutrophils, lymphocytes and histiocytes, with the absence of plasma cells, produce necrosis of the glandular epithelium [95].

1.4.2.4. Submucosal leimyoma

Submucosal leiomyoma is a common cause of abnormal uterine bleeding and should be suspected in an endometrial biopsy when strips of functionalis without or with scattered glandular structure in the absence of smooth muscle fascicles are seen. The agladular functionalis is the consequence of compression atrophy of the underlying tumour.

1.4.3. Endometrium under the exogenous hormones

The endometrium represents a highly sensitive target tissue for endogenous and exogenous steroid sex hormones. Various hormonal therapies are widely used for different conditions. Not infrequently in the routine practice, the pathologist is confronted by confusing histological patterns which do not fit into the classical features of cyclic or involutive endometrium.

The endometrium plasticity under this therapy is expressed by a large spectrum of changes which depend on type of therapy, dose, time of treatment and they can vary from patient to patient. A "mixed" pattern endometrium, defined as an association of proliferative features (tubular glands) with secretory changes of epithelium and stroma, can be seen in various treatments.

In this chapter we proposed to summarize briefly the general histological patterns for specific hormone therapy: oral contraception, HRT, ovulation stimulation and tamoxifen therapy. The hormonal therapy used in the management of leiomyomas, adenomiosis and endometriosis (GnRH agonists, progesterone receptors modulators) are not pertinent to our discussion.

1.4.3.1. Oral contraceptives

This widely used contraceptive method acts on the same principle as "the presence of one pregnancy prevents the onset of another" [59]. Most contain a small, equilibrate dose of oestrogen and progesterone, or progesterone only.

Administration of contraceptive pills produces an arrest of glandular proliferation, secretory changes of the stroma and involution of blood vessels. The glands typically appear as "thin tubules" with some "abortive" secretion, lined by cuboidal epithelium with randomly distributed vacuoles. Initially the stroma is oedematous with spindle cells, later it becomes decidualized with granulocytic infiltration. Against this background, spiral arteries

with thin walls and dilated sinusoidal pattern can be seen. In prolonged therapy, atrophy of both glands and stroma is the rule. [1 96].

This pattern should be discriminated from late menstrual endometrium (coiled hypersecretory glands, blood vessels changes) and from atrophic endometrium (elderly patients, dense fibrotic stroma, different size glands).

1.4.3.2. Progesterone administration

Various progestational agents can be administered for contraceptive (pills, intrauterine) or therapeutic reasons. They can be used in the management of numerous conditions: menorrhagia, endometriosis, adenomyosis, leiomyoma, endometrial hyperplasia, and as concurrent use in women on hormone replacement therapy or tamoxifen [97]. Good results are obtained for treatment of non-atypical hyperplasia based on its ability to inhibit DNA synthesis with arrest and regression of abnormal proliferation [98]. It can be considered as a temporary, but not curative, therapeutic option in cases of atypical hyperplasia or early stage well differentiated carcinoma of young women with hope of fertility preservation [99 100].

Histologically, progesterone induces a reduction of proliferative activity of the glands with varying degree of atrophy and more evident pseudodecidual changes of the stroma than oral contraceptives. In the biopsy specimens there are features that may induce differential diagnostic problems such as: Arias Stella-like changes in older women, cystic or slit-like glands which do not imply necessarily an anovulatory cycle or strips of aglandular functionalis, an indirect sign of submucoasal leiomyoma [1 96].

1.4.3.3. Ovulation induction therapy or controlled ovarian stimulation

Various agents are used to increase the level of endogenous gonadotrophins (recombinant FSH, GnRH antagonists, clomiphene and hCG), which subsequent hyperstimulate the ovaries and induce the ovulation [101].

Morphologically, an asynchrony of endometrial elements maturation is seen. The glands appear less mature (glands of POD 2-3) compared with more advanced stroma (POD8-9) [59]. Recently, it was proved by gene expression profile that controlled ovarian stimulation alter endometrial receptivity with a functional genomic delay of the endometrium. [102 103].

1.4.3.4. Endometrium under hormone replacement therapy (HRT)

More than 20 million women in developed countries [104] are receiving HRT for prevention and treatment of the conditions associated with oestrogen drop levels of menopause.

It is well documented that unopposed exogenous oestrogen is an important risk factor for development of endometrial hyperplasia or even carcinoma, with approximately 20 % of women developing a hyperplasia after one year. [105 106]. The risk ratio varied from 2.3 [107] to 10 [108] being dependent to the daily dose and duration of the treatment [108], and interestingly it persists for many years after the treatment has been stopped [107].

The risk of endometrial proliferation is reduced or even avoided by the addition of progesterone, an important counterbalance of ooestrogens' growth promoting effect [109].

The modern regimens of HRT using oestrogen and progesterone can be administered in two ways: cyclical (sequential) or continuous combined, with differences in morphological endometrial pattern and eventual development of malignancy. The pathologist should be familiar with the endometrial changes since endometrial biopsy is included in the follow-up of women under HRT.

The endometrium presents a wide range of histological patterns [59]. Often weak secretory features with only cytoplasmic vacuolization are present in sequential HRT. This type of therapy has a high incidence of proliferative endometria and in many ways it mimics the normal cycle. 15% of cases receiving less than 9 days progesterone showed a slight proliferative activity with epithelial mitotic figures [110]. Only a small proportion of women (7-8%) have an inactive endometrium [111]. This endometrial pattern contrasts with that induced by continuous combined regimen, in which 2/3 of endometria presented atrophic features, with more than 50% of them being "unassessable or insufficient material" in Pipelle samples. The most frequent findings in the biopsy include: numerous strips of surface epithelium with minimal representation of tubular glands of functionalis, which present an inactive pattern and are associated with variable stromal breakdown. Commonly tubal metaplasia can be seen or its variant eosinophilic metaplasia of surface and/or glandular epithelium and also syncytial aggregates associated with stromal collapse [1].

The atrophic pattern is related to the capacity of continuous progesterone to induce a down regulation of both oestrogen and progesterone receptors [112] and reduce oestrogenic stimulus by conversion of oestradiol into less active oestrone under 17-β dehydrogenase

activity [113]. 25% of cases presented a mild degree of secretory changes, expressed by variable degree of vacuolization, similar to early luteal phase. Proliferative activity is relatively rare in the endometrium under continuous combined HRT and it might result from inadequate progesterone dosage or peripheral oestrogen production [114].

Endometrial polyps are relatively common lesion, especially in the endometrium under the sequential therapy and they can present glandular hyperplasia, but less frequent than in Tamoxifen related polyps.

The most clinicaly relevant endometrial complication associated with HRT is represented by endometrial hyperplasia and carcinoma. Their prevalence differs between the two therapeutic regimes. Sequential therapy is associated with 5.4% and 0.7% prevalence of endometrial hyperplasia without atypia and with atypia respectively, being also influenced by the dose and days of progesterone usage [111].

It has been shown that the continuous combined regimen is not associated with an increased risk of hyperplasia or carcinoma and in fact, this therapy may be capable of transforming a hyperplastic endometrium into an atrophic one [111 114].

1.4.3.5. Tamoxifen therapy

Tamoxifen belongs to the family of selective oestrogen receptor modulators (SERMs) with a competitive antagonism for oestrogen at the oestrogen receptor (ER) level, and is highly used as adjuvant therapy for breast cancer.

The manner of its action on the uterus is complicated and not entirely clear. Tamoxifen molecules behave as agonist and also as antagonist of oestrogen receptors [115].

In a large series of 700 patients treated with tamoxifen approximately 60% did not present pathological endometrial changes. An atrophic/inactive endometrium, similar with that observed in HRT was observed in majority of these cases and 15% presented functional aspects [59]. Pathological alterations were found in the remaining 40% of cases as a result of tissue response to oestrogenic stimulation and included, in order of frequency: polyps, hyperplasia, metaplasia and endometrial carcinoma. Also uterine leiomyoma and andenomyosis were common.

Endometrial polyps are the most frequent pathological findings; they are translucent and oedematous, can reach a large size and may be multiple. Their less cellular, fibrotic or sometimes myxoid stroma contains cystic glands. These are lined by bland epithelium which

frequently shows metaplastic changes [59] [116]. Not infrequently, these inactive glands are associated with hyperplastic ones that occasionally show atypical changes. The latter can be speculatively interpreted as the initial step in a polyp's carcinogenesis [117].

The mechanism of endometrial carcinogenesis induced by tamoxifen is unknown. Biochemical and genetic models strongly favour an oestrogen-dependent pathway with differential gene regulation [118]. Another hypothesis stipulates a possible genotoxic effect of tamoxifen with formation of DNA adducts which were detected in half of the endometrial samples obtained from women treated with this drug [119]. The molecular analysis of tamoxifen related endometrial carcinoma showed interesting features: a higher rate of β-catenin and k-ras mutation but not the high incidence of PTEN and MSI alterations that occur in usual type endometrioid carcinomas [120]. Some of tamoxifen related endometrial cancer can be aggressive, high grade and stage tumours. Tamoxifen-specific gene regulation could be a plausible theory to explain the pathogenesis of these tumours [121]. Not only various types of endometrial carcinomas have been reported in patients treated with tamoxifen, but also malignant mixed Müllerian tumours and sarcomas [122] [123]. The risk increases with time and dosage treatment.

Close gynecological surveillance with ultrasonography, hysteroscopy, with or without biopsies, during tamoxifen treatment is advised. Also, in women with high-risk factors, follow up after discontinuation of therapy is recommended [124] [125].

CHAPTER 2

Introduction - Endometrial and cervical metaplasias

II. Introduction - Endometrial and cervical metaplasias

2.1. Endometrial tubal metaplesia

Despite their common occurrence in the endometrium, epithelial metaplasias and changes are often overlooked and misdiagnosed. Their classification, association and morphological criteria of diagnosis, together with guidelines for their treatment are detailed in "**Endometrial metaplasias and reactive changes: a spectrum of altered differentiation",** a review published in ***Journal of Clinical Pathology*.** We also paid special attention to surface papillary syncytial changes pathogenesis in "**p16INK4A positivity identifies endometrial surface papillary syncitial change as a regressive feature associated with desquamation"** accepted for publication in ***Histopathology,*** and intestinal metaplasia in **"Endometrial intestinal metaplasia: a report of two cases, including one associated with cervical intestinal and pyloric metaplasia"** accepted for publication in ***International Journal of Clinical pathology*.** All these articles are included in the chapter *Publications*.

2.2. Cervical tubal metaplasia

The cervix (endocervix), as a part o Müllerian system manifests the same metaplastic alterations as the endometrium, only the frequency of their appearance is different. Squamous metaplasia is an invariable condition that can be considered within the spectrum of normality and not as an alteration. Its etiology and morphology are well known and are not discussed here. In the literature, less attention has been paid to other types of metaplasia, such as: tubal metaplasia (TM), pyloric/intestinal, or transitional metaplasia.

TM becomes a point of interest in cervical pathology, being considered the most common lesion to be misinterpreted as cervical glandular intraepithelial neoplasia or glandular dysplasia [126 127].

In 1986 Brown and Wells [128] mentioned for the first time tubal metapalsia (TM) in the differential diagnosis of cervical adenocarcinoma and subsequently Suh and Silverberg described extensively this entity [129].

Ciliated non-secretory cells are normal constituents of the endocervical epithelium, being interspersed singly or in small patches between the mucinous cells. They present a higher density in the upper part of the endocervical canal and in the second part of menstrual cycle. Since the normal glands of the uterine isthmus contain a high proportion of ciliated cells, cervical TM should be diagnosed in specimens taken away from this area [93 130].

Cervical TM is similar histologically to the normal Fallopian tube epithelium or to TM involving endometrium [131] or vaginal adenosis [132]. Architecturally, normal glands or with variation in size and shape are lined by three populations of cells (ciliated, secretory and intercalary) with a bland appearance and absence of mucin secretion. In order of frequency TM is seen in the deeper glands of upper endocervix, squamo-columnar junction, surface lining epithelium and lower endocervix. TM can involve the cervical wall in a focal or an extensive pattern [133 134]. Only one segment of the gland can be affected, with a progressive or an abrupt transition from mucinous epithelium to non-mucinous one [127 129]. The latter feature is frequently seen also in endocervical dysplastic lesions and can lead to confusion.

In 1991, Ismail introduced the terminology of “tubo-endometrial metaplasia” to describe changes with intermediate features between tubal and endometrial type epithelium [130]. Architecturally similar endocervical glands were lined by monotonous, pseudostratified columnar cells with high nucleo-cytoplasmic ratio, some of them ciliated or with apical snouts. These types of glands were surrounded by cervical type stroma and lack endometrial hypercellular stroma. Oliva *et al*. described TM coupled with TEM in the same specimen [134]. Occasionally, there is no resemblance to tubal epithelium and the term “endometrial” metaplasia has been used [135]. In our study we prefer to use the terminology tubal metaplasia (TM) and we underline the distinction between ciliated metaplasia, TM and TEM, although there is no difference in their management.

TM is a fairly common incidental microscopic finding in the uterine cervix [133 136] with unknown prevalence, but considered under evaluated. It is often overlooked due to the primary pathological assessment of the more frequent, squamous cervical lesions [129] or due to a lack of familiarity with its morphology. TM rarely produces gross abnormalities [134].

It was often described in the cervix of the premenopausal women [129 133 134]. Its prevalence was higher in specimens following a cone biopsy (26%) than in de novo ones. In this situation, TM was often located in the healed scar area and was considered as an aberrant differentiation after trauma or injury [130]. 31% of unselected cone biopsies and hysterectomy specimens presented cervical TM, with a greater incidence in hysterectomies (62%) than in cone resections (21%). Its frequency also increased with the number of slides evaluated (23% ≤ 12 blocks vs. 52% ≥12 blocks) [133].

Little is known about the histogenesis of cervical TM. Its high prevalence suggests a physiological response of the endocervical glands to oestrogens, similar to the endometrial response [136]. It is not related to menstrual cycle, degree of inflammatory changes, squamous metaplasia or low grade squamous intraepithelial lesions [129]. In the cases without a previous intervention, TM could be considered an idiopathic change [134].

In recent years, it more attention has been paid to this lesion, since it has been confused with endocervical dysplasia or *in* situ adenocarcinoma [128 129 137] or considered as a potential premalignant lesion [138 139].

TM can be misinterpreted as glandular dysplasia, *in situ* adenocarcinoma (AIS) or even an endometrioid variant of minimal deviation adenocarcinoma [140 141]. Multiple morphological features complicate its diagnosis, such as: 1) location at the squamo-columnar junction or lining in a haphazard fashion the deep cervical glands regions also involved by glandular dysplasia [129 134 137]; 2) its architectural pattern with focally crowded glands, occasional with irregular outline, with budding or branching, moderate degree of cystic dilatation, or elongated and curvilinear shape [134 137]; 3) its cytological characteristics with abrupt transition between mucin producing cells and non-mucinous tubal type cells, a feature common in the endocervical *in situ* neoplasia; 4) its low power appearance with a more basophilic epithelium, which can show greater nuclear crowding, a higher nucleo-cytoplasmic ration, and sometimes slight atypia [134 137]; 4) intercalary cells or intraepithelial lymphocytes, can be confused with apoptotic bodies, which are increased in high grade cervical glandular intraepithelial neoplasia/AIS [142]; 5) the stromal periglandular alterations

represented by hypercellularity or myxoid/edematous, slightly inflammatory changes can be mistaken with desmoplastic reaction [134].

Table 2.1. summarizes the comparison of the morphological features of AIS and CTM

Features	AIS	TM
Location in the cervix	Close to the TZ Rarely high up in the endocervical canal Rarely skip lesions	Close to the TZ Frequently up in the endocervical canal
Associated CIN	Often	Incidental
Involved epithelium	Both surface and underlying cryps epithelium	Both surface and underlying cryps epithelium
Transition with normal epithelium	Often abrupt transition between the glands and within an individual gland	Often gradual transition Rarely abrupt transition
Nuclear stratification and loss of polarity	Present, diagnostic criteria	Nuclear stratification may be present, but not with loss of polarity
Nuclear atypia and hyperchromasia	Present	Large, nuclei of the ciliated cells, no hyperchromasia
Nucleoli	Present, macronucleoli	Present, small nucleoli in the ciliated cells
Intracytoplasmic mucin	Decrease→absent	Absent
Mitotic activity and type of mitosis	Increased with >2 mitosis/gland, atypical	Rare scattered normal mitosis
Apoptotic bodies	Increased number	Rare, confusion with “peg” cells
Goblet cells and neuroendocerine cells	Intestinal type CGIN	Absent
HPV related	Most cases	No implication

The majority of the studies considered TM a benign condition, however cervical tubal-type epithelium is not immune to neoplastic transformation [134]. Some reports considered its preneoplastic potential. This was based on the coexistence and even transition between TM with atypical features and AIS. Atypical tubal metaplastic (ATM) glands showed cellular crowding and larger, hyperchromatic nuclei with a varying degree of nuclear stratification [138]. This lesion has been regarded as a precursor of endocervical AIS of tubal type [138]. ATM was previously defined as “atypical tuboendometrial adenosis” and “atypical ectropion” depending on its location and it has been considered an intermediate step between TM and vaginal and cervical clear cell carcinoma of women treated with DES

[143]. Recently, another case of pseudoinfiltrative ATM has been seen in the context of DES treatment, although with undeterminated evolution [137].

Not only the pathologist, but also the cytologist, should be aware of the presence of TM. Novotny et al considered TM the most common entity misinterpreted as AIS in cervical smears [144]. The cytological appearance of TM (flat sheets or cohesive strips or pseudoglands of cells with high nuclear to cytoplasmic ratio, round-to-oval shaped nuclei with evenly distributed, finely granular chromatin and inconspicuous nucleoli) and their location in the area easily sampled by cytological smears (superficial epithelium, squamo-columnar junction) can lead to an erroneous diagnosis of atypical endocervical or endometrial cells [129] [133] [144-146]. The presence of cilia was considered the most useful cytologic finding in order to distinguish TM from AIS. However, the uncommon ciliated carcinoma of endometrium and endocervix as well as the ciliated variant of AIS should be borne in mind and the presence of cilia should not be considered as unshakable evidence of benignity [147]. The presence of ATM should imply extensive sampling of the specimen to exclude an AIS [138].

Therefore, the diagnosis of TM should rely on a compilation of histological findings (no or mild stratification, bland cytologic features, absence of mitotic activity and apoptotic bodies, and prominent cilia in the apical surface) and not just on a single one. Silverberg and co-workers proposed a scoring system to distinguish endocervical glandular neoplasia from lesions that mimic it [148], designed as an aid to correct interpretation and better inter- and intra-observatory agreement. The endocervical lesions are subgrouped in three categories: benign (score=0-3), EGD (endocervical glandular dysplasia) (score=4-5) and AIS (score=6-9) based on nuclear stratification, nuclear atypia and mitosis/apoptosis. TM, considered a benign lesion, should fulfill the criteria of the first category. In the difficult cases, a panel of immunomarkers can be used for the correct diagnosis, and this can include p16, carcinoembrionic antigen (CEA), Ki-67, oestrogen receptor (ER), progesterone receptor (PR), p53, PAX2 and vimentin. All of them will be discussed in the following chapters.

Chapter 3

Aims, background and justification

III. Aims, background and justification

3.1. Aims

a. The entire spectrum of endometrial metaplasia and changes are reviewed and classified and new concepts introduced. A practical morphological approach is proposed, together with clinical guidelines for their management.

b. A new, practical, morphological interpretation of ciliated, tubal metaplasia, the most common endometrial epithelial metaplasia, is outlined. Ciliated tubal metaplasia is subdivided into three categories in order to ascertain if morphological architectural heterogeneity reflects a true biological diversity; the clinical management for each of these subtypes is discussed.

c. The relationship of ciliated, tubal metaplasia with the pathogenesis of endometrial adenocarcinoma is analyzed in order to determine if it has a role of any importance or is an inert lesion that may lead to confusion.

d. The progress of ciliated, tubal metaplasia is investigated in order to identify if it results in an endometrioid or serous adenocarcinoma.

e. A practical morphological and immunohistochemical approach to the differential diagnosis between cervical ciliated, tubal metaplasia and endocervical neoplastic lesions is proposed.

Objetivos

a. Revisar y clasificar el espectro completo de las metaplasias y cambios endometriales introduciendo nuevos conceptos. Se propuso un enfoque morfológico y práctico, junto con las guías clínicas de manejo.

b. Se describe una nueva y práctica interpretación morfológica de la metaplasia tubárica; la metaplasia más común del epitelio endometrial. La metaplasia tubárica se subdividió en tres categorías, a fin de determinar si la heterogeneidad arquitectural refleja una verdadera diversidad biológica. Discutimos la consideración anatomoclínica, tratamiento y seguimiento e cada uno de estos subtipos.

c. Se analiza la relación de la metaplasia tubárica con la patogénesis del adenocarcinoma endometrial, determinando si participa de alguna manera importante o si bien se trata de una lesión inerte que solo conduce a diagnostico erróneos.

d. Se investiga el progreso de la metaplasia ciliada y tubárica para determinar si se trata de un precursor de adenocarcinoma endometrioide o bien de tipo seroso.

e. Se propone un enfoque morfológico e inmunohistoquímico en el diagnóstico diferencial entre las lesiones neoplásicas endocervicales y la metaplasia ciliada y tubárica del cuello uterino.

3.2. Background and justification

The diagnosis of endometrial pathology presents a challenge and there is a lack of consistency, even by experts. Multiple factors contribute of this, such as: 1) the cyclic nature of the endometrium which makes it difficult to establish "norms"; 2) a wide variety of functional and lesional changes; 3) difficult follow-up of preneoplastic alterations, which may be shed spontaneously with the menses, may regress under progestin treatment or may be completely removed with diagnostic curettage; 4) heterogeneity of neoplastic endometrial lesions and their usual blind evaluation from a biopsy, which can provide false negative results.

Diagnostic difficulties in endocervical pathology are also due to the large variation of similar, mimicking conditions.

Cervical and endometrial adenocarcinomas are responsible for the majority of deaths from female genital tract malignancy and their incidence continues to increase. This calls for better prevention and/or early detection. One aspect of cancer prevention resides in the accurate identification of morphologic distinctive precursor lesions or "precancers" [149]. Consequently, from a practical point of view, it is crucial to identify lesions that bear a high risk of progression to or concurrence with an adenocarcinoma, in order to avoid a misdiagnosis or overtreatment, which would lead to the physical and psychological distress of the patient, as well as a waste of valuable funds.

3.2.1. Endometrial carcinoma

Endometrial carcinoma is the most common gynecological malignancy in USA and Western countries [150 151], affecting predominantly postmenopausal women. Almost three decades ago a dualistic model was proposed for carcinogenesis of sporadic endometrial malignant tumours, which comprise 90-95% of endometrial tumours [152]. Each subset presents different rate of incidence, epidemiologic risk factors, precursor lesions, morphology, molecular genetic pathways with alterations of specific genes and clinical outcome.

Type I represents almost 80% of endometrial cancer and has as a prototype endometrioid adenocarcinoma. It arises in relatively young pre- and postmenopausal

women, under the unopposed endo- or exogeneous oestrogenic stimulation [153] and expresses ER and PR [154]. These tumours develop through a transistion of premalignant lesions ranging from endometrial hyperplasia without atypia, to hyperplasia with atypia/endometrial intraepithelial neoplasia (EIN) and finally to well differentiated carcinoma [155]. Generally they have a low to intermediate grade and a relatively favourable prognosis. Their pathogenesis is complex and involves many molecular alterations. The most characteristic molecular defects affect phosphatase and tensin homolog (PTEN) (35-83%) [156], microsatellite instability (MSI) (20-45%) [157], K-ras (10-30%) [158] and β-catenin genes (20% of cases with or without E-cadherin mutations) [159]. PTEN, K-ras mutations and MSI are considered early events. They occur in a subset of atypical endometrial hyperplasia and even in the "normal" endometrium, in the so-called "latent precancer" or the preclinical phase of the disease [160]. On the contrary, p53 mutation is considered a late event and appears during the progression of about 20% to 43% of high grade and advanced stage endometrioid carcinomas [161]. However, a single progression model is unlikely to be applicable to all cases of type I endometrial carcinoma due to the genetic heterogeneity and temporal sequence of the mutations [150]. The rare mucinous adenocarcinoma enters in the same category, since it is frequently reactive for ER and/or PR and has a low histopathological grade.

Endometrial serous carcinoma (ESC) fits into *type II* endometrial cancer. Together with clear cell carcinoma, it follows the oestrogen unrelated pathway and arises in a background of atrophic endometrium [162]. It develops in relatively older women (5-10 years later than endometrioid carcinoma) and is a typically high grade carcinoma with a poor outcome. For many years endometrial intraepithelial carcinoma (EIC) was considered its putative precursor. Recently, this theory has changed. Given the fact that serous EIC and ESC share many features regarding morphology, molecular biology, clinical behaviour and management, EIC is actually seen as an early form of endometrial serous carcinoma [163 164]. It is paradoxal to define EIC as a precancerous lesion in the context of its common extra-uterine disease (17-67%). Endometrial glandular dysplasia (EmGD) is considered a better candidate as a precursor lesion of ESC [164]. EmGD is defined as a multifocal lesion with an intermediate degree of cytological atypia. It involves the endometrial surface, single glands or clusters of glands and presents a pattern of p53 and Ki-67 immunoexpression between

EIC and resting endometrial [164]. Prospective studies are necessary to define its relative risk of progression and/or regression in order to provide guidelines for its clinical management.

In the pathogenesis of serous tumours, the most frequent alteration involves the p53 gene. More than 90% of uterine serous carcinoma, 78% of EIC and 43% of EmGD have p53 mutations [165]. p53 mutations are almost always associated with aneuploidy and do not seem to concur with PTEN mutations in the same tumour [166]. Other molecular alterations include inactivation of $p16^{INK4A}$ (45% of cases), of E-cadherin, loss of heterozygosity located on certain chromosomes as 1p, 17 q [163 167] and overexpression of c-erbB-2 and its product, tyrosine kinase HER-2/neu (18-80%) [168]. MSI, K-ras and β-catenin alteration are rare in serous carcinoma.

The major clinico-pathological features and genetic alterations of type I and II of endometrial carcinoma are summarized in table 3.1 [151 161 169].

However, as in any model, there are exceptions; in daily practice many endometrial carcinomas are in a grey zone with overlapping clinical, morphological, immunohistochemical and molecular features.

Table 3.1. Comparison of major clinco-pathological and genetic alterations of type I and II endometrial cancer

Characteristics	Type I	Type II
Incidence	80%	≤ 20%
Age	Pre-/perimenopausal	>60 years
Unopposed oestrogen	Yes	No
Background endometrium	Hyperplastic	Atrophic
Precursor lesion	Hyperplastic endometrium/EIN	EmGD, EIC
Morphology	Endometrioid, mucinous	Serous, clear cell
Clinical behaviour	Indolent	Aggressive
PTEN inactivation	50-80%	10%
Microsatellite instability	20-45%	0-5%
K-ras mutations	15-30%	0-5%
β-catenin mutations	20-45%	0-5%
p53	10-20%	90%
Her2/neu overexpression	10-30%	18-80%
$p16^{INK4A}$ inactivation	10%	45%
E-cadherin alteration	10-20%	80-90%

3.2.2. Cervical adenocarcinoma

The incidence of cervical adenocarcinoma is increasing and now accounts for approximately 25% of all cervical carcinomas [170]. The reason for this is not completely clear and can not be due entirely to cervical screening, taking into account that cervico-vaginal cytology is less effective for detection of adenocarcinoma and it precursors [171]. However, it could be related to an actual increase of incidence together with better recognition by the pathologist [126 148]. It is accepted that AIS or high grade cervical glandular intraepithelial neoplasia is the precursor of classical endocervical adenocarcinoma [172]. Instead, for endocervical glandular dysplasia (EGD), there is much debate about its existence and the possibility of its being a precursor of AIS. Lack of agreement regarding its terminology (glandular atypia, atypical endocervical hyperplasia, low grade cervical glandular intraepithelial neoplasia, endocevical columnar cell intraepithelial neoplasia), absence of widely accepted diagnostic criteria with no established morphological threshold between dysplasia and AIS, poor interobserver reproducibility, nature and indefinite clinical behaviour [173] contribute to the uncertainity of EGD. Some authors support the theory of multistep development of cervical adenocarcinoma, beginning with EGD [174]. Others consider EGD as a different, transistional morphological expression of a non-neoplastic entity, and sustain that a true prenoplastic lesion of AIS does not exist, or if exists is rapidly overgrown or transformed into AIS [173 175 176]. This latter is supported by the infrequent occurrence of EGD in the vicinity of AIS or invasive adenocarcinoma, low rate of HPV [175 177], low proliferation index, overlapping in the expression of CEA, MIB, $p16^{INK4A}$ with benign mimicking lesions, in particular TM [173]. The diagnostic criteria as “lesser degree of abnormality than AIS” [178] are largely subjective. The objective scoring system proposed by Ioffe et al [148] in combination with a panel of antibodies increase the accuracy of diagnosis and the inter-and intra-observer agreement [173 179] for non-invasive glandular lesion of cervix. This scoring system includes evaluation of three variables: nuclear stratification, atypia, mitosis and apoptosis with subsequent classification of endocervical lesion in three categories: benign (score=0-3), endocervical glandular dysplasia EGD (score=4-5), AIS (score=6-9). Some authors believe that if a diagnosis of EGD is reached after a careful exclusion of the benign mimickers, than the management should be similar to that of AIS,

taking into account that many pathologists do not recognize EGD in the absence of AIS [180]. There is also a proposal to classify a single gland with features of AIS as EGD [180].

Therefore, in routine practice, before categorizing a lesion as preneoplastic or neoplastic a pathologist should pay attention to the mimickers, the incidence and spectrum of which is increasing. The list of potential pitfalls is extensive, but in the daily practice the most frequent are: TM/Tubo-endometrial metaplasia (TEM), endometriosis, microglandular hyperplasia, inflammatory atypia, mesonephric remnants/hyperplasia and radiation effect [180]. They can create diagnosis problems both at cytological and histological level [126 180 181] but their distinction is important for management.

Currently, for both endometrial and endocervical adenocarcinoma there is no unanimity in the classification of their precursors, as there is a lack of criteria that could accurately predict the outcome of the disease. The aforementioned neoplastic lesions of the uterine corpus, AIS or high grade cervical glandular intraepithelial neoplasia for endocervical adenocarcinoma, endometrial hyperplasia or EIN for Type I endometrial carcinoma and EmGD for type II endometrial carcinoma [182] appear to fulfill most of the National Cancer Institute criteria for preneoplastic lesions [149].

Our study focused on TM at the cervical and endometrial level. This subject was chosen because not infrequently this metaplastic lesion is misdiagnosed as cervical glandular intraepithelial neoplasia [170 180 181] or endometrial atypia [3]. Furthermore, there is concern about its preneoplastic potential either at cervical [138 139] or endometrial level [89 183 184]. In order to present an integral picture of TM, a multidisciplinary approach including morphologic evaluation, immunohistochemical and genetic studies (FISH and PCR) were applied. These investigative techniques can also be useful as prognostic determinants for further understanding of its pathogenesis. We intended to establish if TM represents a bridge between normal endocervical or endometrial epithelium and their corresponding adenocarcinomas.

3.2.3. Cell cycle protein, tumour suppressor gene and oncogenes involved in endometrial and endocervical carcinogenesis

Carcinogenesis is a multistep process involving successive stages of initiation, promotion and progression of lesions from normal epithelium to benign and malignant tumours. Each step is accompanied by a variety of quantitative and qualitative gene

alterations that control proliferation, apoptosis, angiogenesis, invasion and metastasis, as well as morphologic and biochemical changes. In endometrial and cervical carcinogenesis various genetic and epigenetic events, including those of the cell-cycle machinery at the G1/S checkpoint, are frequently involved. Four important pathways are implicated: the p21WAF1-p27KIP1-cyclin E-cdk2, β-catenin-Tcf-myc-telomerase, ARF-MDM2-p53 and pRb1-cyclin D1-cdk4/cdk6-p16^{INK4A} [185]. For endometrioid carcinogenesis additional pathways involving PTEN, MSI and K-ras are of special interest.

The morphological problems in the recognition of endometrial and endocervical precursors and ability of immunohistochemistry to provide valuable information regarding the molecular and genetic substrate of the lesions and their biological behaviour led to attempts to establish biomarkers for their diagnosis.

A vast array of biomarkers (61 different antibodies) have been investigated [186] in atypical endometrial hyperplasia, one of the least reproducible diagnoses [187-190], in an effort to establish the lesions that have a high risk of progression to or concurrence with an adenocarcinoma. However, the dynamic nature of the normal cycling endometrium creates a particular difficulty in establishing norms and unique markers.

Endocervical adenocarcinoma precursor lesions are not excluded from such difficulties. Cervical screening cytology is an unreliable method for the diagnosis of glandular abnormalities, having a low sensitivity and specificity [191], largely due to sampling errors and interpretation problems. Subsequently, many reactive endocervical changes are difficult to differentiate from neoplastic ones. Also, colposcopy is ineffective in recognizing the endocervical lesions; minimal invasive biopsy procedure may also be inadequate [192]. All these factors led to the necessity of finding a specific method to improve the detection of glandular lesion. A wide range of immunohistochemical markers has been investigated for the diagnosis of dysplastic endocervical cells/AIS and to differentiate it from benign lesions. They varied between distinct studies and included: p16^{INK4A} [170 176 192-194] ProEx C [195 196], MIB1/Ki67 [170 175 193 194 197 198], bcl-2 [170 194 199], p53 [198 199], CEA [198], Cyclin E [193], Cyclin D1[194], MN, a transmembrane glycoprotein [200]; CD44v5 [201], and mucin gene expression, MUC2 and MUC5A [202], IMP3 [203]; many of them with possible, but limited evidence of diagnostic and clinical aids.

Although some of these antibodies are extremely helpful, none of them is completely reliable in any individual case. The majority of the studies agree that for both

endocervical [170] [179-181] [193] [198] [204] [205] and endometrial lesions [186] [195], a combination of biomarkers in conjunction with meticulous examination of histological features make the distinction between benign precursors and malignant conditions possible.

In endocervical lesions, the preferred panel will include some of the follow antibodies: $p16^{INK4A}$ or ProEx C, bcl2, MIB1, CEA, vimentin, ER [170] [175] [176] [179] [192] [195] [197] [205]. For endometrioid lesions, PTEN [156] [206] [207], PAX2 [208] and bcl-2 [209-211] can be used and p53 and Ki-67 for serous lesions [165] [212].

The ability to establish the biology of TM increases with the number of variables taken into account in the study. Consequently, we analyzed the main incriminator proteins involved in endocervical and endometrial carcinogenesis pathways. Furthermore, we have summarized some of their general features, their implication in pathogenesis of uterine glandular malignancies, controversy regarding their diagnostic usefulness and reasons for their inclusion in our study.

3.2.3.1. p16

p16 is encoded by CDKN2A gene, located on the chromosome 9p21. It is a member of the INK4A family of cell cycle regulatory proteins, which acts as a tumour suppressor protein. Being a cyclin dependent kinase inhibitor, $p16^{INK4A}$ specifically interacts with cyclinD1-cyclin-dependent-kinases (CDK) 4 and 6 complexes formation [213] [214]. These control the activity of retinoblastoma protein (pRb) by phosphorilation, the key regulator of G1 check-point [215]. If pRb is hypophosphorilate, it forms a stable complex with transcriptional activatory members of E2F-family and this complex halts the progression of the cell cycle through G1 phase [214], with downregulation of cell proliferation. If phosphorylation of Rb is achieved, by cyclin D1/CDK4/6 complex in mid G1 and then by cyclin E/CDK2 complex later in G1, release of E2F and transcription of the S-phase genes takes place, with completion of the cell cycle [216]. For an optimal G1-S transition, the cell requires coordinate activation of both cyclin D1 and cyclin E-dependent kinase and subsequent inactivation of pRb, the cell cycle repressor [214]. $p16^{INK4A}$ expression is influenced by the status of pRb, due to the negative feedback loop between them [217].

Its alteration, either up-regulation or inactivation, are encountered in a diversity of tumours, including those of uterine corpus. In the latter situation homologous deletions,

point mutations or promoter hypermethylation, rather than HPV-dependent mechanisms are involved. For cervical cancer it is *vice versa* [218].

Recently, immunohistochemical evaluation of $p16^{INK4A}$, as a marker of HPV-related preneoplastic and neoplastic endo-and exocervical lesions, has started to become routine. The relation between overexpression of $p16^{INK4A}$ and HPV was also documented in the vulva [219 220], penis [221], anus [222], tonsil [223] and rhinosinuses [224].

How is $p16^{INK4A}$ overexpressed in HR-HPV related conditions?

HPV infection can persist, resolve or cause dysplastic and malignant cell transformation [225]. In the majority of situations, viral DNA can remain episomal and contribute to the productive phase. Less often, HPV integrates into the host cell genome and its oncoproteins E6 and E7 will play the key role in cell cycle alterations and malignant initiation. If E6 binds and degrades p53, the "cell custodian", E7 binds and functionally inactivates pRb, the "gate keeper" [226]. Subsequently, E2F is released and activated to transcript the genes necessary for DNA replication. The cell is forced to enter into the S phase and the negative feedback control of $p16^{INK4A}$ is altered, with its up-regulation [218]. Although $p16^{INK4A}$ concentration is high, it does not have an inhibitory effect on the cell cycle, because pRb has already been blocked by E7.

We proposed to evaluate the expression of $p16^{INK4A}$ in the spectrum of endometrial tubal-type lesions in an attempt to establish if its positivity is related to TM aggressiveness, as it was speculated. $p16^{INK4A}$ will be also analyzed in cervical TM in order to validate its usefulness in the differential diagnosis between preneoplastic endocervical lesions and mimickers.

3.2.3.2. Cyclin D1.

Cyclins interact with cyclin-dependent kinases and tumour suppressor gene products and regulate the normal cell cycle. The PRAD-1/cyclin D1 gene is located on the chromosome 11q13. It is considered a critical positive effector of the G1/S control point and a potential oncogene (bcl-1-related oncogene) [227]. In the neoplastic process cyclin D1 participates also in apoptotic pathways, cellular migration and angiogenesis [228]. Its expression is dependent on the phosphorylation status of pRb1 [229] and it is indirectly influenced by β-catenin and p53. Its overexpression leads to cellular proliferation by its effect on progression to S phase. Consequently there is a positive correlation between Ki-67

and cyclin D1 positivity [230]. In mantle cell lymphoma, either t(11;14), bcl-1 rearrangement or cyclin D1 overexpression are present in the majority of cases. These are uncommon findings in various solid malignancies such as breast, urinary bladder carcinoma, head and neck squamous cell carcinoma, small cell lung carcinoma [230 231] and female genital tract neoplasias, including squamous cell carcinoma, ovarian and endometrial adenocarcinoma [230-234].

Some data support the role of cyclin D1 in endometrial carcinogenesis, as part of the pathway β-catenin-TCF/LEF-1-cyclin D1 together with PTEN and p53 [230 231 235 236]. Its expression in endometrioid carcinoma varied between the studies from 40% to 68% [231 234].

We evaluate cyclin D1 expression in TM due to its involvement in cell proliferation and its early implication in endometrial carcinogenesis [231].

3.2.3.3. Bcl-2

Not only cell proliferation, but also cell death, through necrosis or apoptosis, is an indicator of cell turnover. In the cyclic endometrium, as in the other normal tissues, apoptosis or "controlled cell deletion" is under the influence of numerous oncogenes and tumour suppressor genes as bcl-2, Bax, c-myc, ABL, Fas and p53. Among these, the most crucial regulators are members of bcl-2 gene family: bcl-2 and Bax, which present opposite effects of the apoptosis.

Bcl-2 is a proto-oncogene located on the chromosome 18, which encodes a 25kDa protein, mainly localized to the inner mitochondrial membrane. Here, it controls the permeability of mitochondria and inhibits apoptosis by blocking the release of cytochrome c and subsequently activation of caspase [237].

Bcl-2 gene was first described in follicular lymphoma as result of its involvement in the t(14;18) [238]. Subsequently, its aberrant expression was found in several carcinomas and was correlated with treatment response and outcome [239]. In the endometrium, bcl-2 expression is controlled by sex steroid hormones. Oestrogen up-regulates the expression of bcl-2 [240], but progesterone has the opposite effect. Sustained overexpression of bcl-2, under unopposed oestrogen, promotes cell survival with consequently growth and proliferation [241]. bcl-2 provides an increase of survival in cells harbouring mutation [242].

In this study, we propose to analyze the expression of bcl-2 in both cervical and endometrial TM, taking into account two main considerations. First, the controversial role of

bcl-2 in endometrial cancer development, its prognostic value and its contribution to the diagnosis of preneoplastic lesions is discussed. Second, the likelihood of bcl-2 as a reliable antibody in the differential diagnosis of endocervical AIS from mimickers is studied.

3.2.3.4. PAX2

PAX2 is one of the nine members of the PAired-boX gene family (PAX1-PAX). These genes encode transcriptional factors that are expressed in a spatial and temporal pattern during embryogenesis. They play a key role in the control of foetal organogenesis by their involvement in regulation of cell proliferation and self-renewal, resistance to apoptosis, migration of embryonic precursor cells and coordination of specific differentiation programmes [243]. These genes are switched off during the latter phases of terminal differentiation of most structures and maintain this status in the mature organism. However, this phenomenon is not strict, as few normal adult tissues preserve the expression of PAX genes [244].

Deregulated expression and or/activation of specific members of the PAX family appear to play a major role in the pathogenesis of specific cancers of those organ systems in which PAX members exert their developmental functions during embryogenesis [243].

PAX2 is essential for the development of the central nervous system, eye, ear and urogenital tract and consequently is specifically expressed in these organs. In early development, PAX2 is detected in the intermediate mesoderm, from which the Wolffian duct and kidneys are derived [245]. Therefore, its expression in renal cell carcinoma, nephrogenic adenoma and Wilms tumour is not surprising [246 247].

Müllerian duct also originates from intermediate mesoderm adjacent to the Wolffian ducts and PAX expression was identified in all the normal structures derived from this system, with the exception of squamous epithelium [248]. However, the functional and clinical implication of this transcription factor in female genital tract carcinogenesis is not clearly defined. We propose to evaluate its expression in the entirely normal Müllerian ducts derivitives with a specific focus on endometrium metaplastic, preneoplastic and neoplastic lesions.

3.2.3.5. Ki-67

Cancer development and growth are associated with either deregulation of cell proliferation or programmed cell death or both. Cancer research has been focused mainly in cell proliferation as an indicator of tumour kinetics. We used Ki-67 antibody in an attempt to establish the proliferation rate within the TM glands and compare it with normal endocervical and endometrial glands as well as with their respectively preneoplastic and neoplastic conditions.

We should be cautious as to which kind of antibody we use in routine practice for evaluation of proliferation index. The monoclonal antibody Ki-67 reacts with a human nuclear cell proliferation-associated antigen, expressed in all active parts of the cell cycle [249] [250]. It is rapidly broken down at the end of M phase and it is not present in G_0 cell cycle phase. Other markers are available for immunohistochemical evaluation of proliferation rate. MIB1 is a monoclonal antibody which reacts against recombinant parts of Ki-67 antigen [251]. In general, MIB1 counts are higher than Ki-67, these two antibodies are not interchangeable on the measurements of cell proliferation [197]. Another antibody used for proliferation index is PCNA (proliferating cell nuclear antigen). Due to its long half–life, low levels of PCNA may be present throughout the cell cycle [252] and consequently, it is not so tightly linked to proliferation as Ki-67.

In the cervix, the main value of proliferation markers is in the distinction of CIN or preneoplastic endocervical lesions from their mimics (atrophy, atypical squamous immature metaplasia and transitional metaplasia, TM, mesonephric hyperplasia). Also, they are a useful tool in evaluation of cauterized cervical cone or biopsy margins [193].

3.2.3.6. p53

The tumour suppressor gene p53 is located on the 17p chromosome. It is induced in response to DNA damage, hypoxia and oncogene activation. Depending on the severity of DNA alterations, the wild-type p53 (unmutated) will arrest the cell cycle through transcriptional activation of p21, a CDK inhibitor, allowing DNA repair or it will induce apoptosis by activation of bax, the proapoptotic protein and negative trans-regulation of bcl-2, the antiapoptotic protein [209] [253]. Therefore, p53 represents the “guardian” of the cell

cycle, preventing the propagation of cells with DNA damage and minimizes the risk of genomic instability.

p53 gene is mutated in more than 50% of human cancers and represents the most commonly detected genetic abnormalities in endometrial, cervical and ovarian neoplasia [253]. Germ-line mutation of p53 is associated with Li-Fraumeni syndrome.

Mutations of p53 gene produce conformational changes of the protein with resistance to degradation. Subsequently, overexpression of this nonfunctional protein takes place with immunohistochemical detection. However, it should be emphasized that p53 immunonegative reaction does not exclude a p53 mutation. Frame shift mutations and stop codons can lead to a truncated protein, which is not detected by antibodies [212]. Furthermore, the immunoreactivity does not always predict the presence of p53 mutation. In endometrioid tumours changes in other genes that affect p53 function ($p14^{ARF}$, MDM2) are responsible for p53 immunoreactivity [254]. It was also documented that DNA damage induced by irradiation can produce accumulation of wild-type p53 [255]. Consequently, the concordance between immunohistochemical p53 overexpression and the underlying mutation is not absolute, being reported to be approximately 76% for endometrial carcinoma [256].

De novo development of serous carcinoma is tightly related to p53 gene alteration, with its abnormal expression even in the absence of morphological changes [257 258]. Another hypothesis is that serous carcinoma arises secondary to endometrioid carcinoma through p53 mutations, based on its occurrence in mixed tumours [259]. In contrast, p53 presence is a late event in endometrioid neoplasms, being altered in approximately one third of high grade tumours [212]. Thus, p53 mutation is associated with high grade tumours and poor prognosis.

We will evaluate the expression of this protein in the spectrum of endometrial tubal-type lesions due to its early appearance in serous carcinogenesis and speculation that TM is a potential precursor of this lesion [184].

3.2.3.7. Markers of microsatellite instability

Microsatelites are repetitive units of 1-4 DNA base pairs widely dispersed through the genome. The repetitive nature makes them susceptible to errors during replication, with consecutive deletion or insertions responsible for genomic instability [260]. In normal

conditions, the proteins encoded by mismatch repair gene (MMR), such as MLH1, PMS2, MSH2 and MSH6 recognize and correct these replicative errors. Inactivation of any of them leads to microsatelite instability (MSI). This status confers susceptibility to accumulation of other somatic mutations of tumour suppressor gene and oncogene (PTEN and K-ras). Thus, MMR proteins play an important role in tumour initiation and progression [261].

MSI has been reported in a variety of breast, thyroid and brain cancer. It is a constant feature of hereditary non-polyposis colorectal cancer (HNPCC), in which germ-line mutation of mismatch repair genes take place [183]. Endometrial carcinoma is the most common extracolonic tumour associated with HNPCC and, consequently, it presents a high frequency of MSI. However, this molecular phenotype is not so common in sporadic lesions, ranging from 9 to 45% between various studies [157 261 262].

The genetic ground of MSI phenotype in endometrial lesions is largely related to epigenetic silencing of MLH1 gene, secondary to promoter methylation and not to germ-line mutation [263]. Defective MLH1 gene will be followed by loss of expression of MLH1 and its heterodimer PMS2 proteins. MSH2 and MSH6 are rarely involved in MSI of sporadic endometrial cancer [264], but they appear to play a role in tumours with intermediate MSI (those positive for two to three of five markers) [261]. Although the mechanism of MSH6 inactivation seems to be caused by mutations [169], for MSH2 it is still unclear

MSI is regarded as an early event in carcinogenesis of type I endometrial carcinoma, due to its presence within preneoplastic endometrioid lesions and within non-neoplastic endometrium adjacent to MSI positive endometrial carcinoma [160 265-267]. The prognostic relevance of the lesions bearing this alteration remains debatable in the literature [157 260 268].

MSI detection is an inefficient method for precancer routine diagnosis, due to its rare presence in randomly sampled histological normal endometrium [160] and high frequence of microsatellite stable adenocarcinoma. Nevertheless, MSI evaluation remains a useful tool for patients with hereditary defects of DNA mismatch repair gene and for research purposes.

We will analyze the status of MMR proteins in the endometrial tubal-type lesions. The focality of the lesion did not permit molecular analysis mediated PCR and consequently an immunohistochemical study was carried out. Immunohistochemistry predicts MSI in endometrial carcinoma with a sensitivity and specificity only slightly inferior to PCR. The sensitivity was improved by using four MMR proteins in comparison with MLH1/MSH2 pairs [260]. The goal of immunostains is to detect complete absence of expression in tumour cell

nuclei compare with strongly, diffuse positivity of the nonneoplastic endometrium or normal stromal cells, used as internal controls. Loss of MLH1 almost always accompanies loss of PMS2, and loss of MSH2 accompanies loss of MSH6. The case should be reassessed if loss of MLH1 is seen together with loss of MSH2 or with MSH6 [260]. False positive cases can occur in the context of partial methylation with decrease, but not complete loss of the protein expression, or a mutation with the mutant protein still reactive [260].

We did not evaluate the expression of MMR protein within the cervix taking into account that MSI is rare event in cervical adenocarcinoma [269].

3.2.3.8. Phosphatase and tensin homolog (PTEN)

PTEN gene, located on the chromosome 10q23, was identified in 1997 by three independent groups. It was also called MMAC1 (mutated in multiple abvanced cancer) or TEP1 (TGF-β-regulated and epithelial cell-enriched phosphatase) [270-272]. PTEN is a tumour suppressor gene with an important role in cell survival, by its lipid phosphatase activity, and inhibition of cellular migration and adhesion by its protein phosphatase activity [169]. It leads to cell cycle arrest at G1/S point by negative modulation of phosphatidilinositol 3-kinase (PI3K)/protein kinase B (AKT) pathway, up-regulation of CDK inhibitor, p27 and down-regulation of cyclin D1 [273 274]. Also, it has been shown that PTEN controls p53 protein by direct interaction or antagonizing the AKT-MDM2 pathway, with eventual apoptosis induction [275 276].

Inactivation of PTEN gene, either by somatic mutation, deletion or promoter hypermethylation was frequently detected in prostate, endometrium, ovary, breast and brain tumours [277 278]. Instead, its germline mutations are responsible for the appearance of PTEN hamartoma tumour syndromes (PHTS), of which Cowden syndrome is the prototype [279]. The latter is associated with an increased risk of endometrial cancer.

Loss of PTEN protein expression is regarded as the most frequent genetic alteration of type I endometrial cancer, with a rate ranging from 35% to 83% [156 274 280 281]. Also, the prevalence of PTEN alteration in preneoplastic lesions was as common as 20 to 55% [156 274 277]. This variability in its loss could be related to different scoring methods and various antibody clones used for PTEN evaluation [186 282].

PTEN inactivation and acquisition of MSI were indentified very early in endometrial carcinogenesis in the so-called “latent precancer”, a preclinical stage in which only somatic

mutations are present, in the absence of morphological changes [36 150 160]. Therefore, PTEN has been proposed as a marker of the earliest endometrial precancer. In conditions of unopposed oestrogen exposure, its loss could be regarded as an initiator event, although probably not a determinating step in endometrial carcinogenesis [156]. Norimatsu *et al*. favours the utility of this antibody, together with β-catenin and p53, in the cytological diagnosis of endometrial lesions [283].

Taking into account these data, we evaluate the status of PTEN in tubal-type endometrial lesion, by immunostochemistry and FISH analysis, in order to verify the possible preneoplastic potential of TM.

3.2.3.9. K-ras

K-ras protooncogene belongs to retrovirus-associated DNA sequences family (RAS), initially isolated from Kirsten (K-ras) murine sarcoma virus. It encodes a protein ($p21^{ras}$) located on the inner plasma cell membrane, that has GTPase activity and functions as a molecular switch during cell receptor signaling. Activating point mutations, consisting in single aminoacid substitution in critical domains, are predominantly found in exon 1 (codons 12 and 13) and more rarely in exon 2 (codon 61). They are largely related to tumour growth and differentiation in many human cancers [284 285]. The incidence of these mutations varies widely, being documented in 90% of pancreatic adenocarcinoma, 50% of colon cancer, 30% non-small cell lung cancers and rarely found in breast cancer [286].

Activated mutations of K-ras protooncogne have been detected in about 10-30% of endometrioid adenocarcinoma [212 284 287]. Its presence in 16%-23% of endometrial hyperplasia, especially in atypical complex hyperplasia (ACH) and its absence in normal polyclonal tissue, support consideration of K-ras as the earliest detectable alteration of abnormal endometrial proliferation [158 287 288]. Therefore, its presence correlates with progression to malignancy.

The mechanisms of endometrial tumourgenesis induced by K-ras are not completely understood. Experimental studies showed that transcriptional activity of oestrogen receptors was upregulated by alterations of K-ras/Raf signaling pathway [285]. Additionally, long-term use of tamoxifen is associated with a higher prevalence of K-ras mutation and subsequent adenocarcinoma [289]. Also, K-ras plays a role in transferring the mutagenic signals from c-erbB-2 receptors, with stimulation of growth process [284].

Reported data regarding the correlation of K-ras with the clinical behaviour is conflicting [151 284].

We investigated K-ras status in 6 cases of tubal-type lesions, despite contradictory reports related to its mutation in preneoplastic endometrial lesions. Point mutation in codons 12, 13 and 61 of the K-ras gene were analyzed, since they are the most commonly involved.

3.2.3.10. β-Catenin

Another pathway involved in endometrioid adenocarcinoma pathogenesis includes participation of β-catenin and cyclin D1 (β-Catenin-TCF/LEF-1-cyclin D1). This led us to investigate the pattern of their expression in endometrial tubal metaplasia and compare it with normal, hyperplastic and malignant endometria.

Catenins are a family of structurally related cytoplasmic proteins, which in function of their electrophoretic mobility are classified in alpa (α), beta (β) and gamma (γ) catenin. β–catenin plays a dual role within the cells. It mediates the interaction of E-cadherin complex with actin of cytoskeleton, participating to cell adhesion, maintenance of epithelial integrity and polarized states [290]. Secondary, β–catenin binds to APC (adenomatous polyposis coli) and it is able to translocate into nuclei, where contributes to the Wnt signal transduction pathway [291].

In a normal state, β-catenin does not contain a nuclear localization signal and its free cytoplasmic level is low. A stabilization of β-catenin protein takes place in various conditions, such as: mutation of its gene, CTNNB1 [292], mutation or promoter hyperpmethylation of APC gene, decrease of β-catenin degradation or other molecular abnormalities involving Wnt pathway [293]. Further, the ubiquitin proteasome mechanism is overload and β-catenin accumulates within the cytoplasm and secondary translocates into nucleus. Here, β-catenin acts as an oncoprotein by formation of complexes with transcriptional factors of T cell factor/lymphoid enhancer factor-1 (TCF/LEF-1) and regulates expression of c-myc and cyclin D1 gene, promoting cell growth [235 236].

Molecular abnormalities of APC/β-catenin/Tcf pathway, with subsequent nuclear accumulation of β-catenin, have been reported in several human malignancies including colon, ovary, endometrium, prostate, hepatocellular carcinoma, melanomas and medulloblastoma [294].

Nuclear β-catenin is considered to be a molecular feature of type I endometrial adenocarcinoma, where is found with a prevalence of 20-55% [150 159 235 236 292 294] and it is exceptionally encountered in non-endometrioid forms (0-5%) [295]. The abnormal expression of β-catenin in preneoplastic lesions proves its contribution to the early phases of endometrioid carcinogenesis [159 296]. Additionally, activation of APC/ β-catenin/Tcf pathway has been found to be a constant molecular marker of tumours bearing morules not only of the female genital tract, but also of lung, thyroid and colon [292 297 298]. Therefore, β-catenin alteration may represent a common carcinogenetic pathway of tumours with a distinctive morphological appearance, characterized by low grade and stage at presentation and good prognosis [292 294].

The mechanism of β–catenin gene involvement in endometrial carcinogenesis remains unclear. It appears to function as a coactivator of steroid hormone receptors that regulates cell proliferation and differentiation [299]. β–catenin has been showed to be upregulated by oestrogen and tamoxifen [120 300] and, surprisingly, by progesterone therapy [301]. The functional consequences are complex and in the same time opposed. β–catenin may have an oncogenic effect by overexpression of c-myc and cyclin D1, the positive regulators of cell proliferation. Simultaneously, it induces activation of p53-p21WAF1 pathway leading to suppression of cell proliferation or induction of senescence [302]. This could explain the absence of proliferation of morular metaplasia in the context of β-catenin and cyclin D1 coexpression.

We analyzed β–catenin status of tubal-type lesions by immunostains, taking into account that β–catenin mutation has concomitant β–catenin nuclear staining and immunohistochemical assay of this oncoptotein has been proved to be accurate [159].

3.2.3.11. EGFR

Epidermal growth factor (EGF) system is ubiquitous in human organs and plays important roles in embryogenesis, proliferation and differentiation [303]. In cancer, the EGF system is involved in proliferation, migration, differentiation and angiogenesis [304]**.** Its actions are mediated through four receptors. One of them is epidermal growth factor receptor (EGFR or HER-1), a tyrosine kinase transmembrane receptor encoded by c-erbB-1 oncogene. EGFR serves as a receptor not only for EGF, but also for TGFα and therefore, it plays an important role in signal transduction.

EGFR has been found in a variety of normal and neoplastic tissues of bladder, breast, brain, stomach, ovary and endometrium [305]. Its increased expression was involved in the initial stages of cervical squamous cell carcinoma tumourigenesis [306].

A higher expression of EGFR was documented in endometrioid (46-71%), than in non-endometrioid adenocarcinomas (34-56%). EGFR seems to play a dual role in endometrial cancer, being associated with low grade and favourable outcome in type I, but with high grade and poor prognosis in type II endometrial carcinoma [307]. However, others did not find a clinico-pathological correlation of EGFR expression in these tumours [40]. Although, endometrial serous tumours in a high percentage of cases had an EGFR protein overexpression, no gene amplification or activating mutation in kinase domain were found [308]. Thus, it is unlikely that EGFR thyrosine kinase inhibitors will be effective as a single agents in the treatment of these tumours [309].

We will analyze the expression of EGFR in endometrial and cervical TM lesions mainly because of its common expression in endometrioid carcinoma precursors [310 311] and supposition that cervical TM represents a preneoplastic lesion, based on EGFR positivity [139].

3.2.3.12. Oestrogen and progesterone receptors (ER, PR)

Endometrium, one of the most dynamic human tissues, undergoes monthly and lifetime changes under the influence of ovarian steroid hormones, oestrogen and progesterone, which act through their correspondent receptors. Oestrogen (ERα and ERβ) and progesterone (PR-A and PR-B) receptors belong to the steroid/thyroid hormone nuclear receptors superfamily. They are ligand-dependent transcriptional factors which activated, bind to distinct DNA targets sites and modulate the expression of specific genes [312].

It is widely accepted that endometrioid adenocarcinoma is related to unopposed oestrogen exposure. Estradiol up-regulates ER and induces cellular proliferation with inhibition of apoptosis through complex, but not yet completely clear mechanisms [240]. Abnormalities of oestrogen receptors may confer growth advantage through transcriptional activation of ER-dependent genes, in the absence of hormone [151].

Progesterone acts through PR and represents the physiological negative regulator of oestrogen action. Expression of PR is under the control of oestrogen and progesterone, whereas oestrogen induces PR synthesis, progesterone downregulates both receptors. PR-A isoform counters oestrogen action directly by preventing ERα transcription, as also by

limiting PR-B, which acts as oestrogen antagonist [313]. Recently glycodelin, a bioactive substance produced by endometrial cells in response to progesterone, was proved to be partially responsible for the anti-proliferative effects of progesterone. It was speculated that glycodelin could be a target molecule in the management of endometrial preneoplastic and neoplastic lesions [314].

The expression and distribution of ER and PR isoforms appears to play an important role in normal endometrial function. However, their disrupted balance might be critical in endometrial pathogenesis and carcinogenesis [313 315].

In endometrial neoplasia and its precursor lesions, the rate of hormone receptors expression varies widely with the histopathological type, grade and stage on one hand and with methodology used and observer variability on the other hand [313]. The trend is toward a decrease of their expression in type II adenocarcinoma and in high grade and stage endometrioid adenocarcinoma [316 317]. Loss of sex steroid hormones is a common event in endocervical adenocarcinoma, and this feature is used as a useful criteria in making distinction between a well differentiated endometrial adenocarcinoma from an endocervical adenocarcinoma in biopsy or curetting settings [318 319].

We will assess the expression pattern of ER and PR in TM, taking into account that changes in their immunoreactivity are described in preneoplastic endometrial [312] and endocervical lesions.

3.2.3.13. Carcinoembryonic antigen (CEA)

CEA comprises a heterogeneous family of related oncofoetal glycoprotein secreted into the glycocaliceal surface of gastrointestinal cells.

CEA is a constant feature of colorectal adenocarcinoma and, subsequently, it is helpful in discriminating a metastatic tumour with this derivation from a primary non-mucinous ovarian adenocarcinoma. It is also variably expressed in adenocarcinomas of the pancreas and stomach. Nevertheless, it should be kept in mind that a tumour with mucinous differentiation will express CEA regardless of its location [320].

In female genital tract pathology, CEA is mainly used in two situations: as a discriminator marker of cervical preneoplastic lesions from benign mimickers [170 173 204] and as a site specific marker, to ascertain the endometrial or endocervical origin of a well

differentiated adenocarcinoma in curetting specimens [321]. CEA could be also useful in the diagnosis of primary vulvar Paget's disease [320].

The majority of the studies consider TM as the most common benign mimicker of AIS with only scarce, abortive reports suggesting its preneoplastic potential. We believe that CEA can supply evidence to clarify this dilemma.

3.2.3.14. Vimentin

Vimentin is the most widely distributed intermediate filament protein. The intermediate filaments have a well documented role in maintaining cellular structure integrity, but additional activities as its effect on apoptosis, cellular adhesion and migration have been recently reported [322]. Vimentin is a constant feature of normal mesenchymal cells and their correspondent neoplasm, including those of female genital tract. It is particularly assessed in poorly differentiated or anaplastic tumours. Except for these situations, vimentin can be useful in several instances of routine gynecopathological practice. Vimentin helps to distinguish TM (usually positive) from AIS (usually negative) [323 324]; to ascertain the endometrial (usually positive) or endocervical (generally negative) site of origin of a well differentiated adenocarcinoma [318 325 326] and to differentiate cervical microglandular hyperplasia from microglandular surface changes of an endometrioid adenocarcinoma [327]. Additionally, vimentin contributes to distinction of endocervical neoplasia, mainly minimal deviation adenocarcinoma (frequent negative) from mesonephric lesions (constant positive) [328].

3.2.3.15. CD10

CD10, a surface metalloendopeptidase, has been referred as common acute lymphoblastic leukemia (CALLA), nephrilysin or neural endopeptidase 24.11. It is expressed in early B-lymphoid differentiation and in germinal center B lymphocytes. Consequently, it is useful in the diagnosis and subclassification of hematopahological disorders derived from these cells [329]. CD10 is localized at the apical/luminal cell border, on the membrane/Golgi area or in the cytoplasm of the cells.

A wide spectrum has been described of normal tissues expressing CD10, such as: proximal convoluted renal tubules, trophoblastic cells, breast and salivary glands myoepithelial cells, melanocytes, prostatic glandular epithelium, liver canaliculi, bone

marrow stromal cells and pulmonary alveolar cells [330 331]. In non-hematopoietic neoplasms, CD10 was mainly detected in tumours of the genitourinary and gastrointestinal tract and malignant melanoma [332].

In the female genital tract, CD10 is a useful marker. It can be used for the diagnosis of endometrial stromal tumour, confirmation of extra-cervical endometriosis, the diagnosis of mesonephric and trophoblastic tumours and the assessment of the primitivity or metastatic origin (kidney) of a clear cell carcinoma [330 331]. Also, CD10 is a constant feature of morular metaplasia, but not of squamous differentiation [333]. Nevertheless, CD10 is not a specific marker for these lesions and therefore, it should not be used alone and interpreted out of the morphological context.

We assessed the expression of CD10 in an attempt to evaluate: the stromal changes around cervical TM glands, which can be misdiagnosed as endometrial stroma, the CD10 non-specificity for the diagnosis of mesonephric hyperplasia and to confirm the morular metaplastic foci associated with endometrial TM.

Chapter 4

Material and methods

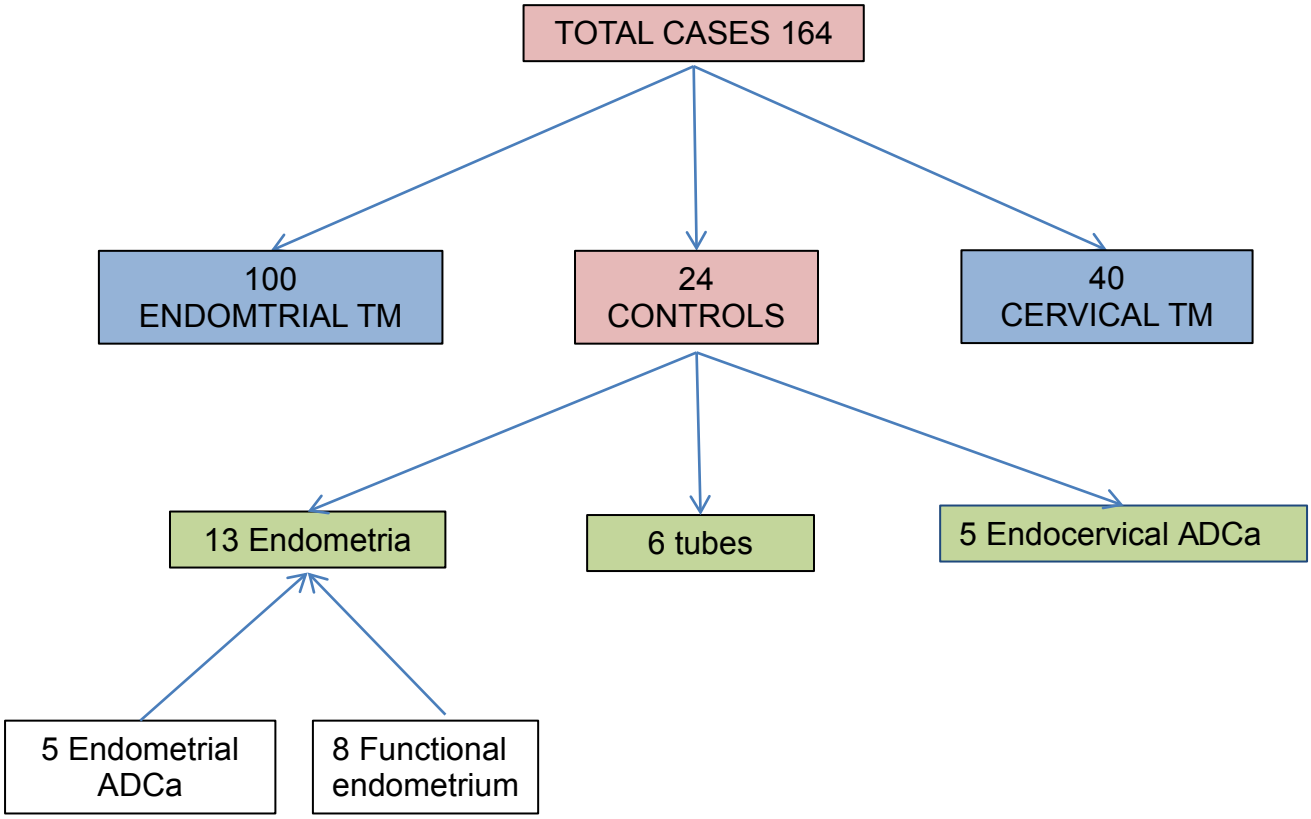
IV. Material and methods

4.1. Case selection

A total of 164 specimens were retrieved from routine and consultation files of Pathology Department of San Cecilio Hospital, Granada, Spain. These cases were diagnosed between1990-2010. 140 cases were chosen based on the coexistence of TM with various endometrial (100 cases) and cervical conditions (40 cases). Benign and neoplastic endometrial and endocervical lesions respectively concurrent with TM served as controls. Furthermore we analyzed 24 independent cases of 8 functional endometria (1 menstrual, 2 proliferative, 3 secretory, 2 decidua), 5 endometrial adenocarcinoma (4 endometrioid and 1 serous adenocarcinomas), 5 endocervical adenocarcinoma and 6 normal fallopian tubes. The distribution of cases used is presented in diagram 1. Clinical data were available for all patients. Anonymous use of redundant tissue for research purposes is part of the standard treatment agreement with patients in our hospital.

The cases included 96 endometrial biopsies (both aspirative and curettings), 6 cervical biopsies, 7 conisations and 49 surgical specimens of uterus (23 simple hysterectomies, 26 total hysterectomies with bilateral anexectomy) and 6 tubal samples from sterilisation procedure. All specimens were formalin fixed and paraffin embedded by conventional techniques. The number of sections, stained with hematoxylin-eosin, available for review ranged from 1 to 12, with a mean of 3. Each individual specimen was assigned and the slide comprising the representative, extensive and/or complex TM areas was selected. The presence of TM was confirmed by two pathologist (FFN, AN). Both in the endometrium and cervix, TM was an incidental microscopic finding associated with various normal, functional and pathological conditions.

Diagram 1. Distribution of the cases used



4.2. Histology

The chosen slides were evaluated histologically for the presence of TM, its extent, location, the size and architecture of the glands involved, relationship with the normal epithelium, association with other metaplastic changes and coexistence of other pathological lesions.

Related to *location,* we evaluated the existence of TM in the surface lining epithelia, glands or both.

The *extension* of the metaplastic change was graded as follows:

(1+) occasional glands were replaced or nearly replaced by metaplastic epithelium;

(2+) more than occasional glands were replaced or nearly totally replaced by metaplastic epithelium, but less than half of the glands available for histological examination;

(3+) greater than half of the glands contained metaplastic epithelium.

Related to *architecture*, we analyzed four distinct architectural groups that shared similar bland cytologic features, presenting either with simple ciliation or tubal type epithelium.

a. Simple TM (STM), defined by tubular glands with smooth contours and/or varying degrees of dilation and/or slightly ramified.
b. Complex TM in isolated glands (CTMI) defined by isolated, eventful distributed highly branched glands or occasional cribriform areas or with papillary buds.
c. Complex hyperplasia tubal-type (CHT), usually a focal endometrial lesion composed by numerous crowded, tubular, irregular glands, sometimes with cribriform or papillary formation.
d. Adenocarcinoma with ciliated features defined by extensive glandular fusion, cribriforme formation and invasive of stroma and myometrium.

The *coexistence of other forms of metaplasia* was also underlined. These included: surface papillary syncytial changes (SPSC), mucinous (MM), oncocytic, morular and squamous (in the endometrioid carcinoma's areas).

The *background stromal changes* were also evaluated. We considered these to be: desquamative, myxoid, inflammatory, atypical, edematous, dense and desmoplastic. The degree of inflammation around TM glands was also noted.

4.3. Immunohistochemistry

The most representative slide was selected and 4µm thick sections of the respective formalin fixed, paraffin-embedded tissue block was used for immunostains. Dako PT Link water bath system allowed the entire pre-treatment process of deparaffinization, rehydration and epitope retrieval. Tissue sections were incubated with a wide panel of antibodies, which included LhS28, $p16^{INK4A}$, bcl-2, Ki-67, p53, cyclin D1, CD10, oestrogen receptor (ER), progesterone receptor (PR), EGFR (epidermal growth factor receptor), β-catenin, PTEN, vimentin and PAX-2, hMLH1, hPMS2, hMSH2, hMSH6 and CEA (carcinombryonic anigen). Table 4.1 presents all the antibodies, their source, clone, dilutions, antigen retrieval and type of tissue used as positive control. When the positive internal control was missing, an external one was included in each immunostaining run. Negative controls were obtained by omission of the primary antibodies from the staining procedure.

Dako Autostainer Link 48 with a HRP polymer staining protocol, as a high sensitivity and signal amplification method, part of the Dako EnVisionTM FLEX Visualization Systems (see also Dako's educational materials on www.dako.com) was carried out. Diaminobenzidine (Dako) was used as chromogen and finally, the sections were counterstained in Harris' haematoxylin.

Table 4.1. Overview of the antibodies used and tissue processing details

Primary antibody	Clone	Dilution	Antigen retrieval	Positive Control	Vendor*
LhS28	Monoclonal mouse Sc-53224	1:200	Tris/EDTA pH 9.0	Normal tubal epithelium	Santa Cruz
$p16^{INK4A}$	Monoclonal mouse E6H4	prediluted	Tris/EDTA pH 9.0	Cervical squamous carcinoma	CINtec histology
Bcl-2	Monoclonal mouse 124	prediluted	Tris/EDTA pH 9.0	Lymphocytes	DAKO
Ki-67	Monoclonal mouse MIB-1	prediluted	Citrate pH 6.0	Tonsil	DAKO
p53	Monoclonal mouse DO-7	prediluted	Tris/EDTA pH 9.0	Serous endometrial carcinoma	DAKO
CyclinD1	Monoclonal rabbit SP4	prediluted	Tris/EDTA pH 9.0	Endothelium	DAKO
ER	Monoclonal rabbit SP1	prediluted	Tris/EDTA pH 9.0	Cervical/Endometrial stroma	DAKO
PR	Monoclonal mouse PgR 636	prediluted	Tris/EDTA pH 9.0	Cervical/Endometrial stroma	DAKO
β-catenin	Monoclonal mouse β-catenin-1	prediluted	Tris/EDTA pH 9.0	Vascular endothelial cells	DAKO
PTEN	Monoclonal mouse 28H6	prediluted	EDTA pH 9.0	Endometrial stroma, Normal proliferative endometrial glands	Master diagnostica
EGFR	Monoclonal mouse EGFR.113	prediluted	Tris/EDTA pH 9.0	Cervical squamous carcinoma	Master diagnostica
CD10	Monoclonal mouse 56C6	prediluted	Tris/EDTA pH 9.0	Normal tonsil	DAKO
CEA	Monoclonal mouse II-7	prediluted	Citrate pH 6.0	Normal colon	DAKO
PAX2	Monoclonal rabbit Z-RX2	prediluted	EDTA pH 8.0	Fetal kidney	Zymed
Vimentin	Monoclonal mouse V9	prediluted	Tris/EDTA pH 9.0	Cervical/Endometrial stroma	DAKO
hMLH1	Monoclonal mouse G168-15	1:10	Tris/EDTA pH 9.0	Normal endometria, Non neoplastic colonic mucosa	BD Biosciences
hPMS2	Monoclonal mouse A16-4	1:10	Tris/EDTA pH 9.0	Normal endometria, Non neoplastic colonic mucosa	BD Biosciences

hMSH2	Monoclonal mouse FE11	1:10	Tris/EDTA pH 9.0	Normal endometria, Non neoplastic colonic mucosa	Calbiochem
hMSH6	Monoclonal mouse 44/MSH6	1:40	Tris/EDTA pH 9.0	Normal endometria, Non neoplastic colonic mucosa	BD Biosciences

** Santa Cruz Biotechnology, Inc. Santa Cruz, California, USA; CINtec Histology (Mtm Laboratories AG) Heidelberg, Germany; Dako, Dako-Cytomation, Glostrup, Denmark; BD Biosciences (BD Pharmigen), San Diego, USA; Master Diagnostica, Granada, Spain; Lab Vision, Fremont, California, USA; Zymed Laboratories Inc (Invitrogen Corporation) San Francisco, USA; Calbiochem (EMD Biosciences Inc).,San Diego, USA.*

4.3.1. Immunoreactivity scoring

Semiquantitative and semiqualitative immunoreactivity was scored by two pathologists involved in the study (AN, FFN). We assessed the antibody distribution pattern between the glands and within the cells of individual gland, percentage of positive cells and intensity of reaction (based on the predominant staining intensity in the target cells). Table 4.2 summarizes the scoring system applied in evaluation of each antibody.

All these antibodies were evaluated in TM and other coexistent form of metaplasias. A comparison with normal functional endometrium, normal endocervical and tubal epithelium, all spectra of hyperplastic endometrial lesions and even endometrial or endocervical adenocarcinoma was reviewed. All antibodies were studied in the cervical, endometrial and tubal specimens. However, the number of cases evaluated for some antibodies was different, depending if a special antibody added or not diagnostic information for cervical and respectively endometrial lesions. For example, PTEN and β-catenin were extensively studied in the endometrium, but only in reduced number of cervical cases and the *vice-versa* situation was for CEA, CD10. We will analyze in detail these circumstances in the next chapter.

Inter-observer differences were resolved by consensus.

Table 4.2. The immunoreactivity scoring system used

Antibody	Cellular pattern	Glandular pattern*	Percentage	Intensity
LhS28	Apical (basal bodies)		<1%= 0, 1-10%=1, 11-50%=2, 51-80%=3, >81%=4	
$p16^{INK4A}$	Nuclear and/or cytoplasm.	mosaic/ heterogeneous/ diffuse	<1%= 0, 1-10%=1, 11-50%=2, 51-80%=3, >81%=4	negative=0, weak=1, moderate=2,

				strong=3
Cyclin D1	Nuclear	mosaic/ heterogeneous/ diffuse	<1%= 0, 1-10%=1, 11-50%=2, 51-80%=3, >81%=4	negative=0, weak=1, moderate=2, strong=3
Bcl-2	Cytoplasm	mosaic/ heterogeneous/ diffuse	<1%= 0, 1-10%=1, 11-50%=2, 51-80%=3, >81%=4	negative=0, weak=1, moderate=2, strong=3
PAX2	Nuclear	mosaic/ heterogeneous/ diffuse	0 (<1%), 1-10%=1, 11-50%=2, 51-80%=3, >81%=4	negative=0, weak=1, moderate=2, strong=3
Ki-67	Nuclear		<1%= 0, 1-10%=1, 11-30%=2, >31%=3	
p53	Nuclear		<1%= 0, 1-10%=1, 11-50%=2, 51-80%=3, >81%=4	negative=0, weak=1, moderate=2, strong=3
PTEN	Nuclear	heterogeneous/ diffuse	0 (<10%) PTEN "Null", 1 (11-50%), 2 (51-80%), 3 (>80%)	negative=0, weak=1, moderate=2, strong=3
MLH1 PMS2 MSH2 MSH6	Nuclear		0 (<10%) MLH1 "Null", 11-50%=1, 51-80%=2 >80%=3	negative=0, weak=1, moderate=2, strong=3
ER	Nuclear		<1%= 0, 1-10%=1, 11-50%=2, 51-80%=3, >81%=4	negative=0, weak=1, moderate=2, strong=3
PR	Nuclear		<1%= 0, 1-10%=1, 11-50%=2, 51-80%=3, >81%=4	negative=0, weak=1, moderate=2, strong=3
β-catenin	Membranous = only membrane positive Cytoplasmic = membrane + cytoplasm Nuclear = cytoplasm + nucleus		<1%= 0, 1-10%=1, 11-50%=2, 51-80%=3, >81%=4	negative=0, weak=1, moderate=2, strong=3
C-erbB-2	Membrane		0 (negative) 1+ (faint staining of a part of cell membrane in >10% of cells 2+ (moderate staining on entire cell membrane in >10% of cells 3+ (strong staining of the entire membrane in >10% of cells	

EGFR	Cytoplasmic	mosaic/ heterogeneous/ diffuse	0 (negative) 1+ (faint staining of a part of cell membrane in >10% of cells 2+ (moderate staining on entire cell membrane in >10% of cells 3+ (strong staining of the entire membrane in >10% of cells	
CD10	Membrane Luminal, Cytoplasmic.		0 (<1%), 1-10%=1, 11-50%=2, 51-80%=3, >81%=4	negative=0, weak=1, moderate=2, strong=3
CEA	Luminal Cytoplasmic		0 – negative 1+ apical 2+ weak, focal cytoplasmic staining 3+ strong diffuse cytoplasmic staining	
Vimentin	Cytoplasmic	heterogeneous/ diffuse		negative=0, weak=1, moderate=2, strong=3

** mosaic or chessboard (alternance of secretory and ciliated cells), heterogeneous (coexistence of positive areas with negative ones in the same gland or between the glands) diffuse (>80% or all the cells within the gland or all cells of totality of the glands were positive).*

Further annotations related to immunohistochemical evaluation methods:

Related to Ki-67, positive cells were defined as any nucleus with detectable staining compare with the negative control background level. The Ki-67 proliferative index was calculated as percentage of positive nuclei in a sample; at least 500 nuclei were counted under 400x magnification (10x ocular and 40x objective). In heterogeneous areas, the percentage of positive nuclei was estimated in at least 15 high power fields, and the average was calculated.

p53 was considered positive if distinct nuclear immunoreactivity was present. At least 500 cells in randomly selected fields were counted. Occasional cytoplasmic p53 staining was ignored.

Any nuclear reaction for cyclin D1 was considered positive, and its unusual cytoplasmic positivity was also recorded.

Due to the highly subjective in the interpretation of cytoplasmic staining, only the β-catenin nuclear pattern was considered as abnormal expression. Nuclear immunstaining irrespective of percent of stained nuclei was considered positive reaction.

The normal staining pattern of hMLH1, hPMS2, hMSH2 and hMSH6 is nuclear. Some cases showed weak cytoplasmic staining, but only the nuclear staining was documented. Loss of protein expression was defined as complete absence of nuclear staining within the

target cells. Cases that were judged to have complete immunonegativity (lack of protein expression) or a weak focal pattern or un-interpretable were reviewed by a third pathologist (JP) and an agreement was reached.

Taking into account that a close relation ($p<0.01$, Spearman correlation test) between intensity of the reaction and percentage of the cells was observed, we decide to use for our study the percentage of immunoreactive cells.

4.4. Mutational analysis

The small size, focality of the lesion and difficulty in recognize ciliated differentiation from the endometrioid one were the main limiting factors of laser microdisection capture and subsequent target molecular techniques. For this reason, we used immunoreaction for MMR proteins to evaluate the microsatelite instability (MSI) and FISH to assess PTEN gene status. For characterizing K-ras mutations PCR was performed.

4.4.1. Evaluation of PTEN by FISH

Interphase fluorescence in situ hybridization (FISH) analysis was applied to the sequential sections of paraffin-embedded tissue to investigate the occurrence of PTEN genomic deletion in 19 of 100 ciliated endometrial lesions (5 STM, 6 CTMI, 8 CHT). Dual-color FISH was performed using commercially available DNA probe for cytoband 10q23 (Spectrum Orange PTEN locus-specific probe) and region 10p11-q11 (Spectrum Green centromere of chromosome 10 probe) (CEP10 α satellite probe 10p11-q11 LSI PTEN/CEP 10; Abbott Molecular Inc.).

Pre-treatment of slides, hybridization, post-hybridization processing, and signal detection were performed as reported elsewhere [278]. Sample showing sufficient FISH efficiency (> 90% nuclei with signals) was evaluated. Signals were scored in at least 100 no overlapping, intact nuclei. Non-neoplastic stromal cells present in the specimen were used as a control. The interest lesion was considered to show hemizygous deletion of PTEN when >20% of its nuclei contained one PTEN locus signal and two CEP 10 signals. Homozygous deletion of PTEN was defined by the simultaneous lack of the both PTEN locus signals and by the presence of control signals in >30% of cells [278].

4.4.2. Evaluation of K-ras mutation by PCR

5 cases of CHT and one extensive STM were chosen for further mutational analysis. The haematoxylin-eosin slides served as map for selection of representative areas. Subsequently the correspondent paraffin embedded tissue was supposed to a punch resection and considered suitable for PCR analysis. After deparaffinisation, the tissue was homogenized for 2 min in lysis buffer using an Ultra-turrax (T10 Basic, IKA, Germany). DNA was isolated according to the manufacturer's protocol of the QIAamp DNA FFPE Tissue Kit of QIAGEN and automated on the QIAcube (http://www.qiagen.com/products/automation /qiacube.aspx).

The quantity and quality of total DNA was estimated using the Nanodrop ND-100 Spectrophotometer (Thermo Scientific, USA). Only samples with a 260/280 absorbance ratio greater than 1.8 were used.

All the DNA samples were treated with EpiTect Bisulfite Kits of QIAGEN that enable complete conversion of unmethylated cytosines to uracils and subsequent purification. The highly sensitive method use innovative protection against DNA degradation that guarantees sensitive results even from 1 ng DNA and ensures high conversion rates of over 99%.

We have used the pyrosequencing assay based on nested polymerase chain reaction (PCR) to characterize K-ras mutation status using formalin-fixed and paraffin-embedded tissues with the QIAGEN KRAS KIT. Mutant and wild-type K-ras DNAs were used as control positive and negative respectively. Pyrosequencing technology, which is based on the principle of sequencing by synthesis, provides quantitative data in sequence context within minutes. PyroMark Q24 is a fully integrated system that provides real-time sequence information and is highly suitable for epigenetics research and genetic analysis. The system includes PyroMark Q24, PyroMark Q24 Vacuum Workstation, PyroMark Q24 Software, PyroMark Gold Q24 Reagents, PyroMark Control Oligo, and PyroMark Q24 Validation Oligo. Sample preparation solutions are also supplied to enable preparation of single-stranded DNA using the PyroMark Q24 Vacuum Workstation.

4.5. Statistical study

To facilitate the interpretation of the results, all the data were recorded in a MS Excel data sheet. Fisher's exact test and Spearman correlation rank test were performed to

determine the statisticsal significance and the relationship between the analyzed variables The SPSS19 software package for Windows (SPSS, Inc., Chicago, IL) was used. $p \leq 0.05$ was regarded as significant. 334. When the cases presented a $p=0.000$, we presented them as $p<0.01$.

Chapter 5

Results

V. Results

5.1. Endometrium

5.1.1. Clinical profile

The age of the patients ranged between 33 and 84 years, with a mean of 53.1 years. 73% of them were perimenopausal or postmenopausal and the remaining 27% were in their reproductive years.

More than half of the women (n=54) presented a history of abnormal uterine bleeding. Ultrasonographic findings revealed thickened endometrium >5mm (n=20), polypoid lesions (n=35) or leiomyomas (n=7). Other findings included uterine prolapse (n=1), cervical HPV-related pathology (n=2), previous diagnosis of atypical hyperplasia (n=1) or endometrioid adenocarcinoma (n=1) and ovarian tumour (n=1). Two patients were treated respectively with GhRH analogues and with tamoxifen. Finally, one patient was pregnant.

5.1.2. Morphological findings

5.1.2.1. Histological criteria

Histological criteria used for the diagnosis of tubal metaplasia included endometrial/endocervical glands in which the normal proliferative/secretory/mucinous epithelium was replaced wholly or partially by tubal-type epithelium. The tubal metaplastic glands with smooth luminal border were lined by the three cellular types: ciliated (non-mucin-secreting or clear cells), secretory (non-ciliated) cells and “peg” or intercalary cells (Fig. 1a). The ciliated cells presented abundant pale or clear cytoplasm with numerous apical cilia, large, oval, sometimes hyperchromatic nuclei with variable nucleoli. The non-ciliated cells presented basal small nuclei, dark, eosinopilic to basophilic cytoplasm, without mucin vacuoles, but with apical cytoplasm protrusions. The “peg” cells were defined by their small

hyperchromatic nuclei located in a clear gap. Also, cases of *ciliated metaplasia,* with a dominant population of ciliated cells, were included in our study (Fig.1b).

Figure 1. A comparison of tubal type with ciliated metaplasia

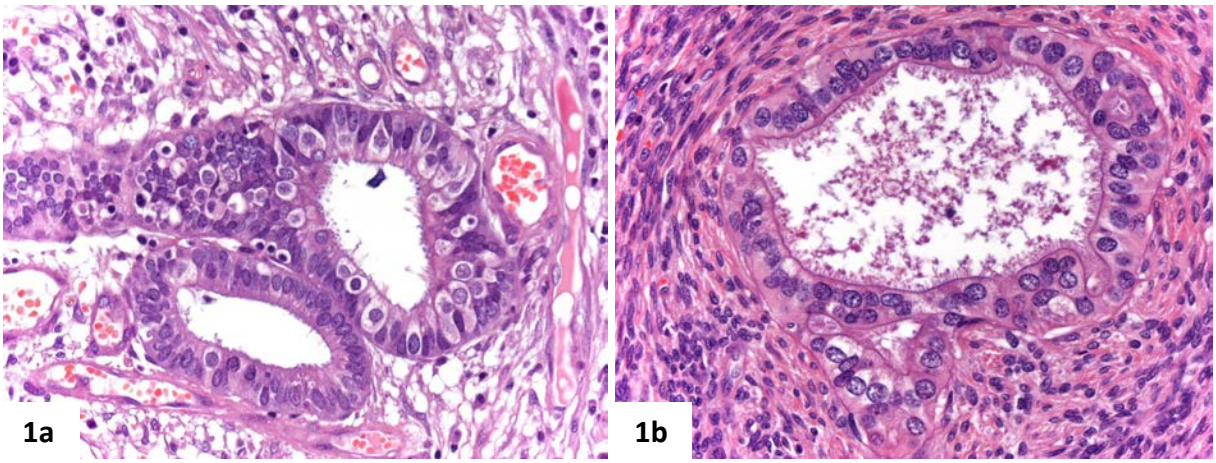


Fig.1a *endometrial glands lined by tubal-type epithelium, with the characteristically presence of ciliated, secretory and intercalary cells;* ***Fig1b*** *endometrial gland lined by ciliated cells.*

5.1.2.2. Topography

TM involved both the surface and endometrial glands in 58% of cases. In the remaining, it was found in the glands in 40% of cases and in only 2% was it limited to the surface epithelium.

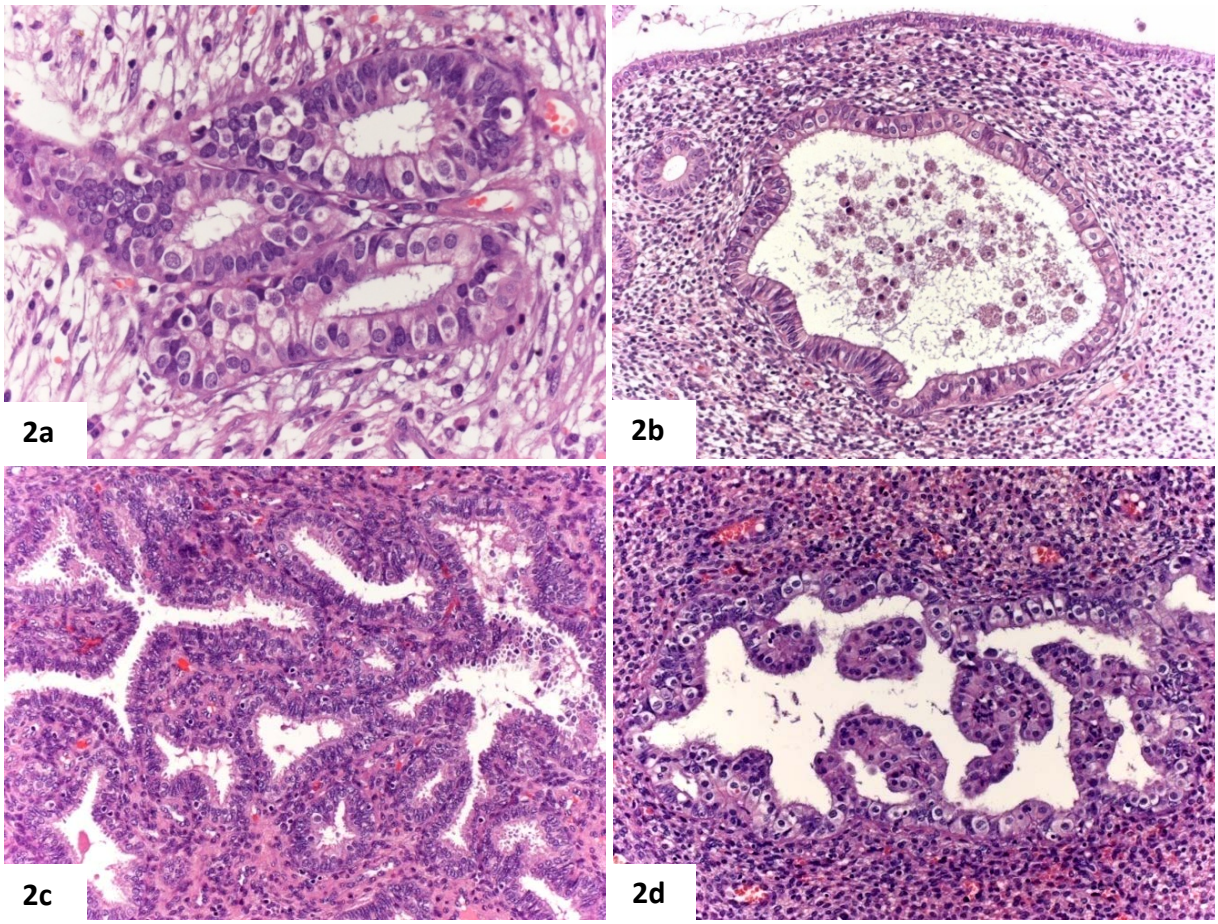
71% of endometrial cases presented more than occasional glands (2+) involved by a tubal type or ciliated epithelium and occasionally (7%) a diffuse involvement (3+) could be seen.

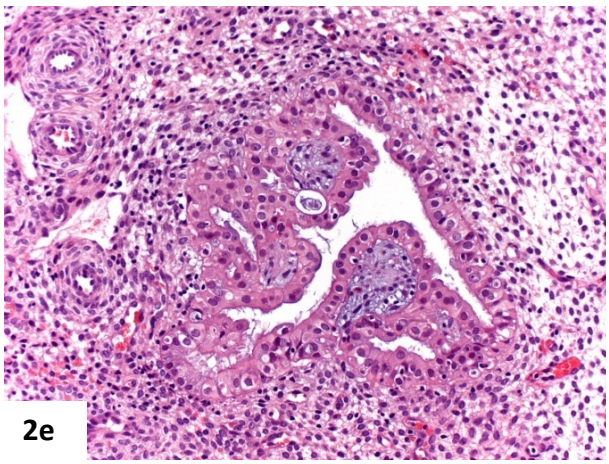
5.1.2.3. Architectural pattern

Endometrial glands lined by a ciliated or tubal epithelium presented a wide range of architectural pattern, ranging from simple to highly complex. We defined three morphological subgroups based mainly on the architectural complexity. *Simple TM (STM)* is defined by normal size (Fig.2a) or cystically dilated glands unevenly distributed (Fig.2b). Also, non-crowded glands with slight stellate or branching, angular contours were included in the same category. The lining tubal-type epithelium was non-stratified or pseudostratified and with bland cytology. A gray zone category with occasional, dispersed and not crowded highly branched glands, (Fig.2c) or with micropapillary (Fig.2d) or cribriforme (Fig.2e) formation was observed, and the term used was *complex TM in isolated glands (CTMI).* When the

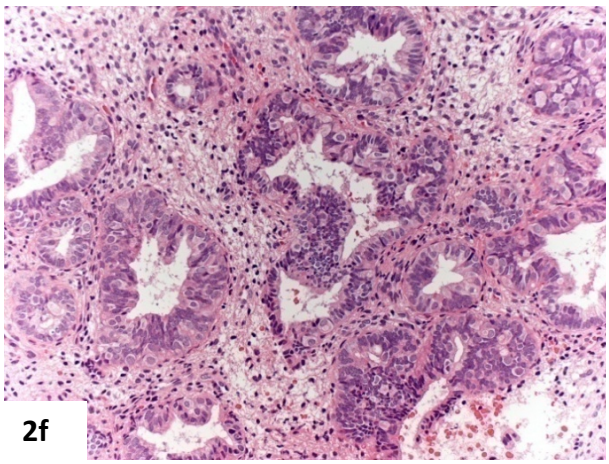
ciliated, tubal glands exhibited a crowded arrangement (Fig.2f), with back-to-back simple tubules (Fig.2g) or with complex changes such as intraluminal tufts (Fig.2h), or micropapillae (Fig.2i), papillae verae with fibrovascular core (Fig.2i, 2j) the term used was *complex hyperplasia tubal-type (CHT).* The same acronym has been applied for cellular stratification that created a cribriform configuration (Fig.2k), focal glandular fusion (Fig.2l) or a solid pattern with microcystic spaces formation (Fig.2m). 72 of cases presented only simple TM, 11 fitted in CTMI and 17 were classified as CHT. These latter cases had also associated areas of STM and consequently the total number of STM cases was 89. 3 of CHT were seen in vicinity of adenocarcinoma, two of them presenting focal and respectively extensive ciliated differentiation (Fig. 2n, 2o). Mild to moderate atipicality and mitotic figures (Fig.2p) were occasionally found. Therefore, no striking cytological differences were observed in the spectrum of ciliated, tubal type lesions from STM to tubal type endometrial adenocarcinoma. Only the extensive areas of meandering glandular structures and associated myometrial invasion help us to differentiated CHT from adenocarcinoma.

Figure 2. Architectural patterns of tubal metaplasia

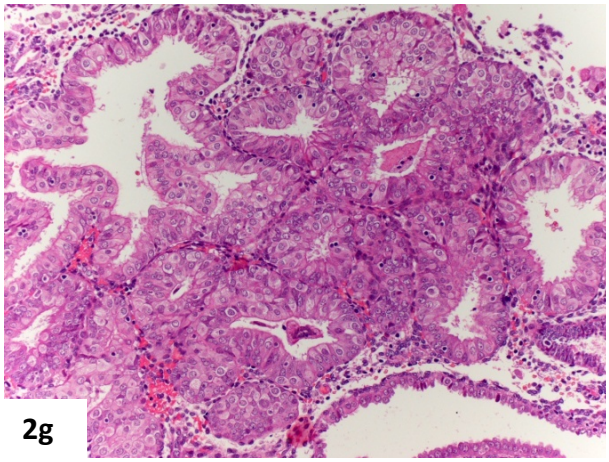




2e



2f



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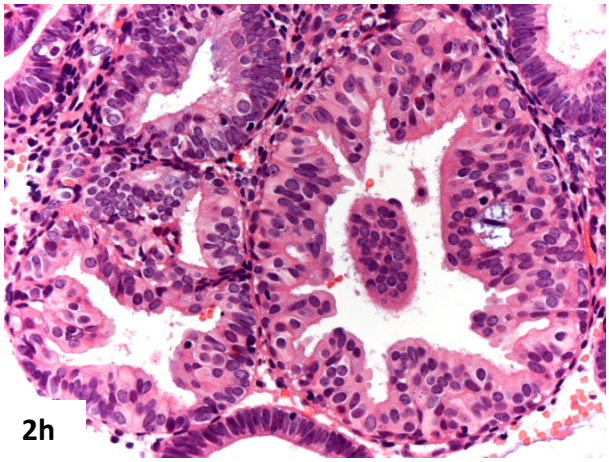
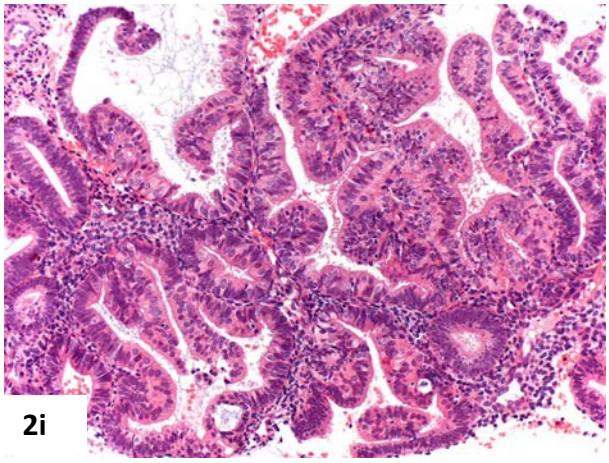
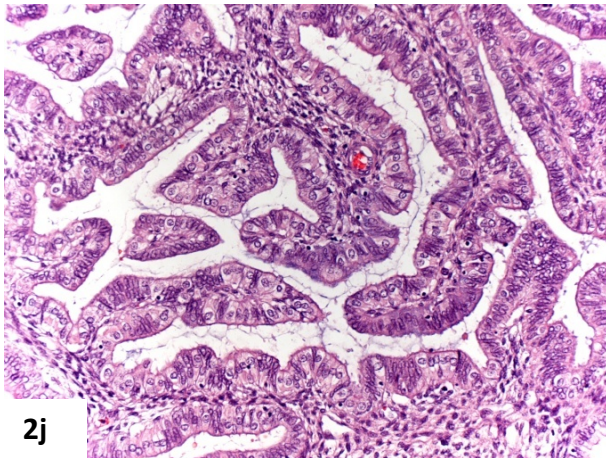


2h



2i



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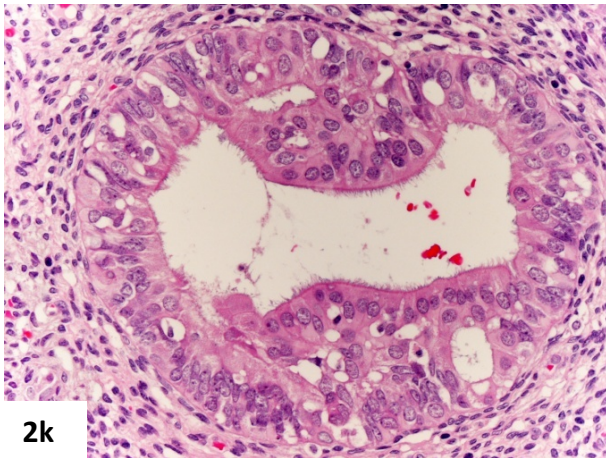

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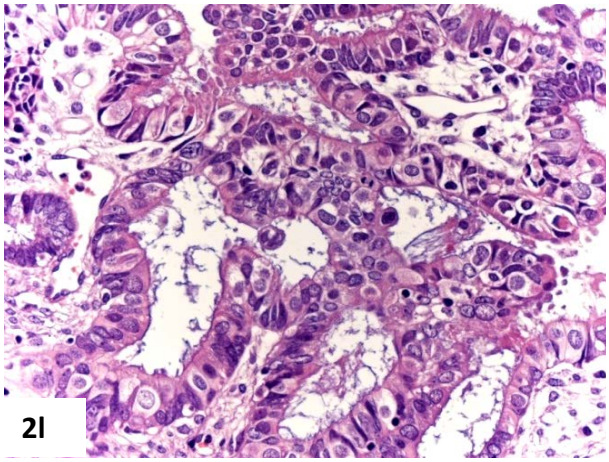


2l

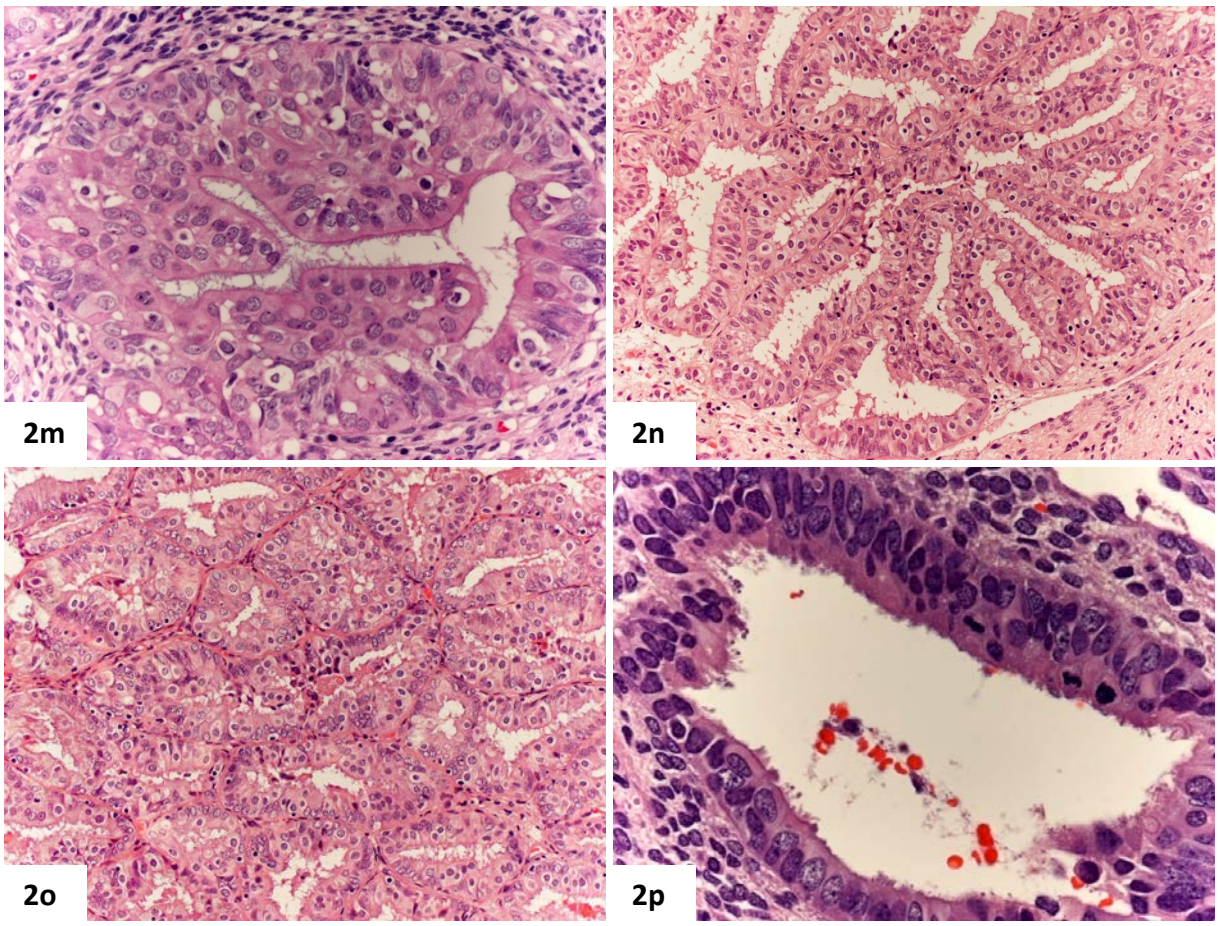


Fig.2a *STM with normal size glands, lined by a monolayer tubal-type epithelium;* ***Fig.2b*** *STM with cystically dilated glands in a case of simple endometrioid hyperplasia;* ***Fig.2c*** *highly branched gland that corresponded to CTMI;* ***Fig.2d*** *micropapillary change in an isolated glands of CTMI;* ***Fig.2e*** *cribriforme formation in an isolated gland of CTMI;* ***Fig.2f*** *crowded arrangement of various sized glands in CHT;* ***Fig.2g*** *back to back tubular glands lined by a tubal type epithelium in CHT;* ***Fig.2h*** *crowded glands with cellular stratification, rising into intraluminal tufts, diagnosed as CHT;* ***Fig.2i*** *micropapillae and papillae verae in CHT; fig.2j intraluminal papillae with a vascular core in CHT;* ***Fig.2k*** *cellular stratification with cribriforme formation in CHT;* ***Fig.2l*** *focal glandular fusion in CHT;* ***Fig.2m*** *stratified cells that almost obliterate the gland lumina, producing a solid pattern in CHT;****Fig.2n*** *extensive glandular fusion in tubal type endometrial adenocarcinoma;* ***Fig.2o*** *extensive back-to-back pattern with absence of endometrial stroma between the glands in a well differentiated tubal-type endometrial adenocarcinoma;* ***Fig.2p*** *moderate nucler atypicality and mitotic figures in a tubular glands of STM.*

5.1.2.4. Morphological context of TM presence

Table 5.1 presents the subcategories of ciliated lesions and the functional and pathological background of their appearance. The majority of STM were found in the context of oestrogen related lesions such as endometrioid hyperplasia (37/72, 51.8%) or endometrial polyps (19/72, 26.38%). More than half of CTMI were found in endometrial polyps, adenomyoma or adenofibrma (6/11). 9/17 CHT were associated in biopsies with scattered glands or areas of proliferative appearance endometria. Five of CHT were

developed in polyps, and two of them were seen in continuity with endometrial adenocarcinoma.

Table 5.1. Cyclic endometria and endometrial lesions associated with TM

Cyclic endometria and endometrial lesions	STM (n=72)	CTMI (n=11)	CHT (n=17)	Total 100
Functional endometrium				**Total 21**
▪ proliferative	4	2	9	15
▪ secretory	2	0	0	2
▪ menstrual	1	0	0	1
▪ atrophy	1	0	1	2
▪ decidua	0	1	0	1
Iatrogenic endometria	0	1	0	1
Hyperplasias				**Total 39**
▪ simple without atypia	27	1	1	29
▪ complex without atypia	3	0	0	3
▪ complex with atypia	7	0	0	7
Polyps	19	3	3	**Total 25**
Adenomyoma/ adenofibroma	3	3	0	6
Endometrioid ADCa	5	0	3	8

5.1.2.5. Other types of metaplasias and changes associated with TM

In the same slide, in 46% of endometrial cases one or more of *other types of metaplasia* coexisted with TM. The most common association was with surface papillary syncytial changes (SPSC) (28%), followed by mucinous (MM) (19%), clear and eosinophilic (5%), morular metaplasia (4%), hobnail (1%) and finally squamous (Sq) (1%). In eight (8) cases two or more type of metaplasia were coupled.

Table 5.2. Cyclic endometria and endometrial lesions associated with various types of metaplasia

Cyclic endometria and endometrial lesions	TM	SPSC	MM	Morules	Sq	Others*
	100	28	19	4	1	6
Functional endometrium	21	4	1	0	0	0
▪ proliferative	15	1	1	0	0	1
▪ secretory	2	0	0	0	0	0
▪ menstrual	1	1	0	0	0	0
▪ atrophy	2	1	0	0	0	0
▪ decidua	1	1	0	0	0	0
Iatrogenic endometria	1	0	0	0	0	0
Hyperplasias	39	21	8	2	0	0
▪ simple without atypia	29	15	2	0	0	1
▪ complex without atypia	3	1	1	0	0	0
▪ complex with atypia	7	5	5	2	0	4
Polyps	25	1	10	1	1	0
Adeno-myoma/fibroma	6	1	0	0	0	0
Endometrioid ADCa	8	1	0	1	0	0

**Others including miscellaneous changes as clear cell, eosinophilic and hobnail*

In the following, we briefly evaluate the morphological features of these endometrial metaplasias and changes that join TM.

Within the endometrium the most frequent association of TM was with *surface papillary syncytial change* (SPSC). This change often accompanies focal signs of breakdown (stromal balls, fibrinoid trombi and acute inflammation) seen in endometrial hyperplasia (21/28, 75%). SPSC has been identified as coalescing aggregates of eosinophilic epithelium, without evident cellular borders (syncytium) that rise into pseudopapillary tufts (Fig.3a) or rarely, in true papillae with a fibrovascular core (Fig 3b). It has been regularly associated with cellular debris and neutrophilic infiltrate (Fig.3c). This alteration was typically confined to the endometrial surface epithelium, encircling stromal balls (Fig.3d), or sometimes, involved the subjacent glands (Fig.3e). Sometimes SPSC cells presented a mild to moderate nuclear atypia in the absence of mitotic activity (Fig.3f).

Figure 3. Morphological features of surface papillary syncytial change

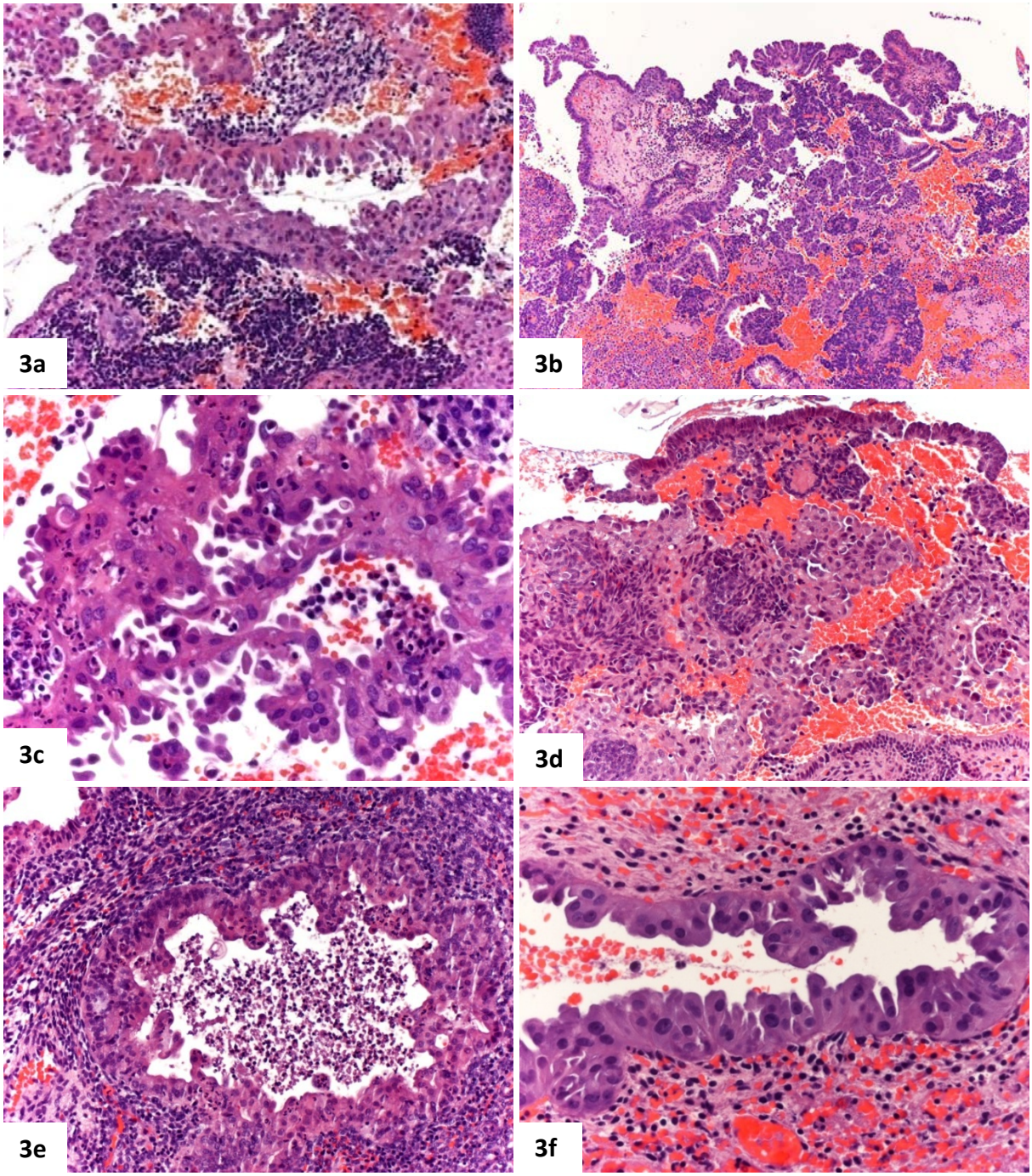
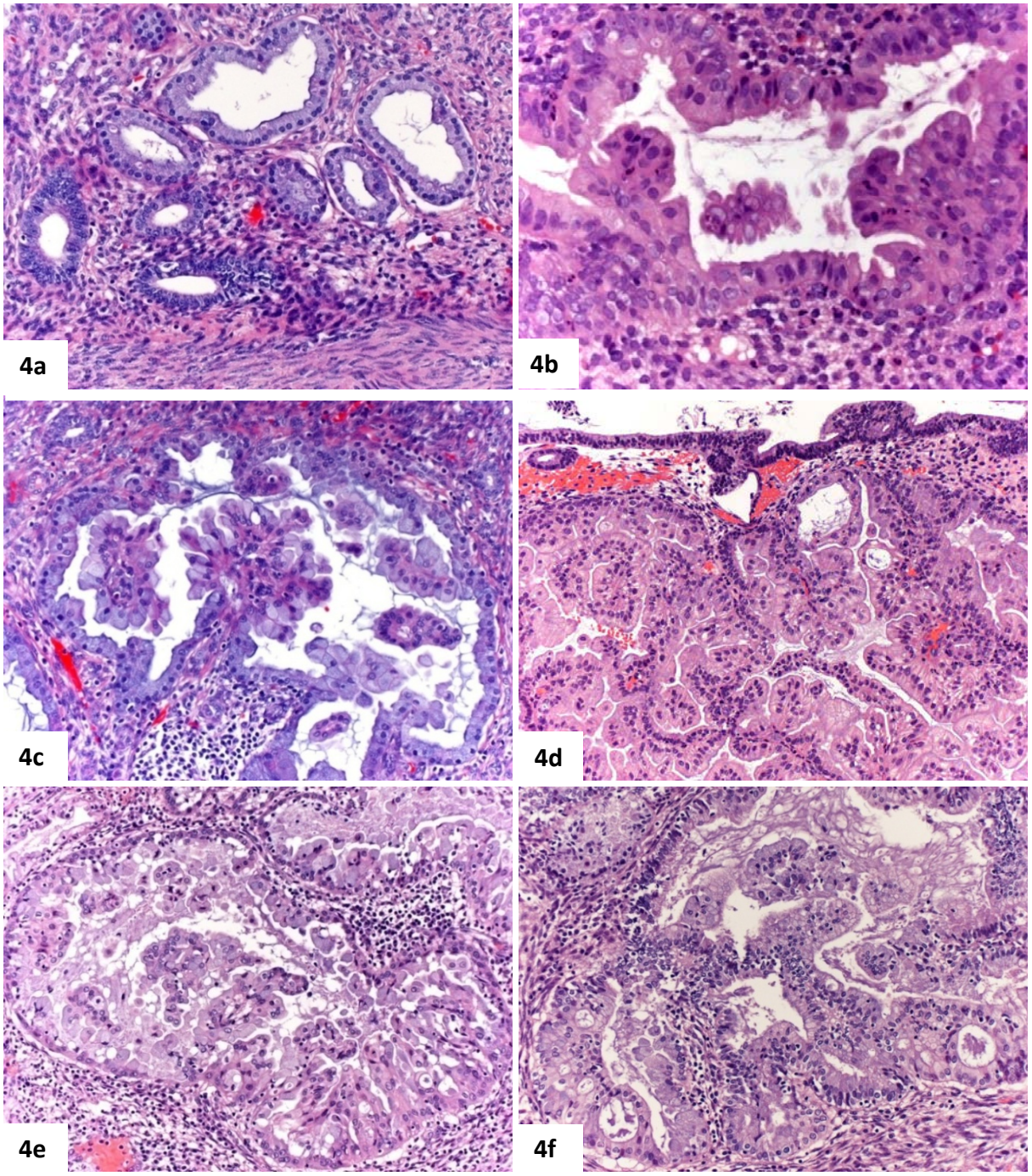


Fig.3a *aggregates of esinophilic cells, without a cellular border replacing surface endometrial epithelium;* ***Fig.3b*** *SPSC represented by short papillae with fibrovascular core;* ***Fig.3c*** *micropapillary aggregates associated with nuclear debris and neutrophils;* ***Fig.3d*** *eosinophilic syncytial aggregates encircling basophilic stromal balls on a hemorrhagic background;* ***Fig.3e*** *micropapillary syncytial changes involving a glandular lumina;* ***Fig.3f*** *moderate nuclear atypia of SPSC cells.*

19 of TM cases had associated *endocervical-type mucinous metaplasia,* characterized by replacement of normal surface and/or glandular endometrial epithelium by tall, mucinous secreting cells of endocervical type. The majority of these alterations correspond to *simple* mucinous metaplasia (Fig.4a) with occasional tufts formation (Fig.4b). Three cases

display a *complex* architecture, with a papillary (Fig.4c, 4d) and micropapillary arrangement (Fig.4e), sometimes fused in a cribriform pattern (Fig.4f). Exceptionally, a polyp was involved by surface complex papillary mucinous lesion (Fig.4g, 4h).

Figure 4. Architectural patterns of endocervical type mucinous metaplasia



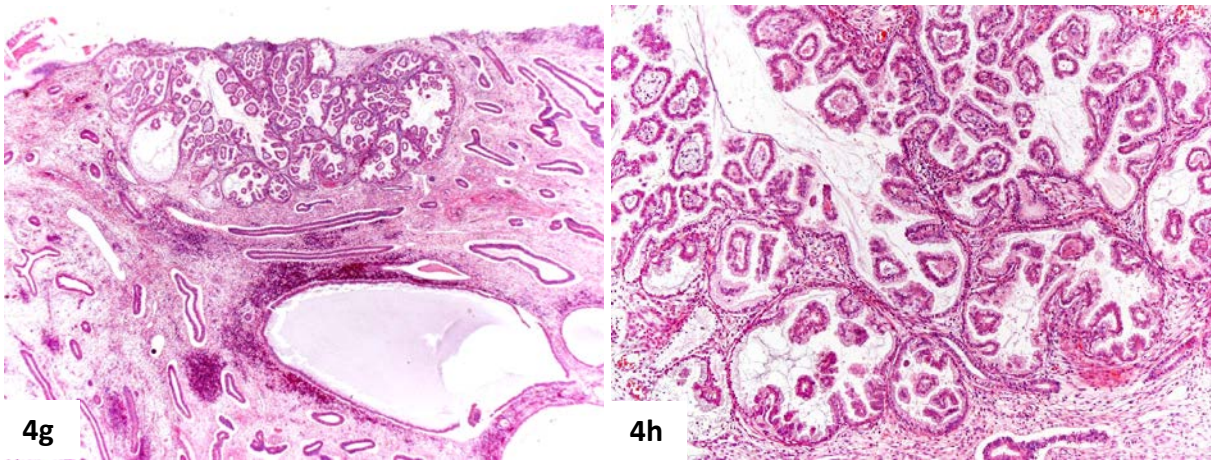


Fig.4a *tubular endometrial glands lined by a monolayer of mucinous endocervical-type cells;* ***Fig.4b*** *focal stratification with tufts formation of mucinous metaplastic epithelium;* ***Fig 4c*** *mucinous metaplasia with intraluminal papillary formation;* ***Fig.4d*** *complex mucinous metaplasia with highly convoluted papillae;* ***Fig.4e*** *complex mucinous epithelium with micropapillary formation;* ***Fig 4f*** *confluence of the micropapillae into a cribriforme pattern;* ***Fig.4g*** *mucinous surface complex papillary changes involving focally an endometrial polyp;* ***Fig.4h*** *detail of the complex architecture of the latter lesion*

Together with mucinous metaplasia, we observed other forms of metaplasia and changes, such as eosinophilic (Fig.5a), tubal (Fig.5b) or clear cells (Fig.5c) that involved the same or different glands.

Figure 5. Mixture of mucinous metaplasia with others forms of metaplasia and changes

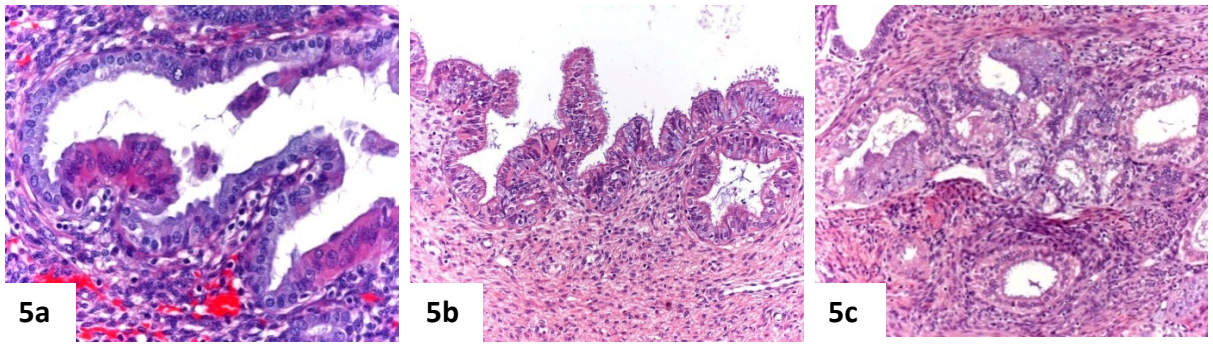


Fig.5a *mucinous endocervical-type cells mixed with eosinophilic, ococytic cells;* ***Fig.5b*** *surface and glandular epithelium of the isthmus present a homogeneous combination of ciliated and mucinous cells;* ***Fig.5c*** *mucinous glands coupled with ciliated, tubal metaplastic glands and foci of clear cell changes.*

An unusual metaplastic phenomena was association of cystic TM with *intestinal type mucinous epithelium*, which presented the characteristically morphology (goblet cells, tall cells with brush border and Panneth cells) (Fig.6a, 6b) and immunophenotype (positivity for CK20 (Fig.6c), CDX2 (Fig.6d), chromogranin (Fig.6e) and vilin (Fig.6f)) as intestinal mucosa.

Figure 6. Intestinal-type mucinous metaplasia of the endometrium

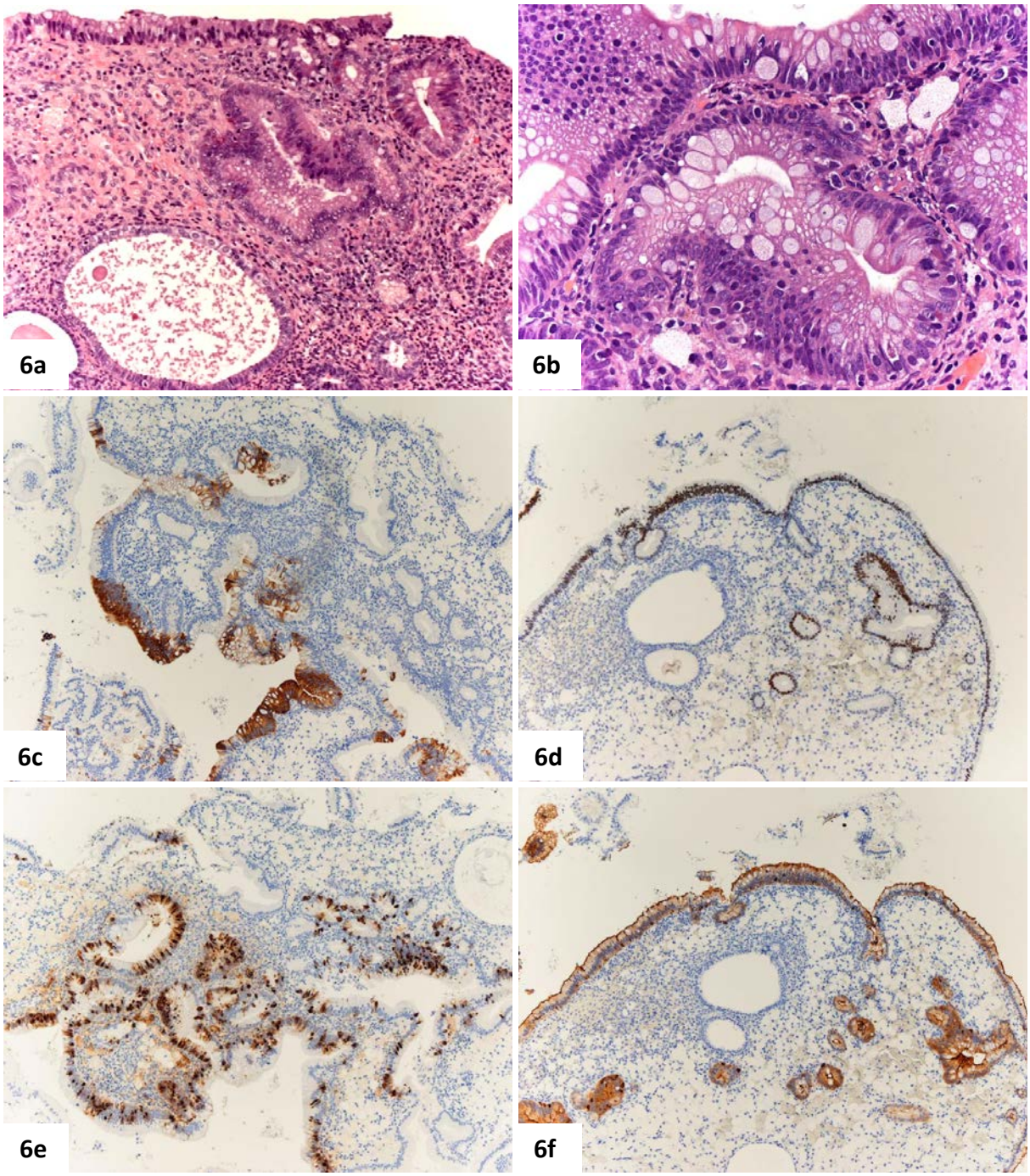


Fig. 6a *the surface and subjacent endometrial glandular epithelium were replaced by intestinal-type cells; in the left corner a cystic tubal-type gland;* ***Fig.6b*** *endometrial gland lined by goblet, tall and Panneth cells;* ***Fig.6c*** *focal positivity for CK20 in the area of intestinal differentiation;* ***Fig.6d*** *CDX2 nuclear immunoreactivity of intestinal metaplasia, the glands lined by tubal-type cells are negative;* ***Fig.6e*** *numerous neuroendocrine cells positive for chromogranin;* ***Fig.6f*** *apical positivity for vilin restricted to intestinal type epithelium.*

Finally, mucinous differentiation was seen as an altered differentiation in two cases of well differentiated endometrioid carcinoma, which belonged to the control group.

Morular metaplasia coexisted with TM in 4 cases. We were able to see various stages of morular development from isolated clusters of cells with eosinophilic cytoplasm (Fig.7a) to well formed mulberry aggregates that filled and deformed the glandular lumina (Fig.7b). Three cases associated preneoplastic (n=1) and neoplastic endometrial lesions (n=2). The remaining case was seen in the absence of architectural complexity, within a polyp (Fig.7c). The transition with the squamous epithelium was evident in a control case of endometrioid adenocarcinoma (Fig.7d).

Figure 7. Morular metaplasia

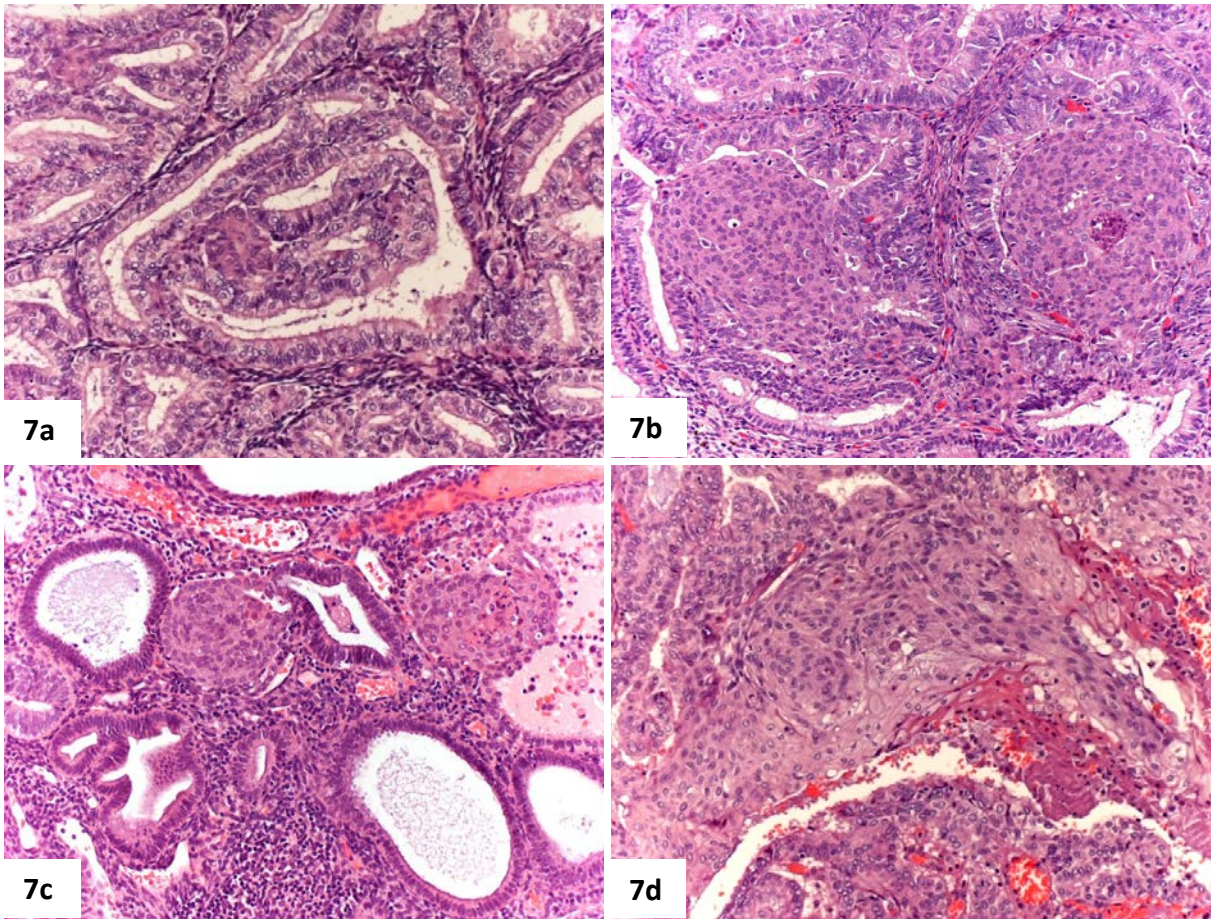


Fig.7a *small, eosinophilic clusters of cells forming incipient morules within a well differentiated ciliated-type adenocarcinoma;* ***Fig.7b*** *mulberry like aggregates filling and distorting the glandular lumina of a complex endometrioid hyperplastic lesion;* ***Fig.7c*** *morules within a stroma of an endometrial polyp, in the absence of complex glandular architecture;* ***Fig.7d*** *transition from morular to keratinizing, squamous metaplasia in a well differentiated endometriod adenocarcinoma.*

An isolated case of benign *squamous metaplasia* was seen in non-neoplastic glands of an endometrial polyp (Fig.8a), a case in which the squamous cells were associated with mucinous metaplastic cells (Fig.8b). In two of the endometrioid adenocarcinoma of the

control group, squamous differentiation was present together with mucinous and respectively morular differentiation.

Figure 8. Squamous metaplasia in non-neoplastic endometrium

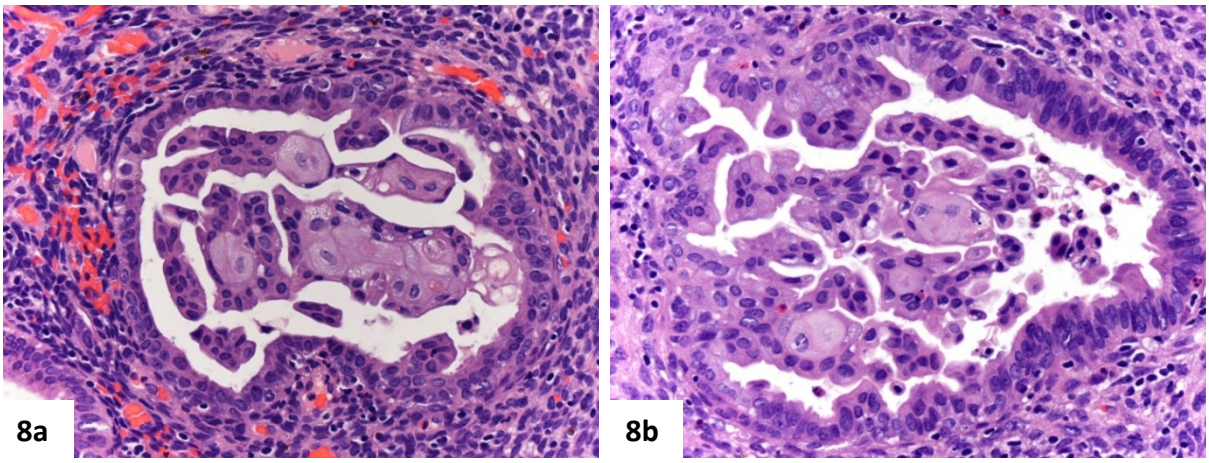
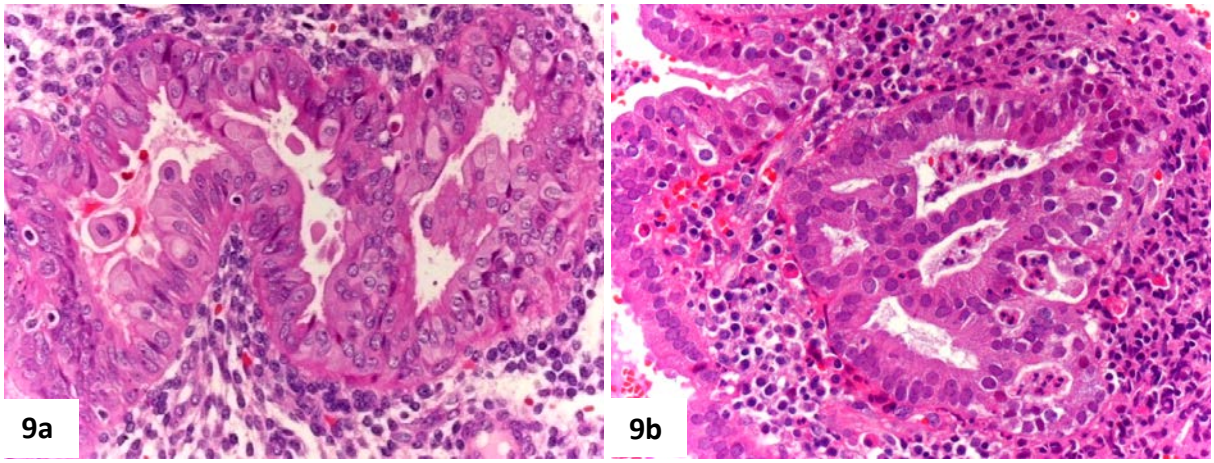


Fig.8a *isolated gland with large, eosinophilic squamous cells differentiated within its lumina;* ***Fig.8b*** *in the same case, another endometrial gland presented mucinous and squamous differentiation.*

Among the *reactive changes,* five TM cases were associated with *eosinophilic changes*. Four of them involved atypical complex hyperplastic glands (Fig.9a, 9b), and the the 5th one was seen in the context of proliferative endometria. In two cases, cells with *oncocytic* features were seen lining endometrial glands (Fig.9c) sometimes exhibiting cilia at their lumina border (Fig.9d). In another, the transition of eosinophilia with clear or mucinous changes was evident (Fig.5a, 5c).

Figure.9. Eosinophilic and oncocytic changes



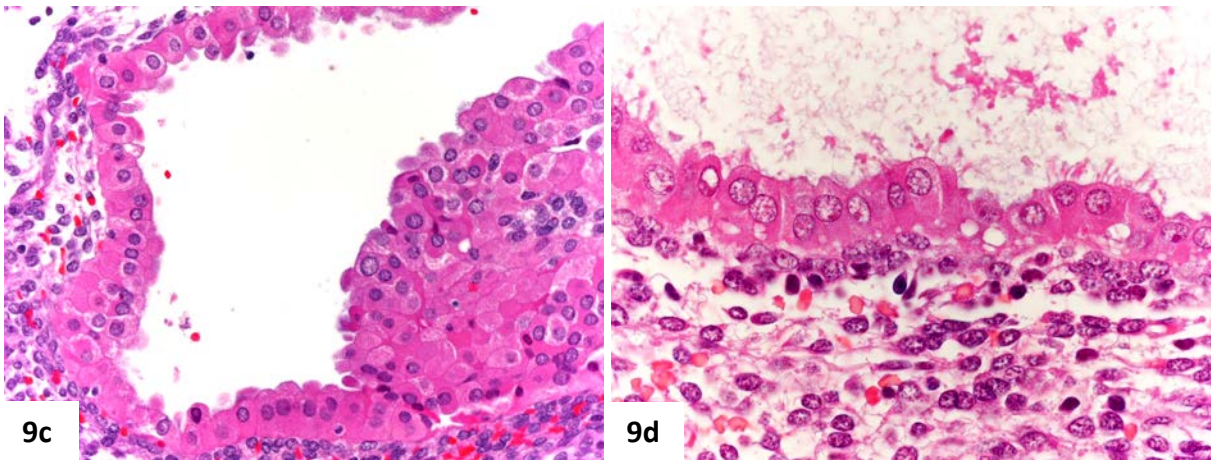


Fig.9a, 9b *complex glands with cribriform pattern lined by atypical cells with eosinophilic changes;* ***Fig.9c*** *endometrial gland with large oncocytic cells, with monotonous nuclei;* ***Fig.9d*** *some of the oncocytic cells present cilia at their luminal border.*

Hobnail change together with TM was seen in a single case of simple hyperplasia. It involved the endometrial surface of a desquamated area (Fig.10a) and was associated with numerous neutrophils (Fig 10b).

Figure 10. Hobnail changes

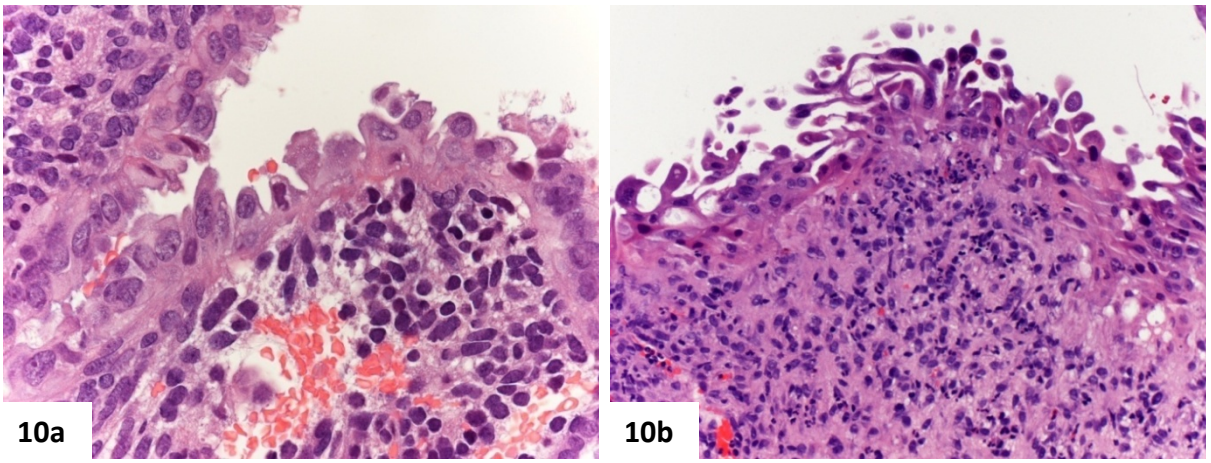


Fig.10a *hobnail shape cells encircling discohesive stromal cells;* ***Fig.10b*** *endometrial surface epithelium replaced by hobnail cells with moderate nuclear atypia, in an inflammatory background.*

None of TM cases had associated stromal type metaplasia.

5.1.3. Immunohistochemical profile

We will outline the expression of each antibody in TM lesions and compare it with the normal endometria, tubal epithelium and entire spectrum of endometrial hyperplasias. 79 benign endometria (41 proliferative, 10 secretory, 2 menstrual, 24 atrophic, 2 decidua) from controls or present in the background of various lesions (atypical and non-atypical

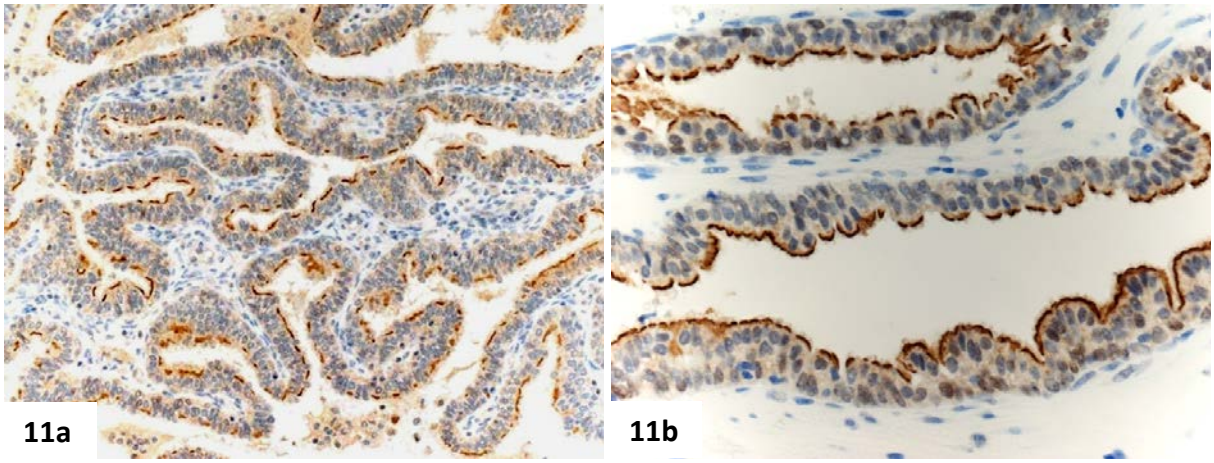
complex hyperplasia, endometrial polyps, adenocarcinomas) were analyzed. Briefly, we will mention also the immunophenotype of the associated endometrial metaplasias. For the immunohistocemical study, we decided to group together complex endometrioid hyperplasia with and without atypia because of their low rate of diagnostic reproducibility and non significant differences in the expression of the antibodies used.

5.1.3.1. LhS28 expression

LhS28, a ciliated cell marker, only stained the apical side of the cell, where the basal bodies of the cilia were located. We were able to identify scattered ciliated cells (<10%) in 27/41 proliferative, 10/24 atrophic, 1/2 menstrual endometria, 2/10 secretory endometria, 21/29 simple and 1/3 complex hyerplastic glands. Decidua and serous adenocarcinoma were completely negative.

We easily confirmed the morphological diagnosis of TM. More than 50% of cells were proved to be ciliated in 78% of STM and 90% of CTMI cases. In CHT the number of ciliated cells was slightly decrease with majority of cases (82%) having more than 10% but less than 50% of cells positive. Two endometrioid adenocarcinomas presented ciliated differentiation, easily identified with this antibody. In figure 11 a comparison is made of this antibody in the normal tubal epithelium (Fig.11a), simple TM (Fig.11b), complex TM in isolated glands (Fig.11c), complex hyperplasia tubal-type (Fig.11d) and tubal-type endometrial adenocarcinoma (Fig.11e). This antibody identifies even TM glands affected by electrocoagulation artifact, contributing to their differential diagnosis with neoplastic glands (Fig.11f).

Figure 11. Lhs28 in normal tubal epithelium and spectrum of tubal-type endometrial lesions



11a **11b**

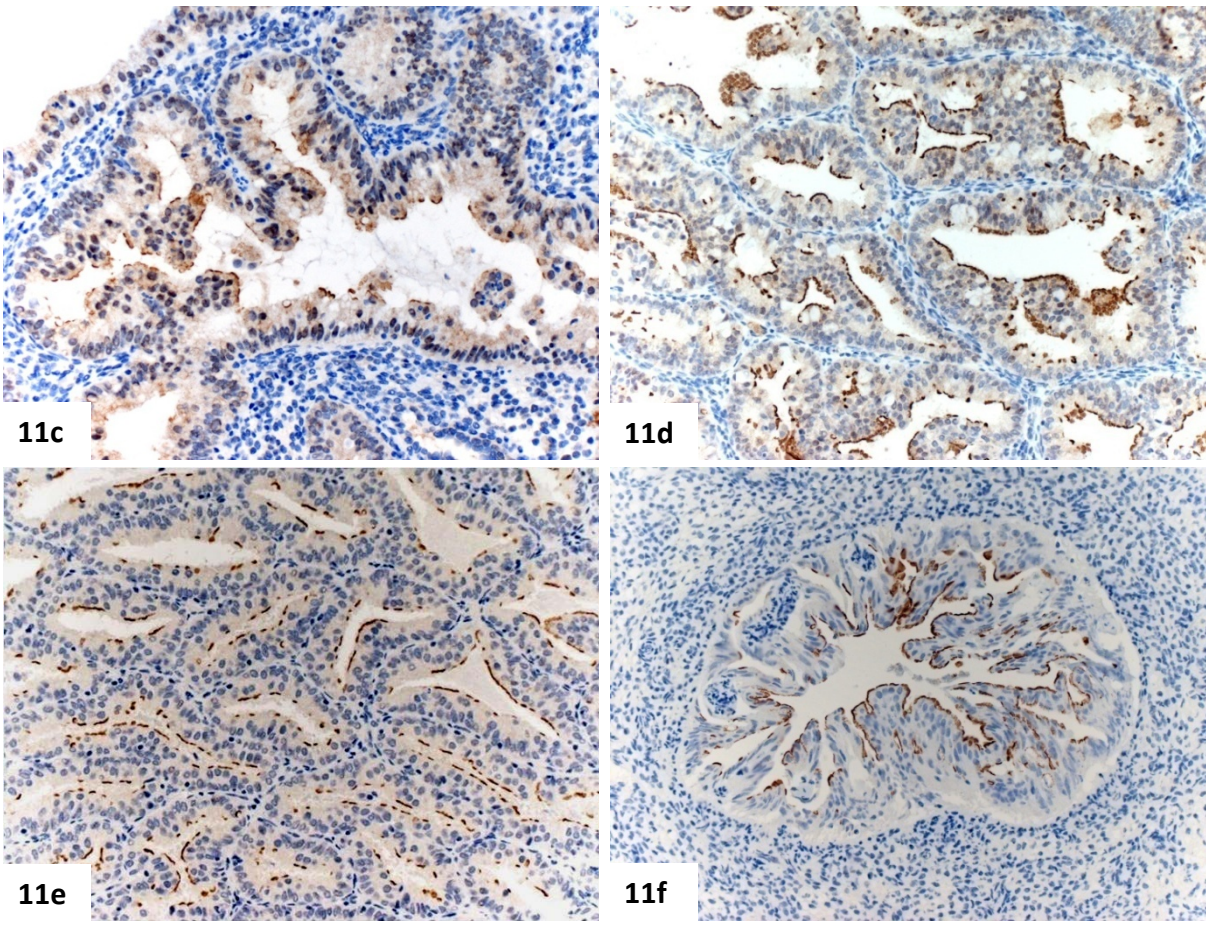


Fig.11a *numerous ciliated cells apical highlighted by LhS28 in the normal Fallopian tube;* ***Fig.11b*** *simple tubular glands lined by numerous ciliated cells, apical positive for LhS28;* ***Fig.11c*** *isolated complex gland with numerous ciliated cells;* ***Fig.11d*** *complex crowded glands with their lumina underlined by LhS28; fig.11e LhS28 confirmed the tubal-type variant of a well differentiated endometrial adenocarcinoma;* ***Fig.11f*** *the presence of ciliated cells was confirmed in an endometrial gland affected by electrocoagulation artifact.*

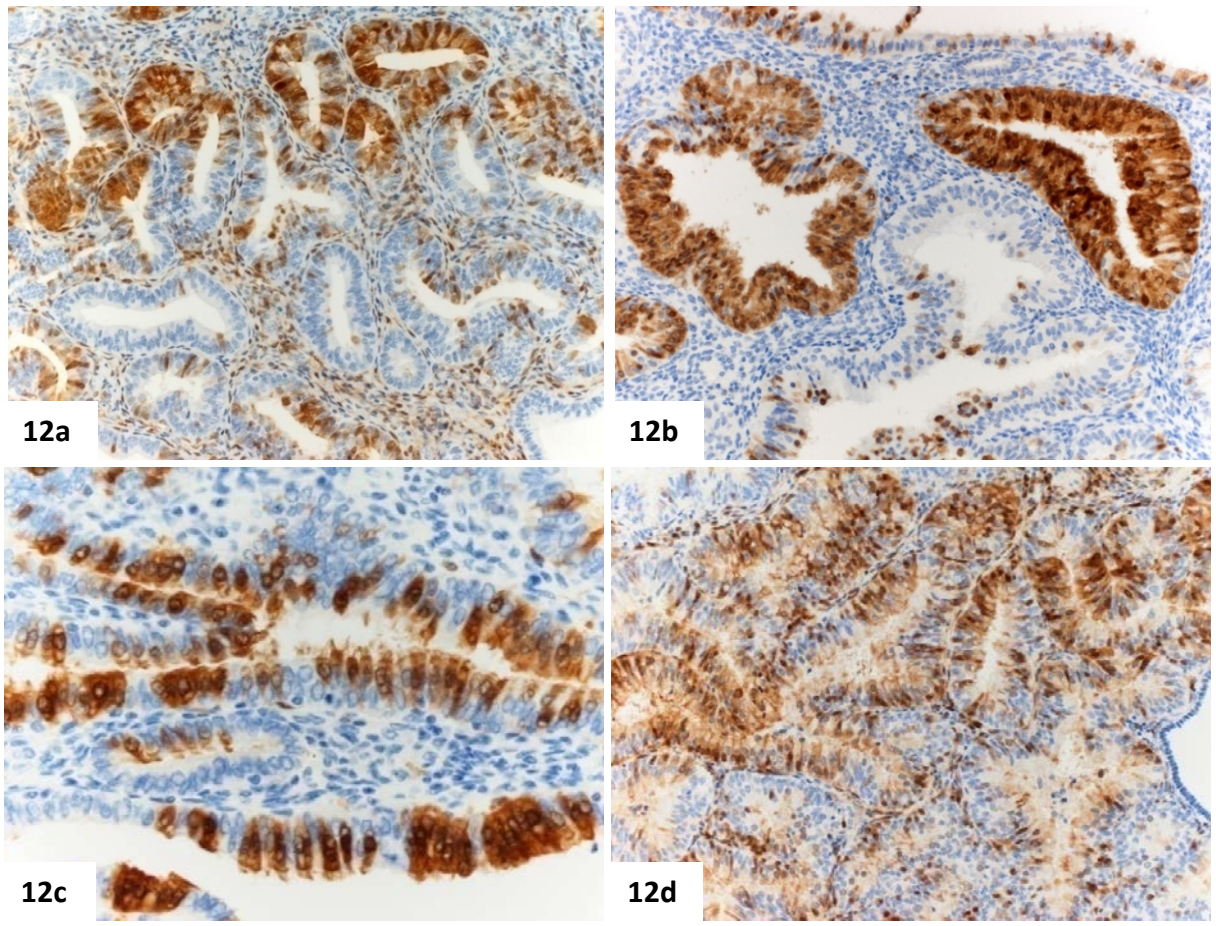
5.1.3.2. p16^{INK4A} expression

The majority of the normal endometria presented a heterogeneous positivity for p16^{INK4A} (62/79), with an extent of staining below 50% (59 cases) (see table 5.3). The brown positivity was located in the cytoplasm in 33 cases, cytoplasm and nucleus 28 cases and only one case presented nuclear stain. Between various phases of menstrual cycle, proliferative and atrophic endometria expressed more often p16^{INK4A}in 36/41 (87.8%) and respectively 22/24 (91.7%), but also scattered cytoplasmic positivity was seen in 4/10 (40%) secretory endometria.

The entire spectrum of ciliated lesion was positive for p16^{IK4A}, with only one exception (116/117), a CTMI case. The extension of staining is detailed in table 5.3. As expected, the highest percentage of positivity was obtained in STM and CTMI, with more than 50% of cells labeled in 74.1% and 81.9% respectively. CHT showed loss of p16

expression, with the majority of cases having less than 50% of cells positive (70.5%). This pattern was statisticsally significant when compared with both CTMI (p=0.016) and STM (p<0.01) (statistics table 5.1). In the spectrum of tubal-type lesions the pattern of staining ranged from heterogeneous positivity, in the glands (Fig.12a) and between the glands (Fig.12b), to mosaic, with a chessboard pattern of positive and negative cells (Fig.12c, 12d) and a diffuse pattern, with >80% of cells being positive (Fig.12e, 12f). But their occurrence was dependent on the glandular architecture. The majority of cases exhibited both cytoplasmic and nuclear positivity (88.03%) (see table 5.4). The intensity of staining was moderate to strong in 94.8% of cases.

Figure 12. p16 expression patterns in tubal-type endometrial lesions



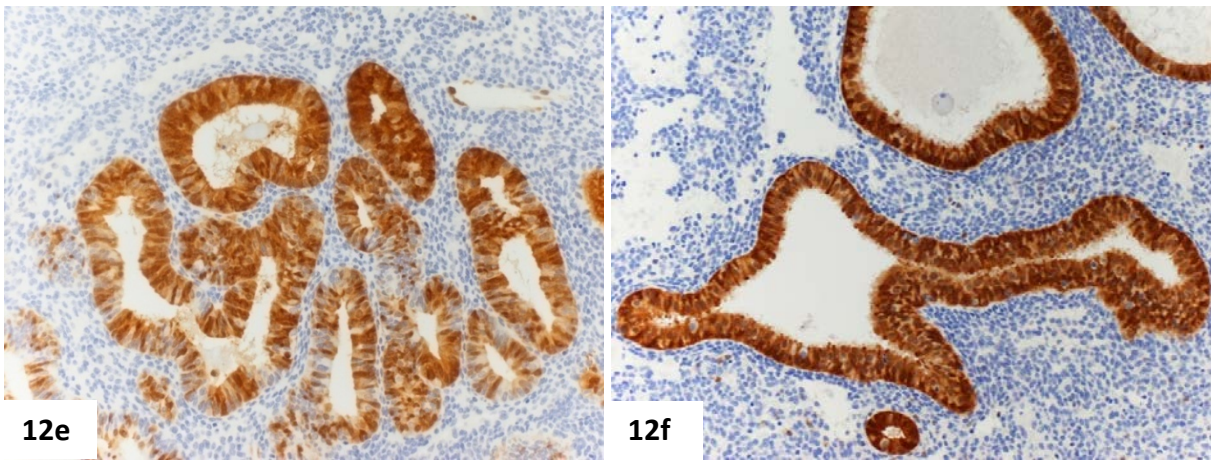


Fig.12a *and* ***12b*** *p16 heterogeneous pattern in CHT;* ***Fig.12c*** *and* ***12d*** *p16 mosaic pattern in CHT and respectively ciliated type adenocarcinoma;* ***Fig.12e*** *and* ***12f*** *p16 diffuse pattern in CHT and respectively STM*

The statisticsal correlation of p16 expression between ciliated lesions and normal, preneoplastic and neoplastic endometrioid lesions are detailed in statistics table 5.1 and 5.2. STM and CTMI expressed a higher percentage of p16 ($p<0.01$) when they are compared with normal proliferative or atrophic endometria as also simple hyperplasia, p16 decrease in CHT but remain superior in comparison with complex endometrioid lesions ($p=0.037$)

A comparison of the expression of p16 in the spectrum of endometrioid lesion is presented in table 5.3 and the statisticsal correlation in statistics table 5.3. For the facility of comparison and because we did not observe any significant differences between complex hyperplastic endometrioid lesion and adenocarcinoma we created a separate category. The only differences noticed were a slightly increased p16 positivity in adenocarcinoma but inferior to 50% ($p=0.037$) and decrease of bcl-2 (statistics table 5.3). None of SH, endometrioid complex hyperplasia and adenocarcinoma were negative. No relevant differences were found between them and normal proliferative endometrium (statistics table 5.3). From two cases of serous carcinoma, one presented a strongly diffuse pattern of staining and the other a focal positivity.

Table 5.3. p16^{INK4A} in cyclic endometria, endometrial lesions associated and spectrum of tubal-type alterations

Histological category*	p16 immunoreactivity					Total
	0	<10%	11-50%	51-80%	>80%	
Proliferative	5 12,2%	23 56,1%	11 26,8%	2 4,9%	0 ,0%	41 100,0%
Secretor	6 60,0%	2 20,0%	2 20,0%	0 ,0%	0 ,0%	10 100,0%
Menstrual	2 100,0%	0 ,0%	0 ,0%	0 ,0%	0 ,0%	2 100,0%
Atrophic	2 8,3%	17 70,8%	4 16,7%	1 4,2%	0 ,0%	24 100,0%
Decidua	2 100,0%	0 ,0%	0 ,0%	0 ,0%	0 ,0%	2 100,0%
STM	0 ,0%	2 2,2%	21 23,6%	40 44,9%	26 29,2%	89 100,0%
CTMI	1 9,1%	0 ,0%	1 9,1%	4 36,4%	5 45,5%	11 100,0%
CHT	0 ,0%	3 17,6%	9 52,9%	2 11,8%	3 17,6%	17 100,0%
SH endometrioid	0 ,0%	18 62,1%	11 37,9%	0 ,0%	0 ,0%	29 100,0%
CH and ACH endometrioid	0 ,0%	7 70,0%	2 20,0%	1 10,0%	0 ,0%	10 100,0%
ADCa endometrioid	0 ,0%	2 22,2%	7 77,8%	0 ,0%	0 ,0%	9 100,0%
ADCa serous	0 ,0%	0 ,0%	1 50,0%	0 ,0%	1 50,0%	2 100,0%
Total	18 7,3%	74 30,1%	69 28,0%	50 20,3%	35 14,2%	246 100,0%

** The histological categories include also control cases*

Graph 1 represents the distribution of p16 immunoreactivity in tubal-type lesion, normal, preneoplastic and neoplastic endometrium, with exclusion of secretory, menstrual and decidua cases.

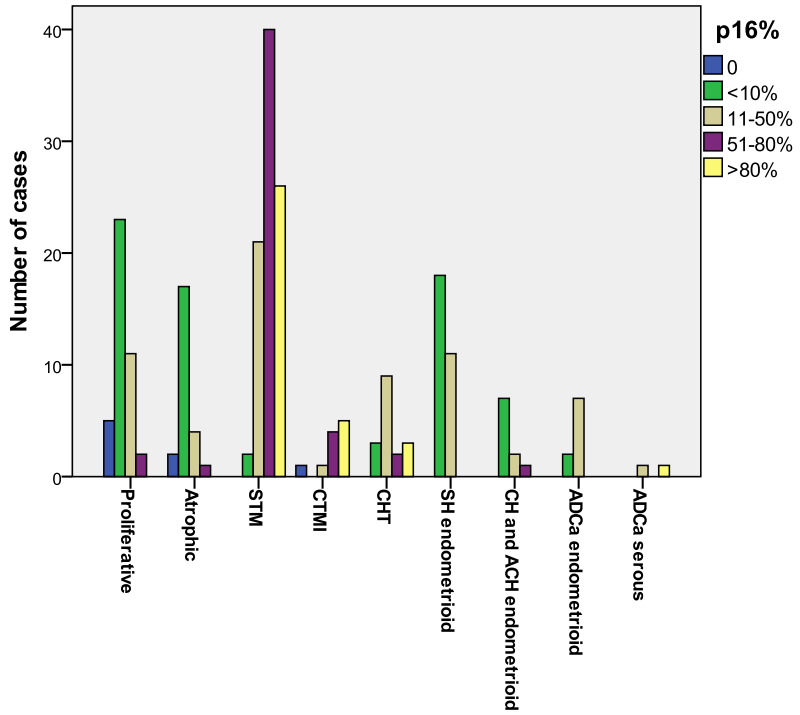


Table 5.4. p16 pattern of expression between the glands and within the cells of tubal-type lesions

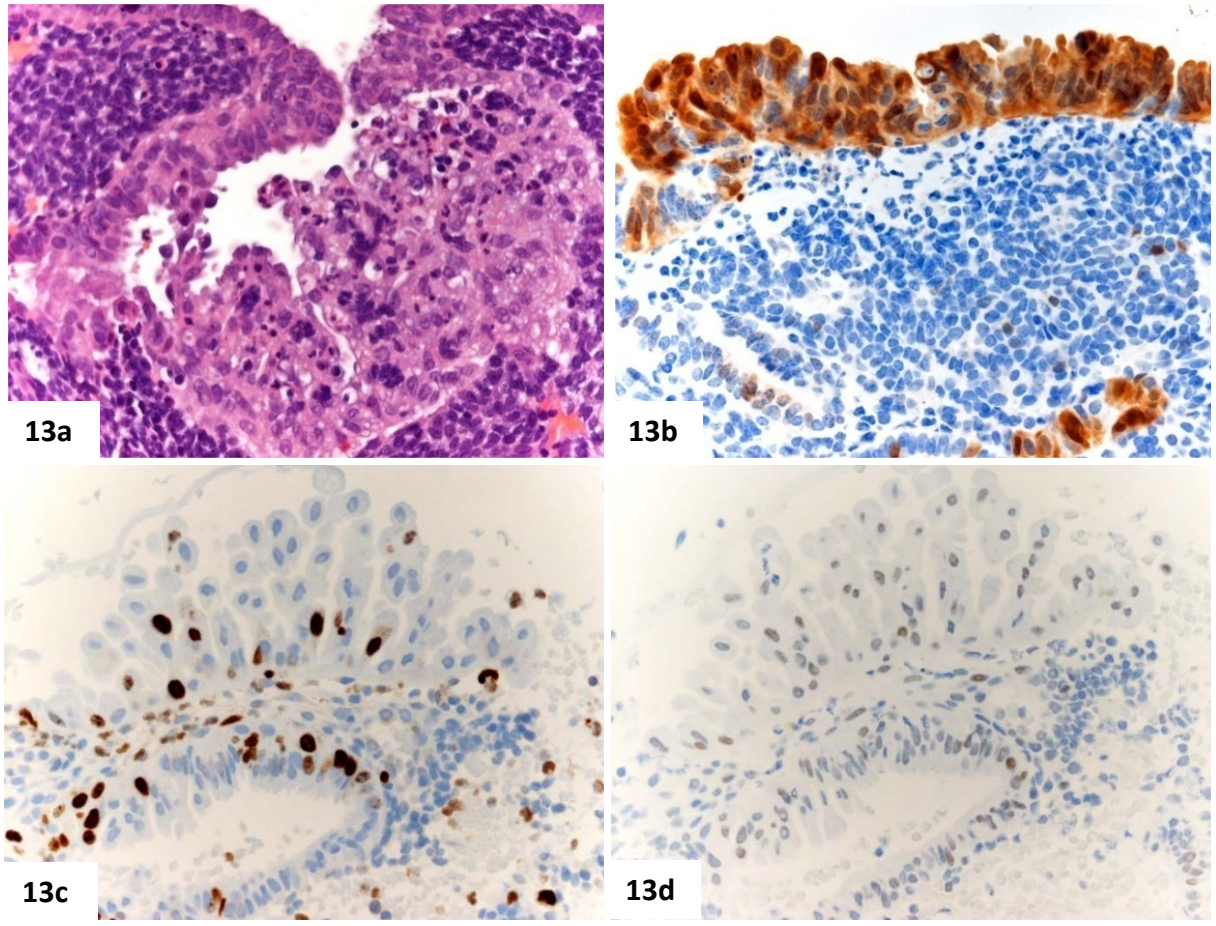
Pattern of p16 within the glands	STM (n=89)	CTMI (n=11)	CHT (n=17)	Total (n=117)
Heterogeneous	51	5	10	66 (56.41%)
Mosaic	17	0	4	21 (17.94%)
Diffuse	21	5	3	29 (24.78%)
Negative	0	1	0	1 (0.85%)
Pattern of p16 within the cells				
Cytoplasmic and nuclear	81	9	13	103 (88.03%)
Cytoplasmic	7	0	4	11 (9.4%)
Nuclear	1	1	0	2 (1.70%)
Negative	0	1	0	1 (0.85%)

The immunoreactivity of p16 in other various endometrial metaplasia and changes is presented in table 5.5.

Surface papillary syncytial changes were present in 29 of 119 cases studied, from which 28 were associated with TM. Being a focal change, 3 of the SPSC areas were not

available for immunohistochemical study, being lost in the further sections. Interestingly, all the SPSC cases were constantly, positive for $p16^{IK4A}$, with a strong, diffuse cytoplasmic and nuclear staining pattern. This feature creates confusion with surface serous carcinoma, which can present a similar morphology, but a different qualitative and quantitative pattern of Ki-67 and p53 expression. Their comparison is presented in figure 13, with SPSC and the correspondent $p16^{INK4A}$, Ki-67 and p53, on the left column (Fig13.a-d) and surface serous carcinoma stained with the same antibodies on the right column (Fig.13e-h).

Figure 13. Morphological and immunohistochemical comparison of SPSC with surface serous carcinoma



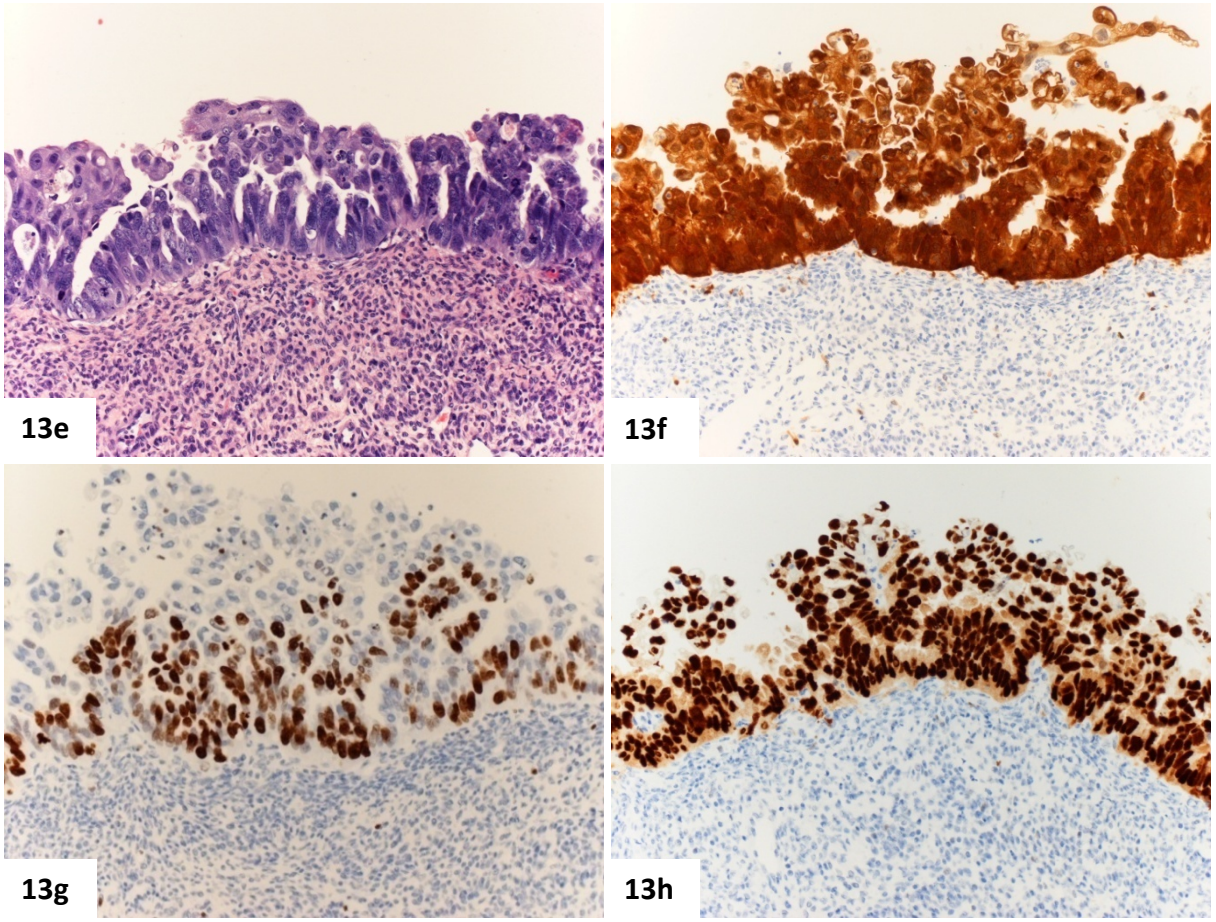


Fig. 13a, 13b, 13c *and* ***13d*** *morphological and immunoreactivity of p16, ki-67 and p53 in SPSC cells.* ***Fig.13e, 13f, 13g*** *and* ***13h*** *morphological and immunoreactivity of p16, ki-67 and p53 in surface serous carcinoma.*

The majority of *mucinous metaplasia* cases were negative or with less than 10% of cell showing weak positivity for $p16^{IK4A}$. The only case of MM with more than 50% of cells positive had a complex architecture (Fig.14a). One case of endometrioid adenocarcinoma presented mucinous differentiation. In these areas $p16^{IK4A}$ was positive in approximately half of the cells, with both nuclear and cytoplasmic patterns.

In our study, we indentified 5 cases of *morular metaplasia*. 4 were also associated with TM and the 5^{th} one was seen in an endometrioid adenocarcinoma control case. The immunoprofile could be evaluated in 4 of them. 3 presented a diffuse, cytoplasmic and nuclear positivity for $p16^{INK4A}$, of moderate to strong intensity (Fig.14b).

Squmaous metaplasia was accessible for evaluation in an isthmic polyp, which shows negativity for $p16^{INK4A}$. Instead, the squamous differentiation of two well differentiated endometrioid carcinomas (control group) showed positivity in more than 50% of the cells with a cytoplasmic, and respectively nuclear and cytoplasmic pattern.

From the 5 cases of *eosinophilic change* visualized in hematoxylin and eosin, only one was available for immunohistochemistry, and showed an almost diffuse positivity for $p16^{INK4A}$ (Fig.14c), as also the area of *hobnail change* (Fig.14d).

Figure 14. p16 immunoreactivity in others type o endometrial metaplasias and changes

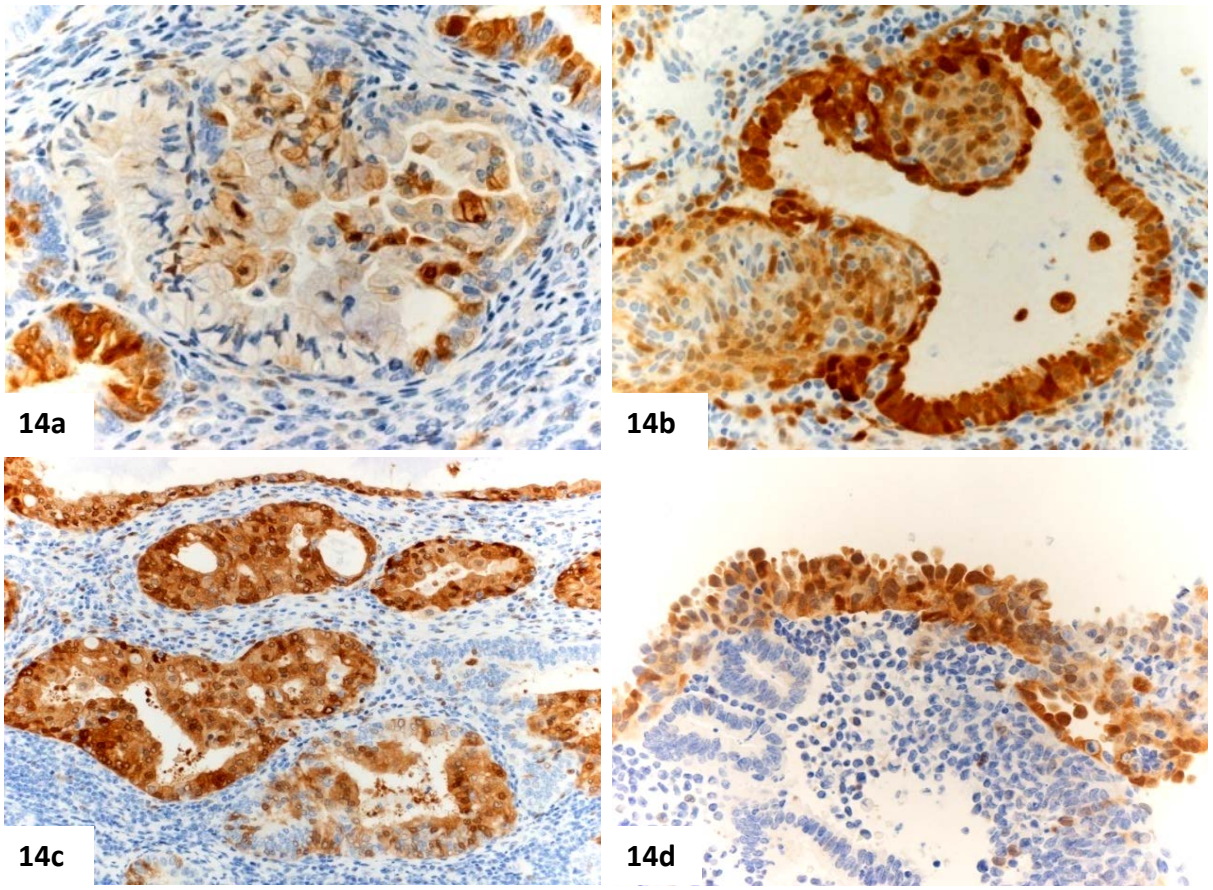


Fig.14a *p16 in complex mucinous metaplasia;* ***Fig.14b*** *p16 in morules,* ***Fig.14c*** *p16 in atypical complex hyperplasia with eosinophilic changes,* ***Fig.14d*** *p16 in hobnail changes associated with endometrial breakdown.*

Table 5.5. p16^{INK4A} staining in various types of metaplasia

Types of metaplasia*	p16 percentage					Total
	0	<10%	11-50%	51-80%	>80%	
SPSC	0 .0%	0 .0%	0 .0%	0 .0%	26 100.0%	26 100.0%
MM	10 52.6%	5 26.3%	3 15.8%	1 5.3%	0 .0%	19 100.0%
Morules	0 .0%	0 .0%	1 25.0%	0 .0%	3 75.0%	4 100.0%
Sq. metaplasia	1 33.3%	0 .0%	0 .0%	1 33.3%	1 33.3%	3 100.0%
Eosinophilic metaplasia	0 .0%	0 .0%	0 .0%	1 100.0%	0 .0%	1 100.0%
Hobnail	0 .0%	0 .0%	1 100.0%	0 .0%	0 .0%	1 100.0%
Total	11 20.4%	5 9. 2%	5 9.2%	3 5.6%	30 55.6%	54 100.0%

** The histological categories includes also control cases*

Finally, endometrial stroma was also evaluated. 45/113 cases showed focal staining for p16^{INK4A} and, interestingly, the majority of these cases were endometrial polyps (28) or adenomyoma/adenofibromas (2).

No case of normal tube exhibited positivity for p16^{INK4A}, neither in the epithelium nor in the stroma.

5.1.3.3. Cyclin D1 expression

More than 50% of proliferative, secretor, and atrophic cells labeled for cyclin D1 in 56.1%, 70%, and respectively 54.2% of cases, but with a weak to moderate intensity. Cyclin D1 expression in menstrual endometria presented a heterogeneous expression between the glands. 58.6% of simple hyperplasia, 40% of complex endometrioid hyperplasia and finally, 55.6% of endometrioid adenocarcinoma exhibited more than 50% of cells positive (see table 5.6). Subsequently, there were no significant differences between the spectrum of endometrioid lesion and also when they are compared with proliferative endometria (statistics table 5.3). Serous adenocarcinoma showed positivity in more than 50% of cells.

Interestingly, TM presented a mosaic pattern of staining with ciliated cells negative or weakly positive and secretory cells moderate to strongly positivity, with an inexplicable cytoplasmic expression together with a nuclear one (Fig.15a). This pattern of expression was highly dominant in STM (63/89, 70.78%) and CTMI (7/11, 63.63%) contrasting with an

isolated case of CHT. None of the TM cases were negative. A similar Cyclin D1 immnunoreactivity was constant in normal tubal epithelia, but the positivity was restricted to the nuclei and did not involve the cytoplasm.

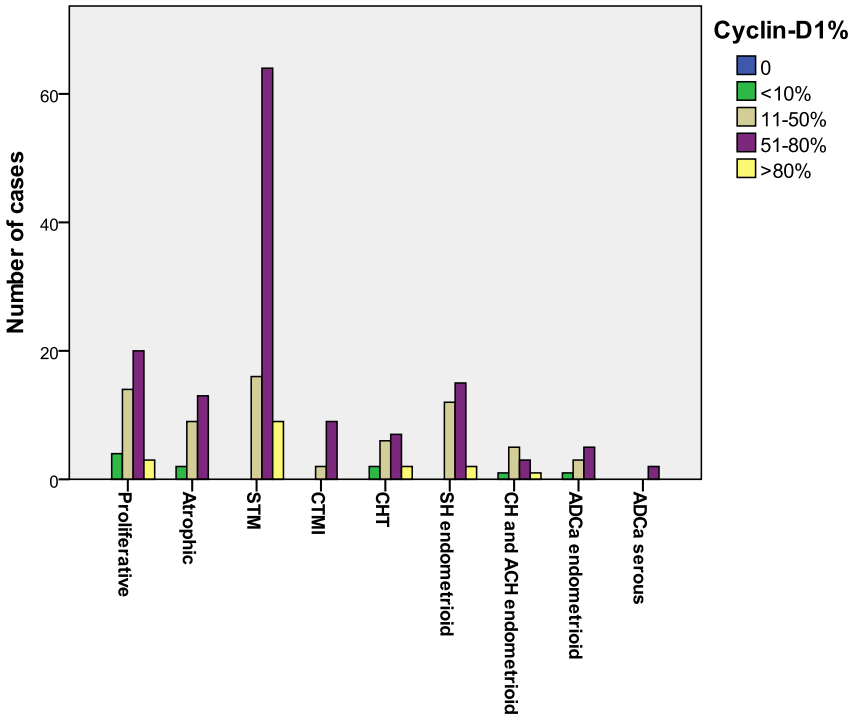
If the threshold of positivity is established at 50%, we observed that CHT had a lower expression of cyclin D1 (Fig.15b) than STM and CTMI. No significant differences between the spectrum of tubal-type lesions as a transistion was obtained. However, they were present between STM and CHT (p=0.001) (statistics table 5.1) and between STM and proliferative (p=0.002) or atrophic endometrium (p=0.005) (statistics table 5.2), with a higher expression in STM glands.

Table 5.6. Cyclin D1 in cyclic endometria, endometrial lesions associated and spectrum of tubal-type alterations

Histological category*	Cyclin-D1 immunoreactivity					Total
	0	<10%	11-50%	51-80%	>80%	
Proliferative	0 ,0%	4 9,8%	14 34,1%	20 48,8%	3 7,3%	41 100,0%
Secretor	1 10,0%	1 10,0%	1 10,0%	4 40,0%	3 30,0%	10 100,0%
Menstrual	0 ,0%	0 ,0%	2 100,0%	0 ,0%	0 ,0%	2 100,0%
Atrophic	0 ,0%	2 8,3%	9 37,5%	13 54,2%	0 ,0%	24 100,0%
Decidua	0 ,0%	0 ,0%	0 ,0%	2 100,0%	0 ,0%	2 100,0%
STM	0 ,0%	0 ,0%	16 18,0%	64 71,9%	9 10,1%	89 100,0%
CTMI	0 ,0%	0 ,0%	2 18,2%	9 81,8%	0 ,0%	11 100,0%
CHT	0 ,0%	2 11,8%	6 35,3%	7 41,2%	2 11,8%	17 100,0%
SH endometrioid	0 ,0%	0 ,0%	12 41,4%	15 51,7%	2 6,9%	29 100,0%
CH and ACH endometrioid	0 ,0%	1 10,0%	5 50,0%	3 30,0%	1 10,0%	10 100,0%
ADCa endometrioid	0 ,0%	1 11,1%	3 33,3%	5 55,6%	0 ,0%	9 100,0%
ADCa serous	0 ,0%	0 ,0%	0 ,0%	2 100,0%	0 ,0%	2 100,0%
Total	1 ,4%	11 4,5%	70 28,5%	144 58,5%	20 8,1%	246 100,0%

** The histological categories include also control cases*

Graph 2 represents distribution of cyclin D1 immunoreactivity in tubal-type lesions, normal, preneoplastic and neoplastic endometrium, with exclusion of secretory, menstrual and decidua cases



Almost all MM presented more than a focal nuclear cyclin D1 positivity (>50%), with two exceptions. The intensity of staining was moderate to strong (see table 5.7).

Another remarkable finding was the strong, diffuse, nuclear and cytoplasmic positivity of cyclin D1 in majority of SPSC (22/26) (Fig.15c), and in the singular case of hobnail changes (Fig.15d). Morular and squamous metaplasia were also positive for cyclin D1 in more of 50% of cells (see table 5.7).

The endometrial stoma presented focal expression of cyclin D1, especially the polyp's stroma.

Table 5.7. Cyclin D1 in various types of metaplasia

Types of metaplasia*	Cyclin D1 immunoreactivity				Total
	<10%	11-50%	51-80%	>80%	
SPSC	0 .0%	0 .0%	3 12.0%	22 88.0%	25 100.0%
MM	1 5.3%	1 5.3%	14 73.7%	3 15.8%	19 100.0%
Morules	0 .0%	0 .0%	4 100.0%	0 .0%	4 100.0%
Squamous metaplasia	0 .0%	0 .0%	2 66.7%	1 33.3%	3 100.0%
Eosinophilic metaplasia	0 .0%	0 .0%	1 100.0%	0 .0%	1 100.0%
Total	1 1.9%	1 1.9%	24 46.2%	26 50.0%	52 100.0%

** The histological categories includes also control cases*

Figure 15. Cyclin D1 expression in metaplasias

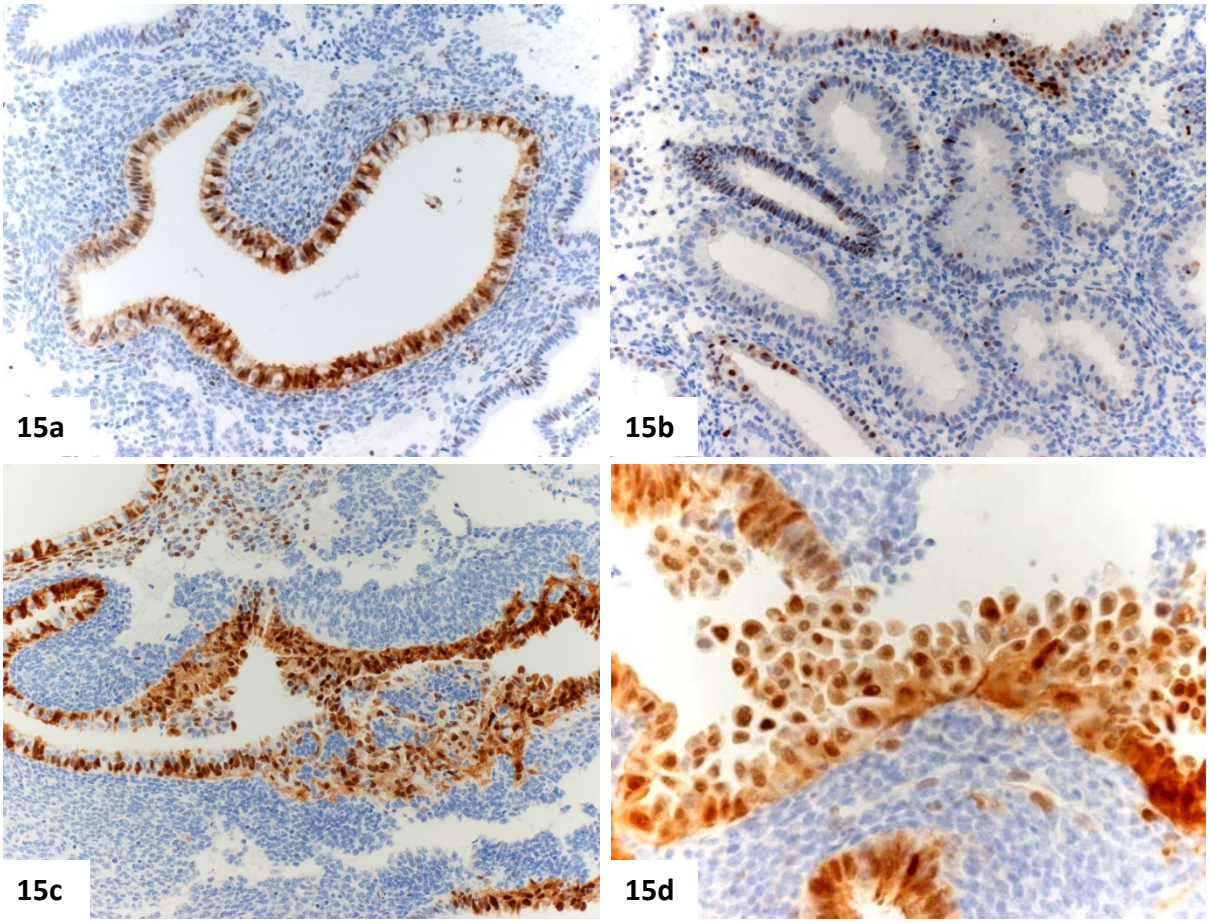


Fig.15a *mosaic pattern of cyclin D1 expression in STM, with positivity restricted to the nuclei and cytoplasm of the secretory cells;* ***Fig.15b*** *decrease expression of nuclear cyclin D1 in CHT;* ***Fig.15c*** *and* ***15d*** *strong nuclear and cytoplasmic cyclin D1 positivity of SPSC and respectively hobnail cells.*

5.1.3.4. Bcl-2 expression

The expression pattern of this antiapoptotic protein was the most interesting feature to be followed.

In the normal functional endometrium bcl-2 showed a diffuse, constant, strong positivity in the proliferative and atrophic endometria, within more than 90% of cases (see table 5.8). Secretory glands exhibited a heterogeneous pattern of staining, from glands completely negative to strongly positive. The same heterogeneity was remarked in menstrual endometria. Remarkable, the glandular component of decidua exhibit a diffuse, strong positivity for bcl-2.

A complete overlap of positivity was reached between normal tubal epithelium (Fig16.a) and STM (Fig.16b) and CTMI. They displayed a mosaic, checkerboard pattern, with a strongly positivity of secretory and intercalary cells and negativity of ciliated cells. Interestingly, we were able to see the same pattern of staining at least in some areas of 14/17 CHT (Fig.16c), but extension and intensity of reaction was lower or heterogeneous, with strong mosaic foci seen in close proximity of slightly positive or negative complex glands (Fig.16d). One case was negative and other two were diffusely positive. Surprisingly, the mosaic pattern was constant in well differentiated endometrioid adenocarcinoma with tubal-type differentiation. One case had strong mosaic positivity (Fig.16e) and the other one presented heterogeneous reactivity with weak and negative areas (Fig.16f). In the continuum spectrum of tubal-type lesions there were no significant differences in bcl-2 expression pattern. But, when we analyze the two extreme of the spectrum, STM and CHT we noticed a decrease of bcl-2 positivity, statisticsally relevant ($p<0.01$) Except for the mosaic pattern, no significant differences were observed between CHT and complex endometrioid lesions ($p=0.064$) (statistics table 5.1).

Similar to proliferative endometria, simple hyperplastic glands demonstrated a diffuse, strong positivity. But, complex endometrioid hyperplasia tends to present a variability of expression, with decreasing of percentage and intensity of immunoreactions in 3/9 cases. Majority of endometrioid adenocarcinoma presented a heterogeneous pattern of staining, with a stronger expression at the invasion front, progressively diminishing toward surface (6/9). Decreased expression in complex endometrioid hyperplasia and adenocarcinomas reached significance when they were compared with proliferative ($p<0.01$) and SH ($p<0.01$) (statistics table 5.3) Serous adenocarcinoma was negative for bcl-2.

Figure 16. Bcl-2 immunoreactivity in the normal and endometrial tubal-type lesions

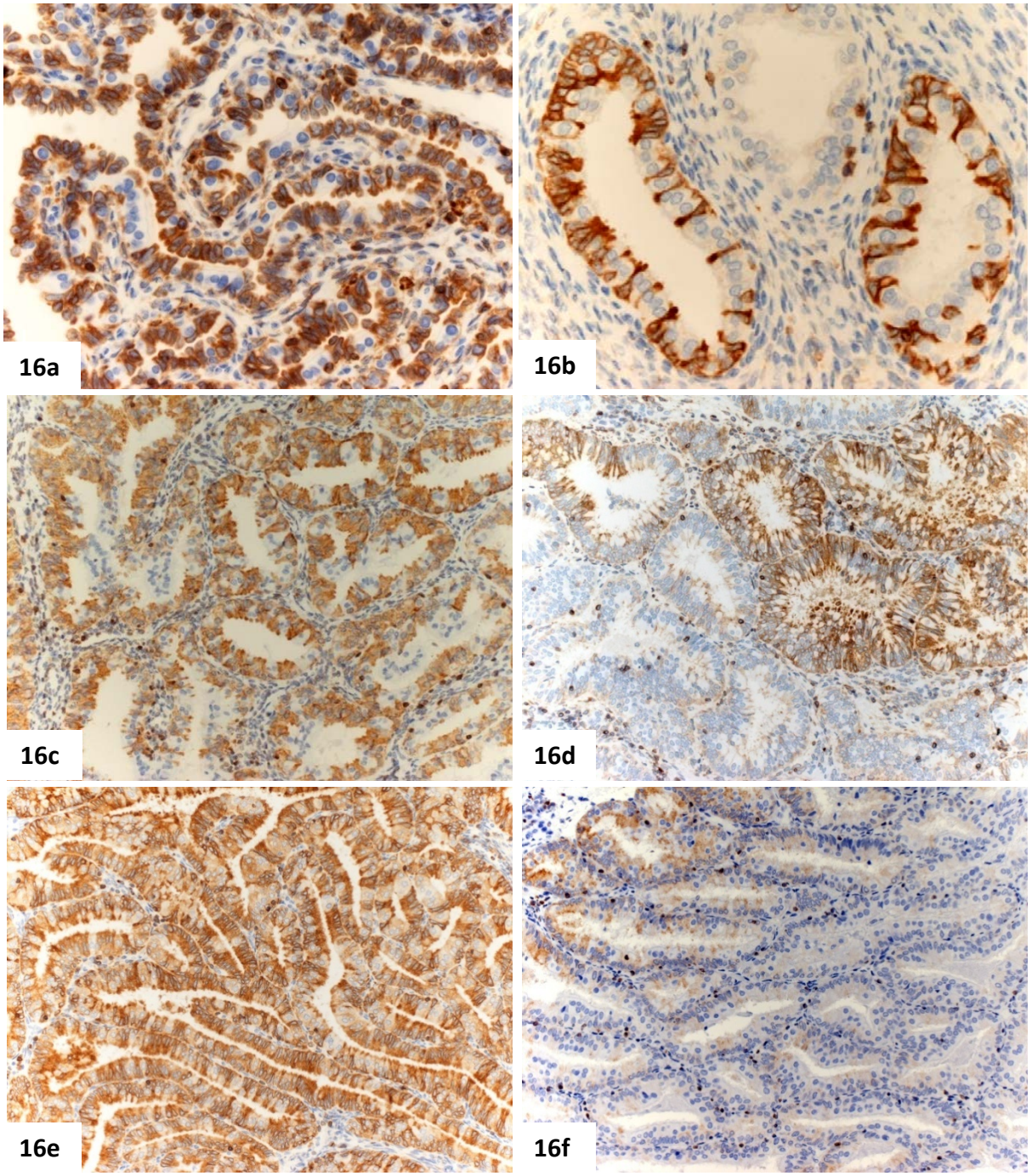


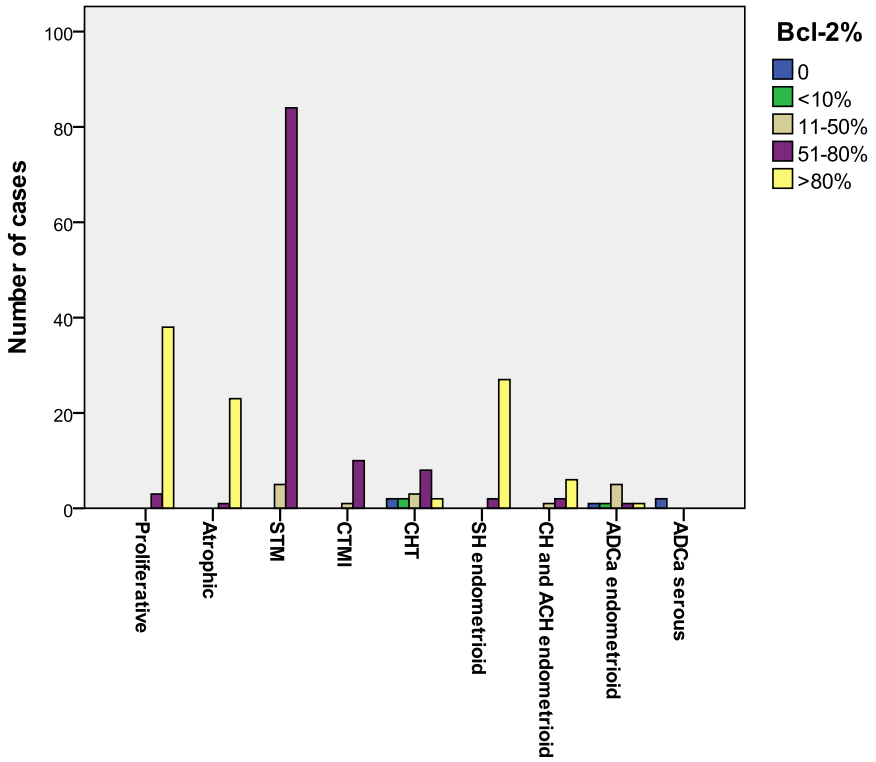
Fig.16a *bcl2 in the normal Fallopian tube;* ***Fig 16b*** *and 16c the mosaic pattern of bcl-2 expression in STM and respectively CHT;* ***Fig.16d*** *weak and strong bcl-2 positive glands in CHT;* ***Fig.16e*** *mosaic bcl-2 positivity in tubal-type endometrial adenocarcinoma;* ***Fig.16f*** *weak and moderate bcl-2 positive glands positivity in tubal-type endometrial adenocarcinoma.*

Table 5.8. Bcl-2 in cyclic endometria, endometrial lesions associated and spectrum of tubal-type alterations

Histological category*	Bcl-2 immunoreactivity					Total
	0	<10%	11-50%	51-80%	>80%	
Proliferative	0 ,0%	0 ,0%	0 ,0%	3 7,3%	38 92,7%	41 100,0%
Secretor	3 30,0%	0 ,0%	3 30,0%	2 20,0%	2 20,0%	10 100,0%
Menstrual	0 ,0%	0 ,0%	1 50,0%	1 50,0%	0 ,0%	2 100,0%
Atrophic	0 ,0%	0 ,0%	0 ,0%	1 4,2%	23 95,8%	24 100,0%
Decidua	0 ,0%	0 ,0%	0 ,0%	0 ,0%	2 100,0%	2 100,0%
STM	0 ,0%	0 ,0%	5 5,6%	84 94,4%	0 ,0%	89 100,0%
CTMI	0 ,0%	0 ,0%	1 9,1%	10 90,9%	0 ,0%	11 100,0%
CHT	2 11,8%	2 11,8%	3 17,6%	8 47,1%	2 11,8%	17 100,0%
SH endometrioid	0 ,0%	0 ,0%	0 ,0%	2 6,9%	27 93,1%	29 100,0%
CH and ACH endometrioid	0 ,0%	0 ,0%	1 11,1%	2 22,2%	6 66,7%	9 100,0%
ADCa endometrioid	1 11,1%	1 11,1%	5 55,6%	1 11,1%	1 11,1%	9 100,0%
ADCa serous	2 100,0%	0 ,0%	0 ,0%	0 ,0%	0 ,0%	2 100,0%
Total	8 3,3%	3 1,2%	19 7,8%	114 46,5%	101 41,2%	245 100,0%

** The histological categories includes also control cases*

Graph 3 represents the distribution of bcl-2 immunoreactivity in tubal-lesion, normal, preneoplastic and neoplastic endometrium, with exclusion of secretory, menstrual and decidua cases.



All the mucinous metaplastic areas were completely bcl-2 negative (Fig.17a), as their correspondent normal endocervical epithelium.

Only 2 of 26 cases of surface papillary syncitial changes presented a weak positivity for bcl-2, the rest of them were completely negative (Fig.17b). Morules were diffusely positive but with weak to moderate intensity of staining. The squamous metaplasia which involved the isthmic polyp, presented the same pattern of positivity as the normal squamous epithelium of the cervix with a diffuse staining of the basal layer.

Figure 17. Bcl-2 negativity in mucinous metaplasia and SPSC

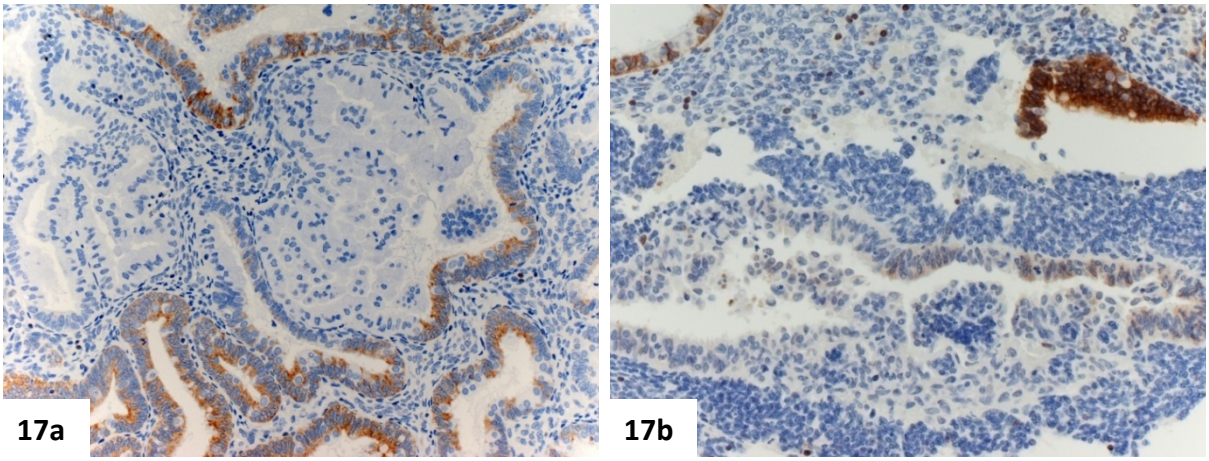


Fig.17a *bcl-2 immnunonegativity of mucinous metaplasia, contrasted with the positivity of secretory TM cells;* ***Fig.17b*** *bcl-2 negativity of SPSC areas*

5.1.3.5. PAX2 expression

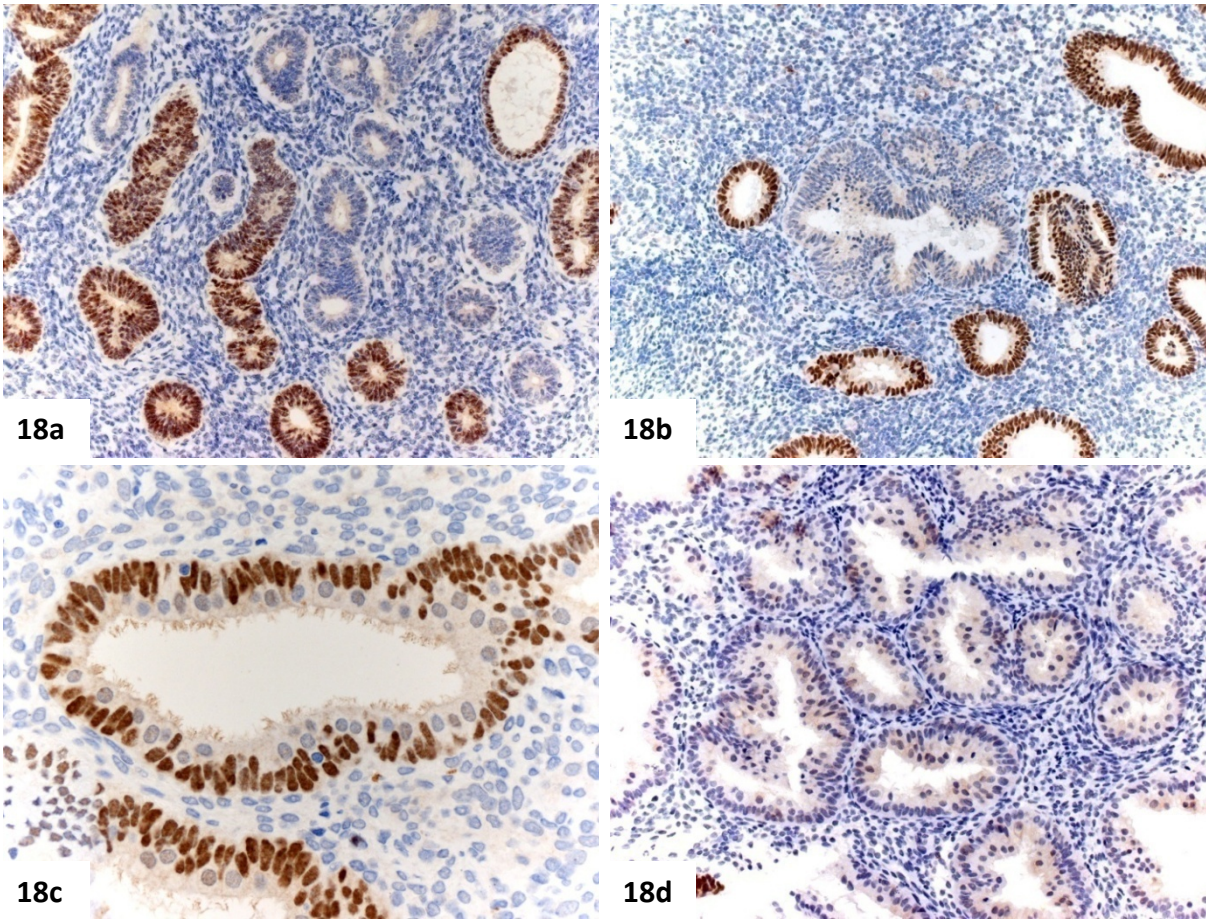
PAX2 was not available in all the cases, due to technical difficulties (related to clone production).

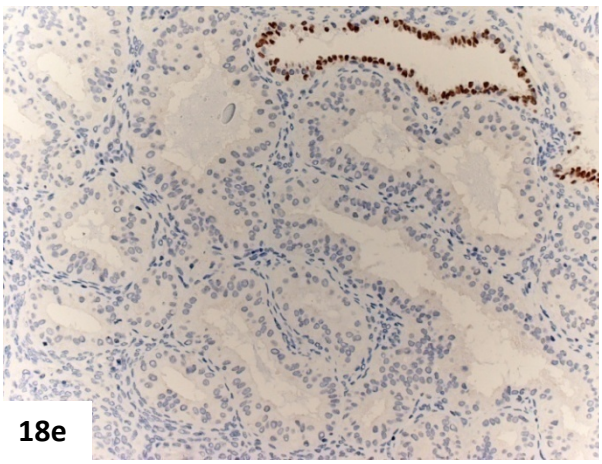
Normal endometrial glands, evaluated in 46 cases, had diffuse, strong PAX2 nuclear expression, independently of the type of functional endometria. Only one proliferative endometria (1/23) presented lack of PAX2 expression in a reduced number of glands (Fig18a). Also 4/19 (21.1%) cases of SH had PAX2 negative isolated glands between uniformly positive ones (Fig18b). Its progressive loss was noticed in complex endometrioid lesions and endometrioid adenocarcinomas, which presented areas of complete loss of PAX2 expression. No statisticsal differences were observed between them (p=0.287) (statistics table 5.3) but relevant decrease was present when compared to both proliferative endometria (p<0.01) and simple hyperplasia (p<0.01) (statistics table 5.3**)**. However, three (33.3%) complex endometrioid lesions and one endometrioid adenocarcinoma preserved PAX2 expression, with more than 50% of cells being positive. Loss of expression was noticed in both serous adenocarcinomas (see table 5.9)

STM was positive for PAX2 in all 53 cases evaluated. Expression was restricted to the secretory columnar cells, whereas ciliated cells and peg cells were negative (Fig.18c). Also the same mosaic pattern was seen in all CTMI (n=10) studied. This pattern completely overlapped with PAX2 expression in normal Fallopian tube epithelium (n=5). bcl-2 was seen to have down-regulation of PAX2 in CHT, with its loss in 64.7% (11/16) of cases (Fig.18d). Five CHT had a heterogeneous pattern of PAX2, with positive glands joining with focal

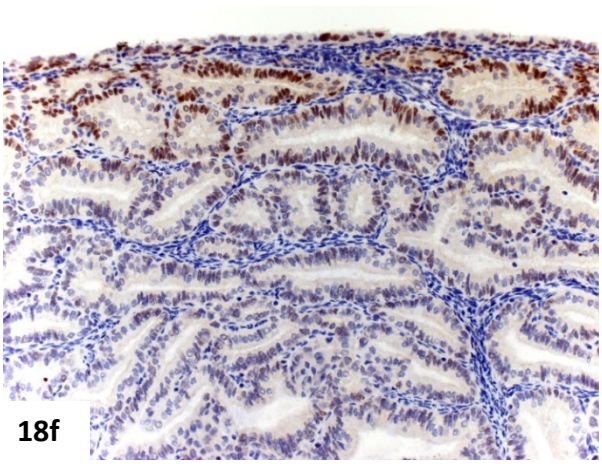
positive and complete negative areas, as in endometrioid complex lesions. In these cases, the dominant pattern was scored. One case preserved a diffuse immunoreactivity. The areas with preserved expression maintained the mosaic pattern. Thus, significant differences were noticed in the expression of PAX2 in STM and CTMI on one hand and CHT on the other ($p<0.01$). The latter expressed PAX2 similarly to complex endometrioid lesions ($p=0.153$) (statistics table 5.1). One of the adenocarcinomas with ciliated differentiation lost the expression of PAX2 (Fig.18e) and the other presented positive mosaic areas and negative ones (Fig.18f).

Figure 18. PAX2 immunoreactivity in normal, hyperplastic and ciliated lesions





18e



18f

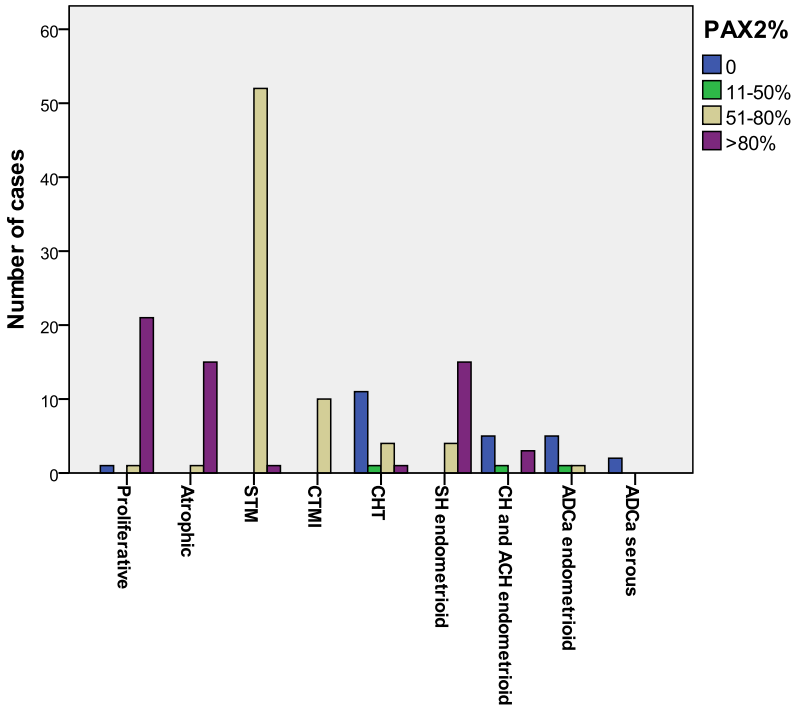
Fig.18a *proliferative endometrium showing scattered PAX2 negative glands;* ***Fig.18b*** *occasional PAX2 null glands in simple hyperplastic endometrium;* ***Fig.18c*** *PAX2 nuclear positivity restricted to the secretory cells of STM;* ***Fig.18d*** *absence of PAX2 staining in CHT;* ***Fig. 18e*** *complete loss of PAX2 expression in tubal-type endometrial adenocarcinoma;* ***Fig.18f*** *transition from PAX2 positive glands with a mosaic pattern to negative glands of tubal-type endometrial adenocarcinoma.*

Table 5.9. PAX2 in cyclic endometria, associated endometrial lesions and the spectrum of tubal-type alterations

Histological category*	Bcl-2 immunoreactivity				Total
	0	11-50%	51-80%	>80%	
Proliferative	1 4,3%	0 ,0%	1 4,3%	21 91,3%	23 100,0%
Secretor	0 ,0%	0 ,0%	1 14,3%	6 85,7%	7 100,0%
Atrophic	0 ,0%	0 ,0%	1 6,3%	15 93,8%	16 100,0%
STM	0 ,0%	0 ,0%	52 98,1%	1 1,9%	53 100,0%
CTMI	0 ,0%	0 ,0%	10 100,0%	0 ,0%	10 100,0%
CHT	11 64,7%	1 5,9%	4 23,5%	1 5,9%	17 100,0%
SH endometrioid	0 ,0%	0 ,0%	4 21,1%	15 78,9%	19 100,0%
CH and ACH endometrioid	5 55,6%	1 11,1%	0 ,0%	3 33,3%	9 100,0%
ADCa endometrioid	5 71,4%	1 14,3%	1 14,3%	0 ,0%	7 100,0%
ADCa Serous	2 100,0%	0 ,0%	0 ,0%	0 ,0%	2 100,0%
Total	24 14,7%	3 1,8%	74 45,4%	62 38,0%	163 100,0%

** The histological categories includes also control cases*

Graph 4 represents the distribution of PAX2 immunoreactivity in tubal-type lesions, normal, preneoplastic and neoplastic endometrium, with exclusion of secretory, menstrual and decidua cases



Mucinous metaplasia was PAX2 positive (n=5), independently of its degree of architectural complexity. SPSC (n=10) and morules had a variable expression of PAX2, ranging from none to less than 50% and from weak to moderate intensity. Unexpectedly, three cases of SPSC showed a diffuse pattern of staining. Squamous differentiation remained unstained for PAX2.

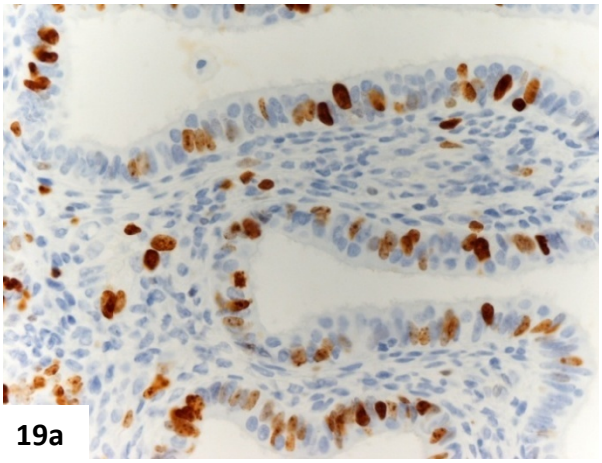
5.1.3.6. Proliferation index Ki-67

The highest proliferation rate was present in normal proliferative endometrium. However, 56.1% of cases had a Ki-67 less than 10%. Scattered nuclei were seen to label for Ki-67 in the secretory glands in 2/10 cases. The majority of atrophic glands (91.6% of cases) had a Ki-67 index <10%. Decidua and menstrual endometrium did not show proliferative activity. The rate of proliferation decreased from normal proliferative and simple hyperplasia to atypical complex hyperplasia (see table 5.10). Approximately 90% of simple hyperplastic glands presented a moderate to high proliferation activity. Instead, 55.6% of endometrioid complex lesions had less than 10% of active nuclei, and this difference was statistically significant (p=0.005) (statistics table 5.3). The endometrioid adenocarcinomas

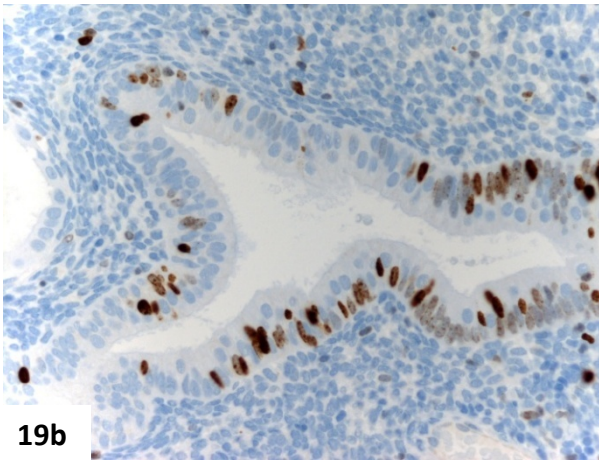
showed active cells at the myometrial invasive border and the rest of tumour presented less than 10% of positive nuclei, with no significant differences compared to complex endometrioid lesions (p=1.00) (statistics table 5.3). This antibody contributes to the decision to unify it with complex endometrioid lesions and analyze them as a separate category (statistics table 5.3). Serous adenocarcinomas and their surface variant (EIC – endometrial intraepithelial carcinoma) had a high proliferation rate, closer to 90% (see table 5.10).

There was no statistically significant differences between STM and CTMI (p=0.443) (statistics table 5.1). 96.6% of STM and respectively 100% of CTMI had a Ki-67 inferior to 10%, with no differences in staining compared with inactive endometrium, when the threshold is established at 10% (statistics table 5.2) or with normal tubal epithelium. Ki-67 invariably labeled the nuclei of secretory cells of both normal (Fig.19a) and metaplastic tubal epithelium. Only three cases of STM exhibited moderate proliferative activity (Fig.19b). Low proliferation rate was also observed in CHT (Fig.19c) and ciliated type adenocarcinoma (Fig.19d) with no significant differences between CTMI and CHT (p=0.205), as also between complex hyperplasia ciliated and endometrioid type (p=0.390) (statistics table 5.1). Although the differences in proliferation between STM and CHT appear significant, (p<0.01), this is negligible if we analyze as a group cases with Ki-67 less than 10% and cases with ki-67 higher than 10% (statistics table 5.1). However, a slight increase of Ki-67 is observed in CHT with 23.5% of cases having a moderate (10-30%) rate of proliferation.

Figure 19. Ki67 in normal tubal epithelium and endometrial tubal-type lesions



19a



19b

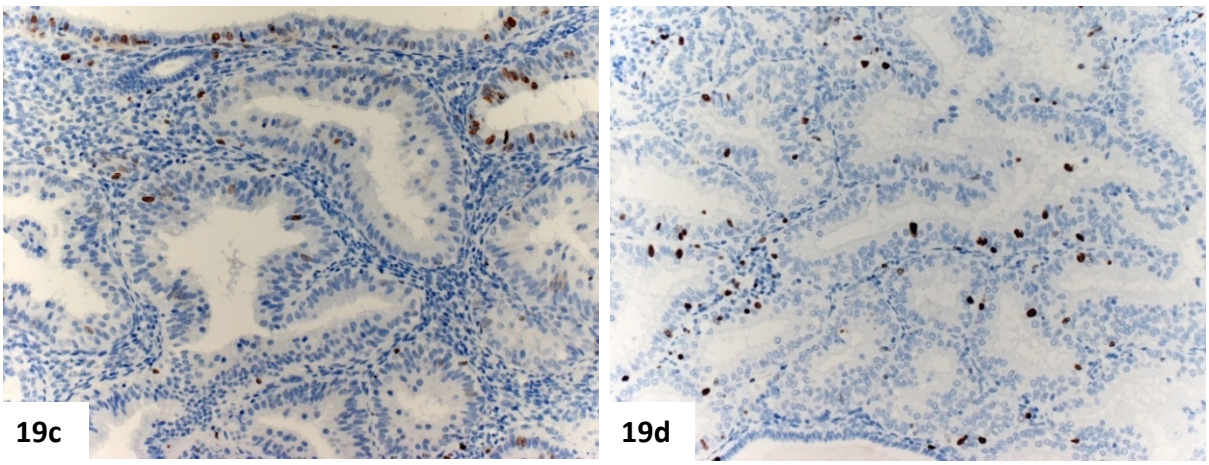


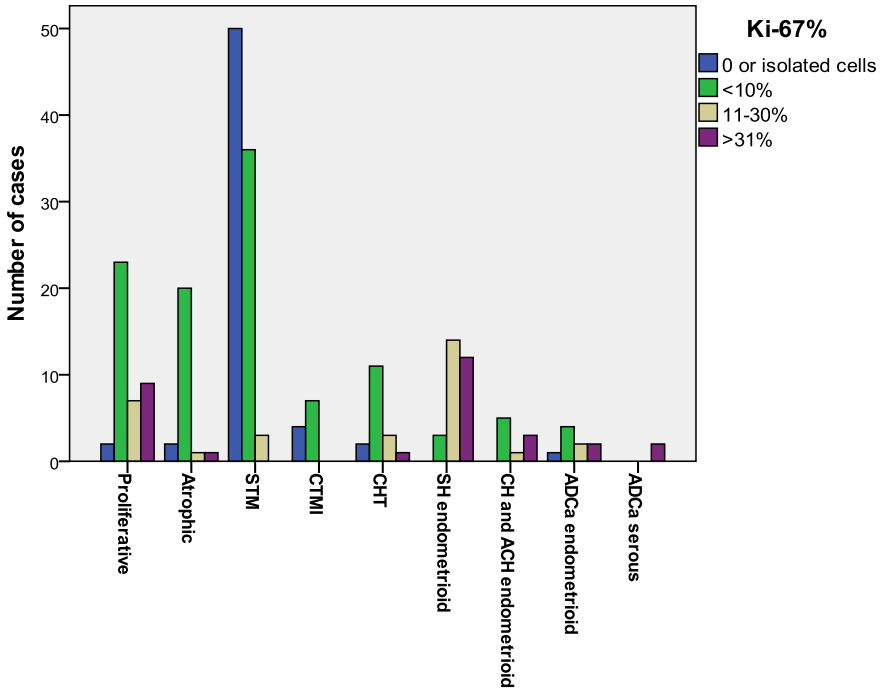
Fig.19a *Ki-67 in normal Fallopian tube;* ***Fig.19b*** *moderate proliferative activity of the secretory cells of STM,* ***Fig.19c*** *and* ***19d*** *low proliferation in CHT and tubal-type adenocarcinoma cells.*

Table 5.10. Ki-67 in cyclic endometria, associated endometrial lesions and the spectrum of tubal-type alterations

Histological category*	Ki-67 immunoreactivity				Total
	0	<10%	11-30%	>31%	
Proliferative	2 4,9%	23 56,1%	7 17,1%	9 22,0%	41 100,0%
Secretor	8 80,0%	1 10,0%	0 ,0%	1 10,0%	10 100,0%
Menstrual	2 100,0%	0 ,0%	0 ,0%	0 ,0%	2 100,0%
Atrophic	2 8,3%	20 83,3%	1 4,2%	1 4,2%	24 100,0%
Decidua	2 100,0%	0 ,0%	0 ,0%	0 ,0%	2 100,0%
STM	50 56,2%	36 40,4%	3 3,4%	0 ,0%	89 100,0%
CTMI	4 36,4%	7 63,6%	0 ,0%	0 ,0%	11 100,0%
CHT	2 11,8%	11 64,7%	3 17,6%	1 5,9%	17 100,0%
SH endometrioid	0 ,0%	3 10,3%	14 48,3%	12 41,4%	29 100,0%
CH and ACH endometrioid	0 ,0%	5 55,6%	1 11,1%	3 33,3%	9 100,0%
ADCa endometrioid	1 11,1%	4 44,4%	2 22,2%	2 22,2%	9 100,0%
ADCa serous	0 ,0%	0 ,0%	0 ,0%	2 100,0%	2 100,0%
Total	73 29,8%	110 44,9%	31 12,7%	31 12,7%	245 100,0%

** The histological categories include also the control cases*

Graph 5 represents the distribution of Ki-67 immunoreactivity in tubal-type lesions, normal, preneoplastic and neoplastic endometrium, with exclusion of secretory, menstrual and decidua cases



Almost all of the other types of metaplasia were inactive, with no proliferative activity. The exception was one case of complex mucinous metaplasia and one case of squamous metaplasia which exhibited a Ki-67 index of 10-30%. Scattered positive nuclei were seen in two cases of SPSC (see Fig.13c).

Table 5.11. Ki-67 in various types of metaplasias

Types of metaplasia*	Ki-67 immunoreactivity			Total
	0	<10%	11-30%	
SPSC	23 92.0%	2 8.0%	0 .0%	25 100.0%
MM	18 94.7%	0 .0%	1 5.3%	19 100.0%
Morules	4 100.0%	0 .0%	0 .0%	4 100.0%
Squamous metaplasia	2 66.7%	0 .0%	1 33.3%	3 100.0%
Eosinophilic metaplasia	1 100.0%	0 .0%	0 .0%	1 100.0%
Total	48 92.3%	2 3.8%	2 3.8%	52 100.0%

** The histological categories include also control cases*

The proliferative activity of the stroma varied over a wide range, depending on the endometrial glandular context; either absent in atrophic endometria and the stroma of the polyps or moderate to high in proliferative and hyperplastic endometria.

5.1.3.7. p53 expression

All the parameters evaluated presented absence or weak intensity of p53 staining, with the exception of serous adenocarcinomas (see Fig.13h), which showed strong and diffuse overexpression. The percentage of cells varied, with more than 50% of nuclei labeled in proliferative, menstrual, hyperplastic endometria and endometrioid adenocarcinoma, less than 50% in secretory, decidua, atrophic, SPSC, morular, squamos and mucinous metaplasia. In the latter, approximately half of the cases were negative. 6 SPSC cases demonstrated a moderate intensity of staining in less than 50% of nuclei (see Fig.13d). A comparison of the staining pattern between SPSC and surface serous carcinoma is presented in figure 13. More than a focal reactivity (>10%) was seen in 92% of TM cases, independently of the architecture, but the intensity of staining was moderate in only 9% of cases, with the rest of cases showing weak positive nuclei. The cases with moderate intensity of the reaction corresponded to STM seen in the context of abnormal uterine bleeding (5/9 cases) and endometrial polyps (4/9 cases).

5.1.3.8. MLH1, PMS2, MSH2 and MSH6 expression

We analyzed the expression of the human mismatch proteins in 24 ciliated type lesions, they corresponded to: two cases of extensive STM, 7 cases CTMI, 12 cases CHT coexistent with STM, and in two cases the full spectrum of tubal-type lesions was present, with STM coexistent with CHT and endometrioid adenocarcinoma with tubal-type differentiation. Finally, in one case STM was seen in the vicinity of an endometrioid adenocarcinoma. Three of 14 CHT (12 CHT and 2 cases associated with adenocarcinoma) showed a concurrent complete loss of MLH1 (Fig.20a) and PMS2 expression (Fig.20b), as also the case of endometrioid adenocarcinomas in proximity to STM. The lack of expression was restricted to the complex areas and did not involve simple tubal-type glands. One of CHT cases developed in endometrial polyp presented loss of PAX2 protein, but no PTEN deletion or K-ras mutation. One CHT with loss of MMR proteins, presented concomitant deletion of PTEN, detected by FISH, but did not show alteration of K-ras gene or of PAX2

protein. The third case showed absence of PAX2 staining, but non evaluable PTEN and K-ras analysis was not available. We did not extend the molecular study for the adenocarcinoma case with alteration of MMR proteins and consequently the status of PTEN and K-ras is not available. In these three cases the proliferation index was inferior to 10% and in only the case with MSI and PTEN mutation, cyclin D1 showed overexpression, with more than 50% of nuclei positive.

The rest of the analyzed variables (functional endometrium: 8 atrophic, 14 proliferative and 2 secretory, 5 mucinous metaplasia, 3 SPSC, 1 morules) diffusely expressed these antibodies. No cases presented a pathological expression of MSH2. All the cases were MSH6 positive, but a focal (<50% of cells), heterogeneous weak to moderate positivity, was remarked in 10 tubal-type lesions (4 STM, 5 CHT, and one endometrioid adenocarcinoma with tubal-type differentiation).

Figure 20. MLH1 and PMS2 in CHT

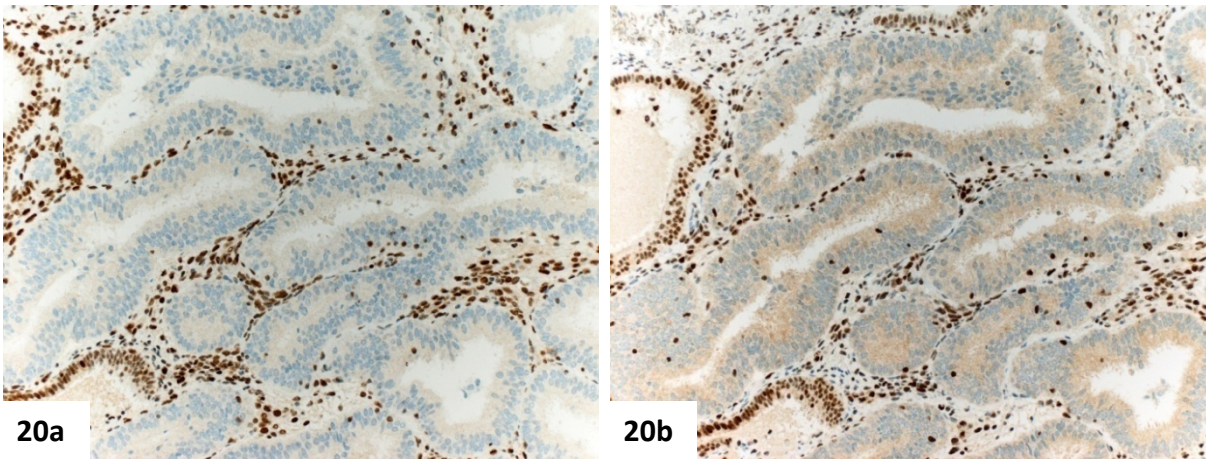


Fig.20a *loss of MLH1 nuclear expression in CHT;* ***Fig.20b*** *loss of PMS2 nuclear immunoreactivity in the same lesion. The negative glands contrasted with the background positivity of endometrial stromal cells.*

5.1.3.9. β-catenin expression

In normal endometrium, constant, strong membranous β-catenin immunoreactivity was observed in glandular cells of normal functional endometrium (proliferative n=41, secretor n=10, menstrual n=2, atrophic n=24, deciduas n=2), with similar intensity independently of the hormonal status. Cytoplasmic staining was also present, mainly in the proliferative glands. The endometrial stroma served as negative control, with the exception of endothelial cells, which showed moderate to strong membrane immunostaining, and

were used as a positive internal control. Nuclear staining was not found in any normal tissue elements.

Membrane β-catenin immunoreactivity in endometrioid hyperplastic lesions was similar to that found in normal glandular components and no differences were noticed along their entire spectrum. Nine well differentiated endometrioid adenocarcinomas (including those with tubal-type differentiation) were studied for the expression of β-catenin. Strong diffuse membranous positivity was invariably present, except one case which showed focal loss of β-catenin reactivity. No significant difference in the intensity of membrane staining was observed along the normal-hyperplastic-adenocarcinoma sequence.

In tubal-type lesions, together with a membranous positivity, we observed a higher immunoreactivity in the cytoplasm of secretory cells compared with negative or weakly positive ciliated cells (Fig.21a). The same pattern was maintained in the entire spectrum and it presented overlap with β-catenin reactivity in normal tubal epithelium. In only one CHT case, small β-catenin positive nuclear aggregates, without clear morphologic aspects of morules, were found.

A total of four cases (one endometrial polyp, one endometrioid ACH and two cases of well differentiated adenocarcinoma, one of which had tubal-type differentiation) presented abnormal nuclear expression of β-catenin. In all of these instances, morular differentiation was present and the positivity was restricted to these areas, or to scattered glandular cells surrounding morules or at a distance from them. This latter feature was present only in adenocarcinoma, a case in which the positive cells were considered as early intraepithelial morular change (Fig.21b). β-catenin distinguished between morules and squamous differentiation in a case of adenocarcinoma (Fig.23a).

SPSC and mucinous metaplasia constantly express β-catenin within the cellular membrane, but the intensity of staining ranges from weak to strong. 73% of mucinous metaplasia (16/19) and 88% (23/26) of SPSC presented a weak to moderate membranous positivity.

Figure 21. β-catenin expression in CHT and tubal-type adenocarcinoma

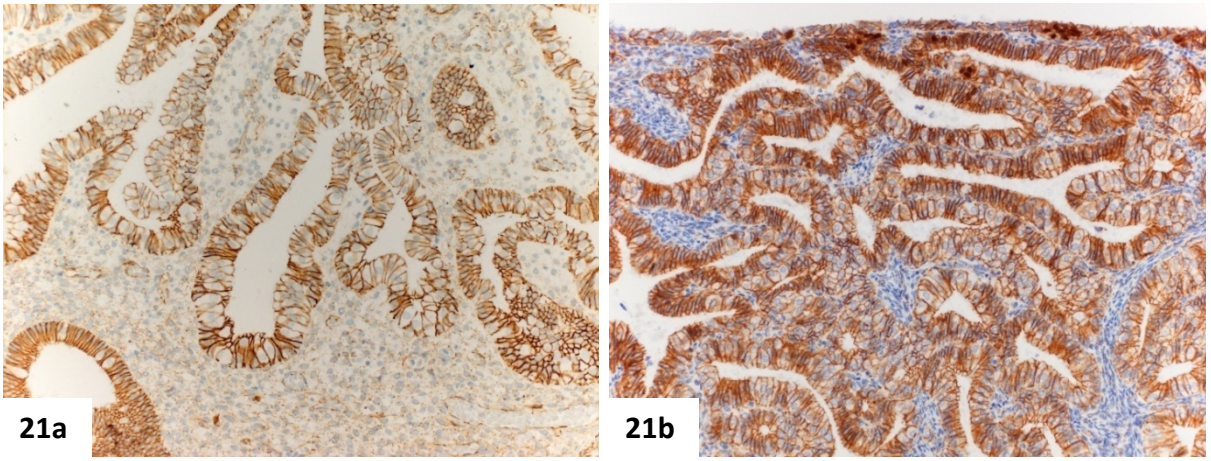


Fig.21a *membranous and cytoplasmic immunoreactivity of secretory cells and only membranous reaction of ciliated cells in CHT;* ***Fig.21b*** *the same pattern together with nuclear β-catenin expression in early morules of tubal-type endometrial adenocarcinoma.*

5.1.3.10. EGFR expression

57 of endometrial cases, including 14 controls, were studied for EGFR. A strike difference was noticed in the normal functional endometrium, with a diffuse, moderate to strong positivity within proliferative and atrophic glands and a weak or absent reaction in secretory, menstrual and decidualized endometria. Simple hyperplastic glands (n=13) and endometrioid ACH (n=3) presented the same diffuse positivity as the proliferative epithelium.

Among the spectrum of tubal-type lesions, 23/38 STM, 6/8 CTMI, 6/11 CHT (Fig.22a) and 1/2 tubal-type endometrioid adenocarcinoma, a mosaic immunoreactivity with pale ciliated cells protruding between moderate positive secretory cells was observed. This pattern was similar with that present in normal tubal epithelium (Fig.22b). The remaining cases were uniformly stained. Taken together, the intensity of reaction in tubal-type lesions was inferior to that remarked in proliferative endometria.

The rest of endometrioid (6) and serous (2) adenocarcinomas had a strong, diffuse or heterogeneous (<50% of cells) (2/6 and respectively 1/2) immunoreactivity. Regarding other associated metaplasias, a complete negativity was observed in mucinous (n=11) (Fig.22c) and in morular metaplasia (n=3) (Fig.22d). Also 5/11 SPSC lack the expression of EGFR and the residual 6 cases presented a very weak immunostaining.

Figure 22. EGFR expression in normal tubal epithelium and various metaplasias

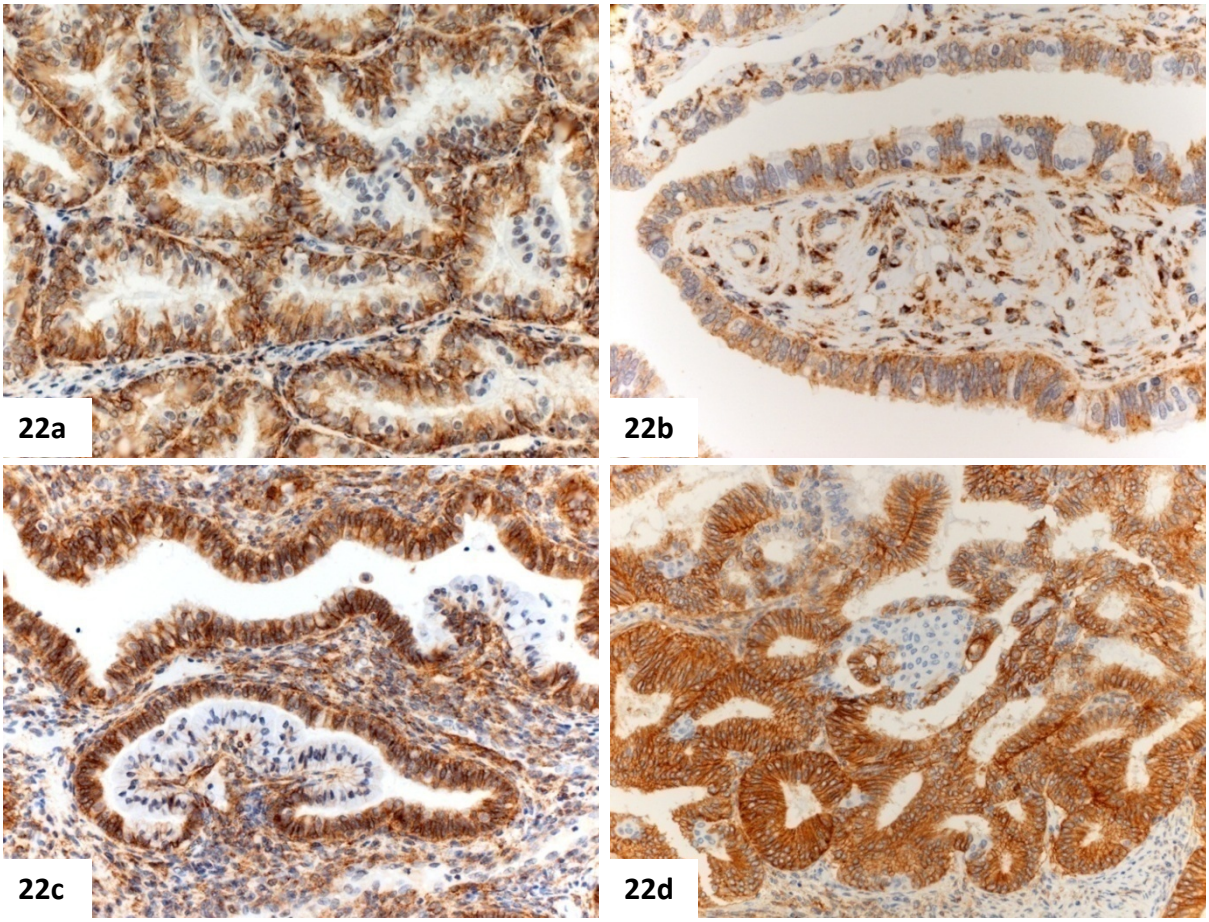


Fig.22a *chessboard pattern of EGFR, with secretory cells positive and ciliated cells negative in CHT glands;* ***Fig.22b*** *the same pattern of EGFR in the normal Fallopian tube epithelium;* ***Fig.22c*** *EGFR negative mucinous metaplastic areas protruding in the lumina of STM glands;* ***Fig.22d*** *EGFR negative morules within an well differentiated endometrioid adenocarcinoma*

5.1.3.11. CD10 expression

All the epithelial components of normal, metaplastic, hyperplastic and neoplastic endometria, as well as normal Fallopian tubes were negative for CD10, with the exception of two cases, one atrophic endometrium and one normal Fallopian tube, which showed a luminal CD10 positivity.

Morular metaplasia presented a strongly positive membranous and cytoplasmic staining. Also early intraepithelial morular changes were easily remarked in the background of negative glandular epithelium.

Nuclear β-catenin (Fig.23a) and CDX2 (Fig.23b) positivity together with the membranous and cytoplasmic staining for CD10 (Fig.23c) are considered specific features of morular metaplasia and discriminate it from squamous metapalsia. Their respective pattern of staining is presented in figure 23. CDX2 was performed only in the cases with morular

changes, in order to complete the panel of their diagnosis. Its nuclear staining was considered positive.

Figure 23. Differential diagnosis between morules and squamous metaplasia

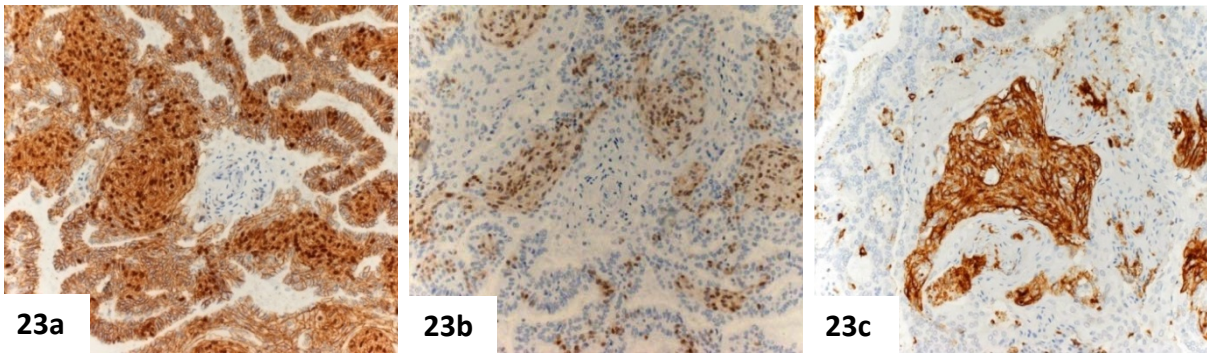


Fig.23a *nuclear and cytoplasmic expression of β-catenin within the morules;* ***Fig.23b*** *immunoreactivity of morules for CDX2;* ***Fig 23c*** *cytoplasmic and membranous CD10 reaction within the morules and its negativity in the squamous metaplasia of a well differentiated endometrioid adenocarcinoma*

5.1.3.12. ER and PR expression

We evaluated these antibodies together due to their close relationship.

Both hormonal receptors are expressed diffusely in normal proliferative, atrophic and hyperplastic endometria. ER and PR were heterogeneously expressed in secretory, decidual and menstrual endometria, with variability in percentage and intensity of staining between the glands and within the same gland. An important decrease of PR in the late secretory phase and decidualized endometria was observed.

Tubal-type lesions expressed constantly and diffusely both sex steroid hormonal receptors. Similar to normal tubal epithelium (Fig.24a), 11 cases: 10 STM and one CHT (Fig.24b) presented slight loss (more than 50%, but less than 80%) or weak expression of ER in the ciliated cells. These differences did not involve PR.

Also, a majority of mucinous metaplasia exhibited diffuse, strong positivity for both ER and PR, with only one case positive for ER in less than 50% of cells. SPSC nuclei revealed low levels of hormonal receptors expression and most cases (19/26) showed a weak to moderate positivity, in less than half of the cells (Fig.24c, 24d). 10-50% of squamous metaplastic nuclei were positive for ER and PR, in contrast to morular metaplastic foci which were completely negative (Fig.24e, 24f).

In well differentiated endometrioid adenocarcinoma ER and PR presented a diffuse (6/9 and 7/9 respectively) or a heterogeneous expression (3/9 and 2/9 respectively), with

more than 50% of cells positive. But the reactivity of both sex steroid receptors decreased, to less than 50% of cells in the surface and invasive components of serous adenocarcinoma, maintaining a higher reactivity at the invasion front.

Figure 24. Pattern of ER and PR in normal Fallopian tube epithelium, CHT, SPSC and morular metaplasia

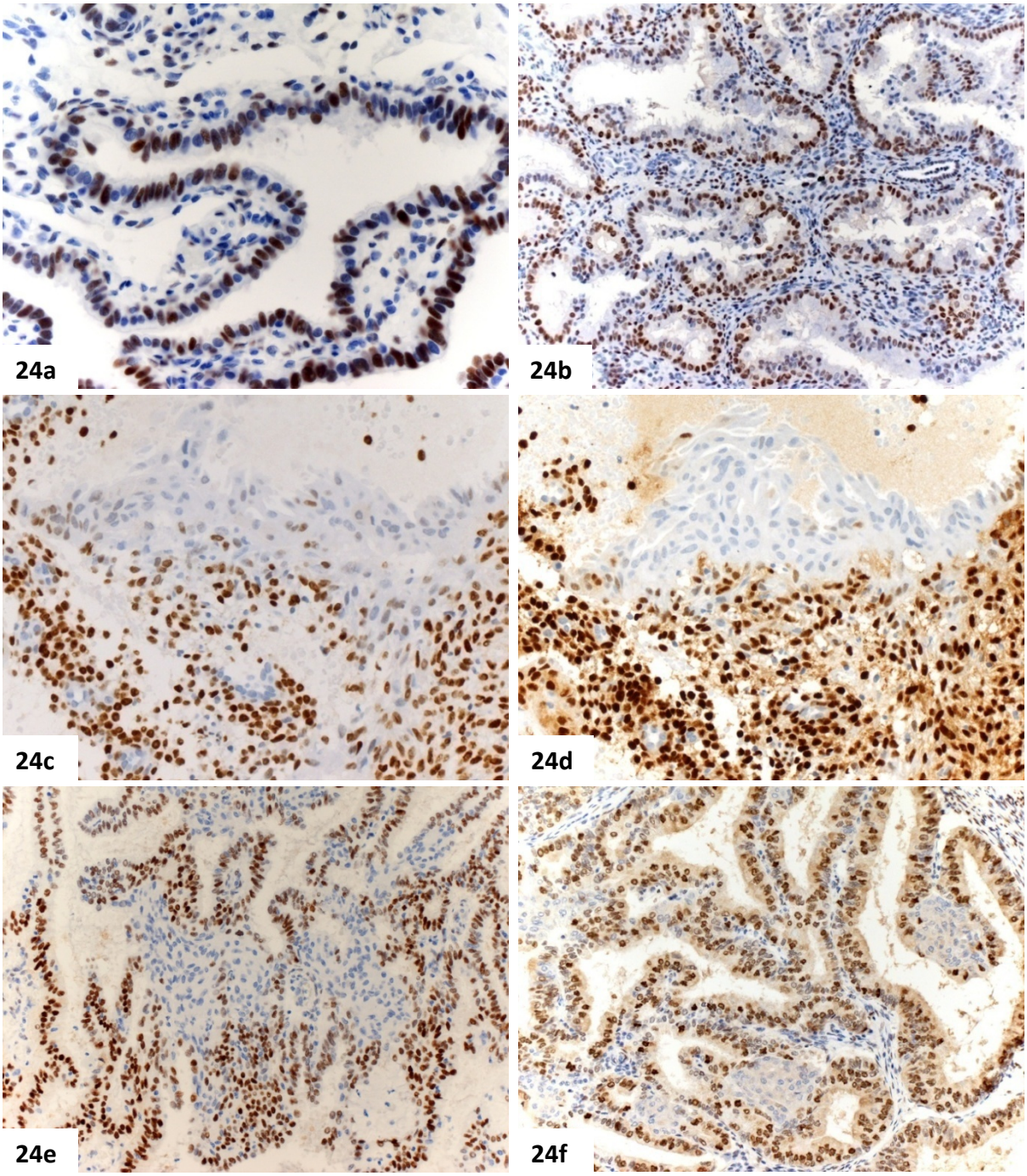


Fig.24a *weak or loss of ER expression in ciliated cells of normal tubal epithelium:* ***Fig.24b*** *the same pattern of ER in CHT glands;* ***Fig.24c*** *and* ***Fig.24d*** *decreased, weak reaction of ER and respectively PR in SPSC;* ***Fig.24e*** *and* ***Fig.24f*** *absence of ER and respectively PR immunoreactivity in morular metaplastic foci.*

5.1.3.13. CEA expression

62 of 113 endometrial cases were studied for CEA. None of the variables taken into the study had a cytoplasmic positivity. The normal functional and hyperplastic endometrial glands, the metaplastic areas and the adenocarcinomas foci were either negative or expressed CEA in a luminal pattern. The latter one was observed in 3/26 proliferative, 2/8 secretory, 1/15 atrophic endometria; 7/9 mucinous metaplasia and 6/49 TM, as also in 3/12 ACH and 1/7 endometrioid carcinomas. Moderate cytoplasmic staining was remarked in the squamous and mucinous differentiation respectively of two well differentiated adenocarcinoma cases.

No case of normal salpinx exhibited positivity for CEA.

5.1.3.14. Vimentin expression

Only 17 cases including controls were stained for vimentin. A uniform strong, diffuse expression was remarked in normal functional endometria (8 proliferative, 1 secretory and 5 atrophic), with exception of one case of decidualized endometria which showed negative glands. Tubal-type lesions, independently of the architectural complexity (9 STM, 1 CTMI and 1 CHT) were diffusely vimentin immunoreactive. The same pattern was seen in endometrioid atypical complex hyperplasia (n=1), endometrioid adenocarcinoma (n=4) and even serous adenocarcinoma (n=1). Heterogeneity of vimentin expression was evident in one case of well differentiated endometrioid adenocarcinoma, with the superficial part extensively negative compared with the strongly positive deeper part. Also, another case showed mucinous areas of differentiation vimentin negative (Fig.25a). Negativity was the rule in squamous (n=2), morular (n=1) (Fig.25b) and mucinous metaplasia (n=3). Two SPSC expressed vimentin in a focal, heterogeneous fashion. Normal tubal epithelium (n=7) had either uniform or 50-80% of cells positive.

Figure 25. Vimentin negativity of mucinous and morular differentiation of well differentiated endometrioid adenocarcinomas

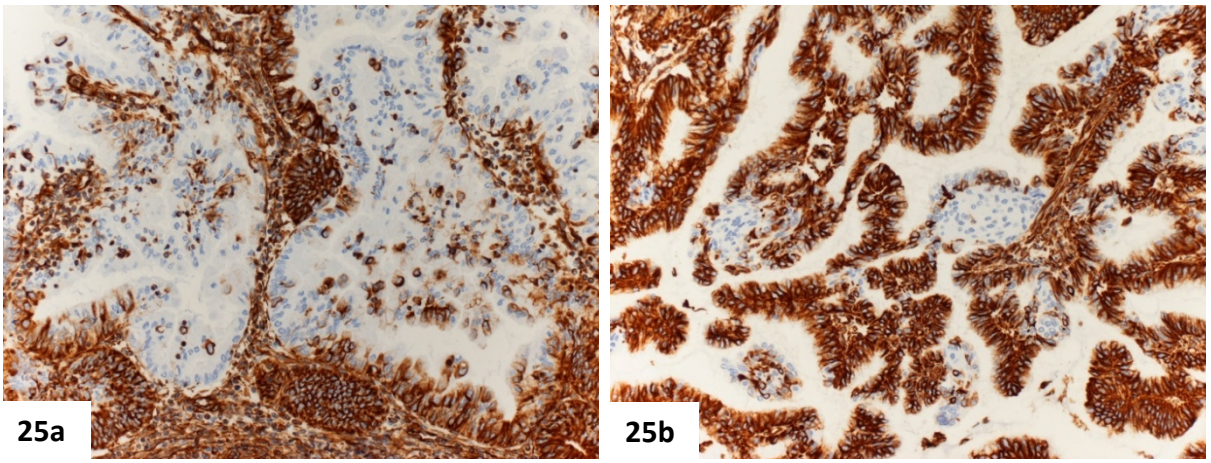


Fig.25a *negative mucinous differentiation areas on the background of strongly positive endometrial stromal cells,* ***Fig.25b*** *morules constantly negative for vimentin arise between positive endometrioid cells of a well differentiated endometrial adenocarcinoma.*

5.1.3.15. PTEN expression

In contrast with the literature, our 113 cases studied for PTEN presented a strong, diffuse nuclear staining, independently of type of variable evaluated: normal, metaplastic, hyperplastic or neoplastic endometria.

5.1.4. Statistical correlations

The statisticsal correlations between various variables taken into the study and previously mentioned in the text are summarized in the comparative tables 5.1, 5.2 and 5.3.

Statistics table 5.1. Immunohistochemical correlations between various architectural subtypes of tubal-type lesions and between complex hyperplastia endometrioid and tubal-type.

Tubal-type lesions	p16 (%)# 0/1/2/3/4	p	X^2	Cyclin D1 (%)# 0/1/2/3/4	p	X^2	bcl2 (%)# 0/1/2/3/4	p	X^2	Pax2 (%)# 0/1/2/3/4	p	X^2	Ki67 (%)* 0/1/2/3	p	X2
STM (n=89)	0/2/24/45/29	0,145	6,810	0/0/18/72/10	0,758	0,709	0/0/6/94/0	0,513	0.209	0/0/0/98/2	N/A	N/A	56/41/3/0	0,443	1,992
CTMI (n=11)	9/0/9/36/46			0/0/18/82/0			0/0/9/91/0			0/0/0/100/0			36/64/0/0		
CTMI (n=11)	9/0/9/36/46	0,016	10,134	0/0/18/82/0	0,204	4,291	0/0/9/91/0	0,202	4,977	0/0/0/100/0	<0,01	15,191	36/64/0/0	0,205	3,950
CHT (n=17)	0/17/53/12/18			0/12/35/41/12			12/12/17/47/12			65/0/6/23/6			12/65/17/6		
CHT (n=17)	0/17/53/12/18	0,001	14,404	0/12/35/41/12	0,006	11,085	12/12/17/47/12	<0,01	26,923	65/0/6/23/6	<0,01	42,891	12/65/17/6	<0,01	16,875
STM (n=89)	0/2/24/45/29			0/0/18/72/10			0/0/6/94/0			0/0/0/98/2			56/41/3/0		
CHT (n=17)	0/17/53/12/18	0.037	7.361	0/12/35/41/12	0.926	0.908	12/12/17/47/12	0.064	7.554	65/0/6/23/6	0.153	4.950	12/65/17/6	0.390	3.564
Endometrioid CH (n=10)	0/70/20/10/0			0/10/50/30/10			0/0/11/22/67¥			56/0/11/0/33¥			0/56/11/33¥		

 # 0=<1%, 1=<10% of cells positive; 2=11-50% of cells positive, 3=51-80% of cells positive; 4=>80% of cells positive

** 0=<1%, 1=<10% of cells positive; 2=11-30% of cells positive: 3=>30% of cells positive*

¥ 9 cases of endometrioid complex hyperplasia (ACH and CH) were stained for bcl2, PAX2 and Ki-67

Statistics table 5.2. Immunohistochemical correlations between STM and normal proliferative and atrophic endometrium

Tubal-type lesions	p16 (%)# 0/1/2/3/4	p	X^2	Cyclin D1 (%)# 0/1/2/3/4	p	X^2	bcl2 (%)# 0/1/2/3/4	p	X^2	Pax2 (%)# 0/1/2/3/4	p	X^2	Ki67 (%)* 0/1/2/3	p	X^2
Proliferative (n=41)	12/56/27/5/0	<0,01	82,974	0/10/34/49/7	0,002	13,123	0/0/0/7/93	<0,01	128,29	4/0/0/4/92(§)	<0,01	71,149	5/56/17/22	<0,01	49,946
STM (n=89)	0/2/24/45/29			0/0/18/72/10			0/0/6/94/0			0/0/0/98/2(∫)			56/41/3/0		
STM (n=89)	0/2/24/45/29	<0,01	66,180	0/0/18/72/10	0,005	11,457	0/0/6/94/0	<0,01	98,636	0/0/0/98/2(∫)	N/A	N/A	56/41/3/0	<0,01	21,549
Atrophic (n=24)	8/71/17/4/0			0/8/38/54/0			0/0/0/4/96			0/0/0/6/94(∑)			8/84/4/4		

 # 0=<1%, 1=<10% of cells positive; 2=11-50% of cells positive, 3=51-80% of cells positive; 4=>80% of cells positive

** 0=<1%, 1=<10% of cells positive; 2=11-30% of cells positive: 3=>30% of cells positive*

§ 23 cases of proliferative endometrium were stained for PAX2

∫ 53 cases of STM were analyzed for PAX2

∑ 16 cases of atrophic endometria were stained for PAX2

Statistics table 5.3. Immunohistochemical correlations in the spectrum of endometrioid lesions

Tubal-type lesions	p16 (%)[#] 0/1/2/3/4[#]	p	X^2	Cyclin D1 (%)[#] 0/1/2/3/4	p	X^2	bcl2 (%)[#] 0/1/2/3/4	p	X^2	Pax2 (%)[#] 0/1/2/3/4	p	X^2	Ki67 (%)* 0/1/2/3	p	X^2
Proliferative (n=41)	12/56/27/5/0	0,125	5,174	0/10/34/49/7	0,451	2,895	0/0/0/7/93	N/A	N/A	4/0/0/4/92(§)	0,158	3,211	5/56/17/22	<0,01	19,002
SH endom (n=29)	0/62/38/0/0			0/0/41/52/7			0/0/0/7/93			0/0/0/21/79(£)			0/10/48/42		
ACH + CH (n=10)	0/70/20/10/0	0,037	6,186	0/10/50/30/10	0,724	2,126	0/0/11/22/67(¥)	0,042	8,159	56/0/11/0/33(¥)	0,287	3,615	0/56/11/33(¥)	1,000	1,739
ADCa endom (n=9)	0/22/78/0/0			0/11/33/56/0			11/11/56/11/11			72/0/14/14/0(†)			11/45/22/22		
SH endom (n=29)	0/62/38/0/0	0,364	2,136	0/0/41/52/7	0,402	2,990	0/0/0/7/93	<0,01	18,041	0/0/0/21/79(£)	<0,01	22,384	0/10/48/42	0,005	11,521
ACH + CH + ADCa endom (n=19)	0/47/48/5/0			0/11/42/42/5			5/6/33/17/39(‡)			62/0/13/6/19(&)			5/50/17/28(‡)		
ACH + CH + ADCa endom (n=19)	0/47/48/5/0	0,236	3,952	0/11/42/42/5	0,965	0,641	5/6/33/17/39(‡)	<0,01	22,496	62/0/13/6/19(&)	<0,01	22,744	5/50/17/28(‡)	0,964	0,645
Proliferative (n=41)	12/56/27/5/0			0/10/34/49/7			0/0/0/7/93			4/0/0/4/92(§)			5/56/17/22		

 # 0=<1%, 1=<10% of cells positive; 2=11-50% of cells positive, 3=51-80% of cells positive; 4=>80% of cells positive

** 0=<1%, 1=<10% of cells positive; 2=11-30% of cells positive: 3=>30% of cells positive*

§ 23 cases of proliferative endometrium were stained for PAX2

£ 19 cases of SH were analyzed were stained for PAX2

¥ 9 cases of endometrioid complex hyperplasia (ACH and CH) were stained for bcl2, PAX2 and Ki-67

† 7 cases of endometrioid adenocarcinoma were stained for PAX2

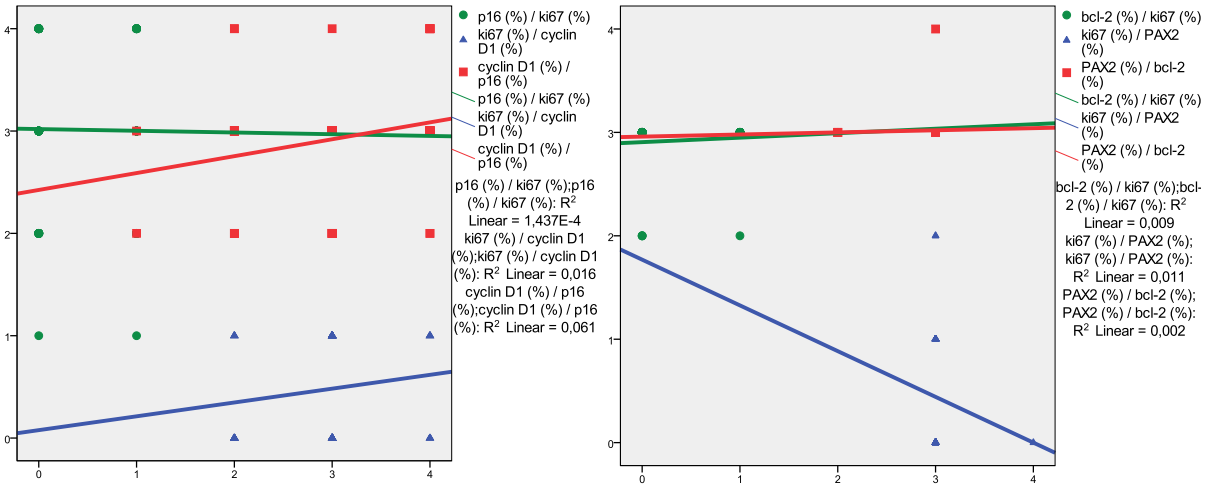
‡18 cases of complex endometrioid hyperplasia and adenocarcinoma were analyzed for bcl-2 and Ki-67

& 16 cases of complex endometrioid hyperplasia and adenocarcinoma were analyzed for PAX2

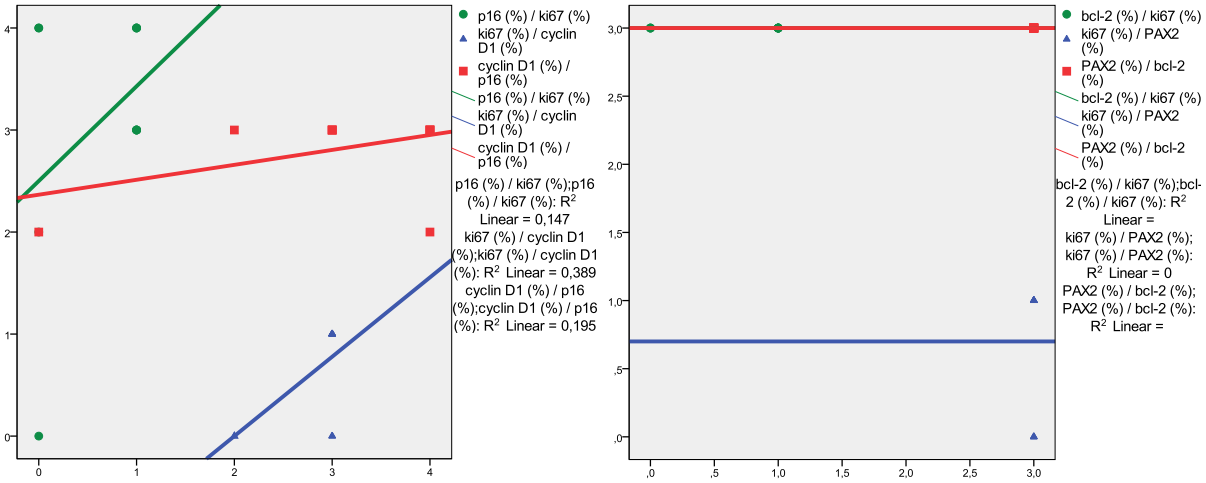
N/A Fischer exact test not applicable

We also analyzed the correlations between various proteins and proliferation rate within the spectrum of tubal-type lesions. Their relationship can be visualized in the linear regression graphics.

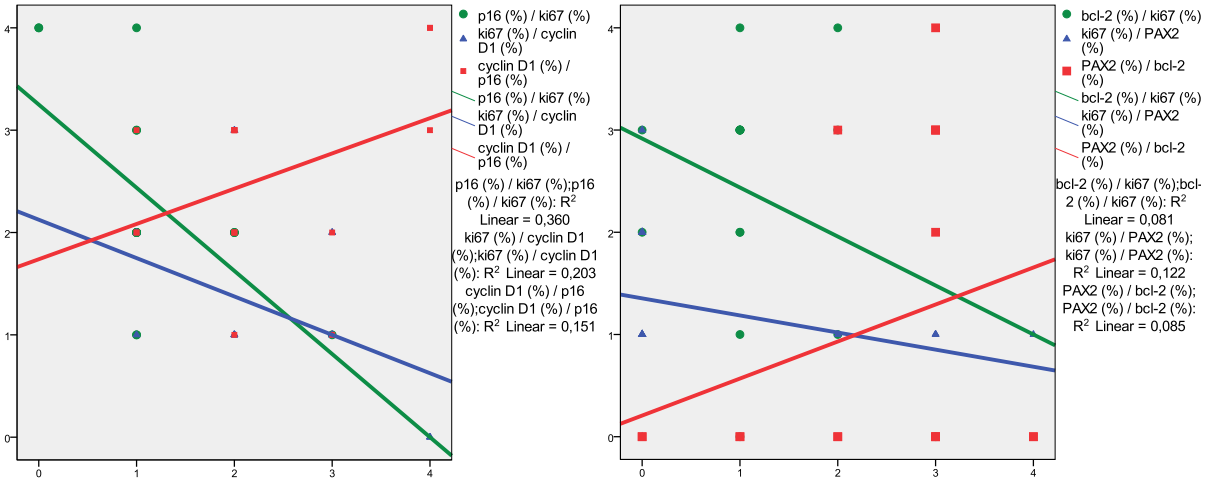
Graph 6. Correlations of p16, cyclin D1 and Ki 67 and correlations of bcl-2, PAX2 and Ki-67 in simple TM



Graph 7. Correlations of p16, cyclin D1 and Ki 67 and correlations of bcl-2, PAX2 and Ki-67 in complex TM in isolated glands



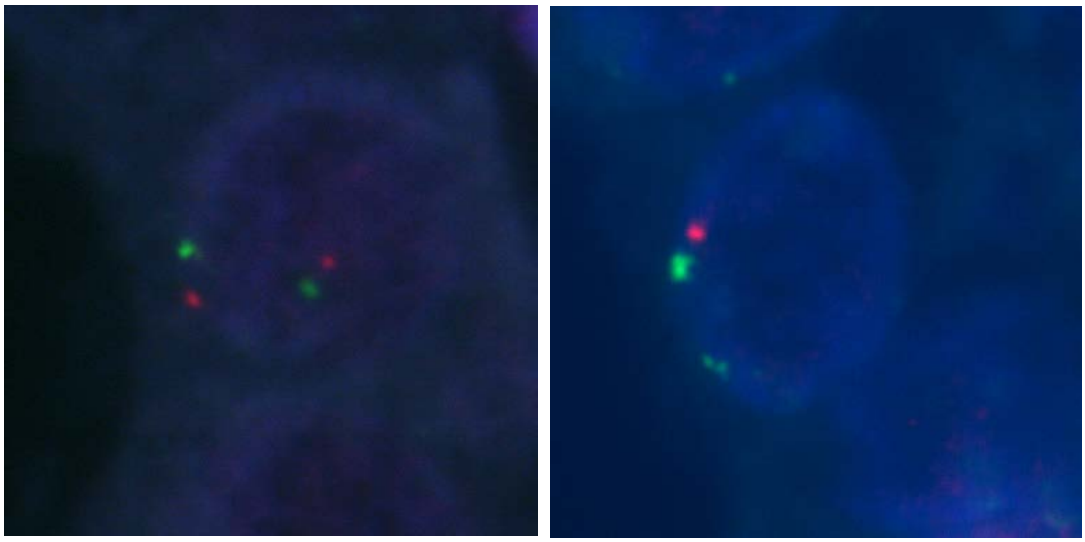
Graph 8. Correlations of p16, cyclin D1 and Ki 67 and correlations of bcl-2, PAX2 and Ki-67 in complex hyperpalsia tubal-type



5.1.5. Evaluation of PTEN mutation by FISH

PTEN hemizygous deletion was found in three of 19 cases studied. One corresponded to CHT (Fig 26), one to CTMI and the third to STM extensive in endometrial adenofibromas. The first presented concomitant loss of MLH1 and PMS2, and did not show K-ras alteration. None of the PTEN-mutated cases demonstrated lack of PAX2 expression.

Figure 26. Normal pattern and hemizygous deletion of PTEN locus

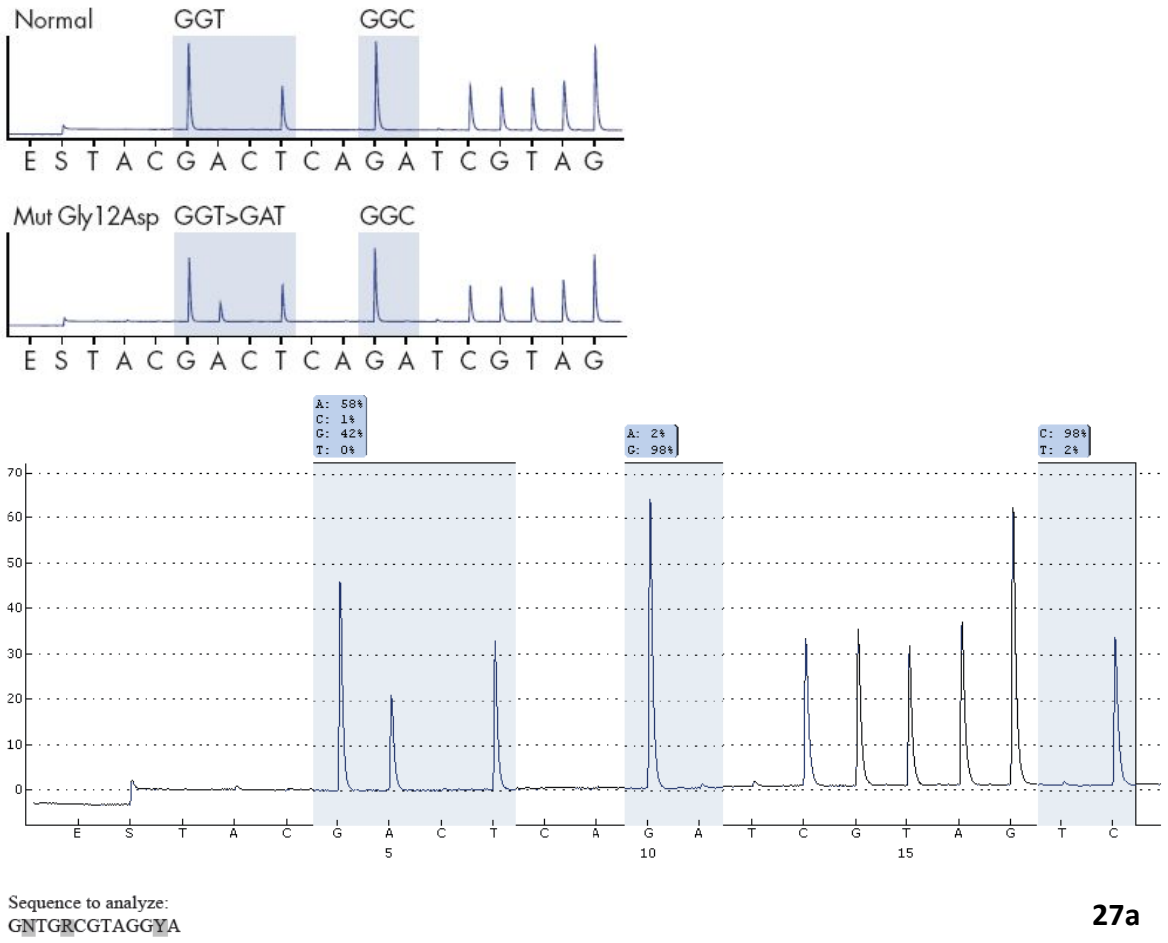


In the right image is exemplified the normal pattern of PTEN gene. In the left, FISH analysis shows loss of one of the signal for PTEN, indicating hemizygous deletion of 10q23/PTEN locus in a case of CHT

5.1.6. Evaluation of K-ras status by PCR

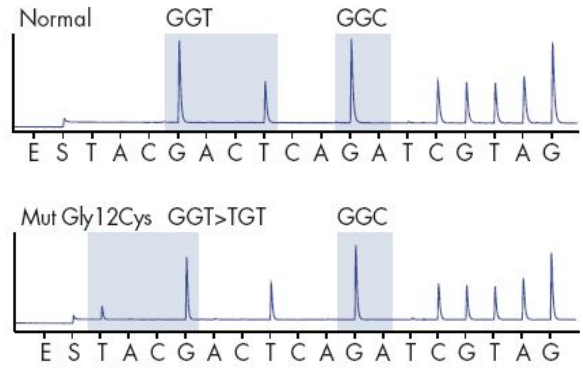
Three of six (50%) tubal type endometrial lesions screened for point mutation in exon1 codons 12, 13 and 61 of the K-ras oncogene were detected to have a mutation. Two of them were morphologically diagnosed as CHT, in 56 and 58 year old patients respectively. One presented a single point mutation in codon 12 (Fig.27a) and the second one had a double mutation in codon 12 and 13 (Fig.27b). The third case corresponded to a 70 year old woman with an endometrial polyp, the glands of which presented extensive STM. In this case a single point mutation in codon 61 was found (Fig.27c). None of these cases presented concomitant alteration of PTEN or showed a MSI status. Only the case with singular mutation in codon 12 had a decrease of PAX2 expression. These three cases with K-ras mutation and 2 of those 3 with PTEN hemizygous status presented overexpression of cyclin D1, but without significant differences from those with normal gene status. Surprisingly, all of them presented a low proliferation index, with a rate inferior to 10%.

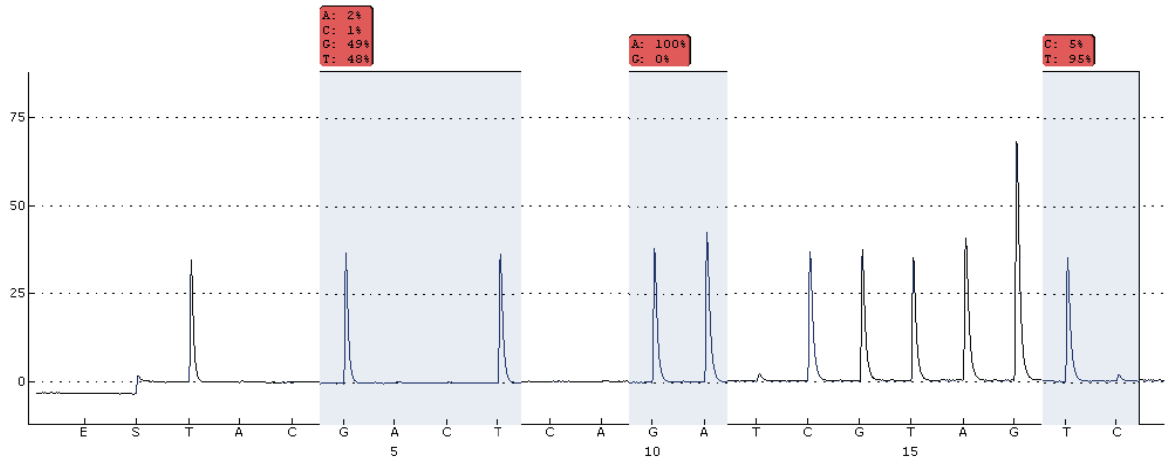
Figure 27. Results of codons 12, 13 and 61 mutation analysis using PyroMark Q24 KRAS

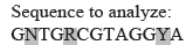


27a

Fig. 27a *Point mutation in codon 12. The upper left Pyrograme trace shows a sample with a normal genotype. The bottom left Pyrograme trace shows mutation analysis in a sample with a G to A mutation in position 2 of codon 12 (GGT>GAT, Gly12Asp), the same mutation observed by us (right Pyrograme trace) in one case.*







27b

Fig.27b *Point mutations in codon 12 and 13. The upper left Pyrograme trace shows a sample with a normal genotype. The bottom left Pyrograme trace shows mutation analysis in a sample with a G to T mutation in position 1 of codon 12 identified post-run by the altered sequence. The right Pyrograme trace exemplified our case with a G to T mutation in position 1 of codon 12 (GGT>TGT, Gly12Cys) and G to A mutation in position 2 of codon 13 (GGC>GAC, Gly13Asp).*

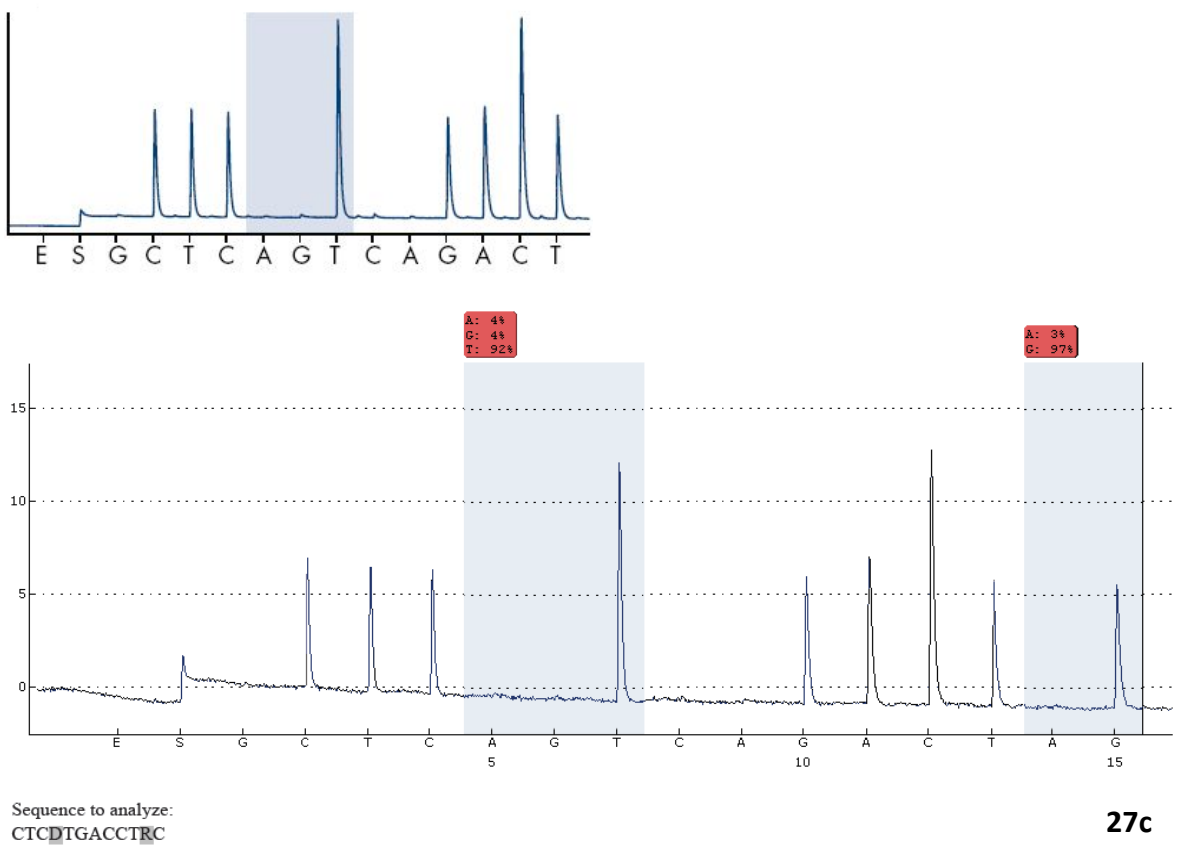


Fig.27c *Point mutations in codon 61. The left Pyrograme trace shows the point mutation in codon 61 refered in the PyroMark Q24 KRAS kit protocol. The right Pyrograme trace shows the third our mutated case.*

5.2. Cervix

5.2.1. Clinical profile

Cervical TM (n=40) was studied within an age range of 36 and 78 years, with a mean age of 45 years. In the majority of cases, main clinical indications for surgical intervention were related to uterine pathology (n=26, 65%). The remaining cases had had previous abnormal cervical cytology (n=2), biopsy (n=7) or endocervical polyps (n=5).

5.2.2. Morphological findings

TM was defined using the same morphological criteria as in the endometrium.

5.2.2.1. Morphological context of TM presence

In the *cervix,* TM was an incidental microscopic finding. The uterine corpus or cervical pathology concurrent with TM is listed in the table 5.12. The majority of the pathological conditions associated with TM were benign (the largest group) or malignant lesions of the

uterine corpus. Concomitant squamous or glandular HPV related lesions were present in a reduced number of cases (n=4) and almost the same number of women presented TM in the surgical specimens following a previous cervical intervention for these types of lesion (n=5). 5 endocervical polypoid lesions (3 polyps and 2 adenofibroma) were also involved by TM.

Table5.12. Conditions associated with TM in the surgical specimens

Histopathological diagnosis	TM cases (n=40)
Leiomyomas	16
Adenomyosis	1
Endometrial endometrioid ADCa	5
Endometrial serous ADCa	2
CIN	3
AIS	1
Endocervical polyps	3
Endocervical adenofibromas	2
Changes for prolaps	1
*Status post previous biopsy of HPV related lesions (CIN or AIS)	4
*Status post QT and RT for Sq.C.	1
*Status post endometrial ACH	1

***For the cases with "status post" no residual lesion was found*

Together with TM, various other lesions were present, such as: tunnel clusters (n=2), Nabothian cysts (n=3), mesonephric hyperplasia (n=1), radiotherapy changes (n=1), endocervical in situ adenocarcinoma (AIS) (n=1), cervical extension of endometrioid adenocarcinoma (n=2) and tubo-endometrial metaplasia (n=3). Furthermore, in some cases (n=8) normal endometrial glands from the isthmus region were observed. The simultaneous occurrence of squamous metaplasia, either immature (n=6) or mature (n=8) was evaluated for all the antibodies.

The histological features of mature and immature cervical squamous metaplasia are well known and need no further description. In one of the cervical cases, TM was associated with a florid proliferation of small to medium size glands, arranged in a lobular pattern, with thick eosinophilic secretion in their lumina. They corresponded to mesonephric remnants hyperplasia and the extent of the process raises diagnostic problems by its location in contact with surface endocervical lining epithelium.

5.2.2.2. Topography

27.5% of cervical TM were located at the squamo-columnar junction (Fig.28a), 40% involved median third, sometimes with deep location (n=2) and 22.5% lined glands placed high in the endocervical canal. In the remaining 10%, TM replaced normal endocervical epithelium of polyps and adenofibromas. Approximately half of the cases also had TM in surface lining epithelia (Fig.28b). Almost 50% of the cervical cases presented more than occasional glands (2+) involved by a tubal type or ciliated epithelium and occasionally (2%) a diffuse involvement (3+) could be seen.

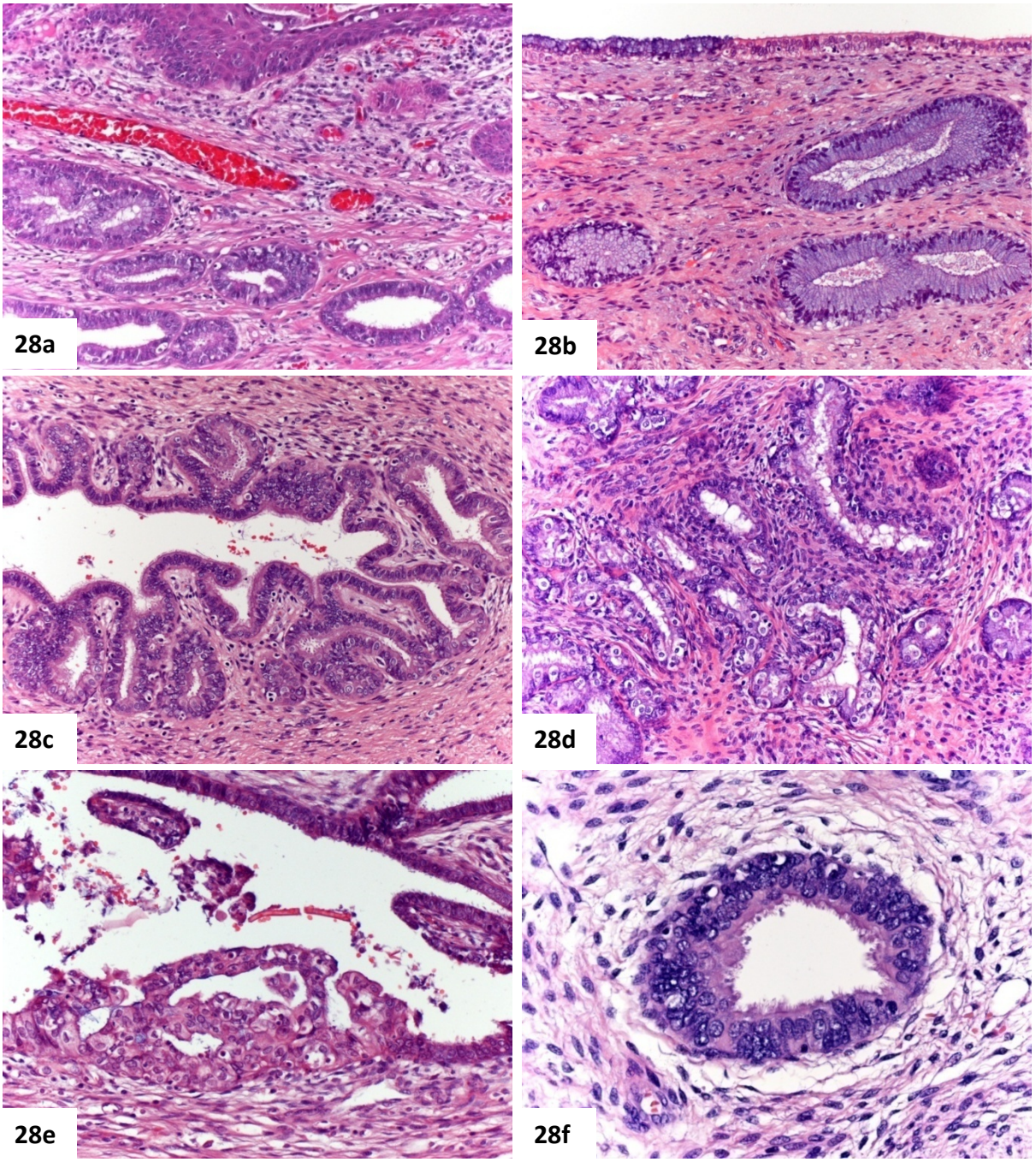
5.2.2.3. Architectural pattern

70% of TM presented a simple architecture, with normal size (Fig.28a) or cystically dilated glands lined by tubal-type epithelia. Rarely, ramified glands were seen (3/40) (Fig.28c), especially in the cases of cervical adenofibromas, where a florid TM proliferation was present. As in the endometrium, TM with more complex architecture, with a back-to-back (Fig.28d), papillary or cribriform arrangement was seen (n=9) (Fig.28e). Within the partially involved glands, a gradual transition from tubal type to secretory mucinous epithelia was frequently observed. In rare instances, an abrupt switch between the two epithelia posed diagnostic problems. Also, pseudo- or true stratification (n=8), mild atypia (n=3) (Fig.28f) and scattered typical mitotic figures (n=2) created difficulties in interpretation. Striking atypia in the absence of proliferative activity, a consequence of radiotherapy, involved endocervical and endometrial TM glands (Fig.28g).

5.2.2.4. Stromal changes

We evaluated also the stroma around TM glands, due to their frequent misdiagnosis with neoplastic endocervical lesions. A desmoplastic-like reaction, with oedematous or myxoid change, sometimes with inflammatory cells was present in 9/40 cases (22.5%) (Fig.28h). Mild to moderate inflammatory background encircled TM glands in 7/40 cases (17.5%) (Fig.28i) and a cambium layer was observed in 2/40 cases (5%), corresponding to adenofibromas (Fig.28j) Diagnostic difficulty was increased by the deep location of the glands with this type of stromal changes (n=2.5%), as the case of an adenofibromas in which the florid proliferation occupied the entire thickness of cervical wall and did not arise in a polypoid lesion.

Figure 28. Cervical TM topography, architectural changes and stromal reaction



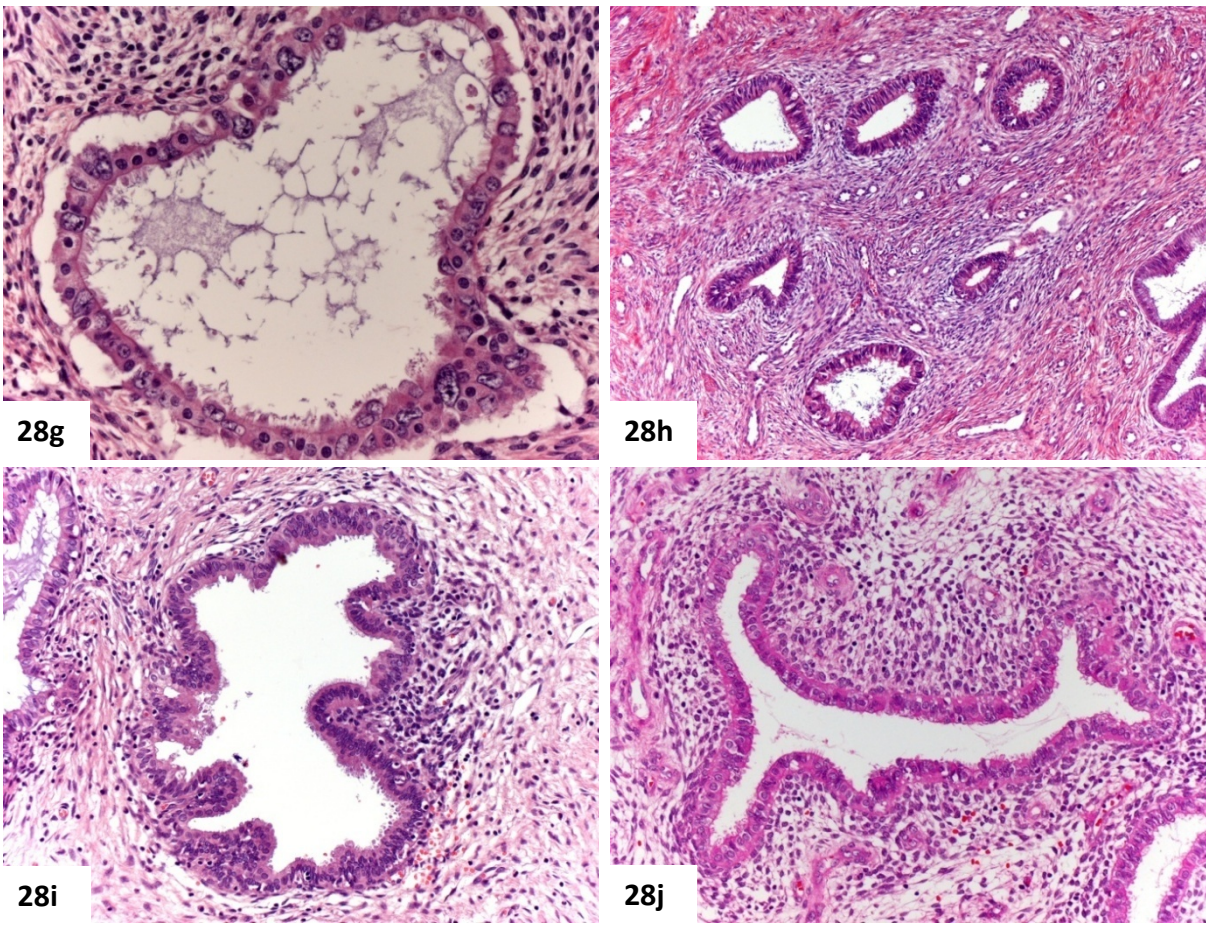


Fig.28a *simple TM located at the squamo-columnar junction;* ***Fig.28b*** *abrupt transition between endocervical and tubal-type epithelium of the surface lining epithelium;* ***Fig.28c*** *complex ramified TM gland;* ***Fig.28d*** *crowded, back-to-back TM glands;* ***Fig.28e*** *cribriforme pattern of TM;* ***Fig.28f*** *stratified TM cells with mild to moderate nuclear atypia;* ***Fig.28g*** *TM gland with marked nuclear atypia induced by radiotherapy;* ***Fig.28h*** *desmoplastic, myxoid-like reaction around simple TM glands;* ***Fig.28i*** *inflammatory infiltrate encircling TM glands;* ***Fig.28j*** *TM with a cambium layer in a cervical adenofibromas.*

For reasons of comparison, we also evaluated 2 in situ and 4 invasive endocervical adenocarcinomas. The latter one included one case of villoglandular adenocarcinoma. Their diagnosis was based on lack of intracellular secretion, nuclear atypicality, increased number of mitosis and apoptotic bodies.

Figure 29. The morphologic overlap of TM with *in situ* endocervical adenocarcinoma is highlighted.

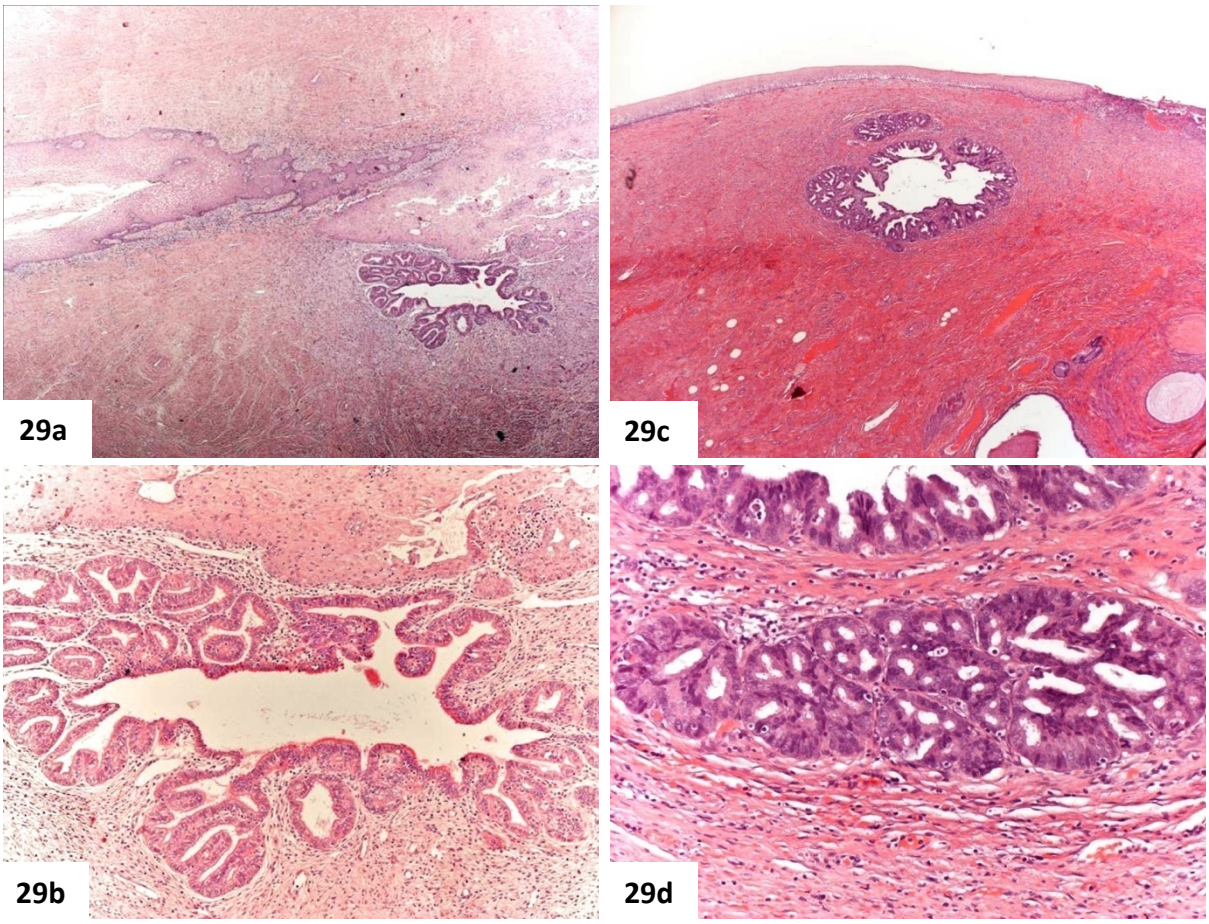


Fig.29a *complex architecture TM (low power), with a lobular appearance, located at squamo-columnar junction;* ***Fig.29b*** *high power of the same area;* ***Fig.29c*** *endocervical AIS located at squamo-columnar junction (low power);* ***Fig.29d*** *detail of the same area.*

5.2.3. Immunohistochemical profile

The expression of each antibody in the normal endocervical epithelia, TM and neoplastic lesion was studied. Also, further annotation will be made regarding normal ectocervix and squamous metaplasia.

5.2.3.1. LhS28 expression

Occasional ciliated cells could be indentified in the normal endocervical epithelium of 6 of 44 cases (Fig.30a), as also within the glands with reactive, actinic changes. None of the benign mucinous lesions or adenocarcinomas presented positivity for this antibody. TM glands were easily recognised due to their apical, granular positivity for LhS28, in more than 50% of cells in almost two thirds of cases (Fig.30b). For the rest of cases a dominant population of secretory cells with less than 50% of cells bearing cilia was present.

Figure 30. LhS28 in the normal and TM glands

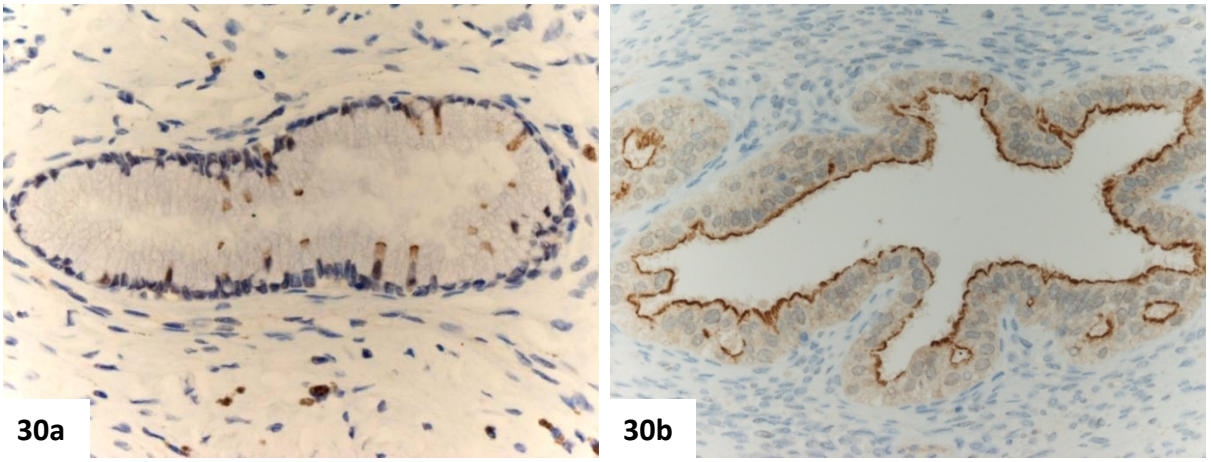


Fig.30a *scattered LhS28 apical positive ciliated cells in a normal endocervical gland;* ***Fig.30b*** *TM glands lined by a majority of ciliated cell population.*

5.2.3.2. p16^{INK4A} expression

The results of immunohistochemical staining for p16^{INK4A} are shown in the table 5.13.

Normal endocervical epithelium was available for evaluation in 45 cases, including control group. In contrast with normal endometrium, the endocervical glands were either negative (60%) or presented rare spots of p16^{INK4A} positivity in individual or small clusters of cells, especially in inflammatory conditions (Fig.31a). There was no expression of p16^{INK4A} protein in normal squamous epithelium, however immature (2/8), mature (2/8) squamous metaplasia and squamous epithelium (1/20) showing inflammatory and reactive changes, sometimes showed focal p16^{INK4A} expression.

All cases of TM were positive, except one. Generally, they presented a moderate to strong positivity in more than occasional cells (>10%), with a heterogeneous pattern. No case labeled in a mosaic fashion. In 35% of cases, >50% of the cells were stained in both their cytoplasm and nuclei (Fig.31b). Two cases showed the same diffuse, strong pattern of staining (Fig.31c) as the neoplastic endocervical lesions, both in situ and invasive, used as controls (Fig.31d). Therefore, p16 had a statistically significant higher expression in neoplastic endocervical lesions compared with normal ($p<0.01$) and TM glands (<0.01). Also, TM presented more p16 positive cells than normal endocervical glands ($p<0.01$) (statistics table 5.4).

Figure 31. p16 expression pattern in TM, normal and neoplastic endocervical cells

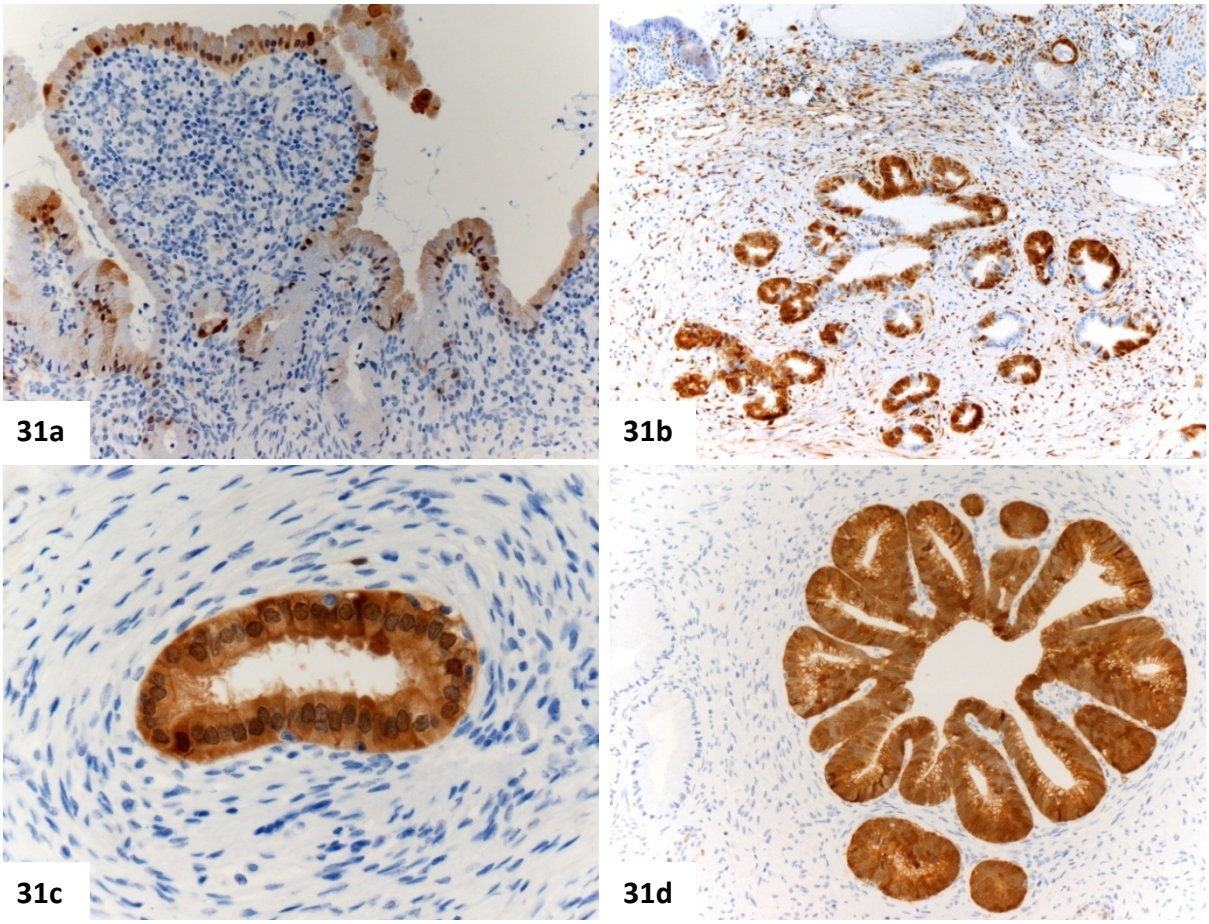


Fig.31a *focal nuclear and cytoplasmic p16 positivity of normal endocervical epithelium placed in an inflammatory background;* ***Fig.31b*** *heterogeneous p16 positivity of TM glands located at squamo-columnar junction;* ***Fig.31c*** *diffuse nuclear and cytoplasmic positivity of TM cells;* ***Fig.31d*** *strong, diffuse p16 positivity of AIS located in vicinity of negative, normal endocervical glands.*

Table 5.13. $p16^{INK4A}$ in TM, normal and neoplastic endocervical epithelium

Histological category*	$p16^{INK4A}$ immunoreactivity					Total
	0	<10%	11-50%	51-80%	>80%	
Normal endoc ep.	27 60.0%	17 37.8%	1 2.2%	0 .0%	0 .0%	45 100.0%
TM	1 2.5%	6 15.0%	17 42.5%	14 35.0%	2 5.0%	40 100.0%
AIS and ADCa	0 .0%	0 .0%	0 .0%	0 .0%	6 100.0%	6 100.0%
Total	28 30.8%	23 25.3%	18 19.8%	14 15.4%	8 8.8%	91 100.0%

** The histological categories include also control cases*

The mesonephric glands, papillary endocervicitis and glandular changes induced by radiotherapy stained focally (10-50%) for $p16^{INK4A}$. The same positivity was seen in half of

the cases that included normal endometrial glands in the same slide. All cases of Nabothian cysts and tunnel clusters were negative.

5.2.3.3. Cyclin D1 expression

Endocervical glands were frequently positive for cyclin D1, with majority of cases showing more than a focal immunoreaction (57.8% of cases more than 50% of cells positive) (Fig.32a, 32b) and with increased expression in the vicinity of inflammatory changes. Cyclin D1 was restricted to the parabasal layer of normal exocervix and mature squamous metaplasia and to the reserve cells of immature metaplasia.

TM was typically cyclin D1 positive, with a heterogeneous and only exceptionally a mosaic (3/40) pattern of staining. Cervical TM glands had cyclin D1 constrained mainly to the nuclei of secretory cells (Fig.32a). Majority of cases had a positivity in less than 50% of cells, in contrast to normal endocervical glands that labeled in more than half of the cells (see table 5.14), and this difference reached significance ($p=0.023$) (statistics table 5.4.). All the endocervical adenocarcinoma cases, either in situ or invasive were negative (Fig.32b), except one case of AIS which had less than 10% of nuclei. In the partially involved glands, the positive endocervical cells were sharply demarcated from the unstained adjacent neoplastic epithelia; the vice versa phenomenon was observed for Ki-67. Cyclin D1 could differentiate between normal and neoplastic endocervical glands ($p<0.01$), as also between TM and malignant lesions ($p<0.01$) (statistics table 5.4).). Benign endocervical lesions had a pattern of cyclin D1 expression comparable to the normal epithelium.

Figure 32. Cyclin D1 in TM, normal and neoplastic endocervical epithelium

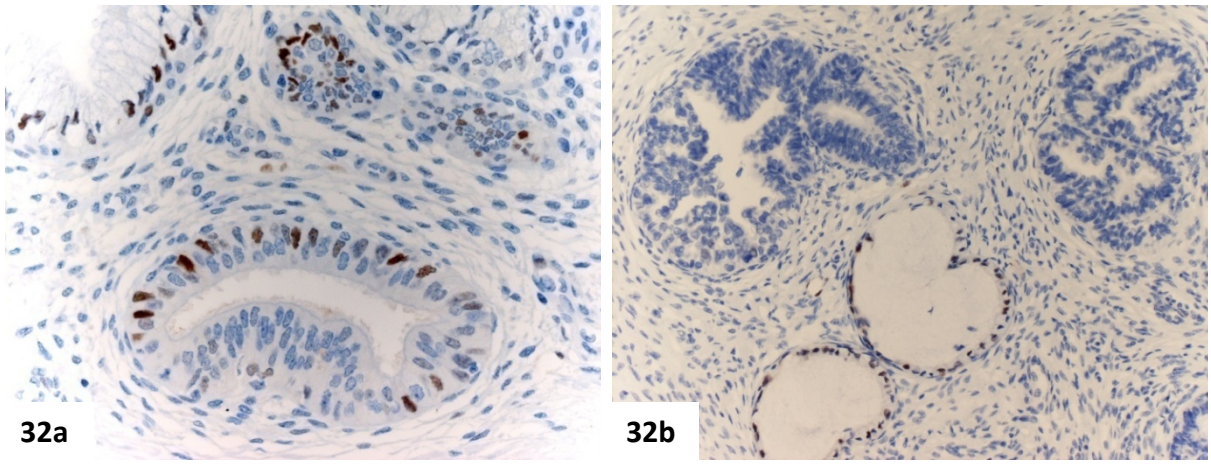


Fig.32a *focal Cyclin D1 nuclear positivity, mainly restricted to the secretory cells of TM;* ***Fig.32b*** *negative cyclin D1 foci of endocervical adenocarcinoma; both lesions present in vicinity diffuse positive normal endocervical glands.*

Table 5.14. Cyclin D1 in TM, normal and neoplastic endocervical epithelium

Histological category*	Cyclin D1 immunoreactivity					Total
	0	<10%	11-50%	51-80%	>80%	
Normal endoc ep.	2 4.4%	4 8.9%	13 28.9%	25 55.6%	1 2.2%	45 100.0%
TM	0 .0%	7 17.5%	22 55.0%	11 27.5%	0 .0%	40 100.0%
AIS and ADCa	5 83.3%	1 16.7%	0 .0%	0 .0%	0 .0%	6 100.0%
Total	7 7.7%	12 13.2%	35 38.5%	36 39.6%	1 1.1%	91 100.0%

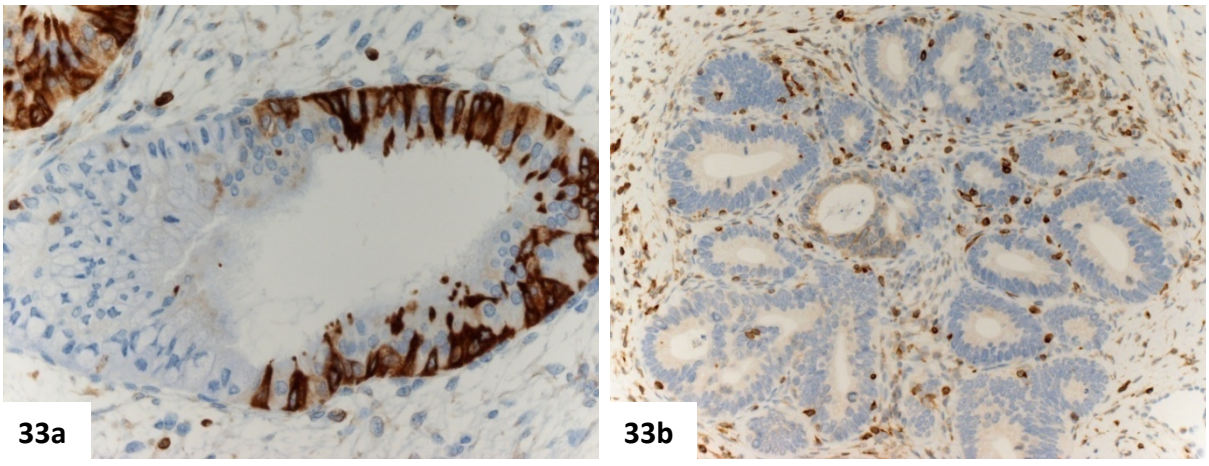
** The histological categories include also control cases*

5.2.3.4. Bcl-2 expression

The normal endocervical epithelium was constantly negative for bcl-2, but a strong positivity was seen in the reserve cells. The ectocervix and mature squamous metaplasia showed consistent bcl-2 expression only in the basal layer. The staining was restricted to the hyperplastic reserve cells in immature squamous metaplasia. Cervical TM presented the same mosaic pattern of staining as their endometrial counterpart (Fig.33a). This was in contrast with normal (Fig.33a), benign and neoplastic endocervical lesions which lack the expression of bcl-2 (Fig.33b). These differences reached statistical significance ($p<0.01$) (statistics table 5.4). Endometrial glands presented in some slides were diffusely positive.

The stroma of both cervix and endometrium showed in majority of cases at least focal immunoreactivity.

Figure 33. Bcl-2 in TM, normal and neoplastic endocervical epithelium



33a **33b**

Fig.33a *endocervical gland negative for bcl-2, partially involved by TM cells which presents the characteristical bcl-2 mosaic pattern;* ***Fig.33b*** *AIS glands complete negative for bcl-2; the positive lymphocytes served as internal control.*

Table 5.15. Bcl-2 in TM, normal and neoplastic endocervical epithelium

Histological category*	Bcl2 immunoreactivity			Total
	0	51-80%	>80%	
Normal endoc ep	45 100.0%	0 .0%	0 .0%	45 100.0%
TM	0 .0%	39 97.5%	1 2.5%	40 100.0%
AIS and ADCa	6 100.0%	0 .0%	0 .0%	6 100.0%
Total	51 56.0%	39 42.9%	1 1.1%	91 100.0%

** The histological categories include also control cases*

5.2.3.5. PAX2 expression

Among 10 specimens which contained normal endocervical glands, PAX2 was uniformly moderately to strongly expressed (Fig.34a). The ectocervical squamous cells (n=8) and mature squamous metaplasia (n=2) did not exhibit positivity for this antibody and variable expression was noted in the immature squamous areas (n=2), in less than 50% of cells positive.

6 cervical TM cases evaluated for PAX2, showed a pattern of staining similar to their endometrial counterpart (Fig.34b). The mosaic pattern, with negative ciliated cells contrasted with a uniform loss of expression within areas of *in situ* (n=2) (Fig.34c) and invasive endocervical adenocarcinoma (n=3). The endocervical glands partially colonized by adenocarcinoma in situ presented an abrupt transition between benign (PAX2 positive) and malignant (PAX2 negative) glandular epithelium.

Benign cervical lesions, such as Nabothian cysts, tunnel cluster and mesonephric hyperplasia (Fig.34d) diffusely expressed PAX2. Consequently, PAX2 facilitates distinction between TM, reactive endocervical epithelium and neoplastic endocervival lesions ($p<0.01$) (statistics table 5.4).

No expression was observed in endometrial and cervical stroma or vascular endothelium respectively.

Figure 34. PAX2 expression in normal, metaplastic and neoplastic endocervical epithelium as also mesonephric hyperplasia

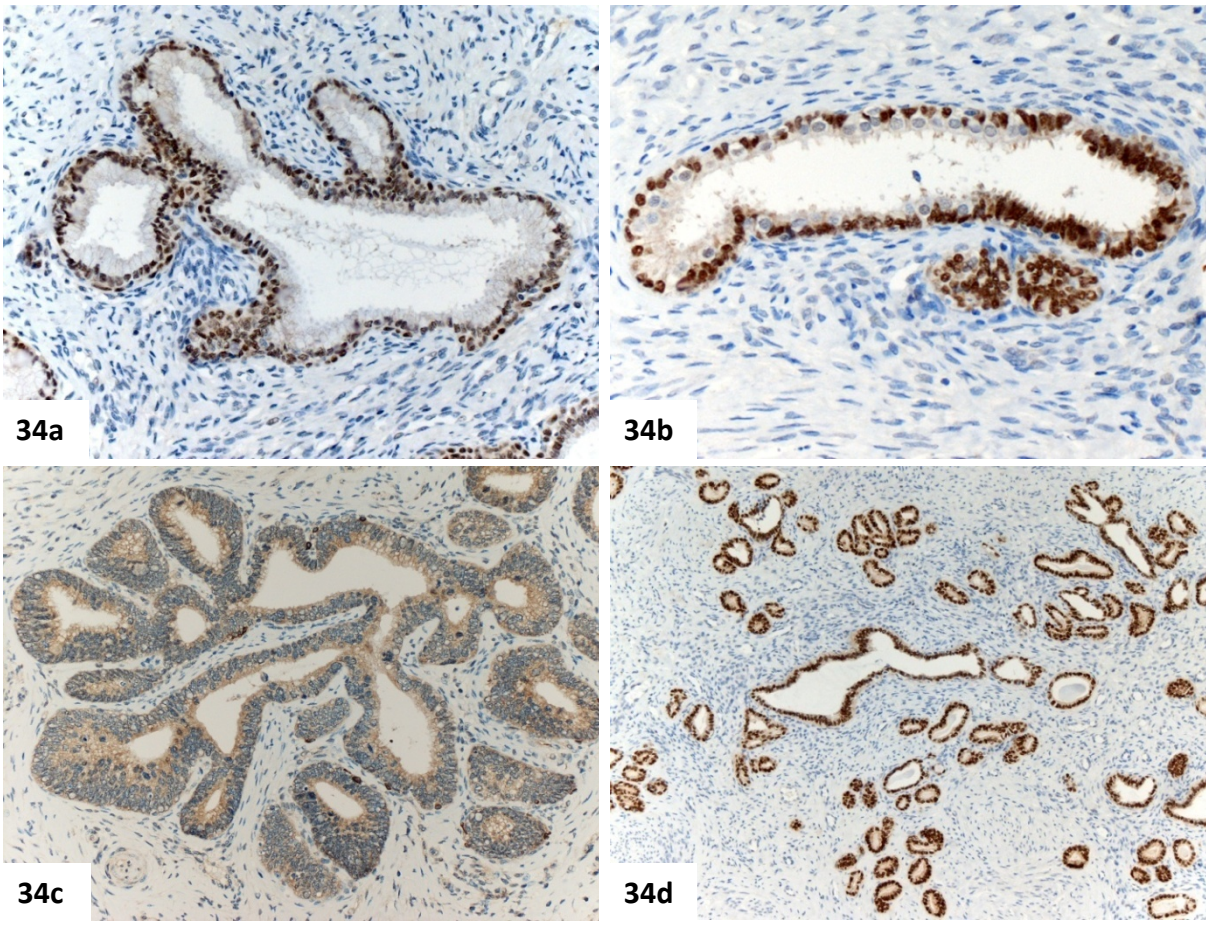


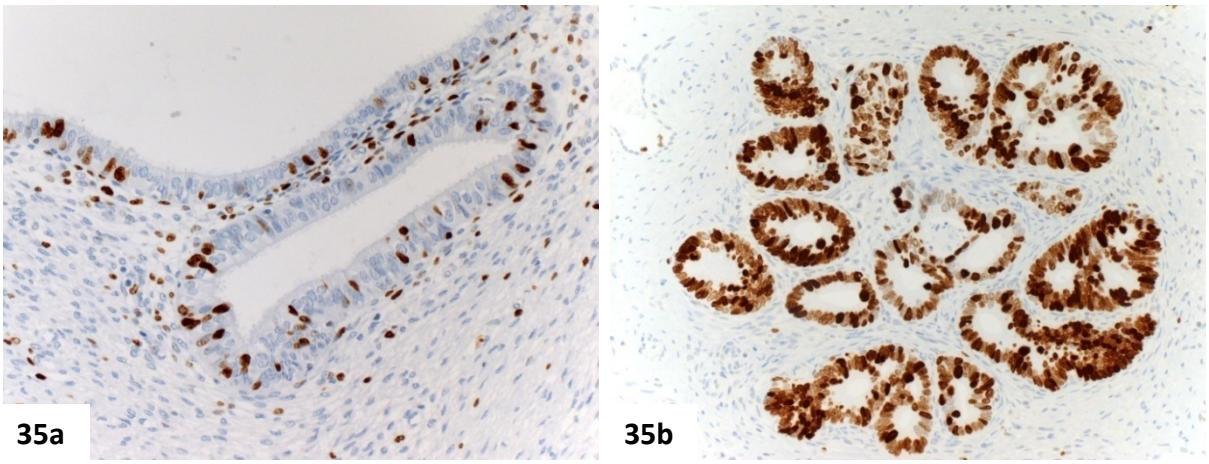
Fig.34a *diffuse PAX2 positivity in endocervical and reserve cells;* ***Fig.34b*** *mosaic pattern of PAX2 expression in TM glands;* ***Fig.34c*** *absence of PAX2 immunoreactivity in AIS;* ***Fig.34d*** *diffuse, strong PAX2 positive mesonephric glands*

5.2.3.6. Proliferation index Ki-67

All endocervical glands were either negative (75.6%) or showed less than 10% of nuclei positive (22.2%). A singular case had a proliferation index of 10-30% (2.2%). Normal and metaplastic squamous epithelium labeled for Ki-67 only in the parabasal or reserve cells. TM glands presented a proliferative activity closer to the normal endocervical glands. 47.5% were negative and other 42.5% exhibited >1% but <10% positivity. No significant differences were noticed at a threshold of 10%. Four cases (10%) exhibited moderate proliferative activity with a Ki-67 index of 10-30% (Fig.35a), from which one was associated with an important inflammatory background. No overlap in proliferation rate was seen between normal endocervical and TM glands or between TM and *in situ* endocervical adenocarcinoma, which labeled for Ki-67 in more than 75% of cells (Fig.35b) ($p<0.01$)

(statistics table 5.4). Occasional positivity was seen in the glands of mesonephric hyperplasia, Nabothian cysts or tunnel clusters.

Figure 35. Ki-67 expression in TM and in situ endocervical adenocarcinoma



35a **35b**

Fig.35a *Ki-67 in TM mainly label the secretory cells nuclei;* ***Fig.35b*** *high proliferation rate of AIS, with almost all of the cells positive for Ki-67*

Table 5.16. Ki-67 in TM, normal and neoplastic endocervical epithelium

Histological category*	Ki-67 immunoreactivity				Total
	0	<10%	11-30%	>31%	
Normal endoc. ep	34	10	1	0	45
	75.6%	22.2%	2.2%	.0%	100.0%
TM	19	17	4	0	40
	47.5%	42.5%	10.0%	.0%	100.0%
AIS and ADCa	0	0	0	6	6
	.0%	.0%	.0%	100.0%	100.0%
Total	53	27	5	6	91
	58.2%	29.7%	5.5%	6.6%	100.0%

** The histological categories include also control cases*

5.2.3.7. p53 expression

In approximately two-thirds (71.1%) of the cases, the normal endocervical epithelium presented a weak, heterogeneous p53 expression in less than 50% of cells; the rest were negative. The pattern of staining was similar in the corresponding benign glandular lesions or in the concomitant mesonephric and endometrial glands. In normal exocervix and squamous metaplasia, p53 presented usually a weak or moderate positivity limited to the basal, parabasal layers and reserve cells.

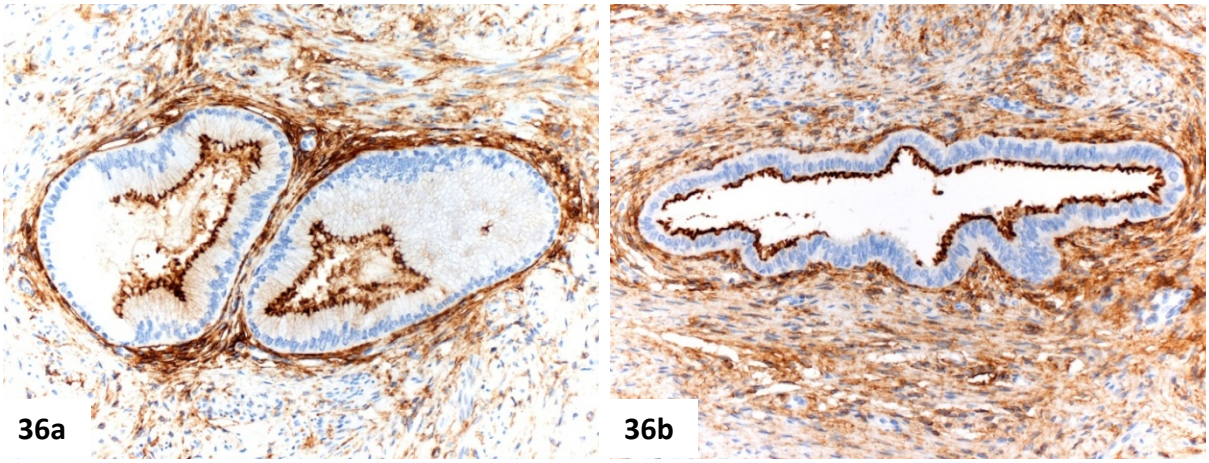
85% of TM cases showed an overlap pattern of positivity with the normal endocervical or endometrial glands, the rest being negative. None of the cases labeled

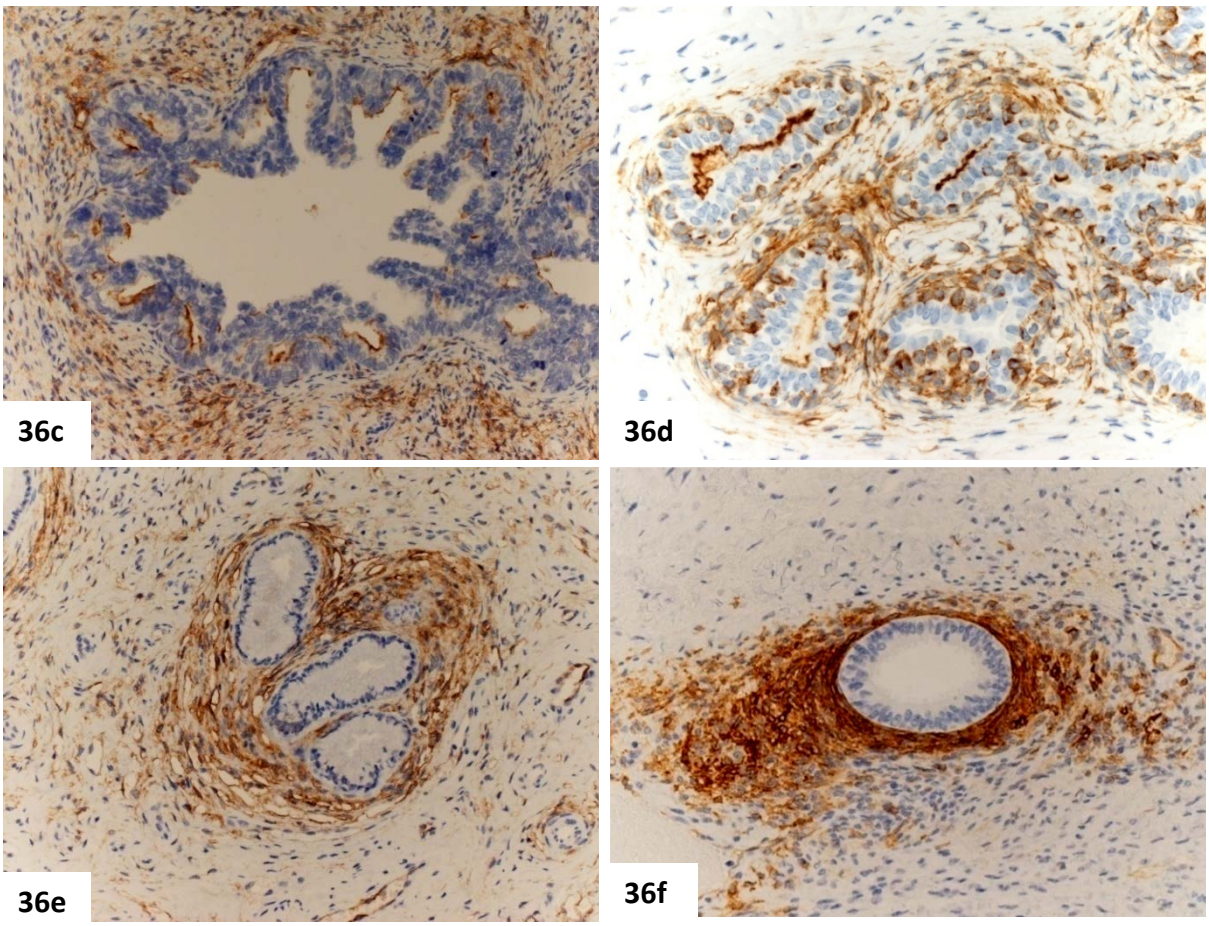
strongly for p53. A moderate intensity of staining was seen in occasional nuclei of mucinous glands with actinic changes and also in 3 of 4 endocervical invasive adenocarcinomas. The other preneoplastic and neoplastic lesions were either negative or presented a focal, weak positivity.

5.2.3.8. CD10 expression

All TM and control cases were tested for CD10. The epithelial components either squamous or glandular were commonly negative, but with some exceptions. A luminal glandular positivity was recorded in 1/45 normal endocervical epithelium (Fig.36a), 7/40 TM glands (Fig.36b), as also in 1/4 invasive endocervical adenocarcinomas (Fig.36c). The single case of mesonephric hyperplasia also presented a CD10 luminal staining (Fig.36d). Another interesting feature was its positivity within the stroma. The stromal cells around the simultaneous endometrial glands showed strong positivity, as expected. However, a moderate stromal densification, highlighted by CD10 was seen around the normal endocervical mucinous epithelium (28/45) (Fig.36e), TM glands (25/40) (Fig.36f) as also around the adenocarcinomatous clusters either in situ or invasive.

Figure 36. Apical and stromal CD10 expression in the cervix





*Luminal, apical positivity of CD10 in normal endocervical glands **Fig.36a**; in TM gland **Fig.36b**; in AIS glands **Fig.36c** and in mesonephric hyperplastic glands **Fig.36d**. Concentric cuffs of stromal cells positive for CD10 around normal endocervical glands **Fig.36e** and TM gland **Fig.36f**.*

5.2.3.9. ER and PR expression

Normal endocervix and benign glandular lesions were diffusely immunoreactive for hormonal receptors. A diminishing of both ER and PR was noticed in the glands with radiotherapy induced changes. Cervical TM was invariable positive for both receptors (Fig.37a, 37b). A negative reaction was remarked in the mesonephric glands. In contrast with normal mucinous and TM glands, two of adenocarcinomas cases (2/6) (one in situ and one invasive) were completely negative for ER and five (5/6) (four invasive and one in situ) for PR (Fig.37c). The remainder preserved these antibodies at least focally, particularly ER (Fig.37d). The exocervix and its corresponding metaplasias had a variable expression of ER and PR. Basal, parabasal and intermediate layer, as well as reserve cell hyerplasia were constantly positive for ER, but less extensively for PR.

Figure 37. Oestrogen and progesterone receptors expression in the normal, TM and endocervical adenocarcinoma

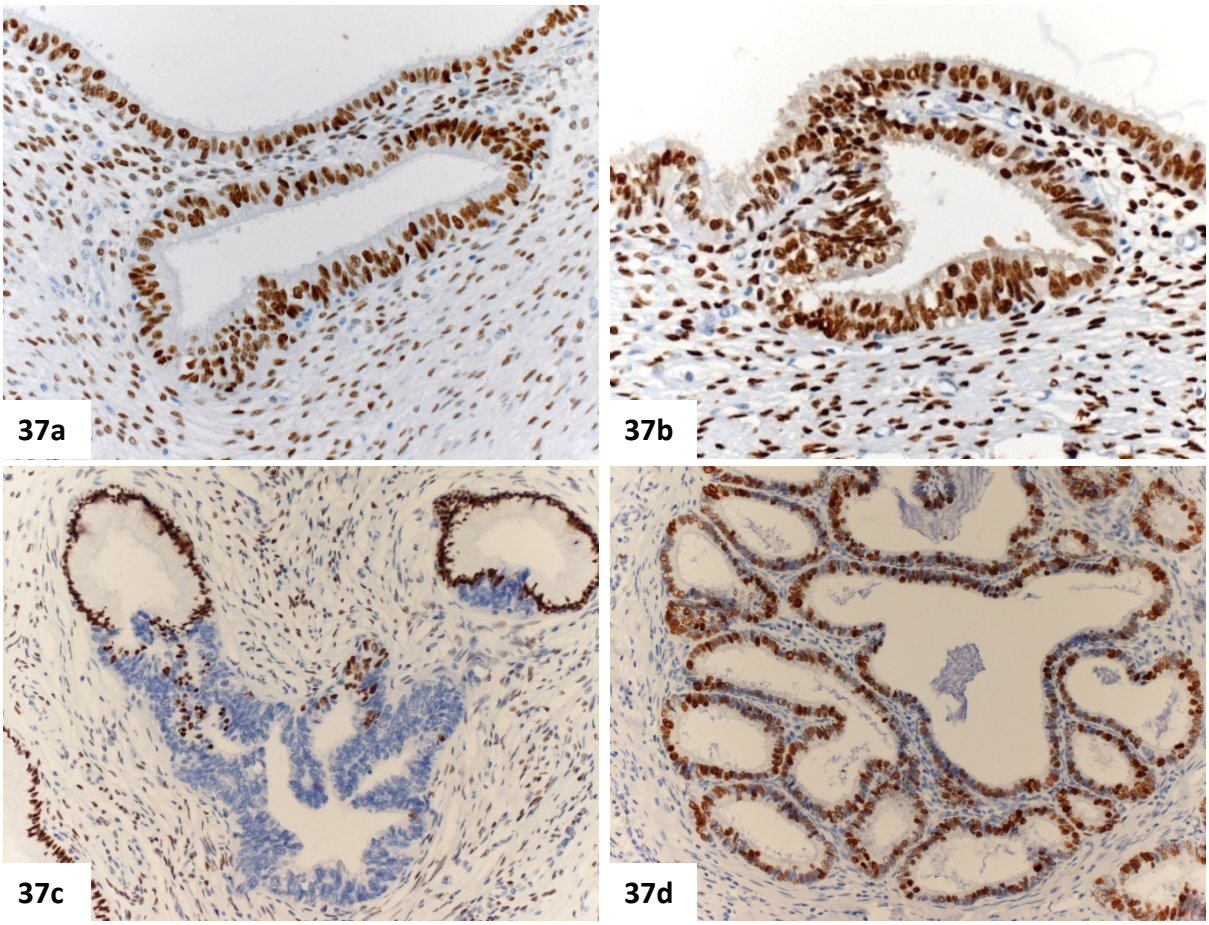


Fig.37a *diffuse expression of ER in TM;* ***Fig.37b*** *constant positivity of PR in TM;* ***Fig.37c*** *abrupt transition between PR positive normal endocervical epithelium and negative AIS cells;* ***Fig.37d*** *preserved ER expression in AIS glands.*

5.2.3.10. CEA expression

All the cervical cases were stained for CEA. The pattern of its expression is presented in table 5.17. TM, as the normal endocervical glands, was either negative or showed a luminal glandular positivity (25/40) (Fig.38a). Only one case had a weak cytoplasmic staining (Fig.38b). The latter pattern was also observed in endocervical epithelium of reactive conditions, such as cervicitis (n=5) (Fig.38c) and actinic changes. Adenocarcinomas showed a variable immunoreactivity, ranging from luminal (Fig.38d), to weak and strong cytoplasmic staining (Fig.38e). This heterogeneity was observed not only between distinct cases, but also between the glands of the same case (Fig.38f).

Figure 38. CEA immunoexpression in TM, normal and neoplastic endocervical epithelium.

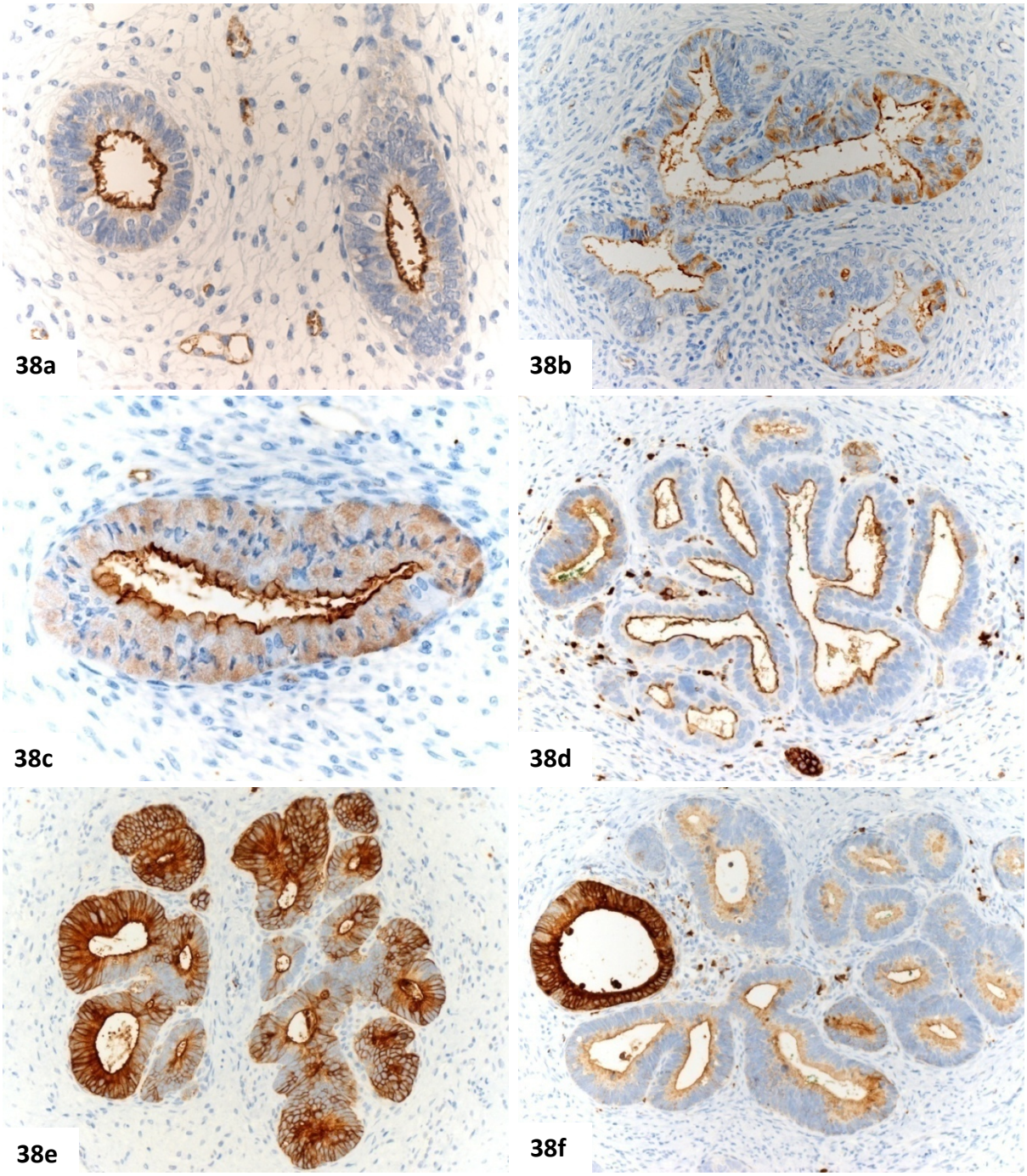


Fig.38a *TM glands with apical CEA positivity;* ***Fig.38b*** *TM presenting weak cytoplasmic CEA immunoreactivity;* ***Fig.38c*** *normal endocervical glands with apical and weak cytoplasmic CEA staining;* ***Fig.38d*** *AIS with CEA positivity restricted to the luminal border of the cells;* ***Fig.38e*** *AIS showing strong cytoplasmic immunoreactivity;* ***Fig.38f*** *AIS with heterogeneous expression of CEA, strong, cytoplasmic positive gland together with only apical positive glands*

Table 5.17. CEA in TM, normal and neoplastic endocervical epithelium

Histological category*	CEA immunoreactivity					Total
	0	1+	2+	3+	4+	
Normal endoc ep	6	34	5	0	0	45
	13.33%	75.56%	11.11%	0%	0%	100%
CTM	14	25	1	0	0	40
	35%	62.5%	2.5%	0%	0%	100%
AIS	0	0	1	0	1	2
	0%	0%	50%	0%	50%	100%
Invasive ADK	0	1	1	1	1	4
	0%	25%	25%	25%	25%	100%

** The histological categories include also the control cases*

5.2.3.11. EGFR expression

As for the endometrium, not all the cervical cases were tested for EGFR. In the normal endocervical epithelium (n=23) and its related benign lesions (n=4), expression of EGFR was restricted to the subcolumnar reserve cells, with mucinous cells unstained (Fig.39a). However, diffuse, moderate staining was observed in all cases of *in situ* and invasive adenocarcinoma (n=5) (Fig.39b). Similar to endometrial TM or normal tubal epithelium, a mosaic EGFR expression was found in 11/19 evaluated cases (Fig.39a). Also, no staining was observed in two TM and an opposite diffuse reaction in other six. A constant positivity was present in hyperplastic reserve cells of immature squamous metaplasia (n=5), as also in the basal, parabasal and intermediate layers of normal exocervix (n=14) and mature squamous metaplasia (n=4). The mesonephric glands were negative.

Figure 39. EGFR expression in TM, normal and neoplastic endocervical epithelium

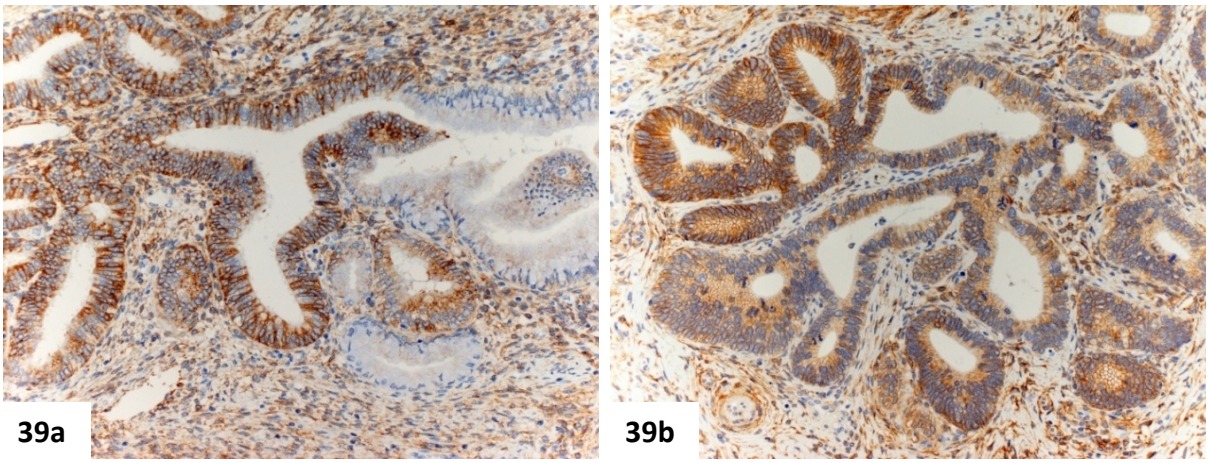


Fig.39a *transition between EGFR negative endocervical cells and positive TM cells, with higher expression within the secretory cells;* ***Fig.39b*** *difuse, moderate immnureactivity of AIS glands.*

5.2.3.12. Vimentin expression

14 cervical cases were evaluated for this antibody. Normal columnar mucinous epithelium was either negative (n=8) or subtly focally positive (n=6). In these latter cases, vimentin showed predominantly a subnuclear (basal) cytoplasmic distribution, together with a delicate staining along the lateral cell borders, reproducing the areas surrounding the mucin vacuole (Fig.40a). The endocervical neoplastic foci were sharply demarcated, appearing as "naked" unstained glands within a strong vimentin positive stromal background (Fig.40b). Also they contrasted with diffusely immunoreactive TM glands (n=10) (Fig.40c). The normal or metaplastic squamous foci were found to be negative for vimentin.

Figure 40. Vimentin expression in normal, neoplastic endocervical epithelium and TM

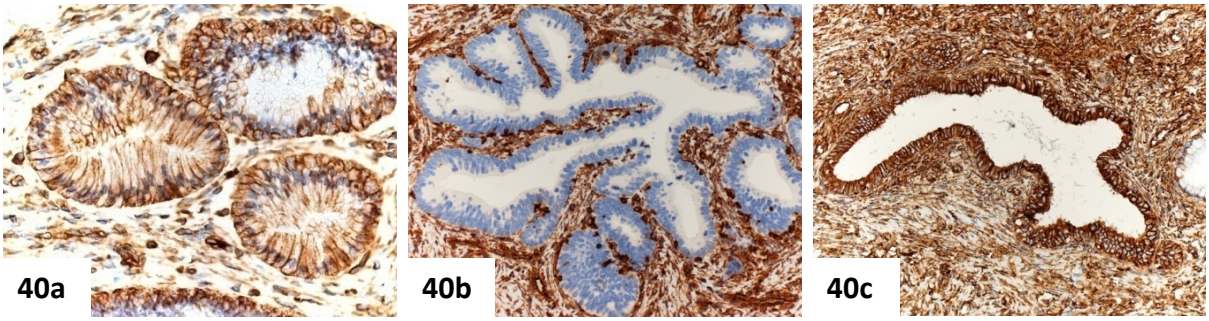


Fig.40a *delicate vimentin staining of normal endocervical glands;* ***Fig.40b*** *"naked" AIS glands on the background of vimentin positive stroma;* ***Fig.40c*** *diffuse, strong vimentin immnureactivity of a TM gland.*

5.2.4. Statistical correlations

The statisticsal correlations between TM, normal and neoplastic endocervical epithelium, previously mentioned in the text are summarized in statistics table 5.4.

Statistic table 5.4.

Histological categories	p16 (%)[#] 0/1/2/3/4	p	X2	Cyclin D1 (%)[#] 0/1/2/3/4	p	X2	bcl2 (%)[#] 0/1/2/3/4	p	X2	Pax2 (%)[#] 0/1/2/3/4	p	X2	Ki67 (%)* 0/1/2/3	p	X2
Normal endoc. (n=45)	60/38/2/0/0	<0,01	59,538	4/9/29/56/2	0,023	11,322	100/0/0/0/0	<0,01	0,707	0/0/0/0/100(†)	<0,01	16,000	76/22/2/0	0,022	7,592
TM (n=40)	2/15/43/35/5			0/17/55/28/0			0/0/0/98/2			0/0/0/100/0(‡)			48/42/10/0		
TM (n=40)	2/15/43/35/5	<0,01	32,775	0/17/55/28/0	<0,01	38,285	0/0/0/98/2	<0,01	46,000	0/0/0/100/0(‡)	0,001	11,000	48/42/10/0	<0,01	46,000
AIS and ADCa (n=6)	0/0/0/0/100			83/17/0/0/0			100/0/0/0/0			100/0/0/0/0(§)			0/0/0/100		
AIS and ADCa (n=6)	0/0/0/0/100	<0,01	51,000	83/17/0/0/0	<0,01	29,531	100/0/0/0/0		Constant	100/0/0/0/0(§)	<0,01	15,000	0/0/0/100	<0,01	51,000
Normal endoc. (n=45)	60/38/2/0/0			4/9/29/56/2			100/0/0/0/0			0/0/0/0/100(†)			76/22/2/0		

 # 0=<1%, 1=<10% of cells positive; 2=11-50% of cells positive, 3=51-80% of cells positive; 4=>80% of cells positive

**0=<1%, 1=<10% of cells positive; 2=11-30% of cells positive: 3=>30% of cells positive*

† 10 cases containing normal endocervical epithelium were analyzed for PAX2

‡ 6 cases of TM were analyzed for PAX2

§ 5 cases of neoplastic endocervical lesions were evaluated for PAX2

In **table 5.18** we summarized the main pattern of staining for each antibody used, in normal, TM, neoplastic endocervical and endometrial epithelium

Antibody	Normal squamous epithelium	Normal Endocervical epithelium	Normal endometrial epithelium	Normal tubal epithelium	TM	Endocervical adenoracinoma	Endometrial adenocarcinoma	Serous carcinoma
p16	Negative (focal staining in the reactive)	Negative (rare single/clusters of cells positive)	Negative (scattered/focal positivity in proliferative and atrophic)	Negative	Positive usually focal, heterogeneous or mosaic (occ diffuse)	Positive diffuse	Negative or patchy and weak positivity	Positive (>75% of cells)
Bcl-2	Basal cells	Negative	Positive (proliferative) Heterogeneous	Positive mosaic	Positive mosaic	Negative	Negative/focal positive Mosaic or	Negative

Antibody	Normal squamous epithelium	Normal Endocervical epithelium	Normal endometrial epithelium	Normal tubal epithelium	TM	Endocervical adenoracinoma	Endometrial adenocarcinoma	Serous carcinoma
			(secretory)				negative in ciliated ADCa	
Ki-67	Parabasal cells (increased in reactive)	Negative (rare single cells positive)	High (proliferative), low (secretory)	Rare single cells, mainly secretory cells	Usually <10% positive (occ >10%), mainly secretory cells	Frequently >50%, occ <30%)	Low (10-30%)	High (>75%)
Cyclin D1	Parabasal cells (increased in reactive)	Positive usually focal (increased in reactive)	Positive usually focal (increases in secretory and decidual endometrium)	Positive focal, mainly secretory cells	Positive usually focal, mainly secretory cells (rarely diffuse)	Negative (focal staining mainly at deep margins)	Positive usually focal	Positive usually focal
CEA	Positive membrane in intermediate and superficial layers	Negative or positive luminal	Negative or occ positive luminal	Negative	Negative or positive luminal	Positive cytoplasmic (occ luminal)	Negative, positive in mucinous differentiation	Negative
p53	Negative or weak/moderate positivity in basal and parabasal layers	Weak positivity or negative	Weak and heterogeneous positivity	Negative or occasional weak positivity	Negative or occ weak/moderate, heterogeneous positivity)	Negative, occ weak positivity	Negative or focal, weak positivity	Positive strong in 90%
β-catenin	Positive membranous	Positive membranous	Positive membranous	Positive mosaic membranous with cytoplasmic in secretory cells	Positive mosaic membranous with cytoplasmic in secretory cells	Positive membranous	Positive membranous or focal nuclear positivity in morules	Positive membranous
Vimentin	Negative (occasional positive)	Negative or delicate positivity	Positive	Positive	Positive	Negative	Positive or focal negativity; Morules negative	Positive
ER/PR	ER positive, PR focal in basal and parabasal layers	Positive diffuse	Positive	Positive, negative or weak positivity of ER in ciliated cells	Positive, negative or weak positivity of ER in ciliated cells	Negative or focally positive	Positive	Negative (occ focal positivity)

Antibody	Normal squamous epithelium	Normal Endocervical epithelium	Normal endometrial epithelium	Normal tubal epithelium	TM	Endocervical adenoracinoma	Endometrial adenocarcinoma	Serous carcinoma
PAX2	Negative	Positive diffuse	Positive diffuse	Positive mosaic	Positive mosaic	Negative	Negative or occ. focal positive	Negative
EGFR	Negative	Negative	Positive	Positive mosaic	Positive mosaic	Positive diffuse	Positive focal or diffuse, mosaic in ciliated type	Positive focal or diffuse
LhS28	Negative	Negative or occasional cell positive	Negative, occasional cells in proliferative, rarely in secretory	Positive in ciliated cells	Positive in ciliated cells (more than 50% of cells)	Negative	Negative (positive in ciliated type)	Negative

Chapter 6

Discussion

VI. Discussion

6.1. Endometrial TM

Endometrial metaplasias are a heterogeneous group of proliferation which reflects the remarkable multipotent capacity of the endometrium to undergo differentiation into almost any Müllerian-type epithelia, urothelium, intestinal or gastric epithelia as well as multiple mesenchymal tissues. However, the frequency with which these occur is heavily in favour of Müllerian tissue. Under various conditions such as hormonal, irritative or mutational, the progenitor or endometrial stem cells and endocervical reserve cells could generate this broad spectrum of lesions [131].

Despite the common occurrence of epithelial endometrial metaplasias and changes, little attention has been paid to them in the literature. Since a comprehensive article was published two decades ago [3], only a few studies have dealt with this subject. In the recent review "Endometrial metaplasias and reactive changes: a spectrum of altered differentiation" accepted for publication in Journal Clinical of Pathology, we proposed a new classification, introduced a practical morphological approach and offered clinical guidelines for the treatment of the entire spectrum of these alterations [131]. In the present study we focused on TM, the most common endometrial metaplasia. Our growing concern about these lesions is due to the scarcity of reliable data, their relationship to adenocarcinoma, which is not yet fully understood, and the fact that they are frequently overlooked and misdiagnosed.

For endometrioid lesions it is well established that the risk of developing an adenocarcinoma is directly related to degree of architectural complexity, extent/distribution (focal, widespread) and the type of atypia [335] [336]. Only few articles emphasize the morphological diversity of endometrial hyperplasia or consider its cytoplasmic

differentiations, including tubal-type [89 265]. This feature was considered as contributing to the poor reproducibility of their diagnosis [89 337].

For many years it was believed that endometrial metaplasia, encountered in the absence of hyperplasia and carcinoma does not have particular clinical significance [338]. It was regarded as a benign mimicker of adenocarcinoma and the difficulty of distinguishing it has been stressed [3 339 340]. In one study, 22% of the patients with metaplasia and hyperplasia were misdiagnosed as having carcinoma and treated accordingly. Including such patients in therapeutic trials can lead to erroneous conclusions regarding the therapeutic outcomes [341].

In 2001, Inoue proposed metaplasia as a distinctive pathway of endometrial carcinogenesis, a precursor of serous, mucinous, clear cell and mixed type adenocarcinoma [183]. Recently the link between morular and mucinous metaplasia and pathogenesis of endometrial adenocarcinoma has started to become clear [333 342 343], but until now only scant, unfounded suppositions have been made regarding the preneoplastic potential of endometrial TM [184].

Our study is unique, detailing the entire spectrum of tubal-type lesions ranging from simple to complex, and even ciliated type adenocarcinoma. With the largest series in the literature, we attempt to offer a complete picture of this lesion, morphologically, immunohistochemically and genetically, and clarify if TM has any importance in endometrial adenocarcinoma pathogenesis or if it only adds to confusion.

In all cases, LhS28, a marker which reacts with basal body of cilia [344] was used for colocalization of tubal-type lesions. Ciliation is a characteristic of Müllerian derivates epithelia and reflects cellular differentiation; consequently, scattered cilia are normally present in the cervix, isthmus and endometrium. Ciliated cells were more frequently found in the proliferative endometria, up to 20% [3 184], and endometrioid hyperplastic glands than in secretory glands. However, to define ciliated or tubal type lesions more than occasional ciliated cells have to be present. When these terminally differentiated cells become the dominant population of the surface or glandular epithelium, the term ciliated metaplasia is appropriate and when all three elements which defined tubal epithelium (ciliated, secretory and intercalary cell) are present the term tubal metaplasia is used. The difference is merely academic, rather than clinically relevant. In this study the eponym tubal metaplasia (TM) will be used.

Its presence is more common than is usually recognized or accepted and in the isthmus is so frequent that can be considered a normal phenomenon [93].

In the present study TM was seen in various endometrial conditions, the majority (72%) being oestrogen related, such as endometrial hyperplasia (39%), polyps (25%) and even adenocarcinomas (8%). As we will discuss later, TM was seen in association with areas of hyperplasia or superimposed on hyperplastic glands.

The fact that TM is an oestrogen driven lesion is well documented in the literature. The first relation between increased number of ciliated cells and oestrogen has been observed in by Novak and Everett in 1928 [345], who noticed this feature in the Fallopian tube of women with "non-secretory hyperplasia" of the endometrium. A few years later, the same authors observed the presence of TM in the endometrium associated with this type of lesion. As a logical sequence, if tubal epithelium responds in this manner to elevated oestrogen levels, it is not surprising that endometrium, also of müllerian duct origin, may undergo metaplastic change in the form of ciliated cells. Similarly, endometrial ciliated cells increased in the context of oestrogen producing granulosa cell tumour [346]. Subsequently, many other studies confirmed the direct relationship between unbalanced exo- or endogenous oestrogen and the appearance of TM in the female genital tract [3 341 346-349]. In some reports, 2/3 of women treated with exogenous oestrogens complained of abnormal uterine bleeding and presented endometrial metaplasia [3 350].

Thus, it is not surprising that it is often associated with polyps, simple or complex endometrial hyperplasia [89] or even andenocarcinoma [341 349 351 352]. Also, it should be borne in mind that endometrial metaplasia and hyperplasia are not mutually exclusive lesions but rather coexistent [3 339 353]. Furthermore, extensive areas of TM were also found in adenofibromas and adenomyomas in which multiple metaplasias coexisted. Its presence in both benign and malignant müllerian mixed tumours is also well known [354]; indeed, TM was a constant feature of dilated glands within these lesions.

Oestrogenic stimulate the centrioles of normal or malignant cells to produce cilia [355]. Eight endometrioid adenocarcinomas included in our study were evaluated for the presence of ciliated cells using LhS28. Two of them could be considered as ciliated or tubal-type variants. Scattered ciliated cells are a frequent finding in usually well differentiated endometriod carcinoma, especially at ultrastructural level [347 356]. However, the definition of the ciliated variant requires more than 75% of the tumoural population to have cilia with a

glandular formation or sheets of cells punctuated by tiny cyst-like lumina resulting in a cribriform appearance [347 355 357 85].

TM was also frequently found lining fragments of surface atrophic endometria [347], where it possibly represents a residual, differentiated lesion that may even remain unchanged or stimulated to develop under radiotherapy (personal findings). Exceptionally, scattered TM foci were found between the normal secretory glands or those of decidual endometrium. These glands probably represent unresponsive foci. As we will discuss later, ciliated cells of TM and normal tubal epithelium sometimes present a downregulation of ER. Decreased ER will interfere with the progesterone response of the cells and consequently their unchanged morphology.

TM frequently involved both surface and glandular epithelium. 71% of cases exhibited more than just occasional glands lined by tubal type epithelium and a diffuse pattern was documented in 7% of cases. In routine practice TM is a common phenomenon, usually involving endometrium focally [3]. Its appearance as an extensive phenomenon is unusual, but is explained in our series by the special selection of the specimens.

In the same endometrial specimens various types of metaplasia and changes often coexist. In our study, 46% of TM cases presented one or more other types of metaplasia. The most common association was with surface papillary syncytial changes (SPSC) (28%), followed by mucinous (MM) (19%). In eight cases two or more types of metaplasia were coupled. This common phenomenon [3 89 342] can result from the fact that more pathways are initiated or a single one can provide the plurality of alterations [342 343 358]. There is a plausible explanation for the frequent coexistence of SPSC with TM. TM is a constant feature of endometrial hyperplasia, which is regularly associated with endometrial breakdown, which generates and associates with SPSC. One study showed that different types of metaplasia can vary between the curettage and the hysterectomy specimens, probably reflecting a greater amount of histological material for the study in the latter [341].

Mucinous and tubal metaplasias are frequently admixed and are perhaps the lesions that pose most interpretation problems, due to the fact that they may occur in both architecturally simple and complex glands and possibly hold the same prognostic significance. Based on the architectural pattern and presence of atypia, Nucci proposed a subclasification of mucinous metaplasia [342] in endometrial biopsy and curetting. The three tier system (A, B, and C) reproduces the multistep progression of mucinous ovarian tumours

[359] and has important clinical and therapeutic implications. The likelihood of carcinoma in the follow-up of these patients was directly related to the degree of architectural and cytological atypia of mucinous lesions. In the absence of concomitant atypical hyperplasia the risk ranged from 0% to 67.4% and 100% for types A, B and C respectively. As with the majority of classification systems proposed for endometrial preneoplastic lesions, this one also has little reproducibility [343] and a simplification is necessary [360].

The mucinous metaplasia subclasification was extrapolated to tubal changes [358]. Accordingly, TM was subdivided into simple, associated with epithelial complexity or with frank neoplasia. Also the latter category was further subdivided into three entities: endometrial intraepithelial neoplasia (EIN) with tubal differentiation, in which crowded glands are lined by focal epithelial tufting and stratification, microglandular pattern and adenocarcinoma with tubal differentiation. But this complex division is not expected to be very popular.

In the recently proposed classification, we subdivided endometrial metaplasia, either mucinous or tubal, into a 2 category scheme, using the terms simple and complex [131]. The same patterns of growth to determine whether an endometrioid lesion is simple or complex were applied. We defined *simple TM* when normal size or cystically dilated glands are unevenly distributed. Also, non-crowded glands with stellate, slight budding, branching or angular contours were included in the same category. The lining tubal-type epithelium was non-stratified or pseudostratified and with bland cytology. When TM glands exhibited a crowded arrangement, with complex changes such intraluminal micropapillae, papillae verae with fibrovascular core, stratification that create a cribriform configuration or a solid pattern, the proper designation was *complex hyperplasia tubal-type (CHT)*.

In order to verify if morphological heterogeneity reflects a true biological diversity we introduced in the present study *complex tubal metaplasia in isolated glands (CTMI).* This is a gray zone category characterized by occasional glands with cribriforme or micropapillary architecture which do not fulfill criteria of crowding in order to be called CHT. Subsequently, we analyzed 72 STM, 11 CTMI and 17 CHT and two endometrioid adenocarcinoma with tubal-type differentiation. All the CHT cases had associated STM; therefore the total number of cases having STM was 89.

More than half of CTMI (6/11) and 5/17 CHT were observed in endometrial polyps, in agreement with a previous study which documented that complex altered differentiations are not a rare phenomenon within the polyps [89].

CHT lack the criteria of invasion, but three of them were seen in the vicinity of endometrioid adenocarcinoma, two of them ciliated variants. This alternative pattern was designated "metaplastic hyperplasia" [3], or altered differentiation of EIN lesion [89 358], and it has been considered to have a risk of developing or associating with an adenocarcinoma [341 347 351]. Recently, one study showed that altered differentiations within the EIN lesion are not rare findings; they appeared in 47% of cases as single or multiple associations. Their frequency and recognition by the pathologist varied. Tubal secretory (round cell) was one of the most common changes, but less reproducible compared with the morular and mucinous ones [89].

Atypical complex hyperplasia or EIN is considered the monoclonal putative precursor of endometrioid adenocarcinoma. It is characterized by structural heterogeneity at both morphological and molecular level [89 265]. However, cytologic atypia in the endometrium is considered a poorly reproducible feature [187 189 361]. Furthermore, the main criteria of EIN diagnosis "cytologically altered architecturally crowded glands" compared with background endometrium, [361] could not be applied for lesions with altered differentiation. Ciliated cells could be large, with pale eosinophilic cytoplasm and round nuclei with conspicuous nucleoli and not always evident cilia. For this reason TM was the most common pitfall in the evaluation of atypicality of an endometrial lesion [3].

The diagnostic challenges of these lesions reside not only in their architectural complexity and bland morphology but also in the presence of cilia. Ciliation are known to reflect cellular differentiation, with their frequent lost in the malignant process, but the dogma "ciliated cells equivalent with benignity" could lead to a false negative diagnosis, since two of our CHT coexisted with a ciliated variant of endometrioid carcinoma. It was proposed that the diagnosis of this rare variant [347 355 357] should be reserved for those cases with clear complex architecture, significant nuclear atypia and/or stromal/myometrial/ vascular invasion [347]. However, in our study there were no cytologically striking differences in the spectrum STM, CHT and ciliated variant of endometrioid adenocarcinoma. In the majority of cases the atypia was absent or mild, with occasional slight nuclear enlargement and very sparse mitotic figures.

In endometrial pathology the atypicality of a lesion can be evaluated from two main points of view: cytological and architectural, and sometimes a complex architecture does not require striking atypia to give the lesion a malignant potential. This is well documented for mucinous metaplasia where complex architecture even with mild atypia bears a high risk of progression or simultaneous association with an adenocarcinoma [342 343]. It was stipulated that the threshold for the diagnosis a mucinous carcinoma in the biopsies is lower compared with endometrioid type [343].

Consequently, as for mucinous lesions, we believe that the architectural pattern is important in deciding if a ciliated, tubal-type lesion is or is not clinically relevant. For the present time it is recommended not to modify the treatment of complex endometrial lesions based on the presence of altered differentiation [89].

The morphological problems in the recognition of endometrial precursors and the ability of immunohistochemistry to provide valuable information regarding the molecular and genetic substrate of the lesions and their biological behaviour gave rise to attempts to establish biomarkers. 61 different antibodies have been investigated [186] in an effort to establish the lesions that bear a high risk of progression to, or concurrence with, an adenocarcinoma. In the present study, we will concentrate on important cell cycle proteins and the main pathogenetic pathways involved in endometrial carcinogenesis. The immunoreactivity was followed along the spectrum of tubal-type lesions and a comparison with normal endometrial and tubal epithelium, preneoplastic and neoplastic endometrial lesions was made.

In all cases, the section stained with LhS28 was used as a map for the location of TM in other slides stained with various antibodies.

Derailments of pRb1-cyclinD1–cdk4/6–$p16^{INK4A}$ pathway, involved in development and progression of both endometrioid and serous adenocarcinoma, has started to be discussed in depth recently [362-364].

We found a significant higher expression of *$p16^{INK4A}$* in STM than CHT, and also when compared to normal proliferative, atrophic, hyperplastic and neoplastic endometrioid lesions. This feature was previously documented and was regarded as a criterion in favour of neoplastic potential of TM [184]. In TM, the pathways implicated in $p16^{INK4A}$ overexpression and its significance has not been yet clarified. We should keep in mind that $p16^{INK4A}$ is a key

regulatory protein of cell proliferation and not a hallmark of HPV infection or a carcinogenic event. Involved in the majority of cell-signalling pathways, in many normal tissues cells $p16^{INK4A}$ functions as a regulator of age-dependent senescence [365 366] and as a constrain of regenerative cell capacity [367]. In our study, one fifth of STM cases had a mosaic pattern of $p16^{INK4A}$ expression, in which ciliated cells were more often positive for $p16^{INK4A}$ than secretory cells. We were able to demonstrate a constant presence of $p16^{INK4A}$ in a strong and diffuse pattern in SPSC, considered a degenerative phenomenon [131 368]. Additionally, morular metaplasia frequently expressed $p16^{INK4A}$, but alone is devoid of malignant potential [193].

Therefore, we believe that the overexpression of $p16^{INK4A}$ in STM and CTMI should be interpreted in conjunction with the low proliferation rate of these lesions and regarded as a feature in favour of the terminal differentiated state rather than a preneoplastic event.

In contrast, CHT showed a statistically significant decrease in expression of $p16^{INK4A}$ in comparison to STM and CTMI, but was maintained in superior to complex endometrioid lesions, probably by a preserved expression of the ciliated cell. A progressive decrease, even if not significant, was observed also in the spectrum of endometrioid lesions, from proliferative to simple and then complex hyperplasia (including atypical). However, with a threshold established at 10%, endometrioid adenocarcinoma had a higher expression ($p=0.037$).

The underlying mechanism that leads to $p16^{INK4A}$ downregulation with progressiveness of architectural complexity is not clear. However, one study documented a diminition of $p16^{INK4A}$ expression in endometrial hyperplasia compared with endometrioid adenocarcinoma. The authors could not state if the transcriptional silencing of $p16^{INK4A}$ observed in endometrial hyperplasia is part of the normal cell function or is an early event in the neoplastic transformation of endometrial cancer [362]. Also, $p16^{INK4A}$ abnormalities at DNA level with loss of its expression in the nucleus was regarded as an early step in the tumourgenesis of a subset of endometrial adenocarcinoma, especially those exhibiting K-ras mutations, pathologic p53 expression, serous papillary/clear cell histological types and an aggressive clinical behaviour [369 370]. Therefore, decreased expression of p16 and not its overexpression could be an indicator of premalignant potential of CHT. However, inactivation of $p16^{INK4A}$ normal function by promotor hypermethylation, point mutation, deletion is not sufficient to induce carcinogenesis but rather cooperation with exogenous

stimuli (carcinogens) or other spontaneous mutation could trigger malignant development [371].

The similar pattern of p16^{INK4A} overexpression in TM and serous adenocarcinoma gave rise to the supposition that TM could be a precursor of the latter lesion [184]. Even if serous adenocarcinoma was described to have a strong, diffuse positivity for p16^{INK4A} [167 372], one out of two of our cases presented a heterogeneous pattern. Also, some studies documented diffuse positivity of p16^{INK4A} in cases of endometrioid adenocarcinoma [180 184] with a consistent expression in squamoid elements [193]. Therefore, p16 cannot be used as a marker to predict a specific type of adenocarcinoma. Its paradoxical overexpression in these tumours is not completely understood, but it is considered to be independent of HPV [372-374].

Finally, p16^{INK4A} was considered a useful tool in determining the endometrial or endocervical site of origin. A very recent study showed that the accuracy of a 3 marker panel including vimentin, hormonal receptors ER or PR and a HPV marker (p16^{INK4A}, ProExC or HPV ISH) is optimal for this discrimination [375]. But the discriminating power of p16^{INK4A} is limited. As we already mentioned, endometrioid carcinoma can be p16^{INK4A} positive with rates varying from 30%-94% and from focal to diffuse between various studies [184 363 376]. Additionally, serous endometrial carcinoma strongly expresses p16^{INK4A}, ProExC and is frequently negative for hormone receptors and vimentin [167]. Therefore, integration of all the clinical, radiological, gross and morphological features is mandatory in order to establish the origin of adenocarcinoma [375].

82% of STM and CTMI showed overexpression of *cyclin D1* with more than 50% of positive cells. This expression was significantly higher in comparison with normal proliferative and atrophic endometria ($p<0.01$) and CHT ($p<0.01$). A previous study documented differences in nuclear cyclin D1 intensity between TM and normal endometria and considered this phenomenon an underlying effect on cellular differentiation with acquisition of a metaplastic phenotype [231]. Also, deregulation of cyclin D1 [231] and p53 [377] in various metaplasias, associated with dysfunctional uterine bleeding, could be responsible for these phenotypical changes of normal endometrial epithelium.

A positive correlation between cyclin D1 and Ki-67 is expected in STM and CTMI taking into account that cyclin D1 promotes progression through the G1-S phase of the cell cycle and it is overexpressed in these lesions. But paradoxically, less than a 10% proliferation

rate was the rule in these lesions. Two mechanisms could explain this. First, we noticed a significantly positive correlation between $p16^{INK4A}$ and cyclin D1 (Pearson correlation r=0.238, p=0.017) in STM and CTMI (taken together). Secondly, previous studies documented increased expression of p21, another cyclin dependent kinase inhibitor in TM [148 184]. Therefore, the proliferative inducing capacity of cyclin D1 was probably overcome by more potent negative regulators of the cell cycle, p16 and p21. This could be seen as a sequestration event of cyclin D1, with its further accumulation in the cytoplasm, as we were able to observe in the secretory cells of 70.78% of STM and 45.45% of CTMI cases. We also observed the peculiar nuclear and cytoplasmic pattern of cyclin D1 in SPSC, an alteration devoid of proliferative activity. It was also described in reactive endocervical epithelium [194] and in some cases of endometrioid adenocarcinoma [364], but no explanations are offered for its presence.

In CHT, $p16^{INK4A}$ decreased immunoreactivity was accompanied by diminished expression of cyclin D1 and its absence in the cytoplasm. Downregulation of cyclin D1 in CHT was statistically relevant compared with STM ($p<0.01$). There were no differences between CHT and complex endometrioid hyerplasia or in the spectrum of endometrioid lesions. This finding contrasted with the previous reports which documented progressive increased expression of cyclin D1 from functional endometria to preneoplastic and neoplastic endometrioid lesions [230 231]. Subsequently, the authors speculated that cyclin D1 could be useful to recognize a subset of precancerous lesions. Our findings suggest that probably other mechanisms independent of cyclin D1 are involved in the possible progression of CHT.

Only slight increase of Ki-67 was noticed in CHT. Even if $p16^{INK4A}$ was decreased in these lesions compared to STM and CTMI, the proliferation rate still appeared to be constrained by this cyclin dependent kinase inhibitor, as was proved by its inverse correlation with cyclin D1 (Pearson correlation r= -0.549, p=0.020).

However, the low proliferation rate within the CHT does not necessarily exclude its preneoplastic potential, because no relevant distinction in proliferation rate was observed between CHT and complex endometrioid lesions (p=0.390).

At the level of endometriod lesions, Ki-67 index appears to contrast with the expected result. The proliferation indices did not follow the morphological disease transistion from benign, hyperplastic and malignant endometria in a linear fashion. The higher proliferation rate was encountered in simple hyperplasia and benign proliferative

endometrium. In contrast to previous reports [241], we found higher Ki-67 in simple hyperplasia than in proliferative endometrium ($p<0.01$) and this could be explained by the increased number of cases in which the proliferative glands were evaluated at the level of endometrial polyps. Ki-67 decreased in the precursor complex lesion (CH, ACH) as it did in well differentiated endometrioid adenocarcinoma compared with SH ($p<0.01$). We did not observe significant differences of proliferation index between CH, ACH and adenocarcinoma. Özuysal *et al.* [230] agrees with our observation but other authors found a gradual increase of Ki-67 from normal through hyperplasia and adenocarcinoma [241 378].

The high proliferation rate of normal proliferative endometrium is expected, due to the necessity to compensate for the monthly desquamation of the endometrium. In complex endometrial hyperplasia the mitotic rate is much lower, but there is not a regular shedding of the tissue which explains the glandular crowding. The drop in proliferation activity is not sufficiently marked to be used as a criterion in the differential diagnosis between benign and preneoplastic lesions [241]. Even if, as we will discuss later, the apoptotic index presents a more straightforward correlation with malignant progression, in hyperplasia the balance is maintained in favour of proliferation.

We also noticed, as others have previously, that at the neoplastic invasive front the proliferation index is much higher than in the rest of the tumour, especially on the surface [184]. The ciliated (one case) and mucinous differentiation (one case) of endometrioid adenocarcinoma showed a slight decrease of proliferation rate in comparison with endometrioid areas. One study showed the same phenomena in conjunction with lower levels of sex steroid hormones receptors and suggested that these differentiated components have undergone terminal differentiation [154].

The tight balance between proliferation and apoptosis, partially controlled by ovarian steroids, is responsible for normal endometrial changes during the monthly menstrual cycle [379]. In normal endometria, there were no significant differences in the pattern of *bcl-2* expression in proliferative and atrophic endometria. These findings were in agreement with some studies [239] but contrasted with others that show a low level of bcl-2 with increase apoptotic index in atrophic glands [210 211 380]. The maximal expression of bcl-2 was lost in the secretory phase and menstruating endometria. Because the endometrial glands do not respond uniformly to hormones, we noticed a heterogeneous pattern of bcl-2

expression, with strongly diffuse glands alternating with mild positive and negative ones. The expression of bcl-2 is down-regulated not only by endogenous progesterone but also by administration of progesterone in endometrial hyperplasia [381]. It was demonstrated that in endometrial epithelial lesions the expression of bcl-2 requires an intact progesterone response pathway [382]. Loss of PR is a likely contributor to the lower levels of bcl-2 expression in various lesions, such as morular metaplasia [383], as we observed in four of our cases. The expression of bcl-2 in simple hyperplasia was similar to that of proliferative endometria, in agreement with prior studies [210 211 239].

Cervical TM has been extensively studied, due to its frequent misdiagnosis with AIS [127] but little attention has been paid to endometrial TM. We report for the first time the expression of bcl-2 in simple and complex endometrial glandular lesions lined by a tubal type epithelium. Endometrial STM and CTMI reproduced exactly the same mosaic pattern observed by us and previously reported by Piek *et al*. in the normal Fallopian tube epithelium [384]. Characteristically, positive secretory and intercalary cells alternate with negative ciliated ones, which were easily to identify morphologically. This contrasted with the diffuse bcl-2 pattern of cervical TM [170 194 199 385]. The difference in the pattern of bcl-2 expression probably reflects the reduced number of ciliated cells in lesions defined as tubo-endometrial metaplasia in prior studies.

Bcl-2 expression in cervical TM was considered an enigma [170]. Its constant presence within endometrial TM, cortical inclusion cysts lined by TM [386] and normal tubal epithelium excludes the possibility of a random phenomenon. This protein is widely expressed in a variety of tissues, most often being limited to proliferating, non-terminal differentiated cells [237 242], as proliferative endometrial cells [211 387]. At the same time, bcl-2 has been considered a protein with differentiation inducing capacities in the Fallopian tube [384]. These data would indicate that secretory cells still maintain proliferative activity, but under various differentiation stimuli they terminally differentiate into ciliated, senescent cells.

When we regarded the spectrum of ciliated lesion as a continuum, no significant differences were observed between simple and complex TM in isolated glands. However, CHT showed a trend towards down-regulation of bcl-2, which was significant in comparison to STM ($p<0.01$), but similar to that observed in complex endometrioid lesions. Thus, we found a progressive decrease of bcl-2 expression, with a more proapoptotic environment, from simple hyperplasia to complex endometrioid lesions and adenocarcinoma, supporting

prior reports [211 239 241]. It has been suggested that bcl-2 persistence within the glands has a pivotal role in the early stage of oestrogen-dependent endometrial carcinogenesis [209]. Its later down-regulation [210 211] implies that bcl-2 related apoptosis is less important and other genetic alterations are required for malignant progression [209 210].

In five CHT a heterogeneous pattern of bcl-2 staining was evident, with positive glands seen in continuity with slightly positive and negative areas. The unique finding of our study was demonstration of preserved mosaic pattern in the spectrum of tubal-type changes ranging from simple TM to complex and even endometrial adenocarcinoma with ciliated differentiation. This feature supports lineage continuity. It is likely that secretory cells are able to induce a monoclonal growth, rather than ciliated ones, which are terminally differentiated.

The common feature of bcl-2 down-regulation in CHT and complex endometrioid lesions supports the malignant potential of CHT and favours both classical and ciliated variants of endometrioid adenocarcinoma, rather than serous carcinoma.

Regarding other type of metaplasias, all cases of mucinous metaplasia lack the expression of bcl-2, as their normal endocervical correspondent. There were no changes in the expression of this antiapoptotic protein in complex mucinous metaplasia.

Another remarkable feature of our study was the demonstration of the loss of bcl-2 expression in SPSC. It is well known that these changes are frequently seen in the context of endometrial breakdown, in which the proapototic field is the main feature. With this observation we add further evidence to the degenerative nature of this type of change [131].

Recently, *PAX2* has been proposed as a useful marker for the diagnosis of endometrioid adenocarcinoma precursors [208].

We were able to observe its constant presence in the normal functional endometria, without a significant different reaction between various menstrual phases, except one case of proliferative endometria (1/23), in which the scattered glands were negative. These findings were different from those recently reported, in which PAX2 presented a temporal and spatial variation of staining, possibly regulated by steroid hormones [247]. Another study observed occasional glands lacking PAX2 expression in more than one third of normal proliferative endometria and considered these foci as “latent precancers” [208]. Theoretically,

the majority of the normal adult tissues loose the expression of pair box gene family, but regular self renewal of endometria can explain the PAX2 preserved expression [208].

STM and its correspondent normal Fallopian tube epithelium had a similar PAX2 immunoreactivity. The characteristically chessboard pattern of staining with positive secretory cells and negative ciliated cells was previously noted by Tong *et al.* [247] in the normal salpinx, and by Rabban et al. in cervical TM [388], but the authors did not offer an explanation for this finding. Instead, on the basis of its constant positivity in all the Müllerian duct derivates, with exception of squamous epithelium, they proposed PAX2 as a marker not only of Wollfian, but also of Müllerian lineage [247].

The mosaic pattern of PAX2 expression in the tubal and tubal-type epithelium results from the main function of PAX gene, that of stem cell maintenance, with resistance to apoptosis and repression of terminal differentiation [243]. The similar pattern of PAX2 and bcl-2 sustains that secretory cells are those cells which preserve the capacity of proliferation and the ciliated ones are terminally differentiated.

No differences in PAX2 staining were detected between STM and CTMI. Instead, 65% of CHT presented a loss of PAX2 expression. As for bcl-2, some of the cases presented a heterogeneous pattern with positive, weak and negative areas. The positive foci maintain the same chessboard pattern. Once again, this feature proves lineage continuity in the spectrum of ciliated, tubal-type lesions.

We also documented a progressive loss of PAX2 in the spectrum of endometrioid lesions, in agreement with the recent reports [208] [389]. There were no significant differences between CHT and complex endometrioid hyperplasia. PAX2 drop off in the latter lesions was shown, not only by immunohistochemistry stains, but also by quantitative RNA advanced techniques [390]. These results appear contrary to the concept that PAX2 persistence and not its loss favours the appearance of tumours, as those in the kidney [391].

Dysfunction of p53/bcl-2/bax apoptosis signalling pathways has been suggested as playing a role in tumourgenesis [209] and overexpression of PAX2 could have a key role in this model, by inactivation of p53 gene [392]. Furthermore, experimental study showed a direct role of PAX2 in tumourgenesis, but it was ambiguous if this transcription factor initiates carcinogenesis or contributes to its progression [393]. PAX2 is diffusely and strongly expressed, as bcl-2, in simple hyperplasia; a condition well known to be associated with unbalanced oestrogen levels. Recently, PAX2 has been proposed as a mediator of cell proliferation in

response to oestrogen and tamoxifen [394]. In the cell culture, PAX2 reduces the cellular levels of functional p53, with a negative regulation of bax (the pro-apoptotic protein) and imbalanced effect of bcl-2 (anti-apoptotic protein). Additionally, oestrogen exercises an up-regulation effect on bcl-2. Subsequently, the proliferative advantages (oestrogen, PAX2) together with the dominant antiapoptotic milieu (bcl-2) confer susceptibility to additional mutation and further neoplastic transformation of the glands. It appears that these proteins have an important role in the initiation of carcinogenesis and their later downregulation suggest that other factors contribute to the tumour progression. Silencing of PAX2 limits the proliferation and induces apoptosis in cell lines [244]. This, together with decreased bcl-2 partially explains the low proliferation index of CHT or complex endometrioid lesion. Therefore, an increased apoptotic index with decreased rate of proliferation demonstrated by ki-67 and PCNA [264] is not a contradiction along the neoplastic range of endometrial lesions.

The possibility that PAX2 could represent a tumour suppressor protein that, if lost, contributes to endometrial carcinogenesis [208] could not totally be excluded, but it is less probable, due to persistent PAX2 expression in various neoplasia of kidney, ovary (serous carcinoma) or breast [244 247 395].

Until now, it is not clear why endocervical and endometrial adenocarcinoma lose expression of PAX2, while others tumours conserve it. Also, the mechanisms of its loss need further investigation. For the present time, the deletion or mutation of PAX2 gene in hereditary renal coloboma syndrome [396], epigenetic silencing in melanoma [397] and hypomethylation of PAX2 promoter region in tamoxifen induced endometrial carcinogenesis [394] have been reported

The similar pattern of PAX2 expression in CHT and endometrioid complex hyperplasia, together with a higher sensitivity than PTEN in detection of precancerous and cancerous endometrial lesions [208] add further support to our belief that CHT could be considered a preneoplastic lesion. Consequently, PAX2 can be used not only for the diagnosis of TM, due to its peculiar expression, but as an important contributor to the decision if a particular lesion bears a high risk of progression to, or concurrent with, an adenocarcinoma.

Almost all TM cases, independently of the architecture, expressed *p53* with a patchy, weak, heterogeneous pattern. The exception was 9 cases of STM which had a moderate pattern of staining. It has been shown that p53 protein overexpression correlates closely with the presence of p53 mutation and its loss of function [316]. In our study no genetic, mutational analysis for p53 gene was available. Nevertheless, a recent study failed to demonstrate alteration of this gene in the microdisected eosinophilic and ciliated glands, even if those cases presented a high p53 immunoreactivity score [398]. The overexpression of p53 in the absence of molecular substrate might reflect DNA damage, with stabilized wild type p53 protein, developed in the context of dysfunctional uterine bleeding [316 377]. This was the case of more than half of our STM with moderate p53 expression.

No obvious differences in p53 expression were noticed between normal functional endometria and TM or between the latter and the entire spectrum of endometrioid lesions. Distinctly, a uniform, intense, homogeneous staining was present in serous adenocarcinoma. Alterations of p53 are regarded as an early event in the development of endometrial serous adenocarcinoma, with its overexpression even in the benign looking endometria, in the so-called "latent precancer" [257 258]. Subsequently, it appears improbable that TM could represent a precursor lesion of this aggressive subtype of endometrial cancer, as has been speculated [184].

We also evaluated surface papillary syncytial changes as an alteration associated with TM. The majority of the cases presented weak p53 positivity, but 6 of them had moderate staining. This feature, together with degenerative atypia observed in some cases and diffuse $p16^{INK4A}$, can lead to the misinterpretation of a serous neoplastic lesion. In these challenging situations, the different proliferation rate is the discriminating factor.

The pattern and intensity of p53 expression are considered the most important parameters in its evaluation, but caution should be exercised in using this protein as an indicator of the malignant or benign nature of the epithelium, because in some instances the differences may only be subtle.

An additional finding that supports our hypothesis related to premalignant potential of CHT is represented by the detection of *MMR proteins* defects, markers of MSI. Concurrent loss of MLH1 and PMS2 expression was found in three of 14 CHT (21.42%). The concordant loss of these proteins reflects the interaction between them. MLH1 and PMS2

and MSH2 and MSH6 respectively, usually form heterodimers [399]; subsequently, alteration of one protein in the heterodimer results in the degradation of its binding partner. STM and CTMI analyzed did not present detectable alterations of MMR proteins.

We found only scarce data in the literature about MSI in ciliated, tubal-type endometrial lesions. MSI was demonstrated in one case (1/75) of anovular endometrial tissue, in which this type of metaplasia was identified in the absence of evident morphological preneoplastic changes [160]. Also, another study using the repetitive genetic marker (HUMARA – human androgen receptor gene) for nonrandom X chromosome inactivation demonstrated monoclonality and MSI in endometrial precancers with various müllerian differentiations, including tubal type [265].

Infrequently, MSI endometria with nondescript morphology was seen to precede by years a diagnosis of carcinoma showing MSI [160] and acquisition of MSI in normal appearing endometria is in general restricted to the cases with hereditary MMR gene defects. None of our cases of functional endometria presented abnormal expression of MMR proteins, in agreement with previous studies [160 262 266]. The absence of sporadic MSI in normal and simple hyperplastic lesion [262] and its presence in precancerous lesions, as also in adjacent non-neoplastic endometrium of MSI positive endometrial carcinoma [160 265-267], points toward consideration of MSI an early event in endometrioid carcinogenesis and a highly specific marker of neoplastic disease.

With this background, the evaluation of MSI in the spectrum of ciliated, tubal-type endometrial lesions allows us to make some pertinent suppositions: 1) CHT could be considered one of the morphological faces of endometrial precancers; 2) due to the fact that loss of pair proteins MLH1/PMS2 is regarded as the dominant pattern of MSI endometrioid adenocarcinoma [260], the end of potential progression of TM will be an endometrioid lesion and not a serous one; 3) three of our cases showed complete loss of the pair proteins MLH1/PMS2, but immunohistochemical detection of MSI phenotype is less sensitive that PCR test [260] and possibly the number of cases with MMR alteration could be higher.

Microsatelite instability has been associated with overexpression of cyclin D1 in endometrioid adenocarcinoma [400]. However, only one of the three MSI CHT lesions showed more than 50% of nuclei positive for cyclin D1. The number of cases is too small to permit relevant connection between MSI status and expression of this cell cycle protein.

In normal endometria, *PTEN* expression was observed to have temporal and spatial changes throughout the menstrual cycle and early pregnancy, being hormonally modulated. Its fluctuations are driven by changes in the physiological requirements for the protein. Strong nuclear PTEN immunoreactivity was observed during the normal proliferative phase, where it probably acts to control proliferation. Subsequently, it decreases to a point where it is no longer detected or restricted to the cytoplasm in the secretory phase and early pregnancy, when its suppressive activities are unnecessary [42 401]. This latter feature requires the establishment of a cutoff value for the frequency of PTEN-negative cells. In endometrial cytology, this threshold was proposed to be ≥50% [283].

In our study all the cases with normal functional endometria (n=79) presented a strong, diffuse expression of PTEN in the glands, stroma and endothelium, independent of the hormonal status of the endometrium.

The same constant positivity was documented along the spectrum of endometrioid and ciliated lesions. This feature is discordant with the literature which describes high frequency of PTEN loss in the endometrioid precursor (20-55%) and malignant lesions (35-83%) [156 274 277 280 281]. This discrepancy is most probably linked to the type of PTEN clone used [282]. By FISH analysis, three (15.7%) of 19 tubal type endometrial lesions analyzed showed hemizygous PTEN genotype. One (1/8) corresponded to CHT, one to CTMI (1/6) and finally one (1/5) has been diagnosed as extensive STM in an endometrial adenofibromas. Loss of one PTEN allele observed by us in these lesions has been reported to be a more frequent pattern than the PTEN homozygosity in endometrioid adenocarcinoma [156]. Therefore, the common phenomenon of PTEN protein loss in endometrioid lesions could not be explained solely by PTEN structural alteration and other non-mutational mechanism are probably involved in the inactivation of its second allele [156].

PTEN deletion in STM should not be seen necessarily as a criterion favouring its preneoplastic potential. PTEN has a limited routine clinical use mainly due to the roughly equal prevalence of PTEN-null phenotype between normal and premalignant lesion [36 207 208 402] and its low sensitivity and specificity to predict progression to carcinoma [206 403]. Furthermore, Baak et al. [206] found that no PTEN-null hyperplasia with morphometric D-score higher than 1 (no significant architectural changes) progressed. Consequently, STM with PTEN alteration almost certainly belong to the largest group of endometrial lesions that probably never progress.

One of six CTMI presented hemizygous deletion of PTEN on a background of proliferative endometrium. PTEN alteration alone is insufficient to cause endometrial cancer [208], but if the proliferative oestrogenic stimulus persists, the probability of further mutations increases, as well as the possibility of malignant transformation. In an appropriate hormonal context, complex TM in isolated glands with PTEN alteration can be an intermediate step through development of complex hyperplasia and adenocarcinoma.

Finally, one of eight CHT cases (12.5%) presented PTEN deletion, associated also with concomitant MMR proteins loss. This association is not fortuitous. Several studies showed that PTEN mutation occurs with higher rates in MSI tumours compared with stable ones [404] and it was suggested that PTEN could be a target in a deficient DNA repair context [405]. The low prevalence of PTEN mutation in our study could be related to the reduced number of cases studied or to alternative pathways of carcinogenesis. However, it was demonstrated that the combination of morphological complexity (D-score lower than 1) with PTEN inactivation highly increases the risk of progression [206]. This supports the hypothesis of the preneoplastic potential of the singular CHT with PTEN gene alteration and favours ciliated or classical endometrioid adenocarcinoma at its possible progression end.

None of the three hemizygous PTEN cases showed loss of PAX2 expression. Recently, it was observed that the double null state better predict the neoplastic potential than inactivation of PTEN alone, but this joint loss was observed in only 31% of precancers [208].

6 cases were analyzed for *K-ras* mutation. We found that half of them presented point mutation; two corresponded to CHT (40%) and the third one to extensive STM. We used punches of the paraffin embedded tissue and not laser microdisection of the glands, which minimized the contamination with non-target cells and dilution of interest DNA. In this context, more than 20% of total DNA presented mutation in the positive cases, the threshold required to score positive for the PCR sequence [406].

Besides the small number of cases, we found a higher prevalence of K-ras mutation (40%) in CTH that previously described for endometrioid lesions (0 to 23%) [158 284 287 407]. The conflicting reports regarding the frequency of K-ras mutation in preneoplastic and neoplastic endometrial lesions are considered related to epidemiological and clinical characteristics of the patients [407]. We presume that our findings may be interpreted in the context of a higher rate of K-ras mutation in endometrial and ovarian adenocarcinoma with

mucinous differentiation than in endometriod type [286 408]. Subsequently, K-ras mutation could be a more frequent pathway involved in carcinogenesis of endometrial adenocarcinoma with altered differentiation.

Both of CHT presented point mutation in codon 12, which is regarded as the codon most frequently involved in endometrioid lesions [158 287 288 407]. Additionally, one of them presented a concurrent mutation in codon 13. A double simultaneous mutation of K-ras has been previously reported in endometrioid adenocarcinoma, where it has been considered to reflect a heterogeneous clone of the same lesion, with at least one of these mutations occurring later during tumour progression [158 284].

Another report described two similar cases defined as complex atypical hyperplasia with tubal cell features; one presented concomitant K-ras and β-catenin mutation and the other only PTEN mutation [292]. K-ras has been regarded as an early event in endometrial carcinogenesis [158 287 407]. However, in contrast with PTEN mutation, frequently found to precede the acquisition of histological stigmata of neoplasia, K-ras activation is regarded as a marker of progression to malignancy, often correlated with morphological changes of endometrial hyperplasia and presence of atypia [287 288]. Despite the absence of evident atypia, both of CHT cases with K-ras mutation should be considered to bear a high risk of progression to adenocarcinoma. Type I endometrial adenocarcinoma is once again the favourite candidate, considering that K-ras activation is an exceptional event in serous adenocarcinoma [212].

The third case corresponded to an endometrial polyp of a 70 year old woman, which presented a point mutation in codon 61 of K-ras gene. Various studies have described a higher rate of K-ras mutation in endometrial polyps, especially in those related to tamoxifen treatment, where mutations in codon 12 but not in 61 were found [289 409 410]. However, in this case no antecedents of tamoxifen therapy were found and this occasional finding is difficult to explain.

Even if MSI accelerates the acquisition of further mutations, none of the MSI lesions presented K-ras mutation; instead all three cases with K-ras activation were microsatellite stable. Our genetic study is too small to allow make any suppositions regarding the relation between these abnormalities, but a previous study showed similar results, with a higher frequency of K-ras mutation in microsatellite negative (32%) than positive (19%) endometrioid adenocarcinoma [212]. However, other reports presented the contrary [262 286] or

even failed to show significant differences of K-ras mutation in microsatellite stable and unstable premalignant and malignant endometrial lesions [287 411].

Also, neither PTEN deletion accompanied K-ras activation or *vice versa.* However, it has been reported that these two mutations do not seem to concur within the same lesion [412].

Activated K-ras, PTEN and β-catenin mutation as well as MSI can cause up-regulation of cyclin D1, contributing to endometrial carcinogenesis [400]. Indeed, five of the cases with genetic alteration (two involving PTEN and three K-ras) presented overexpression of cyclin D1, but the differences were not significant in comparison with the lesion without these genetic abnormalities. Remarkably, even in these conditions the proliferation index was low.

There has been much debate about the discordant pattern regarding *β-catenin* expression in both normal and malignant endometrium. Several studies suggest that sex steroid hormones contribute to the regulation of cadherin-associated cell adhesion system of the endometrium [236 300]. However, we fail to show any differences in the membrane β-catenin expression and no nuclear staining among the various phases of the normal endometria. Our results are in agreement with a previous study [159], but different from others, that found nuclear positivity and changes of the cytoplasmic positivity through various phases of the menstrual cycle [235 236 413]

A progressive higher rate of β-catenin alteration has been reported along the normal-hyperplasia-adenocarcinoma sequence and β-catenin has been regarded as a relatively early event in endometriod carcinogenesis [159 235 402 413 414]. However, we did not observe abnormal expression of β-catenin throughout the spectrum of endometrioid and ciliated lesions and constant strong membrane positivity was the rule. The exceptions were the cases that presented morular differentiation. The prior studies that documented a high prevalence of β-catenin nuclear expression in preneoplastic endometrial lesions do not specify the coexistence of morular metaplasia [159 235 413]. We agree that β-catenin alteration in endometrial lesions without this type of differentiation is a rare event [120 415]. Furthermore, the constant nuclear positivity of morules for β-catenin is well documented and the positivity was usually restricted to the glands with these changes, this was confirmed in our cases. The spot positivity of glandular cells lacking evident morules was interpreted as anearly stage of morular development [292 416]. Taking into account that morules and β-

catenin mutation are the morphological and molecular markers respectively of putative endometrioid malignancy [294] [295] [415], our singular case of CHT with nuclear β-catenin expression could be regarded as a precursor of endometrioid adenocarcinoma.

β-catenin, CDX2 (nuclear transcription factor implicate in intestinal differentiation) [417] and CD10 [333] are considered sensitive markers for distinguishing morular from squamous differentiation, as was exemplified by one of our adenocarcinoma cases.

The mosaic pattern of expression in the tubal-type lesions with increased cytoplasmic staining in the secretory cells, compared with negative or weak positive ciliated cells, could reflect the proliferative potential of the former and terminal differentiated state of the latter. We favour this possibility considering that cytoplasmic β-catenin accumulation accompanies proliferation in normal or pathological endometrial state [235] [236] [413].

Accumulation of the β-catenin through the TCF/LEF-1 pathway may activate cyclin D1 and the expression of these two markers was correlated statisticsally and topographically in endometrial hyperplasia and carcinoma [235] [236]. All our five cases with nuclear β-catenin positivity presented an overexpression of cyclin D1, but no co-localized immunoreactivity could be identified, with exception of morular areas. However, the number of positive cases is too small to permit a statistics correlation.

Even if K-ras mutation produced stabilization of β-catenin in vitro [418], within the present study none of the cases with K-ras alteration associated with β-catenin abnormal expression, in agreement with a previous report [295]. Also, an association between the APC/ β-catenin/Tcf pathaw and MSI has been suggested in colon cancer [419], but we did not find this relationship in our study. Thus, β-catenin alteration appears to play an independent role from MSI, PTEN and K-ras in the pathogenesis of uterine endometrioid adenocarcinonoma [420].

As we have already stated, we observed that the *EGFR* expression in the normal endometrium varies with the stage of menstrual cycle, being up-regulated by oestrogen in the follicular phase and decreased by progesterone in the luteal phase [40] [311]. Therefore, these data support that oestrogen stimulates cell growth through a cascade of events, including elevation of growth factors and their receptors [421]. Consequently, a high expression of EGFR is expected along the spectrum of endometrioid lesions, as observed in

our study. Consistent with previous reports [310], there was no different distribution of EGFR among various hyperplasic lesions, although the number of cases was reduced.

Ciliated lesions, independent of the architecture, had a constant EGFR positivity, but the intensity of reaction was low to moderate compared with the normal proliferative or hyperplastic endometria. Once again, a mosaic pattern of expression was revealed by EGFR immunostains, in which ciliated cells protruded as pale unstained or weak positive areas, between more strongly positive secretory cells. A similar expression was noticed in the correspondent normal tubal epithelium. Downregulation of EGFR in ciliated cells could be related to loss or weak intensity of ER expression in the same cells, in some cases. Although EGF promotes cell proliferation, none of the tubal-type lesions presented a high mitotic index. The positivity for EGFR and low proliferation index could not be necessarily seen as a contradiction, because its positivity was observed also in a high percentage (77%) of inactive, atrophic endometrial [422]. The relatively higher reactivity of EGFR in the secretory of TM, as in the atrophic endometrium, favours a conserved proliferative capacity of these cells, in contrast to the terminally differentiated ciliated cells.

High expression of EGFR in the proliferative phase and its non-discriminatory capacity between various types of hyperplasia, limits its use as a marker of progression of endometrioid or even ciliated lesions.

Other points that we should emphasize are lack of EGFR expression in various metaplasia, including mucinous, morular and SPSC. The absence of EGFR in mucinous metaplasia is not a surprising feature, taking into account that the corresponding normal endocervical epithelium is frequently negative, as we will discuss later. The immunonegativity of SPSC and morules is consistent with their inactive status and absence of hormonal receptors respectively.

Our findings are comparable to reported data regarding the expression of *hormone receptors* in the normal functional endometrium [313 423]. Oestrogen receptors (ER) were present in all cyclical endometrium, with a constant positivity in the glands and stroma. In contrast, progesterone receptors (PR) were downregulated in the secretory and decidualized endometria, with their preservation in the stromal cells. This finding could be regarded as a contradiction, but it is generally accepted that oestradiol up-regulates ER and

PR, while progesterone downregulates both receptors [313]. Subsequently, low levels of PR are expected in progestative conditions.

Tubal type lesions, independently of architectural changes presented a constant positivity of sex steroid hormones. Except in 11 cases, where a simple architecture had a slightly decreased expression of oestrogen receptor. The weak or negative nuclei corresponded mainly to ciliated cells and remarkably the same pattern was observed in the normal Fallopian tube epithelium. An analogy with our findings is the observation of decreased expression of OR, PR and proliferation index in the areas of ciliated type differentiation of endometrioid adenocarcinoma, when these are compared with pure endometrioid components. The authors do not specify which type of cells, secretory or ciliated, lack the positivity of hormonal receptors, but suggested that these foci probably underwent terminal differentiation [154]. Furthermore, SPSC, considered a degenerative phenomenon [131], presented an important decrease of oestrogen and progesterone expression. Therefore, downregulation of OR in TM could be another factor supporting the terminal differentiated state of the ciliated cells.

Absence of bcl-2 protein in the ciliated cells could be explained partially by down-regulation of ER, taking into account that this anti-apoptotic protein is under the hormonal control, with oestrogen increasing and progesterone decreasing its expression [424].

We also observed that morular differentiation is hormonally inert, which explain its resistance to progesterone therapy [333 383].

There were no differences in the expression of sex steroid hormones expression with increasing of endometrial glandular complexity and acquisition of atypia, as was proved by some reports [312]. However, a decrease of ER and PR was observed in 33.3% and 22.2% respectively of well differentiated endometrioid adenocarcinoma, that showed more than 50%, but less than 80% of positive cells. Similar frequencies were previously reported and decreased expression of ER and PR in endometrioid adenocarcinoma appeared to be correlated to tumour grade and stage [236 312 317 423 425]. Endometrioid adenocarcinoma is a hormone dependent lesion; consequently, detection of ER and PR is expected. But, these two markers are also expressed at a lower rate in many serous adenocarcinomas [317 320]. This was the situation of both our serous adenocarcinoma, which expressed hormonal receptors in less than half of the cells, mainly located at the invasive front. Therefore, ER and PR will be effective in the differential diagnosis between papillary endometrioid and serous

adenocarcinoma only together with Ki-67, p53 and p16 and their use as prognostic factors is still controversial.

Summarizing:

We believe that *simple TM* lack preneoplastic potential. Its secretory cells maintain the capacity to proliferate; instead the ciliated ones are terminal differentiated. Our theory is supported by multiple evidences:

a. High expression of p16, especially in the ciliated cells. p16 is a tumour suppressor gene, which constrains the cell cycle and furthermore, regulates the age-dependent senescence.
b. A characteristic mosaic pattern of bcl-2 with a positivity restricted to the cytoplasm of the secretory cells. This protein is mainly limited to proliferating, non-terminal differentiated cells. Additionally, it has a capacity to induce differentiation.
c. The same chessboard pattern was observed for PAX2, with the positivity restricted to the nuclei of secretory cells. This transcription factor has an important role in stem cell maintenance, resistance to apoptosis and repression of terminal differentiation of the cells.
d. Mild proliferative activity restricted to the secretory cells, in contrast with ciliated cells which almost never show mitotic activity
e. Increased β-catenin cytoplasmic staining of the secretory cells compared with weak positivity or negativity of the ciliated ones. Cytoplasmic β-catenin accumulation accompanies proliferation in the normal or pathological endometrium.
f. No alteration of MMR proteins.
g. Stronger expression of EGFR within the secretory than in the ciliated cells. EGF promotes proliferation.
h. A downregulation of ER observed in some cases similar to normal Fallopian tube epithelium.

Additionally, we found in the literature that:

i. TM is negative or showed a very focal, positivity for ProExC [195], a cocktail which targets the cell cycle proteins, minichromosome maintenance protein-2 (MCM2)

and topoisomerase-IIA (TOP2A). These proteins play an important role in regulation of DNA replication and their down-regulation, as in TM, is connected with differentiation or quiescence of the cells [195 196].

j. p21, a cyclin dependent kinase inhibitor was increased in TM [184]. It was frequently expressed in the nuclei of the Fallopian tube ciliated cells, whereas the secretory cells remain negative. p21 previously implication in the differentiation of breast, ovary, striated muscle and intestinal cells [384] points to its involvement in differentiation of ciliated cells.
k. Common occurrence and unremarkable follow-up over a period of 32 months [377].

Complex TM in isolated glands is rather a benign process presenting overlap in the expression of p16, cyclin D1, Ki-67, bcl-2, PAX2 with simple TM. It should raise the possibility of a preneoplastic lesion only in the context of its association with unopposed oestrogen conditions and loss of bcl-2, PAX2, MMR proteins or PTEN alteration.

We believe that *complex hyperplasia tubal-type* represents a clinically relevant and biologically distinctive alteration. It fulfills most of the criteria proposed by National Cancer Institute (NCI) for a lesion to be considered preneoplastic [149] . These include:

a. a precancer differs from the normal tissue from which it arises;
b. a precancer differs from the cancer into which it develops, although it has some, but not all, of the molecular and phenotypic properties that characterize the cancer;
c. when a precancer progresses to cancer, the resulting cancer arises from cells within the precancer, in other words proving lineage clonality;
d. evidence must exist that the precancer is associated with an increased risk of cancer;
e. a method exists by which the precancer can be diagnosed.

By definition ciliated type complex hyperplasia differs from the background endometrium, consequently, we cannot apply the criteria of atypicality used for endometrioid lesions. However, as for mucinous lesions, we assume that extensive areas of highly complex architecture do not require a striking atypia to be considered preneoplastic.

The second and third criteria are supported by the following:

a. CHT had a down-regulation of p16, but maintained higher expression compared with complex endometrioid lesions, probably due to its preservation in the ciliated cells
b. In a similar way to complex endometrioid lesions and adenocarcinoma, complex hyperplasia CHT presented down-regulation of bcl-2 and loss of PAX2. Some cases present a heterogeneous pattern of bcl-2 and PAX2 expression, with positive foci seen in the vicinity of weak or negative areas. The preserved positive areas of ciliated, tubal complex hyperplasia and even endometrial adenocarcinoma with ciliated differentiation had the same mosaic pattern, which proves lineage continuity.
c. We detected MMR proteins defects, markers of MSI and highly specific for neoplastic endometrioid disease in 21.42 % of CHT.
d. PTEN deletion by FISH analysis was encountered in 12.5% of cases. PTEN is the most frequent molecular alteration of preneoplastic and neoplastic endometrioid lesions.
e. 40% of CHT cases presented K-ras point mutation. K-ras activation is regarded as a marker of progression to malignancy, more often correlated with morphological changes of endometrioid hyperplasia and acquisition of atypia.
f. Finally, one case of CHT was associated with nests of β-catenin nuclear positivity.

However, longitudinal follow-up data are necessary to confirm that complex tubal-type hyperplasia has a similar risk of developing an invasive malignancy as their correspondent endometrioid lesions. Our study lacks this data, but three of our cases were found to coexist with endometrioid or ciliated adenocarcinoma. All these facts support that at the progression end of these lesions an endometrioid or ciliated endometrial adenocarcinoma will be found, and not a serous one.

The criteria used to classify endometrial hyperplasia are usually addressed to endometrioid differentiation and consequently many of these lesions are under-evaluated and not recognized as potential precursor of neoplasia. Furthermore, they contribute to the poor diagnosis reproducibility of endometrial lesions. For the present time, the morphologic criteria within biopsy specimens are the most reliable method of CHT diagnosis, and the immunohistochemical study, especially loss of PAX2, PTEN and Bcl-2 expression, can only add further support to their potential malignant behaviour.

6.2. Cervical TM

In contrast to the poorly studied endometrial TM, there in a wealth of studies of endocervical TM in the literature. This is explained by the fact that TM, or tubo-endometrial metaplasia (TEM), was considered the commonest mimicker of preneoplastic endocervical lesions at histopathological [170 180 181 385 426] and cytological level [144 145 427]. In Pavlakis's study [173], five cases diagnosed initially as tubo-endometrial metaplasia were reclassified as endocervical glandular dysplasia (EGD), using the semi-quantitative score system. Nevertheless, others studies made the supposition that endocervical TM could be considered a preneoplastic lesion [138 139].

In this second part of our study, we challenge these two possibilities; TM as only a mimicker of endocervical adenocarcinoma or with a premalignant potential. We also plan to define the best diagnostic approach, by using a comparative morphological and immunohistochemical analysis.

Several features complicate their morphological interpretation, such as: florid deep proliferation, which has been seen in three cases (7.5%), architecturally abnormal glands with crowding, back to back arrangement, papillary or cribriform arrangement observed in nine cases (22.5%), pseudo or true stratification in eight cases (20%), slight degree of cytologic atypia in three cases (7.5%) or scattered typical mitotic figures in two cases (5%). Several other studies also dealt with these morphological pitfalls in TM diagnosis [134 139 176 197]. When the glands were partially involved, a gradual transition between tubal type and secretory mucinous epithelia was frequently observed. In rare instances, an abrupt switch between the two epithelia, similar to that observed in AIS lesion, give rise to diagnostic problems. Cytologically, the lack of mucinous secretion in TM and confusion of intercalary cells (intraepithelial lymphocytes) with apoptotic bodies add further confusion. Other dubious situations can arise in the context of TM associated with previous surgery, when an altered, desmoplastic-like stroma is present [134], as well as in the presence of deep tubal type glands with electrocoagulation artifact. In our study a focal desmoplastic-like reaction with an oedematous/myxoid change, sometimes with the presence of inflammatory cells, was present in 22.5% of cases and was not related to a previous surgical intervention. Also, a moderate inflammatory infiltrate encircling the TM glands (17.5%) or a cambium layer aspect (5%) raise the possibility of a neoplastic process.

The presence of ciliated cells cannot be used as an absolute diagnostic criterium of benignity, because they are not invariably present in TEM. Nevertheless, a ciliated variant of endocervical dysplasia and AIS has been described [138 175]. This rare variant of AIS was associated with typical and atypical tubal metaplasia. Morphologically, it presented residual tubal features in the form of apical cilia. In these cases, it is difficult to differentiate between atypical tubal metaplasia, not infrequent and usually considered to have no malignant potential, and ciliated AIS, a malignant precursor lesion [138]. The diagnosis of AIS ciliated type requires severe nuclear atypia, increased mitotic figures and apoptotic bodies, whereas the presence of cilia in isolated glandular atypia favours a non-neoplastic process [175]. Misdiagnosis of TM with AIS can occur especially in hysterectomy specimens with a previous diagnosis of AIS or adenocarcinoma [170], where the likelihood of finding residual lesions is high [130 136].

There is a little confusion as to the classification of preneoplastic and neoplastic glandular lesions. If AIS can be of both ciliated *and* serous type, the adenocarcinoma refers only to the serous type [180].

The presence of TM cells in cervical or endometrial cytology can represent a diagnostic challenge [144 145 427 428]. They result either from spontaneous desquamation or during cytological sampling. Its frequent location at the transition zone is reflected by its conspicuous presence in the smears.

Due to the frequent presence of TM in a great variety of non-neoplastic and neoplastic conditions in all age groups, their recognition is mandatory to avoid a misdiagnosis or false negative. The fact that these epithelial alterations can line simple glands without any suspicious features, complex hyperplastic glands with some degree of atypicality may also occur.

Well documented and clear involvement of high risk human papillomavirus (HR-HPV) in cervical carcinogenesis, relatively high rates of false negative (15%-50%) and false positive (30%) results of PAP smears in identification of preneoplastic and neoplastic lesions of the cervix (largely determined by sampling and subjectivity of interpretation), significant intra and interobserver variability of the cervical biopsy diagnosis (especially for low grade lesions), call for the necessity of a specific biomarker with a high sensitivity, specificity, simple application, with a good accuracy of evaluation and with an impact on the management of cervical lesions [196 429 430].

p16^{INK4A}, an indirect marker of cell cycle dysregulation, is up-regulated in preneoplastic and neoplastic cervical conditions, especially those associated with HR-HPV infections. In the last decade, the literature has been replete with publications about "*p16^{INK4A} a surrogate marker and a potential screening tool*" for cervical dysplasia and adenocarcinoma. Subsequently, p16^{INK4A} started to be routinely used in the diagnosis of lower female genital tract pathology. Many of these studies exposed confusing and contradictory data [176 181 193 430-434].

In the present study, 40% of cases with normal cervical epithelium presented spots of p16^{INK4A} positivity in individual or small clusters of cells. Numerous other studies reported similar findings [170 194 324 365 434 435]. Also, in accordance with the literature, occasionally weak immunoreactivity was demonstrated in the nuclei and/or cytoplasm of normal squamous epithelium (5%), of both mature (25%) and immature (25%) squamous metaplasia, especially those associated with inflammatory or reactive changes [193 194 433 434]. Furthermore, occasionally strong p16^{INK4A} positivity has been described in reserve cells [434], stromal fibroblasts, endothelial cells, and inflammatory cells (histiocytes), especially around infiltrative malignant lesions [436]. In most normal cells, p16^{INK4A} expression is known to be low at both mRNA and protein level [436] and is detected immunohistochemically only when is physiologically up-regulated. Scattered normal cells expressing p16^{INK4A} have been detected in the cytologic smears [192 429] and this should be kept in mind in order to avoid misdiagnosis with a HPV-related endocervical lesion.

p16^{INK4A} has been proved to be a useful biomarker for both squamous and glandular malignancy [192 429 430 432 434], with a greater specificity than sensitivity for squamous lesions and reduced sensitivity and specificity for the glandular lesions [437]. It should be emphasized that p16^{INK4A} is not a consistent surrogate marker of HR-HPV presence in preneoplastic and neoplastic glandular endocervical lesions. A significant proportion of endocervical adenocarcinomas (25-40%) expressed p16^{INK4A} in the absence of HR-HPV. These cases mainly corresponded to adenocarcinomas of older women or some unusual variants, including minimal deviation adenocarcinoma, gastric, intestinal, mesonephric and clear cell types [438-441]. The etiology of HPV negative adenocarcinoma has not been elucidated as yet. A small fraction appears to be related either to activation of oncogenes (K-ras) or telomerase activation as early event, or inactivation with overexpression of p53 and amplification of c-erb-B2, as late phenomenon [442]. Another possibility can be related to down-regulation of

the cell cycle inhibitors (p16^{INK4A}, p21 and p27) [438]. In the present study we found that all the cases of cervical glandular neoplasia exhibited strong, diffuse positivity for p16^{INK4A}, but the number of cases in our control study was limited to four invasive classical endocervical adenocarcinoma and two *in situ* and we did not asses the presence of HR-HPV. +

The morphologic overlap of TM and preneoplastic endocervical adenocarcinoma is not clarified by using p16^{INK4A} alone. All of our TM cases except one showed constant p16^{INK4A} positivity, with more than occasional cells immunoreactive. Numerous other studies showed this consistent phenomenon within cervical TM, and described a focal, heterogeneous [170 176 179 181 193 194 365 434] or mosaic pattern, with >50% cells positive [203]. It appears that the p16^{INK4A} immunoreactivity pattern is essential to distinguish between TM and a true preneoplastic or neoplastic lesion. If the cells of an adenocarcinoma expressed p16^{INK4A} diffusely and strongly, both in the nucleus and cytoplasm, the majority of TM had a focal, patchy or heterogeneous positivity. However, in routine practice the pathologist is confronted by numerous problematic cases when this clear cut immuno-pattern is not evident or there are exceptions from the rule. A diffuse p16^{INK4A} immunostain has been described recently in 35% of TM glands [194 324], and it was present in 5% of our cases. Furthermore, the characteristic staining patterns (focal versus diffuse) may be difficult to assess in small biopsy and curettage samples, or when minimal lesional tissue is included in the specimen and a consideration should be given to the possibility of false negative results for p16^{INK4A} [375]. Therefore, p16^{INK4A} has a low discriminatory power in differentiate TM from preneoplastic lesions, especially endocervical glandular dysplasia [173 176 181 192 194 380 434]. The immunoreactivity evaluation of p16^{INK4A} must be interpreted in the light of morphological features [443]. In cases of obvious discordance between morphology and immunohistochemistry, not only involving p16^{INK4A} but also other antibodies, the pathologist should be cautious in making a clear cut diagnosis and would be wise to request a re-evaluation of the patient.

The majority of cervical adenocarcinomas are related to HR-HPV [439 441], including tubal type AIS (a single case reported in Lee KR study, in association with HPV16) [175]. Schelsinger, based on the association between typical and atypical tubal metaplasia with ciliated type AIS, suggested that TM cannot be longer considered a benign mimic of AIS, but rather a predecessor of, or a concurrent lesion with, AIS [138]. However, none of TM and ATM (atypical tubal metaplasia) cases showed transcription of viral oncogenes E6/E7 by PCR or in

situ hybridization [176], no DNA HPV was detected in TM cases with MIB1>10% [205], none of ciliated-type dysplasia in Lee KR study (3 high grade dysplasia and 7 low grade dysplasia) was positive for HPV DNA [175] and the majority of the cases are negative or show only scattered positivity for ProExC, a feasible biomarker for cervical HPV-related lesions. All these studies indicate that TM is not associated with HPV infection; neither does it appear probable that TM represents a precursor lesion of AIS tubal type or classic variant [175]. As we have already discussed, there are other unusual types of endocervical adenocarcinoma clearly unrelated to HPV [441]. In the ovary, benign serous lesions are classified on the base of their morhological similarities with tubal-type epithelium and they are considered potential precursor lesions of low grade serous carcinomas. An rather unconventional idea is that TM could represent a preneoplastic lesion of serous cervical adenocarcinomas. This lesion is also positive for $p16^{INK4A}$, but related to HPV [441]. One study suggested that "atypical ectropion" or "atypical tuboendometrial adenosis" may represent transitional states between typical TM or TEM and clear cell carcinoma of the cervix or vagina of patients treated with diethylstilbestrol [143]. Clear cell carcinoma, a very rare lesion within the cervix, can also be positive for $p16^{INK4A}$, but its HPV related status has not yet been clarified. However, this is improbable, as is the possibility that TM could represent a precursor of minimal deviation adenocarcinoma, a tumour with mucinous differentiation.

Why is the utility of $p16^{INK4A}$ limited in terms of screening?

Firstly, $p16^{INK4A}$ is positive in both adenocarcinoma precursors and their benign mimickers. With the finding of an invariable positivity of cervical TM for $p16^{INK4A}$, we agree with the majority of workers in this field and recognise the limitation of this marker as a screening tool in cytology and biopsy specimens [176 181 429 432 433 441]. Furthermore, it is frequently found in other benign endocervical lesions, not related to HR-HPV infection. These include superficial cervical endometriosis, lobular glandular hyperplasia, microglandular hyperplasia [170 194 324 435 437] and mesonephric hyperplasia [444]. Our cases of mesonephric rest hyperplasia, papillary endocervicitis and glandular lesion radiotherapy-induced had more than occasional cells positive for $p16^{INK4A}$.

Secondly, there are still technical, molecular and interpretation problems with $p16^{INK4A}$ and recently there have been numerous contradictory reports in the literature. The most frequent include: factors related to specimen fixation (type, time) and to immunohistochemical technique itself (different clones of antibody, various epitope

retrieval protocols), making it necessity to use positive and negative controls [445 446]. There are difficulties with the microscopical interpretation due to disagreements on the percentage of cells required to consider a reaction positive, pattern of positivity and type of positivity within the cells, either in the nucleus [365] or cytoplasm [176 433] or both [170 181]. Also, there are still unresolved issues about cervical carcinogenesis at molecular level [447].

Thirdly, it has been shown that $p16^{INK4A}$ is focally positive in normal proliferative endometrial glands [176 181 365], in individual or small clusters of endocervical mucinous epithelium (occasional ciliated cells), reserve cells, epithelium lining Nabothian cysts [170 194 365 434 435] and occasional cells of both mature and immature squamous metaplasia, especially with reactive, inflammatory changes [193 194 434]. All of these cells can be present in a cervical smear.

Fourthly, $p16^{INK4A}$ is not a reliable surrogate marker of the presence of HR-HPV. A significant proportion of endocervical and endometrial adenocarcinomas, are $p16^{INK4A}$ positive in the absence of HPV.

Lastly, $p16^{INK4A}$ is far from being considered an indicator of neoplasia. As our study showed, SPSC, a reactive endometrial condition frequent associated with endometrial breakdown, is strongly and diffusely positive for $p16^{INK4A}$.

However, the utility of $p16^{INK4A}$ remains unquestionable mainly in the differentiation of high grade CIN from atrophic epithelium, immature squamous metaplasia, transitional metaplasia and highly inflammatory lesions [448]. In support of this, a recent study showed that approximately one quarter (21.6%) of HR-HPV and biopsy negative cases would be reclassified by using $p16^{INK4A}$ to have different grades of CIN; indeed, more than half will be found to have high grade lesions, with different prognosis and management [449].

Cervical TM, in contrast with its corresponding endometrial lesion, showed a lesser expression of *cyclin D1***.** The pattern of staining was frequently heterogeneous and not mosaic and was restricted to the nuclei. This cell cycle protein had a significantly higher expression in the normal endocervical epithelium, with a striking demarcation from the negative neoplastic cells. For this reason, cyclin D1 has been recently proposed as a useful marker to differentiate reactive from premalignant and malignant endocervical lesions [194]. Therefore, TM presented a cyclin D1 expression closer to the normal endocervical epithelium and benign, reactive conditions than to the neoplastic ones. The absence of

cyclin D1, a proliferation-associated mediator in endocervical neoplasia is a paradoxal finding, explained by the same mechanism which leads to overexpression of $p16^{INK4A}$. Inactivation of pRb by the HPV oncoprotein E7, bypasses the normal requirement for cyclin D1-CDK in the initiation of the cell cycle. Subsequently, the positive feedback loop between pRb and cyclin D1 is altered with downregulation of the latter one. Similarly, the negative feedback loop between pRb and $p16^{INK4A}$ is affected with up-regulation of the later protein, even though functionally inactive [194].

The expression of *Ki-67* was mainly distributed in the parabasal layer and not in the basal cells of the normal and metaplastic squamous epithelium and served as a positive control. This is not a contradictory finding because the basal squamous layer is formed by stem cells with slow-cycling self renewal [450]. Furthermore, basal and parabasal layers express different biomarkers. If the former presents expression of bcl-2 and frequent negativity for ER and PR, the latter is positive for p53 [437]. Cervical TM glands presented a proliferative activity inferior to 10% in majority of the cases (90%), including both cervical adenofibromas with extensive, florid TM. The rate of proliferation within TM was closer to the normal endocervical glands than to endocervical preneoplastic and neoplastic lesions taken for comparison. These latter ones had a Ki-67 superior to 70%. Numerous studies showed that MIB1 had a better discriminatory power between AIS/invasive adenocarcinoma and benign mimickers, including TM than other antibodies. [170 179 193 194 197 198 205 442]. However, there is no agreement regarding the cut-off of proliferation index to distinguish between benign and malignant endocervical lesions. Some investigators considered the percentage at 10% [170], others 16% [197] or 25% [175]. A Ki-67>16% or a MIB1>30% [442] was highly suggestive of neoplasm. Nevertheless, some authors were dogmatic that MIB1<10% implies a benign lesion and MIB1>50% always reflects a malignant process [205]. However, in routine practice this rule cannot be strictly applied, because there are occasional cases of AIS with a low proliferation index, less than 10% [170 176] and also TM with a MIB1 more than 10%, and sometimes around 30% [176 194 205]. 10% of our cervical TM corresponded to this latter situation. In one study, TM with a higher proliferation index was believed to represent a varying degree of endocervical glandular dysplasia [197]. Therefore, the diagnosis of endocervical glandular dysplasia (rarely diagnosed in the absence of associated AIS) should

not be made solely on MIB1 expression, due to its low proliferation index and overlap with tubal metaplasia [179 451].

From the practical point of view, it should be borne in mind that not always does the proliferation index parallel the degree of biological aggressiveness of the lesion or correlate with HPV positivity [175]. There is an overlap of its expression between EGD/AIS and benign mimickers (TM, florid microglandular hyperplasia, glands adjacent to squamous intraepithelial lesions and cases with a history of recent biopsy) [170 176 197 205 451]. Interpretation of this marker should be done with caution in inflammatory associated atypia, which can have an increased rate of proliferation [451]. Intraepithelial lymphocytes which are positive for MIB1 and tangential sectioning can also create confusion.

All of our cervical TM presented a constant positivity for bcl-2, reproducing the same chessboard pattern of expression described for endometrial TM and Fallopian tube epithelium [384]. This finding contrasted with the diffuse pattern of expression previously reported [170 194 199 385] and is probably related to the type of changes studied. TEM cases were previously analyzed and not "true" TM, with easily recognized morphological evidence of ciliated and nonciliated cells. Therefore, TEM terminology should be used for cervical glandular structures lined by an endometrial proliferative-like epithelium, which lack endometrial-type stroma and resemblance with tubal epithelium and diffusely express bcl-2. In agreement with prior reports [170 194 199] none of our endocervical malignant lesions expressed bcl-2. This feature concords with the observation of an almost constant presence of apoptotic bodies in these lesions, a useful criterion for their diagnosis [142]. Therefore, bcl-2 is an important part of the panel used for the differential diagnosis of endocervical neoplasia from TM.

Cervical TM was constantly positive for *PAX2*, independently of architectural complexity. It presented the same mosaic pattern of PAX2 expression as its endometrial counterpart. In agreement with previous data [247 388], the normal endocervix showed diffuse, weak to moderate expression of PAX2. This transcription factor has been regarded as a marker of Müllerian-type epithelium [247]. However, in contrast to proliferative endometrium, the endocervical epithelium is devoid of proliferative activity, expected in the context of PAX2 immunoreactivity.

Constitutive loss of PAX2 was present in all precursor and endocervical neoplastic lesions. Therefore, we support a previous study [388] regarding the usefulness of PAX2 in making distinction between *in situ* endocervical adenocarcinoma and TM. The chessboard pattern of PAX2 together with bland morphology should be interpreted as a clue for benignity; just as its complete loss points to a malignant process.

TM presented overlap of *p53* expression with the normal endocervical epithelium, showing a weak, focal, functional immunopositivity. None of the cases overexpressed p53. The role of HPV in cervical carcinogenesis is well known. The viral oncogene E6 binds p53 and contributes to cell cycle alterations [437]. Subsequently, alterations of the p53 gene are expected in neoplastic endocervical lesions, but we observed only occasional increase of p53, as had a previous study [179]. This could be explained by the frequent degradation of p53 protein in relation with HPV infection. However, widespread staining of p53 in cervical glandular neoplasia can appear and is usually regarded as a negative prognostic factor, being associated with a high grade and high stage lesions [442], or with an un-related HPV tumour, such as minimal deviation adenocarcinoma [452].

Based on the *EGFR* immunoreactivity and aberrant glycosilation, Umezaki et al. proposed TM as a putative precursor lesion of uterine cervical adenocarcinoma [139]. Our study found a constant positivity of TM for EGFR, with a pattern of expression overlapping with that of normal tubal epithelium. Therefore, the immunoexpression of EGFR could not be regarded per se as an indicator of malignant potential, but rather as a contributor to the false positive interpretation of TM lesions.

None of the normal endocervical glands presented expression of EGFR and in some areas the positivity was restricted to the reserve cells. This contrasted with the frequent, moderate positivity of the preneoplastic and neoplastic endocervical lesions that we reported previously [139 453]. EGFR expression in cervical neoplasia was considered as an unfavourable prognostic factor [453 454]. The mechanisms of its overexpression are not completely understood, and appear not to be related to activating mutation in the hotspot region of EGFR intracellular domain, the target for thyrosin kinase inhibitors [454]. This phenomenon could be linked to EGFR gene amplification or deregulation of EGFR internalization and degradation by HPV E5 and E6 oncoproteins [455].

Cervical TM, similar to endometrial TM and to normal tubal epithelium, preserved the expression of *hormonal receptors*. Also, the immunoreaction was analogous to that of surrounding normal endocervical glands. These markers are often used to differentiate TM from AIS, due to the frequent negativity of the latter [179]. However, careful attention should be paid to the interpretation of oestrogen receptors, taking into account that the majority of our neoplastic endocervical lesions preserved at least focal ER expression, in agreement with previous reports [326] [456]. Furthermore, TM had a high degree of ER overlap immunorectivity with EGD [179]. Conversely, PR appeared to have a better discriminatory power in making this distinction [456], being negative in five out of six endocervical neoplasia.

The only case of mesonephric hyperplasia included in our study lacked expression of ER and PR. This, together with vimentin and CD10, can be used to differentiate difficult cases of mesonephric hyperplasia from an endocervical adenocarcinoma [457].

Numerous reports considered *CEA* an important part of the panel used to discriminate preneoplastic and neoplastic endocervical lesion from TM [170] [173] [179] [198]. In the present study, the majority of cervical TM were either negative or expressed a luminal positivity, as the normal endocervical glands. The absence of cytoplasmic CEA immunostaining in tubal metaplasia was considered a criterion in favour of its benignity [198] [323], but it should be stressed that CEA cannot be used as a marker that is totally specific or sensitive for malignancy. We were able to observe weak cytoplasmic staining in one case of TM and 13.3% of benign endocervical glands with reactive inflammatory or actinic changes. Furthermore, not all *in situ* or invasive endocervical adenocarcinomas studied showed a strong cytoplasmic staining; one of six cases had only an apical and others two a weak cytoplasmic positivity. Previous studies also reported negativity or focal positivity in almost 40% of AIS and invasive adenocarcinoma, especially in the minimal deviation variant [323] [442] [451] [458]. Thus, a strongly positive CEA staining suggests carcinoma, whereas a negative reaction is not helpful. Another feature that we should emphasize is the heterogeneity of CEA expression in the same neoplastic lesion, which can prove problematic in small biopsies.

Our study also contributes to the general belief that CEA is a good candidate as a site specific marker for well differentiated adenocarcinoma in biopsies. None of the eight cases of well differentiated endometrioid adenocarcinoma presented a cytoplasmic staining of CEA, with the exception of mucinous and squamous areas. Focal reactivity of endometrial

adenocarcinoma has been reported in less than 20% of cases and in general the positivity was restricted to these types of differentiation [318 451].

In the light of unstained neoplastic endocervical glands and diffuse positive TM areas, our results support the usefulness of *vimentin* as an adjunct to other markers in assessing difficult cases. Some studies considered this intermediate filament a better discriminatory factor than CEA [323], but care should be taken with the tumour invasion front or with the rare occurrance of AIS focal positivity [324]. Furthermore, endometrioid or tubal type AIS arising in the cervix have been described [138], a context in which the value of this stain may be limited.

All of endometrioid endometrial adenocarcinomas consistently expressed vimentin, in contrast with the unstained endocervical neoplastic lesions. However, it should kept in mind that not infrequently endometrial tumours present a heterogeneous pattern of vimentin expression [459] and their mucinous differentiation areas could be negative [451]. These circumstances question the value of vimentin as a discriminator marker of tumour origin in biopsy or curettage material.

For many years, it has been thought, that normal endocervical glands are negative for vimentin. However, both our experience and a recent study noticed the contrary [324]. The normal endocervical cells showed a delicate positivity surrounding the mucinous vacuole, where the intermediate filaments are present.

In female genital tract pathology, *CD10* has been considered a useful marker of mesonephric glands as well as of normal and neoplastic endometrial stroma [330 331]. However, for the diagnosis of mesonephric remnants and their correspondent carcinoma the practical use of CD10 is limited. As we observed, scattered normal endocervical glands, tubal metaplastic glands (17.5%) and even occasional foci of invasive endocervical adenocarcinoma expressed apical CD10. Also, its sensitivity was altered by immunoreactivity of scattered normal atrophic endometrial glands and even normal tubal epithelium. Furthermore, a previous study showed a high frequency of CD10 expression in endometrioid endometrial adenocarcinoma and negativity in some cases of mesonephric adenocarcinoma [457]. All of this is evidence against the use of CD10 as a specific marker for mesonephric origin.

It has been suggested that positive CD10 staining of stromal cells may be useful in confirming a diagnosis of endometriosis [460]. Nevertheless, in agreement with a previous report [457], we found a CD10 positive stromal densification encircling tubal metaplastic and

normal mucinous glands. Consequently, a positive CD10 endocervical stroma does not allow a diagnosis of endometriosis in this location to be made, in the absence of characteristic morphologic findings [331]. Similarly, its positivity around malignant endometrial glands does not necessarily imply involvement of adenomyotic foci, because CD10 can be positive within the desmoplastic stroma. Neither does its absence exclude an adenomyosis due to the possibility of fibroblastic differentiation of endometrial stroma, similar to stroma of the polyps [461].

We demonstrated that an invariable positivity in the morular changes and CD10 can by a useful tool in the discrimination of morular from squamous metaplasia [333 416].

The morphological features (of three types of cells), positivity for bcl-2, PAX2, vimentin, focal $p16^{INK4A}$ expression, low proliferation index, negativity for CEA and for HPV tests would all suggest that TM should be considered a benign mimicker and not a preneoplastic lesion.

Chapter 7

Conclusions

VII. Conclusions

7.1. Endometrium

- Endometrial metaplasias and changes are often focal and overlapping, involving epithelial and stromal components of both eutopic and ectopic endometrium.

- A new classification is proposed, which uses a practical morphological approach and aims to unify terminology and provide indicators of malignant potential.

- Endometrial metaplasias and changes vary from reactive, degenerative lesions (eosinophilic, clear cell, surface papillary syncytial changes) to those occurring in association with malignancy (morules) or having a preneoplastic potential (tubal, mucinous).

- Tubal and mucinous metaplasias are the most frequent epithelial metaplasias. They are often admixed and present the most problems in interpretration.

- *Tubal metaplasia* (TM) is oestrogen dependant and associated with endometrial hyperplasia, polyps, well differentiated endometrioid adenocarcinomas, adenofibromas and adenomyomas.

- A working classification was made by subdividing TM into simple, complex isolated and complex hyperplasia based on the architectural pattern but not atypicality, as this is absent or mild.

- *Simple TM* (STM) is frequent and should be regarded as a benign condition as it does not have a preneoplastic potential.

- A characteristic cytoplasm and nuclear chessboard pattern of bcl-2 and PAX2 expression is restricted to secretory and intercalary cells. This, together with apical LhS28 positivity of the ciliated cells, helps to identify TM. This pattern reproduces the normal Fallopian tube epithelium.

- Secretory cells terminally differentiate into ciliated, senescent cells which often express p16.

- *Complex TM in isolated glands* occurs focally and has an analogous immunophenotype to STM. It is frequently found in endometrial polyps and is probably benign.

- *Complex hyperplasia tubal-type, (CHT)* has a similar architecture and immunophenotype as the corresponding hyperplastic lesions of endometrioid type, showing loss of bcl-2, PAX2, MMR expression as well as a genetic K-ras mutation.

- Even in the absence of atypia, CHT should be seen as a lesion with a high risk of developing or being associated with endometrial cancer. Therefore, its management should be similar to that for atypical endometrioid-type complex hyperplasia.

- Preserved bcl-2 and PAX2 positive areas of CHT and ciliated endometrial adenocarcinoma have the same mosaic pattern as simple TM and the normal Fallopian tube, proving a continuity in lineage. Loss of MMR proteins, markers of MSI and K-ras mutation are characteristic pathways of endometrioid type carcinogenesis. These considerations would suggest that CHT is a precursor more probably of ciliated or endometrioid adenocarcinoma rather than of serous adenocarcinoma.

- Similar conclusions are reached for the equivalent lesion of *Endocervical type mucinous metaplasia.*

- *Intestinal-type endometrial mucinous metaplasia* is rare, but its presence should prompt further investigation of associated lesions in the endocervix. It presents a

characteristic morphological and immunohistocehmical intestinal phenotype, with expression of villin, CK20, CDX2 and chromogranin.

- The heterogeneity of endometrial hyperplasia (tubal-type, mucinous) is one of the factors that contribute to the poor diagnostic reproducibility, as the criteria used to classify endometrial hyperplasia only refer to endometrioid differentiation.

- PAX2 can be used not only for the diagnosis of TM, but as a possible indicator of neoplasia.

7.2. Cervix

- Cervical TM is the most frequent lesion that mimics endocervical in situ adenocarcinoma (AIS) but should not be considered a precursor.

- TM consistently expresses $p16^{INK4A}$ focally or as a mosaic and only rarely diffusely.

- $p16^{INK4A}$ overexpression is not necessarily a marker for HPV-induced endocervical lesions. $p16^{INK4A}$ positive desquamated TM cells, mesonephric hyperplasia, SPSC and even normal endometrial cells constitute a possible source of error in interpretation of cervical smears.

- The mosaic pattern of bcl-2 and PAX2 expression, the low proliferation index and positivity for vimentin are useful to distinguish TM from endocervical carcinoma precursor (AIS). ER, PR and CEA expression do not necessarily discriminate TM from AIS.

- The presence of CD10 positive stromal cuffs around TM and normal mucinous glands does not indicate a diagnosis of endometriosis.

- Apical CD10 positivity is a limited feature in the diagnosis of mesonephric lesions. Normal endocervical glands, tubal metaplastic glands and occasional foci of invasive endocervical adenocarcinoma can also express similar positivity.

Conclusiones

Endometrio

- Las distintas metaplasias y cambios endometriales se solapan y son generalmente focales, involucrando componentes epiteliales y estromales tanto del endometrio eutópico como el ectópico.

- Se propone una nueva clasificación, acompañada de un práctico abordaje morfológico, que tiene como objetivo unificar la terminología proporcionando los indicadores potenciales de malignidad.

- Las metaplasias y cambios endometriales varían desde lesiones reactivas, degenerativas hasta aquellas que ocurren en asociación con tumores malignos (mórulas) y las que tiene un potencial preneoplásico (tubárica, mucinosa).

- La metaplasia tubárica y mucinosa son las metaplasias epiteliales más frecuentes. A menudo se solapan entre si y producen problemas en la interpretación.

- La *Metaplasia Tubárica* (MT) es estrógeno dependiente y se asocia a: hiperplasia endometrial, pólipos, adenocarcinoma endometrioide bien diferenciado, adenofibromas y adenomiomas.

- Se procedió a clasificar la MT mediante su subdivisión en lesiones de tipo simple, compleja eaislada y compleja, basada en el patrón arquitectural, pero siempre en ausencia de atipia, ya que generalmente es esta de tipo leve.

- *La MT Simple* (MTS) ocurre con frecuencia y debe de interpretarse como un hallazgo benigno, ya que no posee potencial de desarrollo neoplasico.

- La positividad citoplasmática y el patrón nuclear en damero por Bcl-2 y PAX2 son característicos de las células secretoras e intercalares. Esto, junto a la positividad apical por LhS28 de las células ciliadas, ayuda a identificar la MT. Este patrón reproduce el epitelio normal de la trompa de Falopio.

- Las células secretoras terminalmente se diferencian hacia ciliadas expresando p16 en estado senescente.

- La *MT compleja en glándulas aisladas* ocurre focalmente y tiene un inmunofenotipo análogo a la MTS. Frecuentemente, se encuentran en los pólipos endometriales y probablemente son de comportamiento benigna.

- La *hiperplasia compleja de tipo tubárico (HCT)* posee una arquitectura e inmunofenotipo similares al de las lesiones hiperplásicas de tipo endometrioide, mostrando una pérdida de la expresión de bcl-2, PAX2 y MMR, así como mutación genética de K-ras.

- Aun en la ausencia de atipia celular, las lesiones de HCT deben de ser interpretadas como una situación de alto riesgo para desarrollar o de estar relacionada con la presencia de un carcinoma endometrial. Por ello, su manejo debe de ser similar al de la hiperplasia compleja con atipia del endometrio.

- En las áreas con persistencia de los marcadores bcl-2 y PAX2 en la HCT y en el adenocarcinoma endometrial de tipo ciliado, se encuentra el mismo patrón en mosaico que en la MT simple y el epitelio normal de la trompa de Falopio, lo cual evidencia su continuidad en la diferenciación genética. La pérdida de las proteínas MMR, MSI y la mutación de K-ras son mecanismos característicos en la carcinogénesis endometrial. Estas observaciones sugieren que es más probable que la HCT sea una lesión precursora del adenocarcinoma endometrioide o ciliado que de un adenocarcinoma seroso.

- Conclusiones similares se han determinado para las lesiones equivalentes en la metaplasia mucinosa endocervical.

- La *metaplasia mucinosa de tipo intestinal del endometrio* es infrecuente, y su presencia obliga a descartar la presencia de lesiones endocervicales asociadas. Se caracteriza por una morfología e inmunofenotipo intestinal con expresión de villina, CK20, CDX2 y cromogranina.

- La heterogeneidad de la hiperplasia endometrial (tubárica y mucinosa) es uno de los factores que contribuyen a la pobre reproducibilidad diagnóstica, debido a que los criterios utilizados en la clasificación de la hiperplasia endometrial sólo se refieren a la diferenciación endometrioide.

- El PAX2 puede ser utilizado no sólo en el diagnóstico de la MT, sino también como un posible indicador de cambio neoplásico.

Cérvix

- La MT cervical es la lesión que simula con mayor frecuencia un adenocarcinoma endocervical in situ (AIS), sin embargo no debe de ser considerada como una condición precursora.

- La MT constantemente expresa $p16^{INK4A}$ focalmente, en patrón de mosaico, o raramente de forma difusa.

- La sobreexpresión de $p16^{INK4A}$ no necesariamente es un marcador de lesión endocervical inducida por VPH. La positividad para $p16^{INK4A}$ en las células descamadas de la MT, hiperplasia mesonéfrica, SPSC e incluso células endometriales normales, constituye una posible fuente de error en la interpretación de los frotis cervicales.

- El patrón en mosaico de expresión del bcl-2 y PAX2, con un bajo índice de proliferación y positividad para vimentina, son una herramienta útil para distinguir entre la MT de un precursor de carcinoma endocervical (AIS). La expresión de ER, PR y CEA no necesariamente sirven para diferenciar la MT del AIS.

- Presencia de manguitos estromales positivos para CD10 alrededor de glándulas mucinosas normales o de la MT no orientan a un diagnóstico de endometriosis.

- La positividad apical por CD10, es una característica limitada en el diagnóstico de lesiones mesonéfricas. Las glándulas endocervicales normales, la MT y focos fortuitos de adenocarcinoma endocervical invasivo pueden tener expresión positiva similar.

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IX. Appendix A: Publications

1. **Nicolae A,** Preda O, Nogales FF. *Endometrial metaplasias and reactive changes: a spectrum of altered differentiation.* J Clin Pathol. 2011 Feb;64(2):97-106. (FI: 2.333).

2. **Nicolae A**, Preda O, Aneiros–Fernandez J, Palacios J, Biscuola B, Nogales FF. *p16INK4A positivity identifies endometrial surface papillary syncitial change as a regressive feature associated with desquamation*. Histopathology 2011 Feb (in press). (FI: 3.855).

3. **Nicolae A**, Goyenaga P, McCluggage WG, Preda O and Nogales FF. *Endometrial intestinal metaplasia: a report of two cases, including one associated with cervical intestinal and pyloric metaplasia*. Int J Gynecol Pathol (in press). (FI: 2.074).

4. **Nicolae A,** Sáez AI, BiscuolaM, Palacios J, Aneiros–Fernandez J, Preda O, Goyenaga P, Nogales FF. *Histological, immunohistochemical and genetic analysis of endometrial tubal metaplasia and its signification.* J Clin Pathol (sent for publication). (FI: 2.333).

5. **Nicolae A**, Goez E, Aneiros-Fernandez J, Stolnicu S, Nogales F.F. *Tubal (tubo-endometrial) metaplasia of the cervix (tem): a possible source of error with endocervical adenocarcinoma* (P3.126). Virchows Arch. 2009; 455 (Suppl 1):S314-315.

6. Nogales F.F, Goez E, **Nicolae A**, Aneiros-Fernandez J, Stolnicu S. Tubal (tubo-endometrial) metaplasia of the endometrium (TEM), a frequent source of misdiagnosis of malignancy, a study of 64 cases (P3.115) . Virchows Arch. 2009; 455 (Suppl 1):S311.

Endometrial metaplasias and reactive changes: a spectrum of altered differentiation

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EDITOR'S CHOICE

Endometrial metaplasias and reactive changes: a spectrum of altered differentiation

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ABSTRACT

Endometrial metaplasias and changes (EMCs) are conditions frequently overlooked and misdiagnosed. The aim of this review is to update current issues and provide a classification with a practical clinicopathological approach. Hormonal or irritative stimuli are the main inducing factors of EMCs, although some metaplasias have a mutational origin. EMCs vary from reactive, degenerative lesions to those able to associate with malignancy or those having a preneoplastic potential. The most common types of EMCs are ciliated tubal metaplasia (CTM) and mucinous metaplasia (MM), which occur in simple and complex glands, and possibly these architectural changes hold the same prognostic significance as they do in hyperplastic endometrioid lesions. Immunohistochemically, CTM is positive for LhS28, bcl-2, PAX2 and $p16^{INK4A}$. Complex CTM is likely to be a precursor of ciliated endometrioid-type carcinomas. MMs should be evaluated architecturally, taking into account that their atypicality is minimal. The differentiation between complex MM and mucinous carcinoma may be extremely difficult. Surface complex, papillary MM in endometrial polyps can be considered as benign. Intestinal-type endometrial MM is rare and its presence should prompt further investigation of associated lesions in the endocervix. Endometrial squamous metaplasia (ESS) is often linked to chronic irritative situations. It should be differentiated from secondary involvement by a human papilomavirus-related cervical lesion. Morular metaplasia is a mutational phenomenon with a distinct phenotype that helps to differentiate it from ESS. Morules are benign, hormonally inert structures that are often markers of complex endometrioid glandular architecture, and they are associated with an attenuated malignancy. Endometrial reactive changes are commonly associated with desquamation or hormonal imbalance. The frequent, $p16^{INK4A}$ positive, benign surface papillary syncytial change may be misdiagnosed, in some cases, as surface serous adenocarcinoma. Eosinophilic, oxyphilic, oncocytic and clear cell changes are non-specific. Rare stromal metaplasias have little clinical significance and should be differentiated from implanted fetal or embryonal tissues.

INTRODUCTION

Endometrial pathology is an important part of the daily routine in histopathology. As a result of its wide morphological variation, the diagnostic interpretation of endometria remains one of the least reproducible fields in gynaecological pathology. In the past,[1] little attention has been paid to endometrial metaplasias and changes (EMCs), and thus they are conditions that are frequently overlooked and misdiagnosed. In this review we analyse the current information on this subject and attempt to provide guidelines for their interpretation and classification that are clinically relevant.

EMCs comprise a morphologically heterogeneous group of proliferations and differentiations found in eutopic and ectopic endometria. Their epithelial or mesenchymal components are replaced either by excessive quantities of homologous cells or partly by heterologous elements. Epithelial EMCs are the most frequent, while mesenchymal EMCs uncommonly occur. The former may occur, mostly focally, in surface and glandular epithelium, but in rare cases they can involve the whole endometrial cavity. While some are usually associated with physiological conditions such as menstruation or pregnancy, most occur in conjunction with pathological situations such as polyps, hyperplasia and adenocarcinoma. EMCs are rarely pure and often various histological types can be seen overlapping in the same specimen.

The tissues derived from the Müllerian ducts and those corresponding to the 'secondary Müllerian system'[2] have a remarkable capacity to undergo multiple differentiations into almost any type of epithelium as well as into various mesenchymal tissues. The endometrium has one of the highest turnovers of cells, only matched by the intestinal lining. From the newborn to late menopause, the endometrium never becomes afunctional, only inactive, and always responds to hormonal stimuli. The continuous endometrial growth, maturation and shedding, performed in short periods of time, involve a myriad of cell cycles and consequently there are multiple opportunities for genetic changes.

Origin

It is likely that endometrial renewal is accomplished by stem cells. Although their identification has not yet been established, various putative candidates have been proposed. These include clonogenic endometrial cells,[3] $CD146^{+}PDGFR\beta^{+}$ [4] or $CD29^{+}CD73^{+}CD90^{+}$ stromal cells[5] and endometrial side population cells.[6] The latter are likely to be responsible for endometrial regeneration and perhaps originate from bone marrow stromal cells.[7] They mainly reside in the vascular endothelial walls and perivascular areas of the basal and functional layers.[8]

Terminology

Metaplasias are adaptive phenomena involving a newly acquired morphology and function. The term change does not necessarily involve true cell transformation, but rather a reactive response of the nucleus or cytoplasm. Neometaplasia, a poorly defined term, usually refers to unusual

differentiations in tumours that reflect their heterogeneity and multipotency. In this review, we will analyse endometrial metaplasias of epithelium and stroma, as well as some frequent reactive epithelial cellular changes.

A proposal of classification for these lesions is presented in table 1.

Associated phenomena and pathogenesis

EMCs are observed in a variety of non-neoplastic and neoplastic conditions in all ages. EMCs and hyperplasia are not mutually exclusive lesions and they often coexist and overlap, since both are related to unopposed oestrogen stimuli.[9] Furthermore, EMCs have also been described in endometria of patients with progesterone-coated intrauterine devices,[10] and even associated with the new selective progesterone-receptor modulators.[11] As a rule, EMCs are frequently seen in endometrial polyps, endometriosis[12 13] and in the benign epithelial component of some tumours such as adenosarcomas.[14] Finally, they can occur in diverse conditions such as chronic inflammation, trauma and vitamin A deficiency.[15 16]

It is clear from the frequent association of EMCs with neoplasia[17–20] that they share some common pathogenetic pathways. However, since EMCs are such a highly heterogeneous group of lesions with different pathogeneses, it would be difficult to attribute them with a generical malignant potential. Although most EMCs are hormonally related and benign, some, such as morules, have a mutational origin and are mostly associated with glandular complexity and atypia.[21–23] The malignant potential of reactive changes and stromal metaplasias is likely to be negligible.

Table 2 summarises the potential risk and association with malignancy of the different types of endometrial metaplasia and changes.

Table 1 Classification of endometrial metaplasias and changes

Endometrial metaplasias and changes	
Epithelial	Ciliary, tubal (simple and complex)
	Mucinous (simple and complex)
	– Intestinal variant
	Squamous
	Morules
	Reactive changes
	– Surface, papillary syncytial change
	– Hobnail variant
	– Oncocytic, oxyphilic, eosinophilic
	– Clear cell, secretory
Stromal	Osseous
	Cartilaginous
	Adipose
	Smooth muscle
	– Myoid, sex-cord like

Table 2 Association of metaplasias and changes with neoplasia and malignant potential

Type of endometrial metaplasia and change	Potential risk and association with malignancy
Morules	Nearly always
Ciliary, tubal complex	Frequent
Mucinous complex (including intestinal)	Frequent
Squamous	Rare
Surface, papillary syncytial change	Rare*
Oncocytic, oxyphilic, eosinophilic	Unknown
Clear cell, secretory	Never
Stromal metaplasia	Never†

*May be present as reactive change in the surface of carcinomas during bleeding episodes.
†May be present in the vicinity of carcinomas.

EPITHELIAL EMCS

Mucinous and tubal metaplasias are frequently admixed and are perhaps the lesions that cause the most problems of interpretation. They may occur in simple and complex glands, and their architectural changes possibly have the same prognostic significance as they do in endometrioid, hyperplastic lesions. Consequently, endometrial lesions with complex architecture are not restricted only to endometrioid-type glands, but may also involve mucinous or tubal glands.

Endometrial ciliated and tubal metaplasia

Ciliation is a characteristic feature of Müllerian epithelia, and its ubiquitous presence in the cervix, isthmus and normal proliferative endometrium could suggest that lesions exhibiting a predominant ciliated component do not represent a true metaplasia but rather a hyperplasia of ciliated cells. Nevertheless, the term 'ciliated metaplasia' is used when the majority of cells of surface epithelium or endometrial glands are prominently replaced by ciliated cells (figure 1). The term 'tubal metaplasia' requires the presence of the three types of cell that constitute the tubal epithelium: ciliated, secretory and intercalary cells (figure 2). However, these differences are merely academic and are not particularly significant.

In the endometrium, ciliated and tubal metaplasia (CTM) is the most common type of metaplasia and also occurs frequently in the cervix, where its location at the squamocolumnar junction, mild atypicality, lack of intracytoplasmic mucin and frequent positivity for $p16^{INK4A}$ can lead to its misdiagnosis as an in situ endocervical adenocarcinoma.[24 25] In the uterine isthmic region, ciliated glands are so common as to be considered normal.

Endometrial CTM is mostly described in conjunction with unopposed oestrogen levels,[1 17 26–29] and its association with simple and complex endometrial hyperplasias[9] and well-differentiated adenocarcinomas is striking.[17–19 29] Residual CTMs can be found in atrophic endometria, where they remain unchanged even after radiotherapy. Endometrial polyps and adenosarcomata also have CTM as a common glandular component.[14] Furthermore, endometriosis is a frequent site of tubal metaplasia[12 13] and when it occurs in the ovary should be differentiated from benign serous neoplasms.

Simple CTM occurs in normal-size or cystic tubular glands and represents the most common type of benign EMC. However, complex CTM occurs in glands that have stellate or

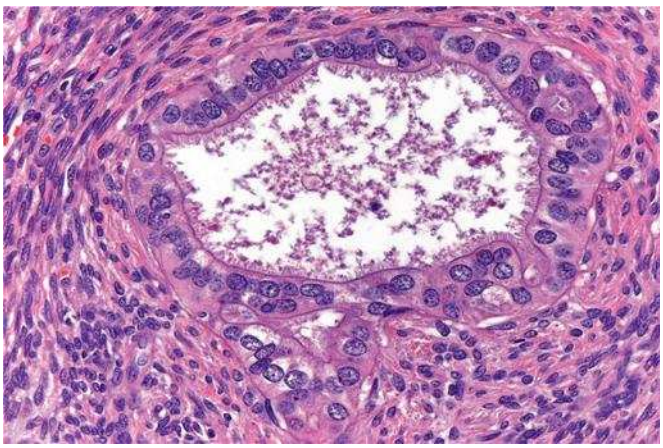

Figure 1 Isolated tubular gland lined by ciliary cells.

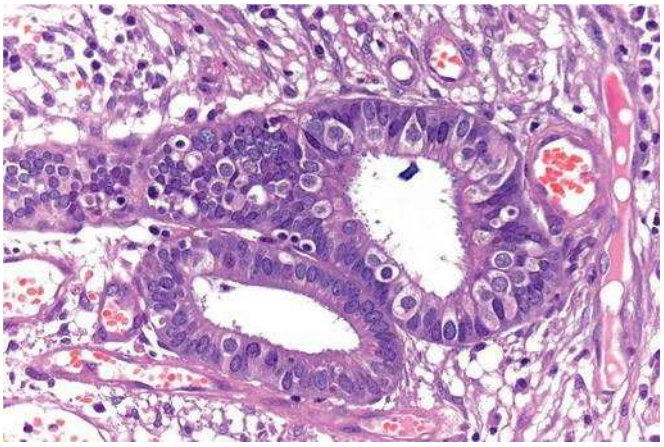

Figure 2 Tubal metaplastic glands showing secretory, ciliary and intercalary cells.

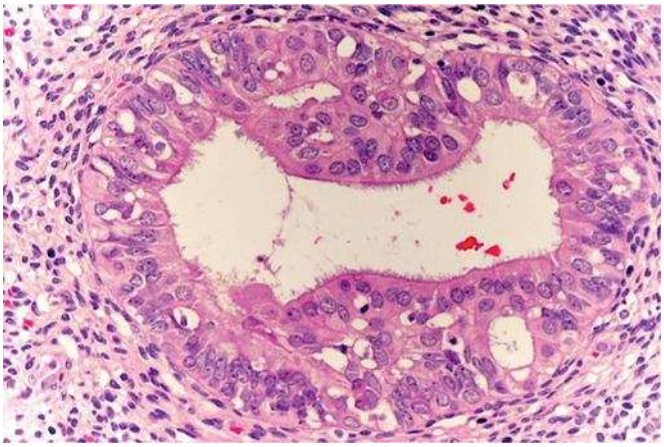

Figure 4 Complex, cribriform glands in tubal metaplasia.

angular contours (figure 3), which are found together with complex changes such as papillae (figure 3) or stratification that may even display a cribriform appearance[1 9 17 18 27 30] (figure 4). We believe that the main discriminating feature between lesions with and without malignant potential is architectural, since atypia is usually minimal, even in ciliated adenocarcinoma. This is evident in the closely related lesion of mucinous metaplasia, where architecturally complex glands, even with no atypia, often associate and merge with endometrioid adenocarcinoma. Consequently, complex CTM of the endometrium should be managed as a complex endometrial hyperplasia.[9 30] However, when focal complex CTM is restricted to endometrial polyps it seems to have little relevance.

Although the CTM immunophenotype has been amply studied in the cervix, there are few immunohistochemical studies of endometrial CTM. LhS28, an antibody that reacts with basal body of cilia, helps to demonstrate ciliary differentiation,[31] while $p16^{INK4A}$ is constantly positive usually in a mosaic or focal fashion[32] (figure 5). CTMs have a low Ki67 index, and p53 shows a weak and heterogeneous pattern.[28] Similar to findings in the fallopian tube epithelium,[33] only the secretory cells of CTM are positive for bcl-2 (figure 6) and also for PAX2.

If complex CTM is likely to be a precursor of an endometrial carcinoma, which type of carcinoma does it precede? The likeliest candidate would be well-differentiated endometrioid carcinoma with extensive ciliary change.[34] There is no contrasted clinicopathological evidence that tubal metaplasias[32] represent precursor lesions of serous carcinoma.

We have been able to demonstrate PTEN and K-ras mutations and microsatellite instability in some cases of complex CTM that had a concurrent weak p53 expression (A Nicolae, personal communication). This profile would support CTM as a precursor of endometrioid but not of serous carcinoma.

Although presence of cilia is associated with a high grade of cellular differentiation, it must be borne in mind that some well-differentiated endometrioid adenocarcinomas are of ciliated type.[27 34 35] This diagnosis should be reserved only for cases with features of invasion,[27] the exception being florid, cystic endosalpingiosis, which is a rare condition in which pseudoinvasive, benign glands with extensive CTM are found deep within the myometrium.[36]

Endometrial mucinous metaplasia

Endometrial mucinous metaplasia (EMM) often occurs in perimenopausal and postmenopausal women, on the surface of atrophic senile endometria and in patients undergoing hormonal treatment, when it may be also accompanied by squamous or tubal metaplasia.[1 37] EMM is frequent in tamoxifen polyps,[38 39] the benign glandular component of adenofibromas and adenosarcomas,[14] and in ovarian and pelvic endometriosis.[12 13]

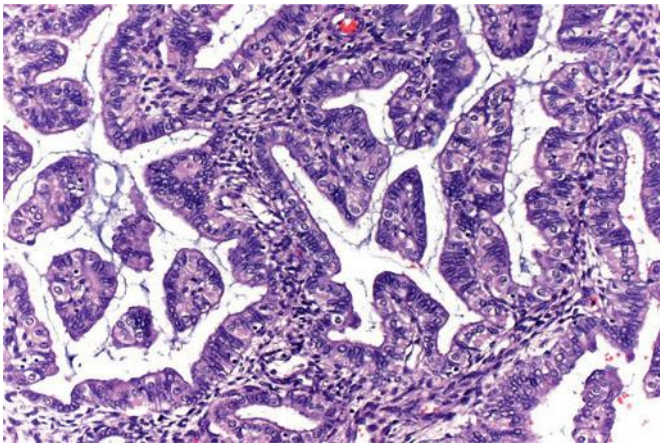

Figure 3 Complex stellate and papillary area of complex tubal metaplasia.

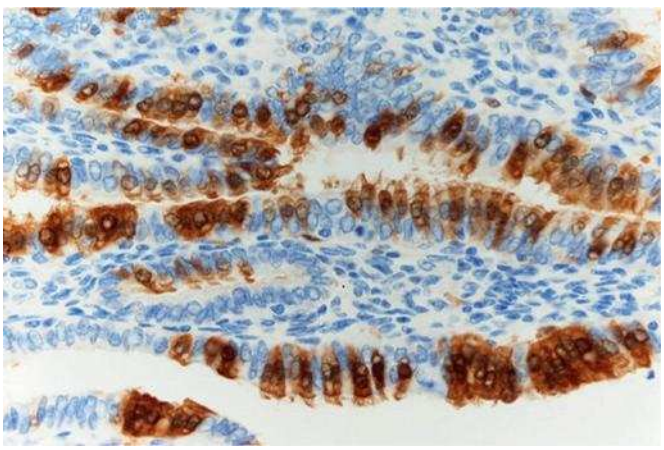

Figure 5 Mosaic p16 positivity in tubal metaplasia.

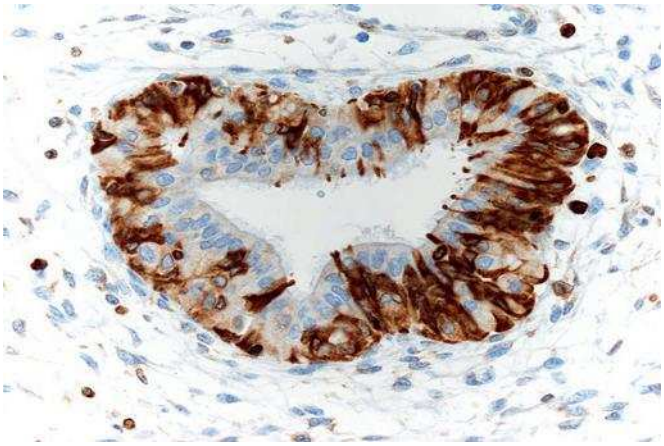

Figure 6 Positivity for bcl-2 in intercalary and secretory cells.

Mucinous metaplasia usually involves the endometrium focally rather than in a diffuse fashion.[1] Rare, extensive EMM can lead to myxometra, an unusual condition defined as accumulation of mucus in the uterus.[40] EMM can also occur as synchronous and multifocal lesions elsewhere in the female genital tract.[41]

In the same way as CTM, EMMs may arise as a response to hormonal stimuli.[1 37] Because of their frequent association with other types of metaplasia (tubal, eosinophilic, papillary change), EMM can be regarded as one of the multiple morphological aspects of the same process.[37 42] EMM can occur as a reactive phenomenon when associated with cervical stenosis or cervical agenesis,[40 43] suggesting a potential irritative role of fluid retention. Nevertheless, mucinous lesions may represent monoclonal alteration of the endometrium,[44] and this would explain their relationship with neoplasia. This is clearly seen in STK-11 gene mutations, especially in patients with Peutz–Jeghers syndrome,[45] where EMM occurs as a spectrum of histological changes ranging from metaplasia to carcinoma, involving synchronously cervix, corpus, fallopian tube and ovary. Multifocality, however, can also occur sporadically.[41 43]

In curettage material, interpretation of EMM can be difficult; indeed, a three-tier morphological classification of progressively architecturally complex lesions has been proposed.[37] This approach, however, has been found to have little reproducibility[42] and, consequently, simplification is preferable.[46] We prefer interpreting EMMs into a two-category scheme using the terms simple and complex EMM.

Simple EMM represents the substitution of the endometrial lining of glands and surface epithelium by tall, bland, columnar mucinous cells of endocervical type (figure 7). It shares common histochemical and immunophenotypical properties with benign endocervical epithelium, such as expression of various mucin core proteins.[29 47]

In complex EMM, the mucinous cells line architecturally crowded, irregular glands. Their columnar lining may present tufting, micropapillary or papillary infoldings (figure 8). More complex proliferations may have a microglandular or cribriform pattern similar to endocervical microglandular hyperplasia, even displaying clusters of neutrophils within intraepithelial microcystic structures. Budding or branching of the glands may also be present. The mucin-secreting cells can be intermixed with tubal-type cells and their nuclear atypia varies from absent to mild. Mitoses are rare. Complex EMMs may occur in association with complex endometrioid lesions (figure 9) and in the vicinity of, and often undistinguishable from, mucinous carcinomas.

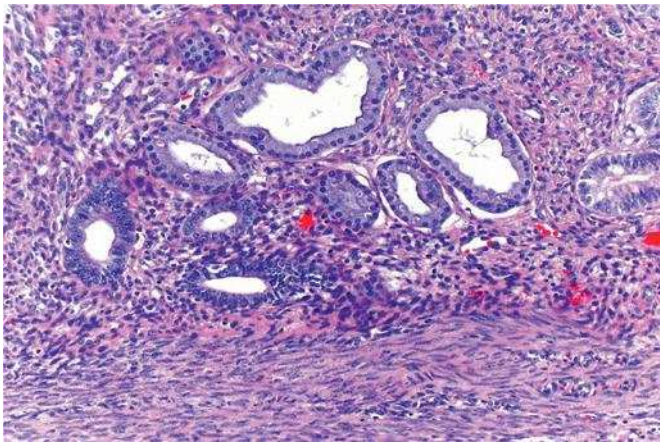

Figure 7 Simple mucinous metaplasia in the basalis of an atrophic endometrium.

Clinicopathologically, we consider simple EMM as benign lesions, while complex EMM should be managed in the same way as complex hyperplastic endometrioid glands, although bearing in mind that in EMM there is little (if any) atypicality. An exception would be cases of endometrial polyps with a surface EMM complex papillary proliferation; these are benign.[48] Frequent misinterpretations of EMMs include microglandular endocervical hyperplasia[49] and the rare microglandular variant of endometrioid carcinoma.[50]

In endometrial biopsy interpretation of complex mucinous lesions, it should be remembered that minimal atypia does not preclude a diagnosis of mucinous adenocarcinoma.

EMM rarely exhibit full intestinal type differentiation, which can occur in the endometrial surface and in polyps.[51] This lesion is characterised by glands lined by columnar cells with a brush border, goblet cells and sometimes a variable number of neuroendocrine cells (figure 10). Similarly, their immunophenotype is characteristically intestinal with expression of villin, CK20, CDX2 and chromogranin. Intestinal metaplasia is commoner in the cervix where it is nearly always associated with in situ or invasive adenocarcinomas. For this reason, intestinal EMM should be managed with caution and any endocervical neoplastic lesions should be excluded.

Endometrial squamous metaplasia

In the Müllerian system, the incidence of squamous metaplasia increases caudally. While squamous metaplasia of the cervix is

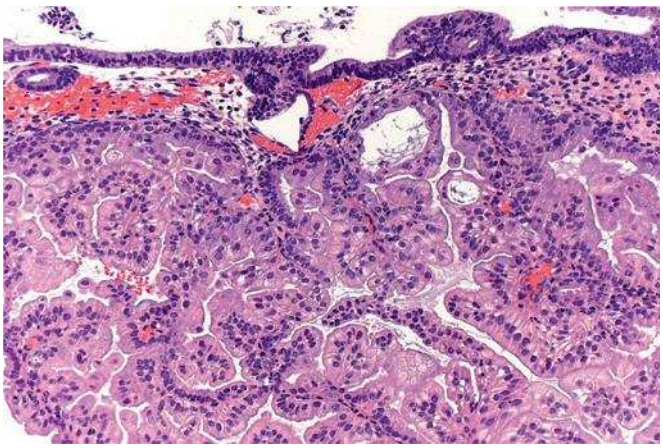

Figure 8 Complex papillary mucinous metaplasia in curettings.

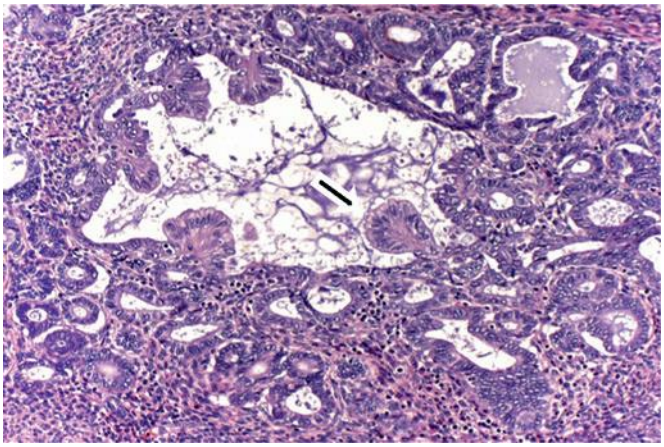

Figure 9 Complex endometrioid hyperplasia with a glandular space lined by micropapillary mucinous metaplasia (arrow).

common, it is rare in the non-neoplastic endometrium and exceptional in the fallopian tube.[52]

Endometrial squamous metaplasia (ESM) replaces the surface or endometrial glands with mature, non-keratinising, well-differentiated squamous cells. The term squamous metaplasia should be restricted to benign lesions, while the term squamous differentiation should be applied preferentially to carcinomas.[53 54] ESM is usually a focal process but can be diffuse, involving extensive areas of the endometrial cavity, when it is known as ichthyosis uteri.[55]

ESM may occur as a response to chronic irritative situations, such as cervical obstruction, chronic endometritis including tuberculosis,[56] pyometra,[57 58] and foreign bodies such as the earlier intrauterine devices.[59] Various other situations including uterine artery embolisation of leiomyoma,[60] vitamin A deficiency[61] and even progestin therapy[62 63] have been reported as possible aetiopathogenetic factors of ESM.

The malignant potential of ESM is low, with only a few reports of isolated cases of pure squamous carcinoma developing from ichthyosis uteri.[64] These should be differentiated from the endometrial involvement by cephalad extension of human papillomavirus-related squamous cervical lesions such as verrucous carcinoma, cervical condylomata and squamous intraepithelial neoplasia.[65 66]

ESM is frequently related to endometrioid carcinoma. It has been proposed[67] that the finding of an extensive (>2.1 mm) solid squamous proliferation in a curettage specimen would imply a diagnosis of carcinoma, although this may be an exaggeration.[68]

The relationship of ESM with morules is interesting. Although ESM originates de novo from neoplastic glands, in a fifth of cases it coexists with morules (see below), where transitions between the distinct two types of metaplasia occur.[1 23 53 58] This does not mean that neither all ESMs are necessarily neoplastic nor that all ESMs necessarily originate from morules.

Morules

Squamoid endometrial nodules and their morphological differences with ESM were reviewed as early as 1930 by Robert Mayer.[69] The current term of morule, which refers to its morphological similarity with a mulberry, was subsequently coined in 1959.[70]

Morules are rounded, well-circumscribed aggregations of uniform, oval or spindle-shaped cells with regular, inactive nuclei and an eosinophilic cytoplasm. They merge with ESM in 20% of cases. Morules originate within the glandular epithelium, protrude and eventually plug the entire lumen, and this leads to epithelial atrophy, forming 'free' stromal nodules. Central necrosis of comedo type is more frequent in the larger morules, probably due to ischaemia. Optically clear nuclei are frequent.[23 54]

Although the occurrence of ESMs in normal endometria or polyps is rare[23] (figure 11), they are nearly always associated with focal complex endometrial glandular lesions in the eutopic and the ectopic endometrial tissue.[23 53 58] Morular metaplasia is also seen in endometrial polyps with complex glands and in atypical polypoid adenomyoma, where they are considered a requisite for diagnosis.[46] Rarely, morules are associated with chronic endometritis, submucosal leiomyoma, irradiation and intrauterine devices,[53] but this is likely to be coincidental rather than a pathogenetical relation.

Does morular metaplasia represent an early step in squamous differentiation, or do they represent two distinct phenomena? Some authors consider them an immature stage of squamous differentiation.[1 57 58] Nevertheless, we believe they are separate entities based on their different biological potential,[23] and their morphological[70] and immunohistochemical features.[23 53 54] Table 3 highlights the main immunophenotypical differences between morules and ESM. From a practical viewpoint, positivity for diffuse and membranous CD10, nuclear CDX2 and β-catenin is constant in morules and usually differentiates them well from ESM. Empty, clear nuclei contain biotin[23 53 54 75 78] and are identical with those present in pregnancy, where they

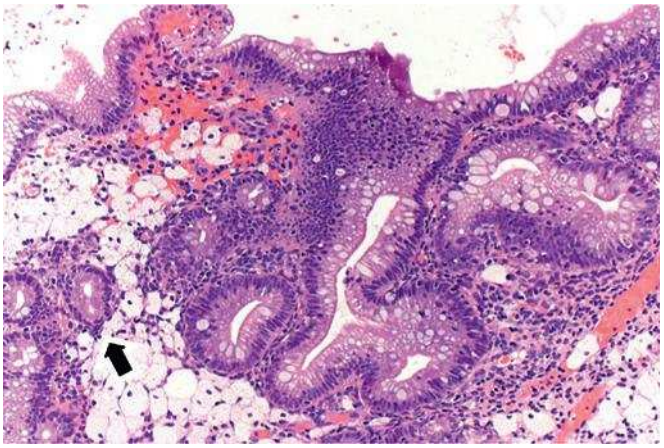

Figure 10 Complete intestinal metaplasia of endometrium. Isolated normal endometrial glands are present (arrow).

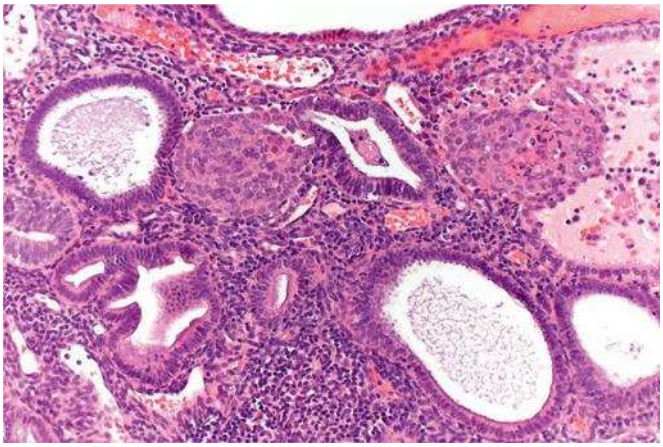

Figure 11 Morular metaplasia in a polyp associated with simple glands.

Table 3 Immunohistochemical differences between morular and squamous metaplasia

Marker	Morular	Squamous
CD10	Positive diffuse[23 58 71]	Negative[23 71]/focal positive[58]
β-Catenin	Positive diffuse, strong cytoplasmic and nuclear positivity[21–23 53 58 71–78]	Positive (membrane)/rare positive nuclei[21 23 53 58 71 74 77]
CDX2	Positive diffuse[58 76 77]	Negative/focal positive basal and parabasal cells[58 77]
ERα, PR	Negative/focal positive[23 53 58 75 79]	Positive focal (most cases)[23 53 58]
p63	Negative/focal peripheral positivity[58]	Positive[58]
Involucrin	Negative[53 54]	Positive[53 54]
EMA	Negative[53 54]	Positive[53 54]
Biotin	Positive[23 53 54 75 78]	Negative[53]
Neuroendocrine markers	Positive focal (some cases)[53 54]	Negative[53 54]
NSE	Positive[53 54]	Negative[53 54]
HPV DNA	Negative[53]	Positive (some cases)[53]

EMA, epithelial membrane antigen; ER, oestrogen receptor; HPV, human papillomavirus; NSE, neuron-specific enolase; PR, progesterone receptor.

are related to steroid hormone hyperstimuli.[78] The reactivity for various cytokeratins (not included in table 3) varies widely between studies.[23 53 54 58]

An interesting immunohistochemical feature of morular metaplasia is a neuroectodermal aberrant phenotype represented by the expression of neuron-specific enolase, synaptophysin, S-100, somatostatin and acethylcholinesterase.[53 54] We have seen cases of massive morular metaplasia in endometrioid lesions with chromogranin and synaptophysin positivity (figure 12) that prompted a differential diagnosis with neuroendocrine carcinoma.

Endometrial morules display identical histology and immunophenotype to those found in tumours of various sites such as gastrointestinal, lung and thyroid. They are highly specific in rare neoplasms such as low-grade adenocarcinoma of fetal lung type and the cribriform morular variant of papillary thyroid carcinoma.[71] It has been proposed that tumours containing morules reflect a common pathway of carcinogenesis involving molecular alterations of the Wnt-signalling pathway with nuclear β-catenin accumulation.[54 71 75 80 81] Also, all morules have in common nuclear expression of CDX2[58 76 77] and β-oestrogen receptor,[75] which appear to be related to their pathogenesis.

Morules are hormonally inert, with absence of α-oestrogen and progesterone receptors[23 53 58 75 79] and a very low proliferative index. Consequently, they neither participate actively in the neoplastic transformation compared with their oestrogen-sensitive glandular counterpart nor are they influenced by progestative therapy.[23 79] Thus, complex glandular lesions with morules will eventually result in adenocarcinoma but not in squamous carcinoma.[79] The proportion of endometria with morular metaplasia that may progress into adenocarcinoma will be dependent strictly on the architecture and cytological atypia of the glandular component. In the rare cases, where there is absence of complex glandular changes, they are likely to have a benign outcome. In contrast, lesions associated with complex glandular proliferation, with or without atypia, can develop adenocarcinomas.[30 79] These are usually well differentiated and have an attenuated malignancy.[22 23 57 71 75 80 81]

Apart from the aforementioned differences with squamous metaplasia, morular changes can mimic granulomata, stromal or smooth muscle lesions, and may be indistinguishable from the spindle cell areas[82] present in some endometrioid carcinomas.

In summary, although morules are a benign metaplastic differentiation, they are often markers of glandular complexity and attenuated malignancy. Their presence in apparently normal glands or in aspiration biopsies should prompt curettage in order to exclude complex glandular lesions. Their immunophenotype is complex and different from squamous metaplasia, which is a lesion into which they may differentiate.[23] Biopathologically, they may reflect a special pathway of cancer development common to various organs, involving the β-catenin gene.

Endometrial reactive changes

Endometrial reactive changes comprise a heterogeneous group of lesions that can be associated, among others, with desquamation, hormonal imbalance, ischaemia and the vicinity of tumours.

Endometrial surface papillary syncytial change (SPSC) has been known under a host of terms such as papillary metaplasia,[1] surface syncytial change,[68] syncytial papillary hyperplasia,[83] papillary syncytial change[84] and eosinophilic syncytial change.[85]

SPSC is frequently associated with endometrial breakdown, occurring in normal conditions such as physiological, cyclic desquamation, and it is also present in abnormal endometrial conditions such as polyps, hyperplasias and even in biopsies of endometrioid adenocarcinoma taken during metrorrhagia.[86]

Microscopically, SPSCs are found as surface syncytial aggregates of eosinophilic cells occasionally extending into the subjacent glands. They often surround basophilic, stromal naked aggregates ('stromal balls') (figure 13). They frequently form micropapillae and even papillae containing fibrous cores. Sometimes, moderate to marked nuclear irregularity and occasional mitoses can be present. Neutrophils are often incorporated into the eosinophilic epithelium. Changes usually associated with endometrial breakdown such as haemorrhage, stromal balls, acute inflammation and fibrin thrombi are almost invariably associated.

The significance of SPSC is yet unknown. Two main pathogenetic possibilities have been considered. Some[87 88] favour the possibility of a cellular change associated with endometrial regeneration, while others[84 85] interpret it as a degenerative phenomenon secondary to the ischaemia that occurs during endometrial shedding. Its strong cytoplasmic and nuclear $p16^{INK4A}$ positivity[89] (figure 14) would support the latter. Others have considered it as a metaplastic[1] or hyperplastic[83] phenomenon.

SPSC may reveal atypical cellular and architectural features that may be misinterpreted as malignant. Its main differential diagnosis is with papillary serous carcinoma, as this highly aggressive tumour is also a surface lesion in its early stages; however, it is much more commonly found in the atrophic

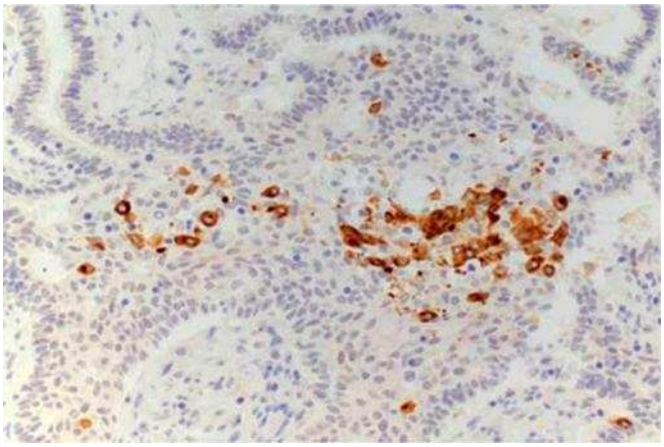

Figure 12 Synaptophysin positivity in morules. Other neuroendocrine markers were also expressed.

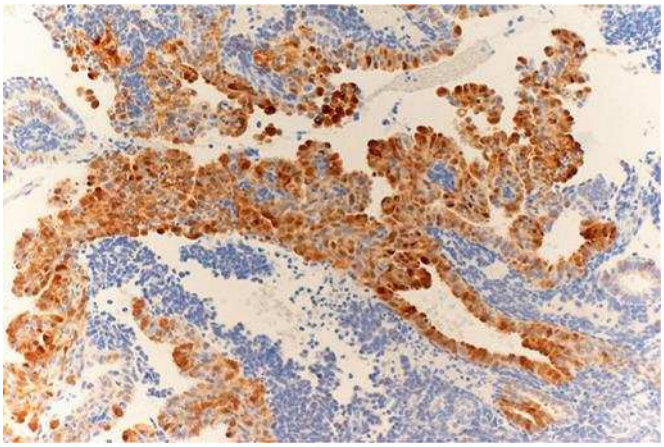

Figure 14 Positivity for p16 highlights surface papillary syncytial change areas.

endometrium of older patients and only rarely in the premenopausal menstrual mucosa. Although SPSC and serous carcinoma can be p53 positive, their staining pattern is different, being heterogeneous and weak in SPSC, and strong and diffuse in serous carcinoma and its precursors. Furthermore, the Ki67 index is high in serous carcinoma and very low or absent in SPSC.[28]

Hobnail change

This change often appears as a reactive phenomenon after curettage or abnormal bleeding, and is a type of SPSC. Histologically, the surface and/or glandular epithelia are replaced by teardrop-shaped cells with an appearance reminiscent of the Arias-Stella phenomenon, clear cell carcinoma or the detached eosinophilic cells of a serous adenocarcinoma. They have scanty eosinophilic or clear cytoplasm and prominent apical nuclei that bulge into the luminal spaces. Hobnail change can also be associated with radiotherapy. In gestational and puerperal endometria hobnail change represents an Arias-Stella phenomenon. Similarly, it can be found in Mirena coil endometria.[10]

Oxyphilic, oncocytic and eosinophilic changes

All these terminologies have the non-specific histological features of eosinophilia as their common denominator. These changes can be considered as reactive alterations rather than true metaplasias. They can occur in a host of normal, non-neoplastic and neoplastic endometria[1 19 57 90] where they may be associated with other types of epithelial metaplasias and changes, especially with SPSC and CTM. Among them, oncocytic cytoplasmic changes are the most unusual (figure 15) and can present with nuclear atypia, which is not necessarily a criterion for malignancy, since they may only represent degenerative phenomena.[68] Oxyphilic or oncocytic changes can involve architecturally complex glands and even adenocarcinomas of the eutopic[30 90–92] and ectopic endometrium.[12 93]

The nature of these eosinophilic cells is poorly understood, being considered by some as a form of immature mucinous metaplasia,[29 94] while for others, they represent a surface degenerative change.[85] This is supported by their presence in the vicinity of endometrial granulomatous lesions.[56 95]

The importance of recognising eosinophilic metaplasia resides in distinguishing it from oxyphilic or oncocytic adenocarcinoma, especially when the latter shows only minimal cytological atypia.[90 92] Hepatoid areas associated with endometrioid carcinomas may also have oncocytic-type changes.[96]

Clear cell or secretory change

The finding of secretory glands with vacuolated or apocrine-like cells in an endometrium with proliferative features points to a hormonal imbalance (spontaneous or iatrogenic) rather than to

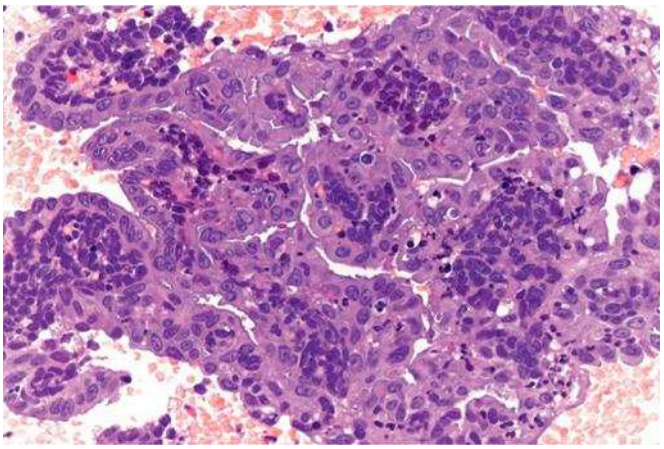

Figure 13 Surface papillary syncytial change enveloping collapsed stromal balls.

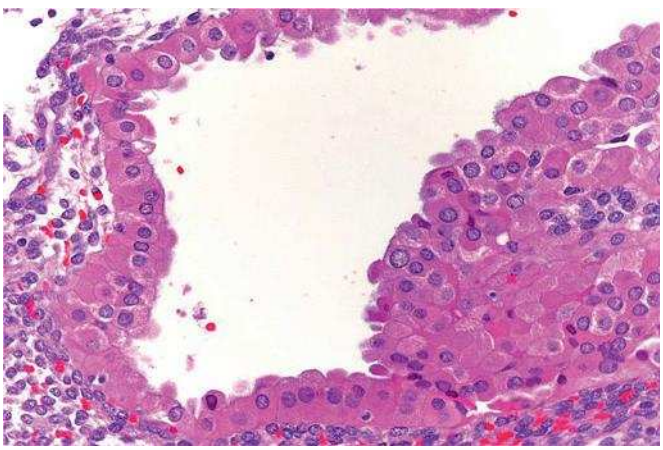

Figure 15 Oncocytic metaplasia.

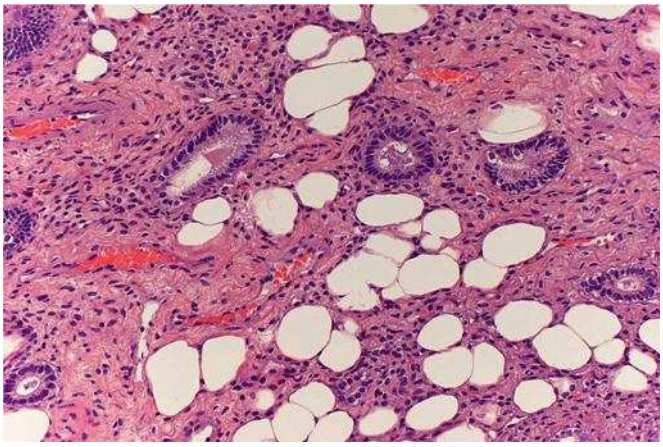

Figure 16 Adipose metaplasia in the stroma of an endometrial polyp.

a true metaplasia. This may also occur in some simple and complex hyperplasias,[97] and even in well-differentiated adenocarcinomas, especially in those treated by progestogens.

Distinction from a neoplastic proliferation such as clear cell carcinoma or a secretory variant of endometrioid carcinoma is based on the absence of architectural complexity, stromal invasion, cellular pleomorphism and mitotic figures.

STROMAL ENDOMETRIAL METAPLASIA

Although most endometrial metaplasias involve the endometrial epithelium, there are rare cases of mesenchymal metaplasias that are usually incidental findings with little clinical significance.

The presence of heterotopic tissues in the normal endometrium is often explained as embedded fetal or embryonal tissue as a result of pregnancy termination. This may be the cause of unusual lesions such as glial nodules in the endometrium.[98] In the absence of an organised histology of mesenchymal tissues and a history of previous obstetrical manipulation, metaplasia is the alternative to be considered.

As mentioned above, endometrial stem/progenitor cells are the most likely candidates for the development of a wide range of differentiations, including mesenchymal-type cells. Mesenchymal or stromal stem-cell-like precursors, isolated from the menstrual blood, show an in vitro multipotent capacity to induce myocyte,[99 100] chondrocyte[100 101] and adipocyte differentiation,[99 100 102] as well as osteogenic tissue.[99 100] Unusual heterotopic cell types can also be differentiated.[99] The multiple expressions of complex mesenchymal, epithelial and even neuroectodermal[103] components are characteristic of malignant mixed Müllerian tumours.

Osseous metaplasia

Bone within the uterine cavity may have various origins. It can be a dystrophic phenomenon secondary to chronic endometritis[104] or cervical surgery,[105] and it may also occur in the vicinity of adenocarcinomas.[106] The metaplastic nature of this condition is proved by genetic analysis[107] and morphologically by its continuity with stromal cells. It should be differentiated from in utero fetal bone retention after pregnancy termination.[108] Osseous metaplasia can be deeply embedded in the uterine mucosa and may present the same contraceptive effect as an intrauterine contraceptive device.[109 110]

Cartilaginous metaplasia

Rare, benign appearing nodules of cartilage can be present in the endometrium.[111] Transition between stromal and cartilaginous cells is helpful to identify them as metaplastic lesions. Although very rare, cartilaginous metaplasia of the uterus has been identified as a heterologous element of a metastatic metaplastic breast carcinoma and thus should be considered in the differential diagnosis of malignant mixed Müllerian tumour.[112]

Adipose metaplasia

Contrary to the myometrium and ovarian cortex, the endometrium rarely develops fatty tissue. Its presence has been described as a reactive phenomenon in the vicinity of endometrial tumours[106] and polyps. Histologically, it appears as clusters or nodules of mature fat cells (figure 16), which blend at their periphery with the endometrial stroma, sometimes surrounded by a mild inflammatory reaction.[113]

Smooth muscle metaplasia

Smooth muscle cells can be identified in the endometrium as isolated short fascicles or even as well-defined nodules. The latter can be considered as an intraendometrial leiomyoma or, possibly, an extension of the surface myometrium. Myoid differentiation possibly takes place from endometrial stromal cells, exemplified by its presence in ovarian endometriosis[114] where it can induce, in an extreme form, the so-called uterus-like mass.[115]

Small sex-cord-like structures with myoid differentiation,[116] also known as plexiform tumourlets, are represented by sex-cord-like trabeculae that coexpress CD56, cytokeratins and myoid markers. They originate from the stroma of eutopic and ectopic endometria including adenomyosis.

Take-home messages

- Endometrial metaplasias and changes (EMCs) are a heterogeneous range of differentiations involving epithelium and stroma. The former are the more frequent, especially ciliated tubal and mucinous metaplasias and surface syncytial papillary change.
- Hormonal stimuli are the main inducing factor, although some metaplasias develop mutational phenomena.
- EMCs vary from reactive degenerative lesions such as eosinophilic clear cell and surface syncytial papillary change to others with a potential to associate with malignancy (morules) or have a preneoplastic potential (mucinous and tubal).
- The latter may occur in simple and complex glands and their architectural changes possibly hold the same prognostic significance as they do in endometrioid, hyperplastic lesions.
- Morules are markers of putative malignancy and their presence in an endometrial aspiration biopsy should prompt curettage in order to exclude complex glandular lesions.
- Recognition of endometrial epithelial metaplasias is necessary in daily diagnostic practice and should be included in the histopathological report especially when they have complex architectural features.

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Letter to the Editor

p16^{INK4A} positivity identifies endometrial surface papillary syncitial change as a regressive feature associated with desquamation

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Sir: Surface papillary syncitial change (SPSC) is a morphological feature that is frequently associated with endometrial breakdown occurring with normal physiological cyclic desquamation or with uterine bleeding of dysfunctional or organic origin.

Although the significance of SPSC is not yet understood, there are two main hypotheses to explain its pathogenesis. Some authors[1,2] propose a cellular change associated with endometrial regeneration, whereas others[3,4] suggest that SPSC is a degenerative phenomenon secondary to the ischaemia that occurs during endometrial shedding. Some cases of SPSC show atypical cellular and architectural features that may be misinterpreted as malignant.

p16^{INK4A} is a cell cycle protein that is irregularly expressed in proliferative endometrium.[5] Some types of endometrial neoplasms, such as transitional[6] and serous carcinomas, frequently express this protein. Furthermore, p16^{INK4A} positivity is used to differentiate endocervical adenocarcinomas from endometrioid endometrial adenocarcinomas.[7]

Recently, we have been able to identify constant overexpression of p16^{INK4A} in SPSC associated with various physiological and pathological conditions of the endometrium. We believe that this finding is not only a marker for the presence of SPSC in endometrial biopsy diagnosis, but also helps in understanding its pathogenesis.

We analysed 32 cases of SPSC in endometrial biopsy specimens of patients experiencing abnormal uterine bleeding, with an age range of 17–80 years. SPSC was associated with menstrual (nine cases), proliferative (seven cases), secretory (three cases) and postgestational (two cases) endometria (total of 21 cases). The remaining cases were found in atrophic endometria (five cases), simple hyperplasia (four cases) and endometrial polyps (two cases). Six patients were undergoing hormone replacement therapy for menopausal symptoms, and four patients were being treated with tamoxifen. Immunohistochemistry for p16^{INK4A} (clone E6H4; CINtec, Heidelberg, Germany), p53 (clone DO-7; Dako, Denmark) and Ki67 (clone MIB-1; Dako) was performed.

Microscopically, SPSC was identified as surface syncitial aggregates of eosinophilic cells occasionally extending into the subjacent glands or lining basophilic stromal naked aggregates ('stromal balls'). (Figure 1A,B). Frequently, they formed papillary tufts or micropapillae lacking fibrovascular stromal cores (Figure 1C). Sometimes, moderate to marked anisokaryosis (Figure 1B,C) and isolated mitoses were detected. Neutrophils were seen, often incorporated into the epithelium (Figure 1A). Changes usually associated with endometrial breakdown, such as haemorrhage, shrunken endometrial stromal aggregates, acute inflammation and fibrin thrombi, were observed.

Immunohistochemistry revealed that all SPSC foci had strong nuclear and cytoplasmic p16^{INK4A} positivity that contrasted with both the negative desquamated glandular epithelia (Figure 1D–F) and the occasional cytoplasmic staining of the proliferative glands. Only 1–2% of SPSC cells were labelled by Ki67, whereas p53 showed a weak and heterogeneous staining pattern.

SPSC is a characteristic feature of endometrial desquamation, being found in the early phases of menstruation as early as 36 h after the onset of cyclical bleeding.[1] It becomes prominent in prolonged, profuse abnormal uterine bleeding of hormonal origin, and can also be present in association with polyps, various types of hyperplasia and even carcinoma.[3,8]

SPSC has been known as 'syncitial papillary hyperplasia',[9] 'papillary metaplasia',[10] 'surface syncitial change',[11] 'papillary syncitial change'[3] and 'eosinophilic syncitial change'.[4] Its significance, besides serving as a marker of endometrial breakdown, is not yet completely understood. Since its initial description as a hyperplastic phenomenon,[9] it has been variously described as a metaplastic,[10] degenerative[3,4] or reparative[1,2] change. The possibility that it is a regenerative or reparative phenomenon is supported by its early appearance in the first days of menstruation, when endometrial epithelial regeneration takes place. It has been described as an epithelium, originating from the glandular stumps and capable of relining and restoring the denuded surface of the endometrial cavity after shedding.[1] This is supported by the presence of SPSC foci in endometria with abnormal breakdown (persistent proliferative endometrium), where the abnormal hormonal environment induces proliferative, regenerative changes with mitoses that can be seen in up to 19% of cases.[3]

It seems unlikely that SPSC represents a metaplastic phenomenon,[10] as it is confined to the superficial epithelium and is associated with stromal breakdown.

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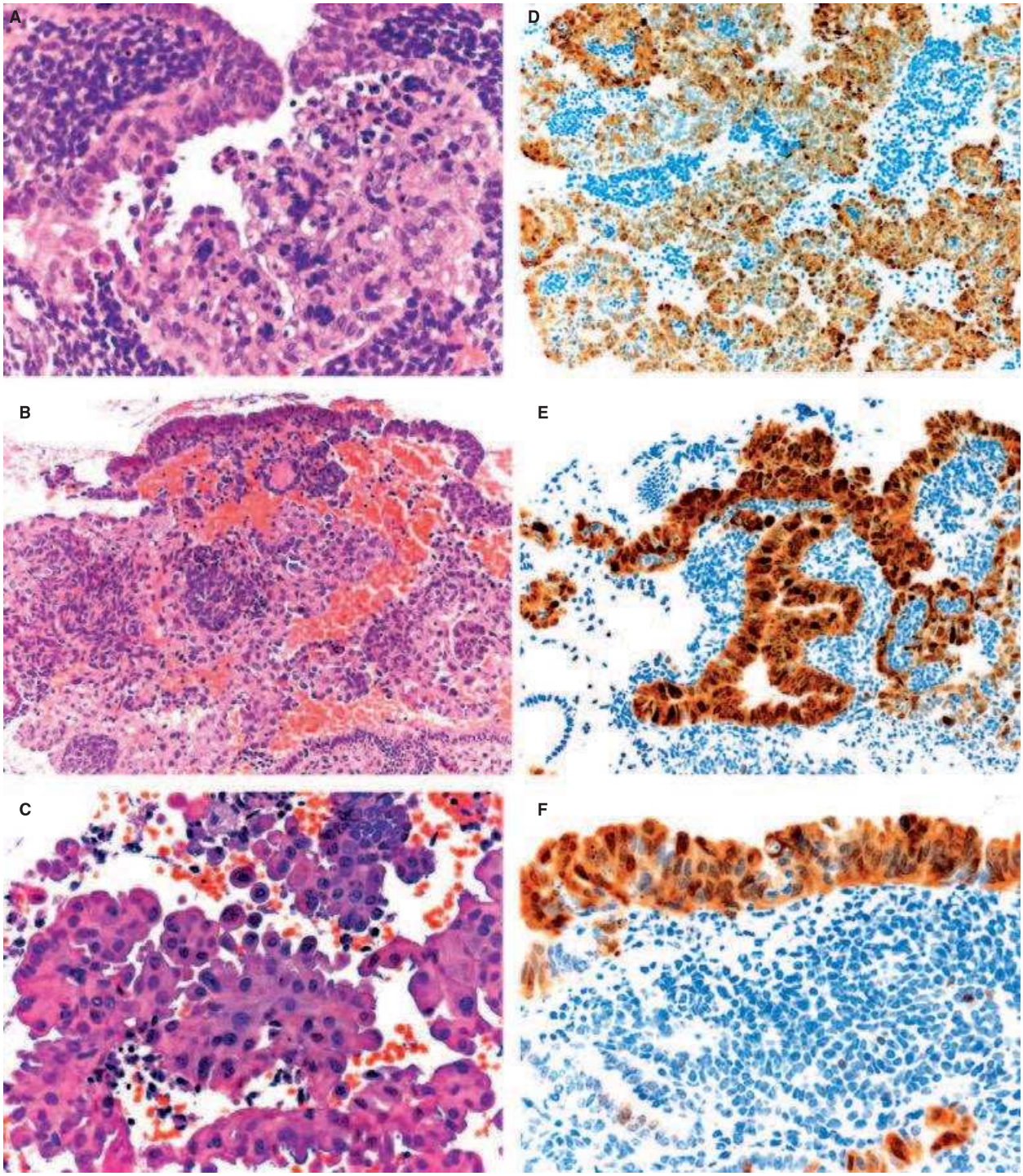

Figure 1. **A,** Syncytial arrangement of eosinophilic epithelial cells of surface papillary syncitial change (SPSC), characteristically associated with neutrophils and karyorrhectic debris. **B,** Sheets of papillary syncytial change covering clumps of dark stromal cells. **C,** Prominent micropapillary projections of eosinophilic cells with a moderate degree of nuclear atypia mimicking serous carcinoma. **D–F,** Strong $p16^{INK4A}$ positivity of SPSC contrasts with its negativity in the stroma (**D**) and glands (**E**, lower left, and **F**). Glandular clefts are substituted by SPSC (**E**).

Furthermore, the possibility that SPSC could represent an incipient form of squamous or morular metaplasia[12] seems remote.

However, some authors have proposed that SPSC represents a regressive or degenerative change.[3,4] The collapse of the stroma during desquamation, with loss of

its vascular supply, would reduce the confluence of the surface epithelium into syncitial–papillary aggregates that can be subsequently modified by ischaemia or necrosis.[8] Recently, a study using proliferation markers[4] demonstrated a very low proliferation rate of SPSC, with a Ki67 index of 1.3% and no concurrent expression of phosphohistone H3, a mitosis-specific antibody, thus supporting a regressive or degenerative pathogenesis for this lesion. SPSC immunohistochemistry for $p16^{INK4A}$ provides additional support for this proposal, as $p16^{INK4A}$ is an indirect marker of cell cycle dysregulation. $p16^{INK4A}$ is a key regulatory protein of cell proliferation, but not a hallmark of human papillomavirus (HPV) infection. It has been demonstrated that the expression of $p16^{INK4A}$ increases remarkably with ageing, owing to oxidative stress in many normal tissues, and that it is also overexpressed in the apoptotic cells of breast ductal epithelium.[5] Consequently, $p16^{INK4A}$ is regarded as regulator of age-dependent senescence,[5] and constrains cellular proliferation and regeneration.[13] Thus, $p16^{INK4A}$ expression in SPSC, coupled with its low Ki67 proliferative index, supports the notion that SPSC represents a regressive phenomenon.

Nevertheless, its early presence during menstruation, together with the absence of other morphological candidates for the role of a regenerating epithelium involved in the lining of the extensively denuded endometrial surface, requires further investigation.

In practical differential diagnosis, however, $p16^{INK4A}$ positivity identifies SPSC but does not differentiate it entirely from serous carcinoma of the endometrium. Although p53 is expressed in both serous carcinoma of the endometrium and SPSC, the staining pattern is different, being strong and diffuse in serous carcinoma and its precursors, but weak and heterogeneous in SPSC. The Ki67 index, however, is high in serous carcinoma and very low in SPSC.[14]

Furthermore, the strong $p16^{INK4A}$ positivity present in desquamated SPSC cells that may be found in cervical cytological samples could be a pitfall in the differential diagnosis of HPV-related lesions. Finally, it must be borne in mind that both mesonephric and tubal metaplasia are also $p16^{INK4A}$-positive, and these conditions could therefore cause additional false-positive results in $p16^{INK4A}$ screening of cytological specimens.

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Case Report

Endometrial Intestinal Metaplasia: A Report of Two Cases, Including One Associated With Cervical Intestinal and Pyloric Metaplasia

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Summary: Intestinal metaplasia of the endometrium is extremely uncommon with only a single earlier case report. We describe 2 cases of endometrial intestinal metaplasia, one of them involving an endometrial polyp, characterized by the presence of intestinal-type epithelium containing goblet and neuroendocrine cells, which were positive with CK20, CDX2, chromogranin, and villin. In 1 case, there was concomitant intestinal and pyloric metaplasia in the endocervix. Together with the observation of the earlier reported case of endometrial intestinal metaplasia, there was also intestinal metaplasia in the cervix. This suggests a possible association between intestinal metaplasia at different sites in the female genital tract. **Key Words:** Endometrium—Intestinal metaplasia—Immunohistochemistry.

Although Müllerian (endocervical)-type mucinous metaplasia is frequent in the endometrium, there is only 1 earlier report of intestinal metaplasia (IM) in this location (1), and little is known of its behavior and optimal management. However, in the cervix, IM is a well-known entity closely related to cervical adenocarcinoma and adenocarcinoma *in situ* (2). Indeed, as associated premalignancy or malignancy is almost invariable, further clinicopathological investigation is mandatory.

In this study, we report 2 cases of endometrial IM and describe its immunophenotype. We also discuss the significance of endometrial IM given that 1 case was associated with concomitant pyloric (lobular)-type metaplasia and IM in the endocervix.

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CASE REPORTS

In case 1, an obese, hypertensive 74-year-old woman presented with abnormal uterine bleeding. Endometrial aspiration biopsy showed, in its scanty fragments, a complex glandular lining composed of tall, basophilic cylindrical cells with well-defined apical brush borders and many interspersed goblet and neuroendocrine-type cells. Numerous foamy histiocytes were present in the stroma. A diagnosis of endometrial IM was made, and a hysterectomy with bilateral salpingo-oophorectomy was recommended due to the possibility of concurrent preneoplastic, neoplastic cervical, or ovarian lesions. The patient had no stigmata of the Peutz-Jeghers syndrome (PJS). Total abdominal hysterectomy and bilateral salpingo-oophorectomy were performed.

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In case 2, a 63-year-old woman presented with postmenopausal bleeding. Hysteroscopy was performed and a 1 cm endometrial polyp was removed.

Pathological Findings

In case 1, the specimen consisted of a uterus, cervix, bilateral ovaries, and fallopian tubes. The gross specimen showed a 10 cm subserosal and 2 further 3.5 and 1.5 cm intramural leiomyomas. The endometrial cavity was irregular with 3 sessile polypoid excrescences, the largest measuring 8 mm, which on cut section were uniform and light yellow (~~Fig. 1~~). The cervix and ovaries were grossly unremarkable.

Histology showed an atrophic endometrium with glandular changes that included tubal metaplasia and mucinous endocervical-type metaplasia. Focal areas of endometrioid glands with complex architecture were evident, in keeping with complex nonatypical hyperplasia. However, the most notable finding was numerous foci of IM with goblet cells (Fig. 2A) and neuroendocrine cells containing eosinophilic cytoplasmic granules involving the polypoid areas, thus confirming the biopsy diagnosis.

Various cell changes were seen in the glandular component of the cervix, including discrete lobules of pyloric-type glands consisting of closely packed round glands lined by tall, clear, mucinous cells (Fig. 2B). There were also intestinal-type glands similar to those present in the endometrium (Fig. 2C). Atypia was minimal and no mitoses or apoptotic bodies were found. In addition, a focus of tubal metaplasia and a small area of transitional metaplasia were also present at the squamocolumnar junction.

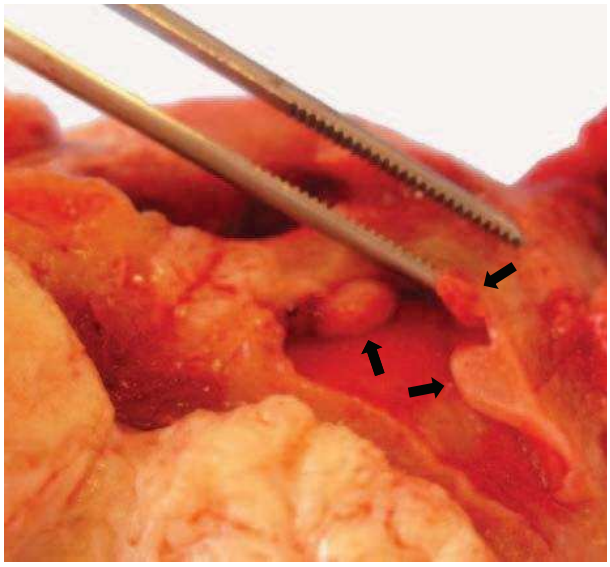

FIG. 1. Case 1. Three polypoid excrescences (arrow) protrude into the endometrial cavity. A sectioned intramural leiomyoma (lower left corner) is also present.

The ovaries both exhibited a moderate degree of cortical stromal hyperplasia with isolated luteinized cells. Hilus cell hyperplasia was present in the left ovary. The presence of benign leiomyomas was confirmed.

In case 2, the specimen consisted of a polyp measuring 1 cm. Histology showed a benign endometrial polyp composed of atrophic, sometimes dilated, endometrial glands in a fibrous stroma containing thick-walled blood vessels. There was no glandular crowding or nuclear atypia. There was IM with glands focally lined by goblet cells (Fig. 3). No nonpolypoid endometrium was represented.

Immunohistochemical and Molecular Findings

Immunohistochemistry was performed according to standard procedures. The endometrial IM in both cases and the cervical IM in case 1 exhibited strong nuclear CDX2 and apical villin positivity (Figs. 2D–E), and many chromogranin-A-positive cells interspersed among the columnar and goblet cells as well (Fig. 2F). There was coexpression of CK7 and CK20. p16 was negative in the IM in both endometrial and cervical locations. The pyloric metaplasia in case 1 was negative for chromogranin A, p16, CDX2, villin, and CK20, but was positive for CK7. p16 positivity was only found in the endometrial and cervical glands showing tubal metaplasia.

Human papillomavirus (HPV) detection, using the GenoFlow HPV Array Test Kit (DiagCor Bioscience, Hong Kong) and polymerase chain reaction on formalin-fixed and paraffin-embedded material, was negative in the cervical section containing IM in case 1.

In both the ovaries in case 1, there were numerous groups of α-inhibin–positive, luteinized cells, scattered in the cortical stroma. The left ovarian hilus cell hyperplasia was also strongly α-inhibin positive.

DISCUSSION

We report 2 cases of endometrial IM, an exceedingly rare phenomenon with only a single earlier case report in the literature (1). Case 1 exhibited a wide spectrum of metaplastic changes, including tubal,

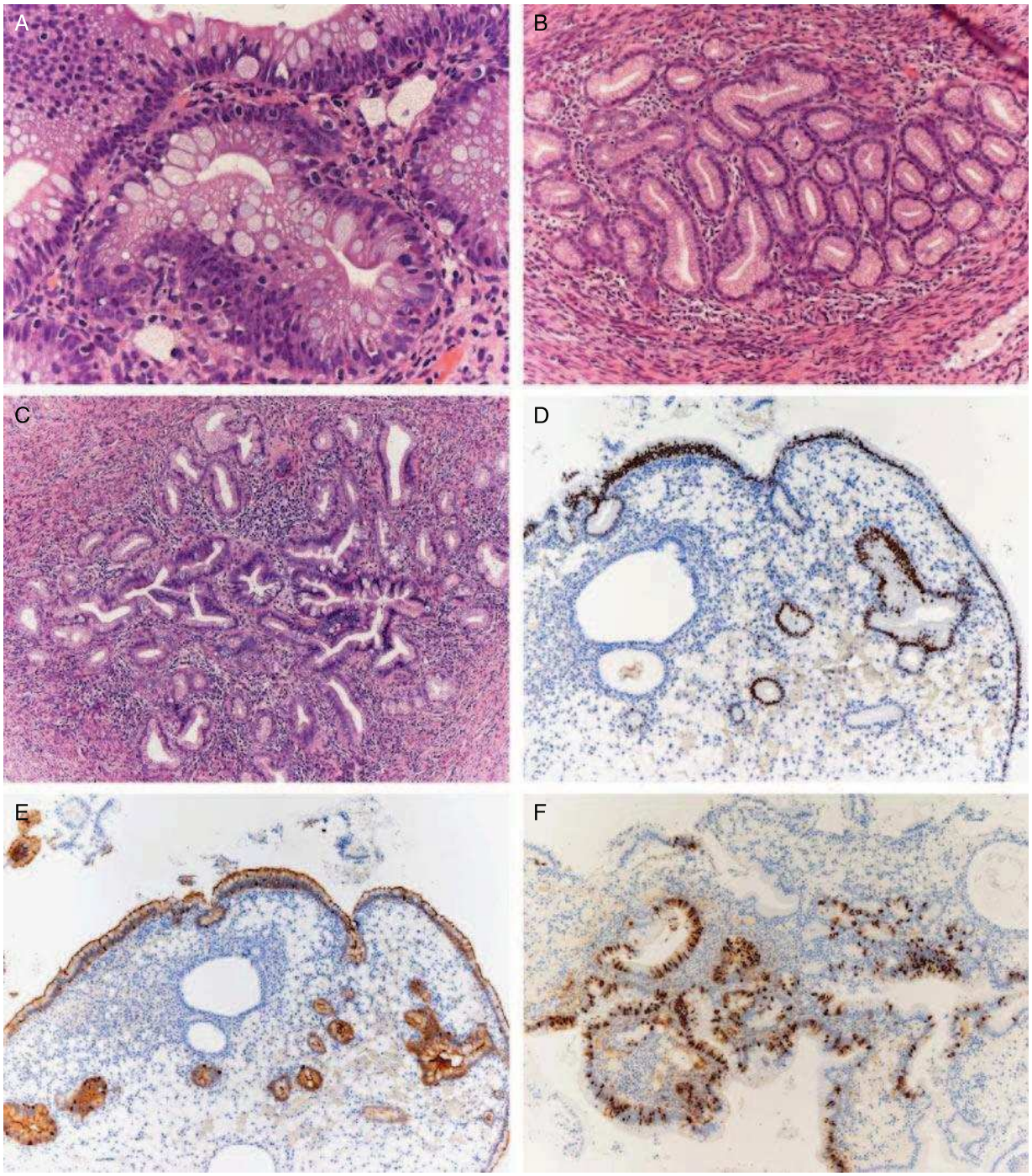

FIG. 2. Case 1. Intestinal-type epithelium present in the endometrium (A). Lobular, pyloric-type glands (B) together with intestinal metaplasia (C) are also present in the cervix. Immunohistochemically, CDX2 (D) and villin (E)-positive epithelia delineate the extent of the intestinal metaplastic lesion. Intestinal metaplasia shows abundant chromogranin-positive cells (F).

müllerian mucinous, pyloric, and intestinal, in both the endometrium and cervix. We believe that these lesions may have occurred secondary to unopposed estrogenic stimulation, which was possibly related to the presence in both ovaries of luteinized cortical stromal hyperplasia. Furthermore, the concomitant leiomyomas and endometrioid complex hyperplastic lesion are also likely to be related to hyperestrogenism (3,4). In case 2, the endometrial IM occurred within a benign endometrial polyp; endometrial

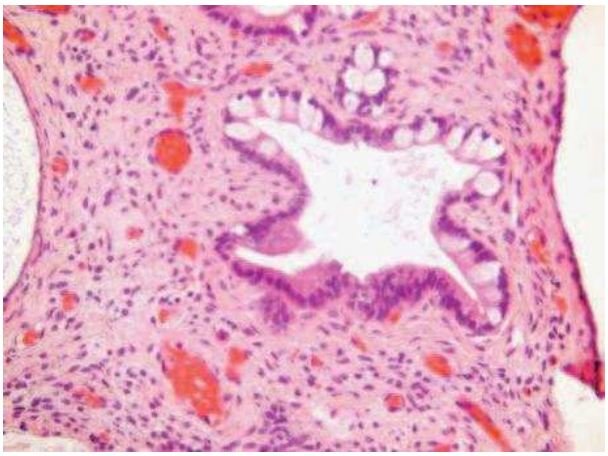

FIG. 3. Case 2. Intestinal metaplasia focally involving glands of a benign endometrial polyp.

polyps are also thought to be related to hyperestrogenism. The only earlier reported case of endometrial IM involved a polyp (1) in a menopausal woman who was receiving long-term tamoxifen therapy, which is frequently associated with estrogen-like activity.

The intestinal character of the lesions is confirmed by the demonstration of an enteric immunophenotype with positivity for CK20, villin, and CDX2. Chromogranin-positive neuroendocrine cells were present in both cases; these are common in intestinal-type epithelium. The hybrid immunohistochemical profile with coexpression of CK7 and CK20 is also characteristic of intestinal-type mucinous lesions in the cervix (5), ovary (6), and vulva (7), and even in the heterologous intestinal elements of Sertoli-Leydig cell tumors (8).

An interesting feature was the negative staining for p16 in the cervical IM in case 1. Most cases of cervical IM (which are associated with adenocarcinoma or adenocarcinoma *in situ*) are p16 positive due to the presence of high-risk HPV. Negative staining with p16 and a failure to identify HPV in the cervical IM in case 1 suggests a non-HPV–related lesion with a different histogenesis to the vast majority of cases of cervical IM. The cervical IM in case 1 did not contain mitotic figures or apoptotic bodies and there was an absence of nuclear atypia, raising the possibility that this may represent an extremely rare form of "benign" IM in the cervix.

Simultaneous mullerian mucinous lesions in the female genital tract involving the ovarian surface, fallopian tube epithelium, endometrium, and cervix may occur in both PJS and secondary to sporadic mutations of STK11 gene, the gene implicated in PJS (9,10). Furthermore, it is well known that PJS can be associated with endocervical minimal deviation adenocarcinoma (11). However, none of the characteristic lesions of PJS were found in our cases and neither of the patients had stigmata of PJS. We believe it is likely that in case 1, the IM in the endometrium and cervix represent synchronous independent lesions as part of a "field-change" effect.

Another mechanism that could explain concurrent involvement of the endocervix and endometrium by IM in case 1 is cephalad extension of the cervical lesion into the endometrium. This rare phenomenon is occasionally found in cases of squamous carcinoma *in situ* of the cervix extending into the endometrial cavity and fallopian tube or ovary (12,13).

On account of the scarcity of reported cases, we do not know the implications of endometrial IM. However, it is perhaps significant that in case 1 in our study and in the earlier reported case of endometrial IM (1), there was associated cervical IM, a lesion that, as discussed, generally does not occur in benign glands and is usually indicative of a premalignant or malignant lesion (14,15). In addition, a rare association of both pyloric and IM in the cervix was found in our case 1. Only 1 similar case has been earlier reported in the endocervix but not with coexistent endometrial IM (16).

Both lobular, pyloric-type metaplasia and adenocarcinoma *in situ* with a gastric phenotype have been considered as possible precursors of cervical minimal deviation adenocarcinoma (9,17,18). In our case 2, the cervix was not biopsied, and thus we do not know whether there was IM at this site.

For these reasons, and taking into account that both in case 1 and in the earlier reported case of endometrial IM (1), there were similar synchronous cervical lesions, we propose that the endocervix should be evaluated and biopsied in cases of endometrial IM to exclude a coexistent cervical glandular lesion.

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Histological, immunohistochemical and genetic analysis of endometrial tubal metaplasia and its signification.

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SUMMARY

AIMS: To clarify the relationship between endometrial tubal metaplasia (TM) and neoplasia.

METHODS: We studied the morphology and immunophenotype (LhS28, p16, cyclin D1, bcl-2, PAX2, p53, Ki67, β-catenin, PTEN and MMR) of 100 cases of TM with increasing degrees of architectural complexity. PTEN and K-ras analysis was also performed in selected cases. TM was classified into simple TM (STM), non-crowded, complex isolated TM, (CTMi), crowded complex hyperplasia tubal-type (CHT), and tubal-type adenocarcinoma (TTA).

RESULTS: We analyzed 72 cases of STM, 11 CTMi and 17 CHT. Half of STMs were found in endometrioid hyperplasias and over half of CTMi in polyps. All CHTs were associated with STM and two of them were related to TTA developing within polyps.

STM and CTMi had a similar immunophenotype with low Ki67 index, p16+ ciliated cells and cyclin D1+ secretory cells. Bcl-2 and PAX2 stains were similar to tubal epithelium. p53, PTEN and MMR proteins were normal.

Analogous to complex endometrioid atypical lesions, CHT presented a diminished expression of bcl-2, p16, cyclin D1, loss of PAX2, MLH1 and PMS2. 1/8 had PTEN deletion and 2/5 K-ras point mutations (codons 12, 12&13).

CONCLUSIONS: Simple TM is a condition which develops under oestrogen stimulus. Complex TM in isolated glands is a lesion characteristic of endometrial polyps, although it may occur in endometrial hyperplasia. Both lesions have similar immunophenotype and CTMi is possibly without clinical significance. Complex tubal-type hyperplasia is architecturally similar to complex endometrioid and mucinous lesions and should be considered to have a high risk of developing, or associating with, an endometrioid or ciliated adenocarcinoma.

THIS PAPER PROVIDES NEW FINDINGS IN THE HISTOLOGY AND IMMUNOPHENOTYPE OF TUBAL ENDOMETRIAL METAPLASIA WITH SPECIAL CONSIDERATION OF THE CLINICOPATHOLOGICAL EVALUATION OF COMPLEX CILIATED LESIONS

Introduction

Despite their frequent occurrence, little attention has been paid to endometrial metaplasias [1], of which tubal metaplasia (TM) is the commonest variety. TM occurs in association with normal and reactive lesions. However, its relationship with neoplasia is not clear and has possibly been underestimated, since TM has only been considered as a mimic which lacked any premalignant potential [2-4]. In some cases, the presence of ciliary differentiation does not exclude the preneoplastic nature of these lesions [5 6]. A recent paper [7], supported the hypothesis of preneoplastic change in TM based not on morphological complexity but only on the overexpression of some regulatory cell proteins in a short number of cases.

We believe, however, that TM with complex glandular growth patterns may be related with neoplasia. In order to determine its significance, we analyzed the morphology and immunophenotype of 100 cases of TM with various degrees of architectural complexity and carried out a genetic study in selected cases with both simple and complex architecture.

Material and methods

1. Case selection

A total of 100 cases of TM diagnosed in the period from 1990-2010 were analyzed. As well as the internal controls, 19 cases, comprising functional endometria (1 menstrual, 2 proliferative, 3 secretory, 2 decidual), endometrial adenocarcinomas (4 endometrioid and 1 serous adenocarcinomas) and 6 normal fallopian tubes were used as controls. All samples and clinical information were collected by the Tumour Repositories Network of Andalusia, following the technical and ethical procedures of the network, including anonymity. Approval was obtained from the San Cecilio University Hospital ethics committee.

We analyzed 96 endometrial biopsies, 17 hysterectomy and 6 tubal resections. The number of H&E sections available for review ranged from 1 to 12, with a mean of 3.

2. Histology

A diagnosis of TM was made when ciliated cells or tubal type cells were the main component in the glands or surface epithelium. We analyzed its location, architecture of the

involved glands, association with other metaplastic changes and coexistence with other lesions. We evaluated its atypicality and analyzed the cases in four distinct architectural groups:

a) *Simple TM* (STM), defined by simple tubular glands, often cystically dilated (Fig 1).

b) *Complex TM in isolated glands* (CTMi) corresponding to occasional non-crowded, branched glands with minimal cribriform areas or with micropapillary buds (Fig 2a, 2b).

c) *Complex tubal-type hyperplasia* (CTH), composed of foci of crowded tubular or irregular glands, with a cribriform or papillary architecture (Fig 2c).

d) *Tubal-type adenocarcinoma* (well differentiated) showing a marked crowding, fused glands without intervening stroma or with myometrial invasion (Fig 2d).

3. Immunohistochemistry

Immunohistochemistry was performed according to standard procedures using the antibodies listed in table 1.

Only 24 cases were analyzed for mismatch repair proteins (MMR): 2 with extensive STM, 7 with CTMi, 12 with CHT. In 2 cases the full spectrum of tubal-type lesions including adenocarcinoma was present. Finally, 1 STM case was seen in the vicinity of an endometrioid adenocarcinoma.

All antibodies were evaluated and scored by two pathologists (AN, FFN). The intensity of reaction was evaluated as following: negative=0, weak=1, moderate=2, strong=3; the percentage of positive cells was scored as: 0 (<1%), 1 (1-10%), 2 (11-50%), 3 (51-80%), 4 (>81%). PTEN and MMR proteins were assessed as: 0 (<10%) PTEN or MMR “Null”, 1 (11-50%), 2 (51-80%), 3 (>80%). Ki67 was evaluated as: 0 (none or only scattered nuclei), 1 (1-10% of nuclei), 2 (11-30%) and 3 (>30%). Interobserver differences were resolved by consensus.

The pattern of antibodies expression was recorded as: mosaic or chessboard (alternate secretory and ciliated cells), heterogeneous (coexistence of positive areas with negative ones in the same gland or between the glands) and diffuse (>80% to 100% the cells within the gland, or all cells of all the glands were positive).

4. Genetic analysis.

Laser capture microdissection and subsequent targeted molecular techniques are limited by their small size, the focality of the lesion and difficulty in distinguishing tubal-type

differentiation from endometrioid. For this reason, we used immunoreaction for MMR proteins to evaluate the microsatelite instability (MSI) and FISH to assess PTEN gene status. K-ras mutations were analyzed by PCR.

Evaluation of PTEN by FISH

Interphase fluorescence in situ hybridization (FISH) analysis was applied to the sequential sections of paraffin-embedded tissue in order to investigate the occurrence of PTEN genomic deletion in 19 of 100 tubal-type endometrial lesions (5 STM, 6 CTMi, 8 CHT). Dual-colour FISH was performed using commercially available DNA probes for cytoband 10q23 (Spectrum Orange PTEN locus-specific probe) and region 10p11-q11 (Spectrum Green centromere of chromosome 10, CEP 10 probe). Pre-treatment of slides, hybridization, post-hybridization processing, and signal detection were performed as reported elsewhere [8]. Signals were scored in at least 100 non overlapping, intact nuclei. Non-neoplastic stromal cells present in the specimen were used as a control. The lesion was considered to show hemizygous deletion of *PTEN* when >20% of its nuclei contained one *PTEN* locus signal and two CEP 10 signals. Homozygous deletion of *PTEN* was defined by the simultaneous lack of the both *PTEN* locus signals and by the presence of control signals in >30% of cells [8].

Evaluation of K-ras mutation by PCR

5 cases of CHT and 1 case of extensive STM were chosen for further mutational analysis. Selection of representative areas was done from haematoxylin-eosin slides. Subsequently, the correspondent paraffin embedded tissue was punched off. After deparaffinisation, DNA was isolated according to the manufacturer's protocol of the QIAamp DNA FFPE Tissue Kit of QIAGEN. The quantity and quality of total DNA was estimated using the Nanodrop ND-100 Spectrophotometer (Thermo Scientific, USA) using only samples with a 260/280 absorbance ratio greater than 1.8. All the DNA samples were treated with EpiTect Bisulfite Kits of QIAGEN for complete conversion of unmethylated cytosines. Pyrosequencing assay was conducted with the QIAGEN KRAS KIT. Mutant and wild-type K-ras DNAs were used as control positive and negative respectively.

5. Statistical study

Fisher's exact test and Spearman correlation rank test were performed to determine the statistical significance and the relationship between the analyzed variables. SPSS19 software package for Windows (SPSS, Inc., Chicago, IL) was used. $p \leq 0.05$ was regarded as significant.

Results

1. Clinical findings

The age range of the patients was from 33 to 84 years, with a mean of 53.1. 73% of them were perimenopausal or postmenopausal and the rest were in their reproductive years. More than half of the women (n=54) had a history of abnormal uterine bleeding. Ultrasonographic findings revealed thickened endometrium >5mm (n=20), polypoid lesions (n=35) or leiomyomas (n=7). Other findings included uterine prolapse (n=1), cervical HPV-related pathology (n=2), previous diagnosis of atypical hyperplasia (n=1), endometrioid adenocarcinoma (n=1) and ovarian tumour (n=1). Two patients were being treated with GnRH analogues and tamoxifen. One patient was pregnant.

2. Histopathological findings

TM involved both the surface and endometrial glands in 58% of cases; was restricted to the glandular epithelium in 40% and to the surface epithelium in 2%.

Endometrial glands lined by a tubal-type epithelium presented a wide architectural variation, ranging from simple to highly complex, as defined in material and methods. The majority, 72 cases, presented only STM. A further 11 corresponded to CTMi and 17 to CHT. The latter also coexisted with areas of STM and consequently, STM was present in 89 cases. 3 cases of CHT were seen in vicinity of adenocarcinoma, 2 of them with extensive tubal-type differentiation. Atypia was minimal in all lesions, ranging from STM to tubal-type adenocarcinoma.

The different types of functional endometria and endometrial lesions associated with TM are shown in Table 2. The majority of STMs were found in lesions such as endometrioid hyperplasias (37/72) or endometrial polyps (19/72). More than half of CTMi were found in endometrial polyps, adenomyoma or adenofibroma (6/11). 9/17 CHT were associated in the

biopsy with scattered glands of proliferative appearance, 5 had developed in polyps of which 2 were seen in continuity with tubal-type adenocarcinoma.

46% of cases had one or more different types of metaplasia. The most common association was with surface papillary syncytial changes (28%), followed by mucinous (19%), clear and eosinophilic (5%), morular (4%), hobnail (1%) and finally squamous metaplasia (1%). In 8 cases there were more than two types of metaplasia.

3. Immunohistochemical findings

Taking into account that a close relation ($p<0.05$, Spearman correlation test) between intensity of the reaction and percentage of the cells was observed, we decided to use the percentage of immunoreactive cells.

Cyclic endometria. Scattered LhS28 positive ciliated cells were observed in proliferative (59%) and atrophic endometria (42%) and, less frequently, in secretory-type glands (20%). Less than 10% of cells showed a cytoplasmic or cytoplasmic and nuclear reaction for p16. This was a common finding in proliferative (56%) and atrophic (71%) glands. However, isolated cytoplasmic positivity was observed in 40% of secretory endometria. Nuclear cyclin D1 expression remained constant throughout the various phases of the menstrual cycle. Bcl-2 showed a strong, diffuse pattern of expression in proliferative, atrophic and also in decidual glands. In secretory and menstrual endometria, strongly positive bcl-2 glands coexisted with weak and negative ones. All cases evaluated for PAX2 had a diffuse expression, except for one proliferative endometrium which showed scattered negative glands. No abnormal expression of p53, MMR, PTEN and β-catenin was evident.

Normal tubal epithelium. All cases were negative for p16. Cyclin D1 nuclear expression was weak or absent in ciliated cells. Secretory cells showed cytoplasmic staining with bcl-2 and β-catenin and a nuclear reactivity for PAX2. This contrasted with the negativity of the ciliated cells imparting a chessboard appearance with alternation of positive and negative cells. Secretory cells had less than 10% Ki67 positivity. Ciliated cells showed weak or absence of ER expression. PR was constantly positive.

Tubal-type lesions. Presence of cilia was confirmed by LhS28 staining and their immunoreactivity for p16, cyclin D1, bcl-2, PAX2, and Ki67 is presented in table 3.

a. **STM.** All cases were positive for p16, the majority showing a heterogeneous or mosaic pattern in more than half of the cells, mostly in ciliated cells. In almost 30% of cases a diffuse pattern was present. A significantly higher expression of p16 was present in STM glands when compared with normal proliferative ($p<0.05$) or atrophic endometria ($p<0.05$). 71% of cases had a mosaic pattern of cyclin D1 expression, with both nuclear and cytoplasmic positivity, mainly in the secretory cells and its positivity was higher than in normal proliferative and atrophic endometria ($p<0.05$). STM showed a significant positive correlation between cyclinD1 and p16 expression ($r=0.245$, $p=0.020$). A characteristic chessboard pattern, similar to the one found in the normal tubal epithelium, was present for both bcl-2 and PAX2. Ki67 index was <10%. Focal, moderate staining for p53 occurred in 10% of cases. PTEN and MMR expression was not altered in the analyzed cases. Only 10% of cases showed weak or absence of ER. PR expression was diffuse.

b. **CTMi**. In these cases, p16, cyclin D1, bcl-2, PAX2, p53, Ki67, β-catenin, PTEN and MMR expressions were similar to those found in STM.

c. **CHT.** LhS28 confirmed the presence of ciliated cells (Fig.3a). Compared with cases of STM and CTMi, CHT showed a decreased p16 expression, with 70% of cases having less than 50% positive cells. Data were statistically significant when CHT was compared with both CTMi ($p=0.016$) and STM ($p=0.001$). Also, in comparison with complex endometrioid lesions, p16 occurred in a higher percentage of CHT cells ($p=0.037$).

   Cyclin D1 expression was decreased in CHT when compared with STM ($p=0.006$) but not with CTMi. Instead, a similar percentage of positive cells was observed when CHT was compared with its endometrioid counterpart. Only one case presented a mosaic pattern of cyclin D1 expression.

   The mosaic pattern of bcl-2 expression persisted in CHT but there was a decrease of intensity and percentage of positive cells (Fig 3b). Diminishing bcl-2 expression was significant when CHT was compared to STM ($p<0.05$), but not when contrasted to

endometrioid lesions. In 5 cases, strongly positive glands were seen in continuity with weakly positive or negative glands. CHT presented a complete loss of PAX2 expression in 65% of cases (Fig 3c) and only 6% preserved positivity for PAX2. In the rest of the cases, positive glands were seen together with negative ones. The loss of PAX2 was significant when CHT was compared with STM ($p<0.05$) or with CTMi ($p<0.05$) but there were no differences with complex endometrioid lesions. CHT had a slightly higher proliferation rate than STM and CTMi. There was a negative correlation between proliferation rates and p16 expression ($r=-0.549$, $p=0.022$), however, no significant correlation was observed between p16 and cyclin D1 or cyclin D1 and Ki67. There were no alterations of p53 and PTEN patterns. Only one case had abnormal nuclear expression for β-catenin in small nuclear aggregates, without the morphological aspects of morules, while the remaining cases had the same pattern as STM and CTMi. CHT had a diffuse expression of hormonal receptors as did CTMi, except one case, which showed a similar pattern to normal salpingeal epithelium.

3 (21.42%) of the 14 cases of CHT showed a complete loss of MLH1 (Fig 3d) and PMS2 expression. They corresponded to CHT lesions not associated with adenocarcinoma; one of them was found in an endometrial polyp. The lack of expression was restricted to the complex areas and did not involved STM.

d. **Tubal-type endometrial adenocarcinoma:** The two cases studied had less than 50% p16 positive cells, but more than 50% were positive for cyclin D1. One case presented a constant chessboard pattern for bcl-2 and the other a progressive loss, with higher expression at the invasive areas. One of the adenocarcinomas lost PAX2 expression, while the other had mosaic positive glands and negative areas. Ki67 index was 10%. There was a diffuse positive expression of PTEN and MMR proteins with no aberrant p53 expression. One of the cases had abnormal nuclear expression for β-catenin, but it was restricted to the incipient or well formed morules that were associated with ciliated differentiation. Both cases were diffusely ER and PR positive.

4. Evaluation of PTEN by FISH

PTEN hemizygous deletion was found in 3 of 19 cases studied. One was CHT, one CTMi and the third extensive STM in an endometrial adenofibroma. The CHT case presented concomitant loss of MLH1 and PMS2 and did not show K-ras alteration. None of the PTEN-mutated cases demonstrated lack of PAX2 expression.

5. Evaluation of K-ras status by PCR

Three of six (50%) tubal type endometrial lesions screened for point mutation in exon1 codons 12, 13 and 61 of the K-ras oncogene were detected to have mutations. Two were morphologically diagnosed as CHT in 56 and 58 year old patients. One presented a double mutation in codon 12 and 13 and the second one had a single point mutation in codon 12. The third case corresponded to a 70 year old woman with an endometrial polyp, the glands of which presented extensive STM with areas of focal crowding, but without evident complex architecture. In this case a single point mutation in codon 61 was found. None of these cases had concomitant alteration of PTEN or showed MSI status.

Discussion

For many years, it was believed that endometrial metaplasia occurring in the absence of hyperplasia and carcinoma was not clinically significant [2 9]. However, Inoue proposed in 2001 that metaplasia was a distinctive pathway of endometrial carcinogenesis, being a precursor of non-endometrioid or mixed type adenocarcinomas [10]. Recently, morular and some types of mucinous metaplasia have been considered to play a potential role in the pathogenesis of endometrial adenocarcinoma [11-13].

Only few articles have focused on tubal-type differentiation of endometrial lesions [4-6 14], one of which has proposed that it may have a preneoplastic potential [7]. In this paper, we analyze 100 cases from a wide spectrum of tubal-type endometrial lesions with simple to complex architecture, including well differentiated tubal-type adenocarcinoma.

Our study supports the notion that TM is an oestrogen-related lesion [6 15 16] since we found a frequent association of TM with endometrial hyperplasia, polyps and well differentiated endometrioid adenocarcinomas; in all of these, TM was a constant feature in dilated glands. Nevertheless, TM was present in atrophic endometria, possibly as a residual,

differentiated lesion. Finally, scattered TM glands were rarely seen between normal secretory glands or in decidualized endometria.

46% of TM cases were found together with one or more different metaplasias or changes. The most common association was with surface papillary syncytial changes (SPSC), followed by mucinous metaplasia (MM). Their frequent coexistence [2 6] indicates that more than one differentiation pathway is simultaneously initiated by various stimuli [11 12 17].

In endometrioid lesions, the risk to develop an adenocarcinoma is related to the degree of architectural complexity and atypia [18]. However, there is ample evidence that atypia is a poorly reproducible feature [19-21]. In endometrial mucinous metaplasias, the architectural complexity of glands clearly discriminates between benign and premalignant lesions, while atypia is almost invariably absent [11 12]. In our study we found a similar correlation for tubal metaplasia, a lesion frequently associated to mucinous metaplasia, which possibly has a similar pathogenesis. We have recently proposed a classification of both types of metaplasia, mucinous and tubal, into *simple and complex [1],* in a similar way to endometrioid lesions. In order to verify if morphological architectural heterogeneity reflects a true biological diversity, complex lesions were further subclassified into complex TM in isolated glands and complex hyperplasia tubal type.

A recent study, analyzing only the aberrant expression of various regulatory proteins and not taking into consideration the architectural changes of tubal-type lesions, proposed TM as a potential precursor lesion, possibly of serous adenocarcinoma.[7]. Our results, however, based on the analysis of cell cycle proteins and the main genetic pathways involved in endometrial carcinogenesis, fail to support this hypothesis.

Simple TM had a characteristic chessboard pattern of bcl-2 and PAX2 expression, with a positivity restricted to the secretory and intercalary cells. This pattern is identical to that found in the normal Fallopian tube [22 23]. The main biological roles of these two proteins involve both resistance to apoptosis and repression of terminal differentiation [24 25]. Simple TM had a marked expression of p16, which was particularly strong in ciliated cells. The pathways implicated in p16 overexpression and its significance in TM are still not clear. However, the high expression

of p16 in simple TM should be interpreted in the context of a low proliferation index and of p16 involvement in age-dependent senescence [1 26 27].

The secretory cells of simple TM have a mosaic pattern of bcl-2 and PAX2 as well as a mild proliferative activity. It is possible that they maintain a capacity of proliferation, eventually terminally differentiating into ciliated, senescent cells which have conspicuous p16 expression. The high expression of p16 and p21 [7] could be responsible for the sequestration of cyclin D1, that may accumulate in the cytoplasm of the secretory cells, blocking proliferative activity, as demonstrated by the low Ki67 index.

All our cases failed to show any PTEN negative glands; this may be due to the type of monoclonal antibody used [28]. However, 1 of the 5 cases of simple TM studied by FISH, presented a hemizygous deletion of PTEN. This case corresponded to an adenofibroma with extensive tubal-type changes. Even if PTEN is considered as the most frequent genetic alteration of endometrioid preneoplastic and neoplastic lesions [29 30], it has a limited value, due to its low sensitivity and specificity to predict progression to carcinoma [31]. Consequently, PTEN mutation alone is insufficient to cause endometrial cancer [32]. Furthermore, we found a point mutation in codon 61 of K-ras in a case of extensive simple TM in an endometrial polyp. Various studies have described a higher rate of K-ras mutation in endometrial polyps, especially in those related to tamoxifen treatment, where mutations in codon 12 but not in 61 were found [33]. In the present case this occasional finding is difficult to explain.

Simple TM is frequent and lacks any microscopic features suggestive of a possible neoplastic transformation. In a series of 5 cases [15] a 32 month follow-up proved unremarkable.

Complex TM in isolated glands occurred in more than half the cases in endometrial polyps and was often associated with mucinous metaplasia and SPSC. In these complex glands, there were no significant differences compared with simple TM in the expression of p16, cyclin D1, bcl2, PAX2, Ki67, β-catenin and MMR proteins. Therefore, we believe that this lesion is probably similar to simple TM. Its presence in polyps possibly bears the same benign significance as complex papillary proliferation found in postmenopausal, endometrial polyps [34]. However, one of six cases of complex TM in isolated glands occurred in a proliferative endometrium and presented a hemizygous deletion of PTEN.

Complex hyperplasia tubal type was analyzed in 17 cases and showed an architectural complexity similar to that of endometrioid complex hyperplasia, but lined by a tubal type epithelium with minimal or no atypia. These complex patterns have been named “metaplastic hyperplasia” [2] or altered differentiation of endometrial intraepithelial neoplasia (EIN) [6 17] and have been found in association with adenocarcinoma [2 35 36]. A recent study showed that approximately half of EIN lesions had altered differentiation, the most common of which was tubal secretory change, although less reproducible [6].

The diagnostic challenge of these lesions resides not only in their architectural complexity and bland morphology, but also in the presence of cilia. Cilia should be considered as a terminal cellular differentiation, rather than a feature associated with benign behaviour.

Immunohistochemically, CHT showed a trend towards down-regulation of bcl-2; although this has been previously reported in complex endometrioid lesions [24 37], its role in endometrial carcinogenesis remains uncertain [38 39]. In contrast with STM and CTMi, CHT presented a loss of PAX2 expression in 65% of cases, similar to complex endometrioid lesions and adenocarcinoma, where its loss of expression appears to have a better specificity than PTEN in detecting preneoplastic endometrial lesions. [32]. Generally, the proliferation index of CHT was low, similar to that reported in its endometrioid counterparts. [40]. Consequently, it appears that the antiapoptotic milieu and proliferative advantages induced by bcl-2 and PAX2 confer susceptibility to tumour initiation and that their later silencing would suggest that other factors contribute to tumour progression. Also, down-regulation or loss of these proteins could be partially responsible for the low proliferation index of CHT or complex endometrioid lesions [41].

We found a statistically significant, decreased expression of p16 in CHT when compared with STM and CTMi. The underlying mechanism that leads to p16 downregulation in lesions with progressively complex architecture is not clear. p16 abnormalities at DNA level with loss of its expression in the nucleus are, however, regarded as an early step in the tumorigenesis of a subset of endometrial adenocarcinomas [42].

CHT presented a diminished cyclin D1 expression. This finding contrasted with its increasing expression in preneoplastic and neoplastic endometrioid lesions [43 44], thus proposing

that cyclin D1 may be useful to recognize a subset of precancerous lesions, but this raised expression does not occur in complex tubal-type lesions.

Further support for the possible malignant potential of CHT is found in the concurrent loss of MLH1 and PMS2 expression, markers of MSI, which was found in 21.42% of our cases. Indeed, MSI is considered an early event in the pathogenesis of endometrioid adenocarcinoma, being a highly specific indicator of neoplastic disease [5 45 46]. PTEN FISH analysis revealed deletions in one of our cases which also had concomitant MMR proteins loss; this is similar to previous findings showing that PTEN mutation occurs more frequently in tumours with MSI than in stable ones [47]. Furthermore, the risk of progression is highly increased by the combination of morphological complexity and PTEN alteration [48].

Furthermore, we found K-ras mutations in 2 of the 5 cases analyzed; both presented point mutation in codon 12, which is regarded as the codon most frequently involved in endometrioid lesions [49-51]. Additional codon 13 mutation was present in one of them. This simultaneity has been explained as an later mutation occurring during tumour progression [52]. Two cases of complex atypical hyperplasia with tubal cell features have been reported to present concurrent K-ras and β-catenin mutation in one case and PTEN mutation alone in the other [53]. K-ras has been regarded as an early event in endometrial carcinogenesis [49 51] and a marker of progression to malignancy, often correlated with the morphological changes in endometrial hyerplasia and atypia[49 50].

Taking into account the complex architecture of CHT together with the altered expressions of bcl-2, PAX2, MMR proteins and the genetic changes in PTEN and K-ras, we believe that these complex lesions have a similar premalignant potential as its endometrioid counterpart. The malignant phenotype that may eventually develop is more likely to be either a tubal or endometrioid well differentiated carcinoma rather than a serous carcinoma, as we found in three of our CHT cases.

Longitudinal follow-up data are necessary to demonstrate that complex TM has a similar risk of developing carcinoma as their corresponding endometrioid lesions.

TAKE HOME MESSAGES

Simple TM represents a benign condition developed under oestrogen stimulus.

Complex isolated TM is a lesion characteristic of endometrial polyps, although it may occur in endometrial hyperplasia. It has an identical immunophenotype as simple TM and possibly no clinical significance .

Complex hyperplasia tubal-type is architecturally similar to complex endometrioid and mucinous lesions and should be considered to have a high risk of developing, or associating with, an endometrioid or ciliated adenocarcinoma.

ILLUSTRATIONS

Figure 1. Simple tubal metaplasia showing ciliated, intercalary and secretory cells.

Figure 2. Complex tubal metaplasia in isolated (non-crowded) glands of endometrial polyps with cribriform **(A)** and micropapillary areas **(B)**. Complex hyperplasia tubal-type with crowding of irregular glands **(C).** Tubal-type adenocarcinoma. Surface myometrial invasion was present.

Figure 3. Immunohistochemistry of complex hyperplasia tubal-type. Despite glandular crowding, cilia are prominently stained by LhS28 **(A)**. Marked decrease of bcl-2 in complex glands contrasts with positivity in a simple TM **(B)**. Glands are PAX2 negative (inner control an atrophic cystic gland -arrow-) **(C).** MLH1 negative glands set in a background of positive stromal cells, PMS2 had a parallel distribution **(D)**

Fig. 1

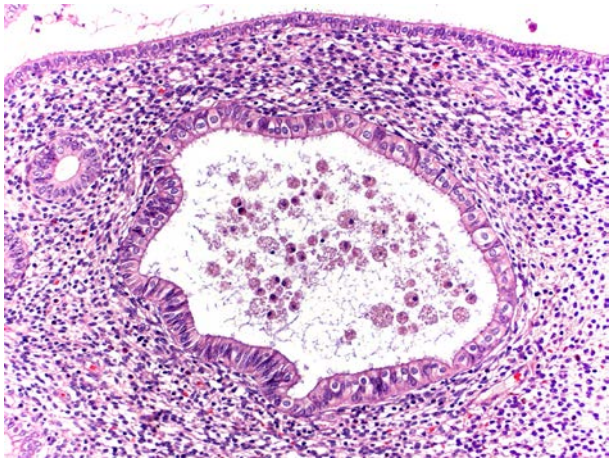

Fig. 2

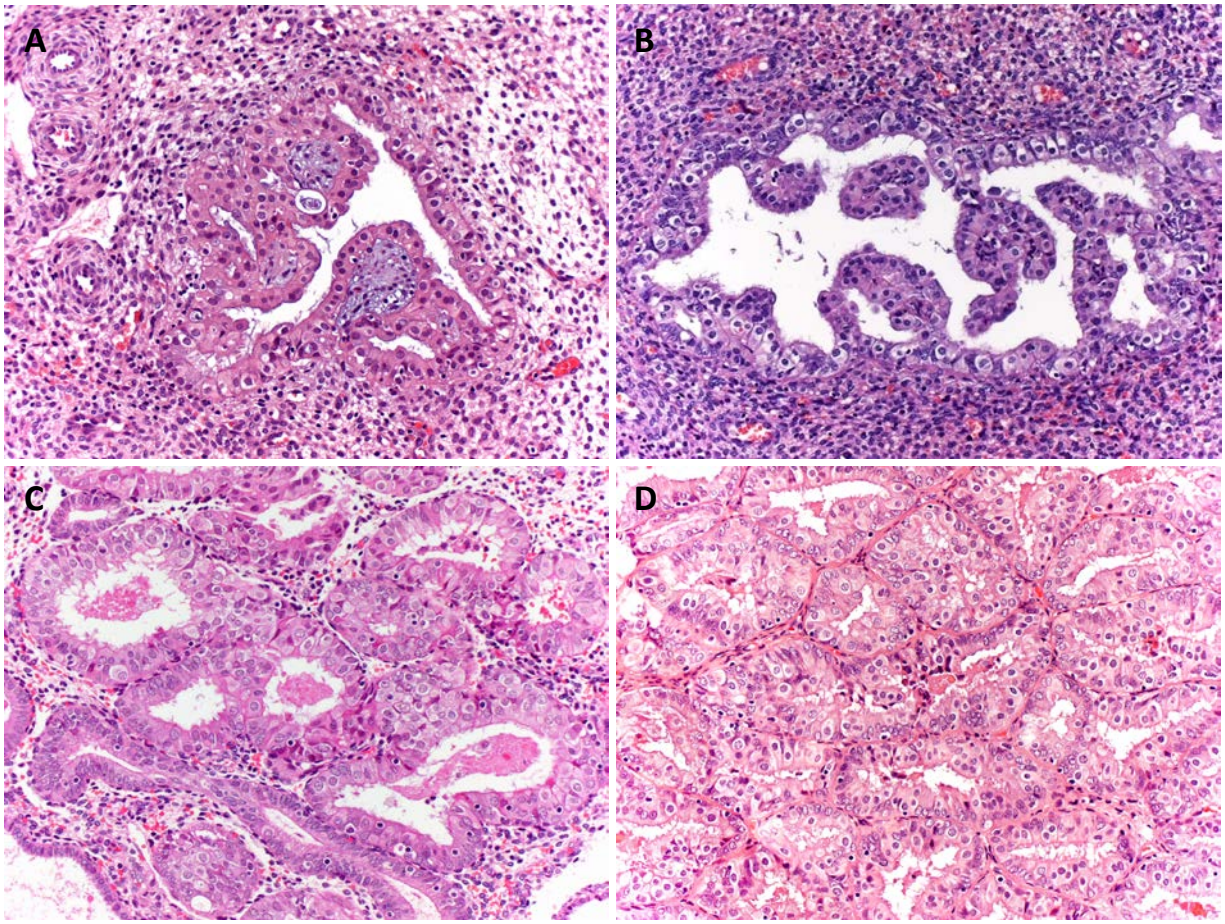

Fig. 3

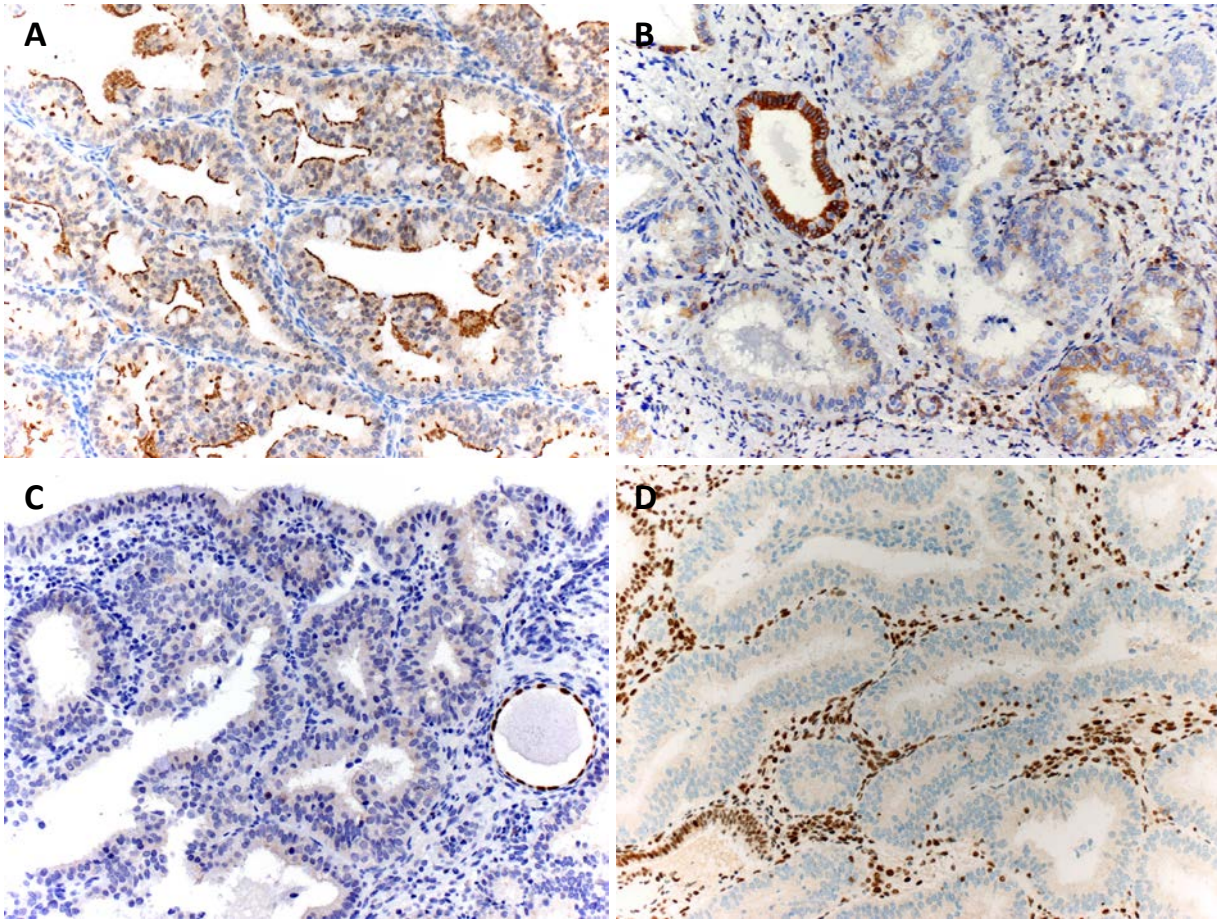

Table 1. Antibodies used and tissue processing details

Primary antibody	Clone	Dilution	Antigen retrieval	Positivity	Vendor*
LhS28	Sc-53224	1:200	Tris/EDTA pH 9.0	apical	Santa Cruz
p16^{INK4A}	E6H4	prediluted	Tris/EDTA pH 9.0	nuclear and/or cytoplasmic	CINtec histology
Bcl-2	124	prediluted	Tris/EDTA pH 9.0	cytoplasmic	DAKO
Ki-67	MIB-1	prediluted	Citrate pH 6.0	nuclear	DAKO
p53	DO-7	prediluted	Tris/EDTA pH 9.0	nuclear	DAKO
Cyclin D1	SP4	prediluted	Tris/EDTA pH 9.0	nuclear	DAKO
ER	SP1	prediluted	Tris/EDTA pH 9.0	nuclear	DAKO
PR	PgR 636	prediluted	Tris/EDTA pH 9.0	nuclear	DAKO
β-catenin	β-catenin	prediluted	Tris/EDTA pH 9.0	membranous, cytoplasmic and/or nuclear	DAKO
PTEN	28H6	prediluted	EDTA pH 9.0	nuclear	Master diagnostica
PAX2	Z-RX2	prediluted	EDTA pH 8.0	nuclear	Zymed
hMLH1	G168-15	1:10	Tris/EDTA pH 9.0	nuclear	BD Biosciences
hPMS2	A16-4	1:10	Tris/EDTA pH 9.0	nuclear	BD Biosciences
hMSH2	FE11	1:10	Tris/EDTA pH 9.0	nuclear	Calbiochem
hMSH6	44/MSH6	1:40	Tris/EDTA pH 9.0	nuclear	BD Biosciences

*****_Santa Cruz Biotechnology, Inc. Santa Cruz, California, USA; CINtec Histology (Mtm Laboratories AG) Heidelberg, Germany; Dako, Dako-Cytomation, Glostrup, Denmark; BD Biosciences (BD Pharmigen), San Diego, USA; Master Diagnostica, Granada, Spain; Lab Vision, Fremont, California, USA; Zymed Laboratories Inc (Invitrogen Corporation) San Francisco, USA; Calbiochem (EMD Biosciences Inc).,San Diego, USA._

Table 2 Cyclic endometria and endometrial lesions associated with TM

	STM (n=72)	CTMi (n=11)	CHT (n=17)	Total 100
Functional endometrium				
▪ Proliferative	4	2	9	15
▪ secretory	2	0	0	2
▪ menstrual	1	0	0	1
▪ atrophy	1	0	1	2
▪ decidua	0	1	0	1
Iatrogenic endometria	0	1	0	1
Hyperplasias				
▪ simple	27	1	1	29
▪ complex without atypia	3	0	0	3
▪ complex with atypia	7	0	0	7
Polyps	19	3	5	27
Adenomyoma/ adenofibroma	3	3	0	6
Endometrioid ADCa	5	0	1	6

STM – simple tubal metaplasia; CTMi – complex tubal metaplasia in isolated glands, CHT –complex hyperplasia tubal – type
ADCa - adenocarcinoma

Table 3. Immunoreactivity of various types of tubal metaplastic lesions

Tubal-type lesions	p16 (%)* 0/1/2/3/4	p	X2	Cyclin D1 (%)* 0/1/2/3/4	p	X2	bcl2 (%)* 0/1/2/3/4	p	X2	Pax2 (%)* 0/1/2/3/4	p	X2	Ki67 (%)# 0/1/2/3	p	X2
STM (n=89)	0/2/24/45/29	0,145	6,810	0/0/18/72/10	0,758	0,709	0/0/6/94/0	0,513	0.209	0/0/0/98/2	N/A	N/A	56/41/3/0	0,443	1,992
CTMi (n=11)	9/0/9/36/46			0/0/18/82/0			0/0/9/91/0			0/0/0/100/0			36/64/0/0		
CTMi (n=11)	9/0/9/36/46	0,016	10,134	0/0/18/82/0	0,204	4,291	0/0/9/91/0	0,202	4,977	0/0/0/100/0	<0,001	15,191	36/64/0/0	0,205	3,950
CHT (n=17)	0/17/53/12/18			0/12/35/41/12			12/12/17/47/12			65/0/6/23/6			12/65/17/6		
CHT (n=17)	0/17/53/12/18	0,001	14,404	0/12/35/41/12	0,006	11,085	12/12/17/47/12	<0,001	26,923	65/0/6/23/6	<0,001	42,891	12/65/17/6	<0,001	16,875
STM (n=89)	0/2/24/45/29			0/0/18/72/10			0/0/6/94/0			0/0/0/98/2			56/41/3/0		
CHT (n=17)	0/17/53/12/18	0.037	7.361	0/12/35/41/12	0.926	0.908	12/12/17/47/12	0.064	7.554	65/0/6/23/6	0.153	4.950	12/65/17/6	0.390	3.564
Endometrioid CH (n=10)	0/70/20/10/0			0/10/50/30/10			0/0/11/22/67‡			56/0/11/0/33‡			0/56/11/33‡		

** 0=<1%, 1=<10% of cells positive; 2=11-50% of cells positive, 3=51-80% of cells positive; 4=>80% of cells positive*

 # 0=<1%, 1=<10% of cells positive; 2=11-30% of cells positive: 3=>30% of cells positive

‡ 9 cases of endometrioid complex hyperplasia (CH) (with and without atypia) were stained for bcl2, PAX2 and Ki-67

N/A – Fischer exact test not applicable

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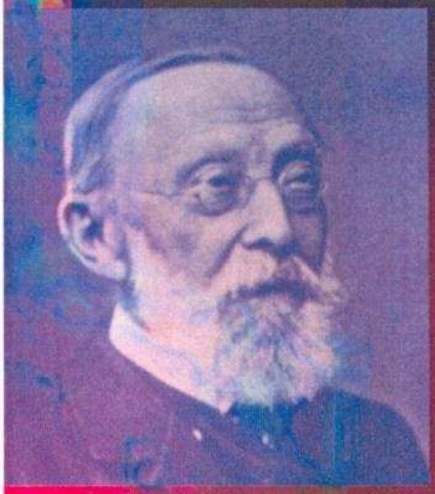

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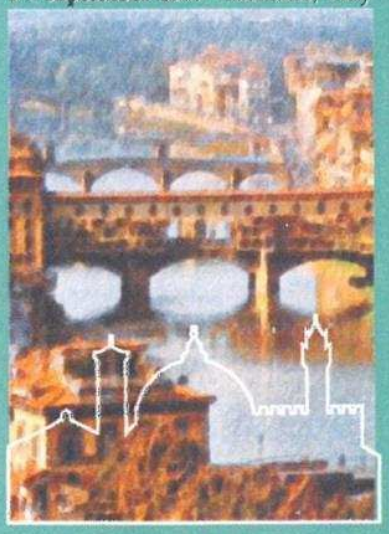

ABSTRACTS

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respectively. In our designed model, age (OR 1.13 [1.03 – 1.27]; p=0.01) and the presence of echoic or multilocular cyst (OR 18.8 [1.3–250]; p = 0.031) in sonographic study greatly influenced the diagnosis of malignant from benign ovarian lesions.

Conclusion(s) Our macroscopic pathological findings revealed that ultrasonography effectively results in earlier diagnosis and decreases unnecessary surgical staging procedures. This study showed that advanced age and the presence of echogenicity and multilocularity in sonographic study are two independent predictors for malignant ovarian lesions.

P3.115

Tubal (tubo-endometrial) metaplasia of the endometrium (TEM). A frequent source of misdiagnosis of malignancy. A study of 64 cases

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Background TEM is a condition of uncertain significance that is not frequently recognized or reported and consists of glands with eosinophilic, ciliated and intercalary cells. Its real incidence and associations are not known. Formerly, many such lesions were considered as "CIS". Its architecture may be complex and can be confused with atypical hyperplasia or well differentiated adenocarcinoma. Recently, p16 has proven to be a reliable marker of TEM that may be useful in identifying TEM in cases with a diagnostic difficulty.

Methods Routine and consultation material comprising 64 endometrial biopsies and hysterectomies. Immunohistochemistry performed for p16-Bcl-2-p53-ER-PR-Ki67.

Results TEM in endometrial biopsy material associated to: 13 cyclic, 12 polyps, 2 adenomyomas and 6 adenocarcinomas and 19 simple, 4 complex without atypia and 8 atypical hyperplasias. Histologically TEM was reduced to isolated simple glands 53, 9 had a complex papillary or cribriform pattern. Atypia present in 2. Coexistent mucinous metaplasia in 3 from these cases. Misdiagnosis of atypical hyperplasia or carcinoma occurred in 6 cases. All of the cases positive for p16.

Conclusion(s) TEM to be recognized as a differential element in the diagnosis of atypical hyperplasia and adenocarcinoma in lesions with eosinophilic cells and a cribriform- papillary architecture, including cases with additional mucinous papillary metaplasia. TEM is frequently present in various types of hyperplasia and in the vicinity of adenocarcinoma and thus, its significance is to be assessed. P16 positivity helps in establishing a differential diagnosis.

P3.116

Umbilical endometriosis in pregnancy without previous surgery: a case report

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Background The presence of extrapelvic endometriosis has been reported in many organs. Up to 40% of patients present with umbilical lesions. In 75% of the women, umbilical endometriosis develops spontaneously, as it did in the present case. Objective: To illustrate the influence of pregnancy on primary umbilical endometriosis.

Methods Case report: We report a case of umbilical endometriosis in a pregnant woman at 20 weeks of gestation. The patient revealed rapid enlargement of a reddish-brown polypoid nodule within the umbilical depression with the typical history of monthly bleeding from the umbilicus. She reported now permanent umbilical bleeding as well as continuous umbilical pain. A total excision of the umbilic and a transabdominal ultrasound examination were performed. The excised tissue measured 1,5 cm in diameter.

Results Histologically, it showed endometrial glands surrounded by endometrial stroma with strong decidual reaction. The diagnosis of endometriosis was confirmed. The pelvic ultrasound examination did not identify ovarian cysts of a possible endometriotic nature. No therapy was given. At 2-month follow-up evaluation, there were no signs of recurrence of the disease.

Conclusion(s) Spontaneous umbilicus endometriosis is a rare disease that can worsen during pregnancy.

P3.117

Use of immunohistochemistry criteria in definition of the prognosis of superficial cancers of ovaries

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Background Taking into account high morbidity of cancer of ovaries, including young women, and as importance of elaboration of morphological criteria of the prognosis of morbidity of cancer.

Methods 112 cases of the superficial cancers of ovaries removed by surgical way (endometrial, serous and mucinosis) have been investigated. Research was carried out with the traditional morphological methods, the full stereometric analysis of a tissue, research of proliferous activity (PCNA, Ki67) and factors of a progression (Bcl2 and p53).

Tubal (Tubo-endometrial) metaplasia in endometrial pathology. A frequent source of misdiagnosis of malignancy

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Background:

Tuboendometrial metaplasia (TEM) is defined as the presence of glands lined by extensive ciliated and intercalary cells in the endometrium, cervix and endometriosis. Its significance is unknown, although it is likely to be a clonal lesion since it is associated with various neoplastic conditions. However, its presence is not usually recognized and due to its complex architectural patterns, it can be confused with atypical lesions of the endometrium including well differentiated carcinoma. Recently, p16 has proven to be a constant marker of TEM and although a nonspecific marker, it can be of use in identifying TEM areas in problem endometria.

Material and Results:

72 endometria from aspiration biopsy and surgical specimens from both routine and consultation material were analyzed. TEM was associated with the following histological diagnoses (see table 1). Topographically, TEM was found focally in cases of various hyperplasias, polyps, adenomyomata and in the uninvolved endometria adjoining adenocarcinomas. It was also present in cyclic endometria (2 secretory, 9 proliferative, menstrual 2) and in 4 atrophic. Histologically TEM patterns were the following: isolated simple glands 54/72, pure papillary pattern 4/72, papillary and cribriform pattern 4/72 and pure cribriform 2/72. Mild atypicality only occurred in three cases. Minor mitotic acivity was sporadic and isolated.

Immunohistochemistry for p16 was performed in all cases. Additionally, Ki67, RE, PgR, p53 was performed in some. All TEMs were positive for p16, most frequently in a strong, diffuse pattern, although focality was noted. p16 highlighted and differentiated TEM from surrounding glands, which in the case of normal endometria, were usually negative or focally positive. Ki67 index was lower than 5% and p53 was consistently negative.

Coexistent mucinous metaplasia occurred in 2 cases.

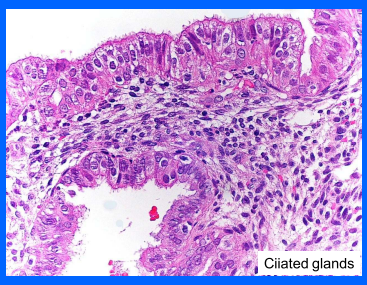
Misdiagnosis of atypical hyperplasia or incipient carcinoma occurred in 11/72 cases.

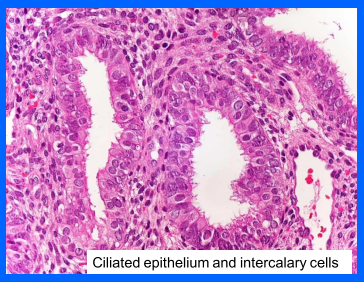
Associations	*# cases*
Simple hyperplasia	21
Cyclic	13
Polyps	12
Atypical hyperplasia	11
carcinomas	6
Atrofic	4
Complex hyperplasia	3
adenomyoma	2
Total	72

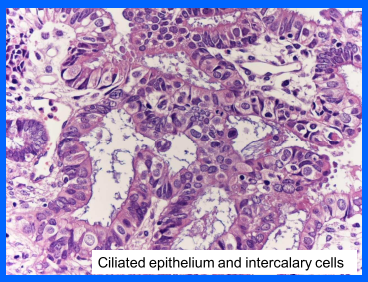
Table 1

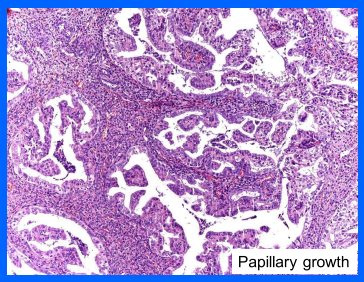
TEM Patterns	# cases
Isolated glands	54/72
Pure paillary	4 /72
Papillary and cribiform	4/72
Pure cribiform	2/72

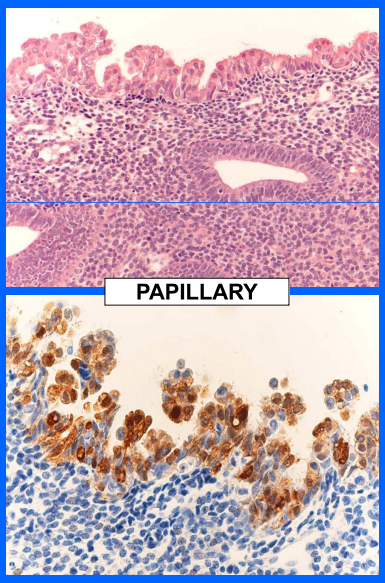
Table 2

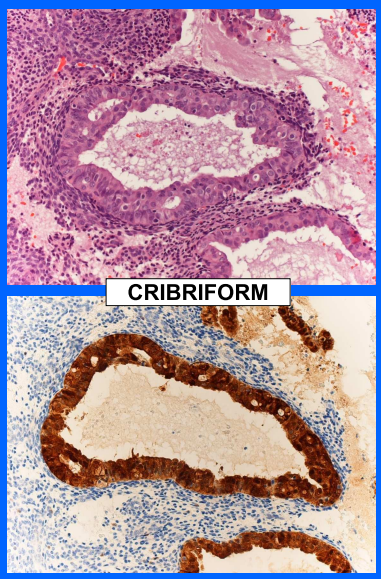


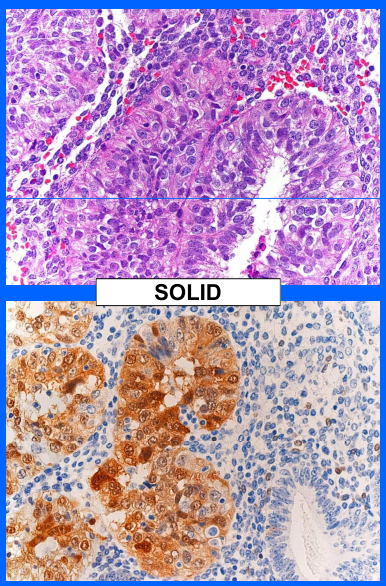












Discussion and conclusion:

TEM is not an unusual situation in endometrial pathology and it is present in eutopic and ectopic endometria. Due to the presence of significant architectural distortion in TEM displaying patterns such as papillary, cribriform and solid that are the hallmark of differentiated carcinomas, misdiagnosis are frequent. TEM represents a frquent pitfall in the diferential diagnosis of atypical hyperplasia and incipient carcinoma. Moreover the association with mucinous papillary metaplasia add further complication to the interpretation of lesions. P16, although a non specific marker is characteristic and constant in TEM and its positivity may help in identifying TEM and differentiating it from the usually negative carcinoma and hyperplasias.

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hyperplasia (SH), in 211 cases the complex hyperplasia (CH), in 53 cases the simple atypical hyperplasia (SAH) and in 165 cases the complex atypical hyperplasia (CAH). We performed the indirect tristadial ABC method of IHC for 3 antibodies: PTEN, Ki-67 and PCNA on formalin fixed embedded tissue taken by biopsies from 80 cases (14 SH, 12 CH, 54 SAH and CAH).
Results PTEN was focal positive for SAH, diffuse for CAH and for some cases of SH and CH. Ki-67 and PCNA were also very frequent in group SAH and CAH.
Conclusion(s) PTEN, Ki-67 and PCNA take part in the process of endometrial carcinogenesis following probably molecular pathways and determine the malignity potential of atypical hyperplasia of endometrium.

P3.124
Sertoliform endometrioid carcinoma of the ovary

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Background Sertoliform endometrioid carcinoma of the ovary (SEC) is an uncommon variant that bears histologic similarity to Sertoli and Sertoli-Leydig cell tumors (SLTs). We discuss the elements of the differential diagnosis and insist upon the value of epithelial membrane antigen in identifying SEC.
Methods A 59-year-old postmenopausal woman presented with a one year history of progressive enlargement of an abdominal mass. Pelvic sonography and abdominal computed tomography showed a pelvic mass measuring 12 X 10 X 10 cm. A staging operation with total abdominal hysterectomy, bilateral salpingo-oophorectomy, infracolic omentectomy and pelvic lymph node dissection was performed. Microscopically, the right ovarian tumor consisted of small hollow tubules, anastomosing cords and trabeculae, and tightly packed nests. Component of typical endometrioid carcinoma was noted only focally. The tumor cells were diffusely immunoreactive for epithelial membrane antigen and negative for inhibin and calretinin.
Results Discussion: Clinically, SEC affects an older population, while patients with Sertoli-Leydig cell tumors have an average age of 25 years and may exhibit endocrine manifestations. The most important histologic features used to distinguish the 2 entities is the presence of areas with the usual pattern of endometrioid carcinoma. The cells in the sertoliform component are immunoreactive for cytokertin, epithelial membrane antigen, while inhibin and calretinin stain negatively.
Conclusion(s) Adequate sampling, a careful search for areas of conventional endometrioid carcinoma, and immunohistochemical studies are helpful in the evaluation of ovarian tumors with sex cord-stromal features.

P3.125
The expression of p16, p53 and Ki-67, in uterine smooth muscle tumors: an immunohistochemical analysis

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Background Recent studies reported the overexpression of P16 protein in uterine leiomyosarcomas. However the potential role of immunohistochemistry of P16, P53 and Ki-67 in differential diagnosis of leiomyosarcomas and leiomyoma variants has not been well assessed.
Methods In this study we have compared the expression of three biomarkers p16, p53 and Ki-67 in 38 cases of uterine smooth muscle tumors, including 15 usual LM, 8 BLM and 15 LMS. All specimens had been routinely fixed in formalin and processed in paraffin wax. Each case was scored on the extent of staining: negative (< 5%), focal (5–25%), mediate (26–75%) and diffuse (> 75%).
Results Negative immunoreactivity for p16 was seen in100% of usual LM, 50% of BLM and 40% of LMS. Among p16 positive neoplasms 50% of BLM and 13,3% of LMS shows focal expression. 46% of LMS shows mediate to diffuse expression of p16. P53 positive expression was observed in 6% of usual LM, 12% of BLM and 53% of LMS. Negative immunoreactivity for p53 was seen in 93,3% of usual LM, 87% of BLM and 46% of LMS. Ki-67 positivity was seen in approximately 47% of LMS, while showed negative expression in 100% of usual LM, 100% of BLM and 53% of LMS.
Conclusion(s) P16 in combination with p53 and Ki-67 may be of value in the assessment of problematic uterine smooth muscle tumors, although further studies with follow-up are necessary to confirm this.

P3.126
Tubal (tubo-endometrial) metaplasia of the cervix (TEM): a possible source of error with endocervical adenocarcinoma

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Background TEM is a p16+ condition of uncertain significance. In the cervix it is not often recognized and may have cellular and architectural features similar to endocervical adenocarcinoma such as multistratification, loss of polarity and papillary formations. Its diagnosis may

be difficult in cervical biopsies involving squamo-columnar junction and surgical specimens with deep parietal involvement.
Methods 2 cervical biopsies, 2 polyps and 9 hysterectomy specimens containing TEM were analysed. All patients were perimenopausal. Immunohistochemistry for p16-CEA-Bcl-2-Ki67-ER-PR-CD10 was performed.
Results Foci of TEM were localised preferentially in squamo-columnar junction. 4 cases had papillary "florid" architectural complexity and were localised deeply into the cervical muscular wall. 1 case was continuous with mesonephric hyperplasia and other occurred in a post-irradiation cervix. Glands were not surrounded by endometrial stromal cuffs. All cases were p16 positive, CEA negative and exhibited a low Ki67 index.
Conclusion(s) Squamo-columnar junction TEM can mimic in situ adenocarcinoma, as it is found in similar location. When TEM occurs in an architecturally complex "florid" fashion with multiple deep intramuscular foci in the cervical wall, it may be confused with minimal deviation adenocarcinoma, mesonephric rests and tunnel clusters. Diagnosis should be strictly done by demonstration of ciliated and intercalary "peg" cells in complex glandular structures. P16 positivity does not mean necessarily that this is a HPV related lesion, as it is a constant marker of endometrial TEM. In such cases, CEA negativity and low ki67 would support a diagnosis of TEM.

Head & neck pathology

P3.127
An unusual case of meningioma with osseous metaplasia (review of metaplastic meningiomas)

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Background Meningiomas comprising scattered or sheets of cells with lipomatous, chondroid, osseous or xanthomatous like matur mesenchimal differentiations called metaplastic meningiomas. While lipomatous change is common, osseous metaplasia is very exceptional. Among meningiomas in the archive of our institute, only 6 (3 lipomatous, 2 xanthomatous, 1 osseous) of 280 cases were metaplastic. This very unusual osseous metaplastic meningioma is presented with clinical, radiological and pathological features.
Methods Case: 60 years old woman with complaint of back and leg ache for nearly 3 years applied neurosurgery clinic. By examining weakness of right knee flexion-pollex dorsiflexion and hipoesthesia under thoracal 7 dermatome the patient had lomber MRI and cranial CT. On MRI 12x14 mm. hipointense, nodular lesion bulging back at thoracal 11 level existed. Cranial CT revealed nodular lesion on the right frontal region which consequent for parasagittal meningioma. The patient didn't permit the excision of the parasagittal lesion, so only the spinal nodule was excised.
Results Microscopic evaluation of the spinal nodule revealed osseous metaplastic focus among the classical meningomatous areas. The lesion was diagnosed as meningioma with osseous metaplasia.
Conclusion(s) This very unusual osseous metaplastic meningioma is presented with clinical, radiological and pathological features.

P3.128
Diagnosis of myotonic and myofascial syndroms of acute and chronic neck pain

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Background The prevailing myotonic syndromes were identified which were the musculus obliquus capitis inferior syndrome (39.4%); superscapular area syndrome (33% of patients); musculus scalenus anterior and musculus scalenus medius syndromes (18.9%); musculus pectoralis minor syndrome (9.7%). Hypodynamia caused system disorders were noted in 78.3% patients including excessive body mass and fat content; reduced blood circulation rate and heartbeat volume and the pronounced decrease of PWC170. The most informative spondylographic findings were reduced thickness of posterior areas of intervertebral disks from CI to CVII (77.9%), cervical lordosis impression (76.4%) and uncovertebral arthroses (58.2%).
Methods The dynamic monitoring of 195 patients. An extended neurological examination was carried out which included roentgenometry of cervical and vertebrocranial areas of spinal column, electromyography of 7 to 9 relevant muscles, finding of the "key" muscle and the overall computer aided assessment of osteomuscular, cardiorespiratory and oxygen transport system disorders.
Results Clinical and electromyographic criteria for diagnosis of myotonic and myofascial syndromes of neck pain were identified based on the occurrence rates. The role of major system disorders in pathogenesis of neurological manifests of neck pain was studied. New therapeutic approaches to stopping pain and myotonic syndromes were developed; the effectiveness of early rehabilitation measures was demonstrated.
Conclusion(s) The most seriously affected ("key") muscles in neck pain patients were found. Diagnosis and treatment strategies for neck pain patients were developed.

Tubal (tubo-endometrial) metaplasia in cervical pathology. A source of error with endocervical adenocarcinoma.

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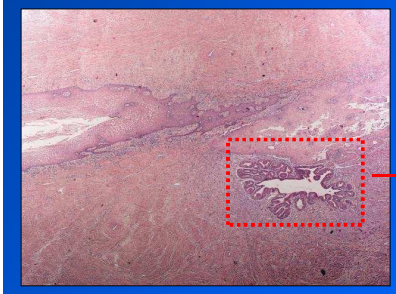
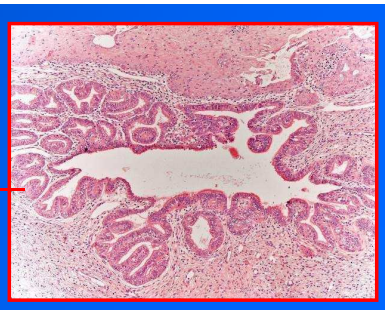
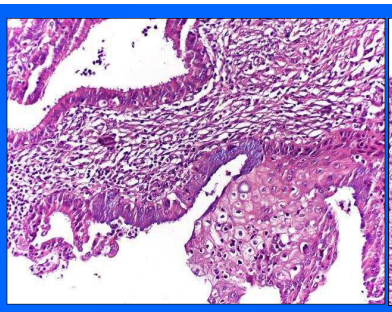
From the Departments of Pathology of Universities of Granada; Spain and Targu Mures(*), Romania

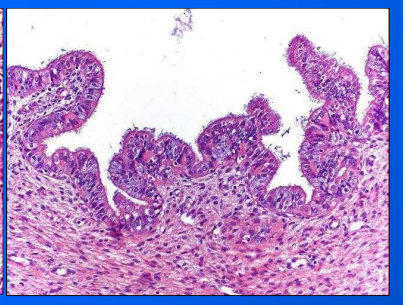
Background:
Tubo-endometrial metaplasia (TEM) is a condition of uncertain significance that can be also found in the cervix. It may have cellular and architectural features resembling adenocarcinoma and other neoplastic conditions, creating diagnostic difficulties, especially in biopsy specimens. Its diagnosis can be complex in small cervical biopsies. p16 is a constant marker for TEM.

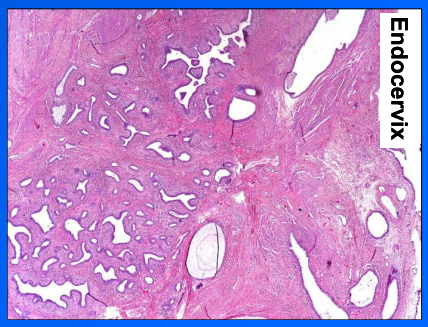
Methods:
3 cervical biopsies specimens and 12 TEM containing hysterectomy specimens were analysed.
Immunohistochemistry for p16, CEA, bcl-2, Ki67, ER, PR was performed.
Results :
TEM was localized preferentially in squamo-columnar junction where it merged with endocervical epithelium where ciliated cells became interspersed with columnar cells. Although most presented as isolated glands, papillary change with architectural complexity occurred in 3 cases. TEM glands also had complex architecture and were closely packed with little intervening stroma. In 2 cases, TEM was localised deeply into the cervical muscular wall and had a florid, complex architecture. Immunohistochemically, cases were p16, RE, RP and bcl-2 positive, had a low Ki67and apical positivity for CEA.

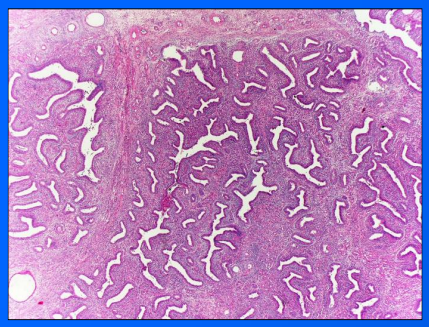




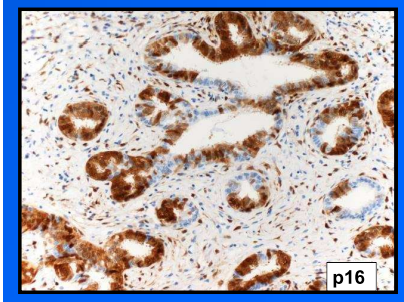


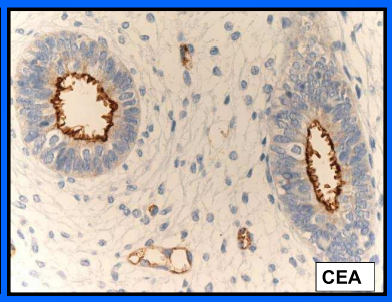


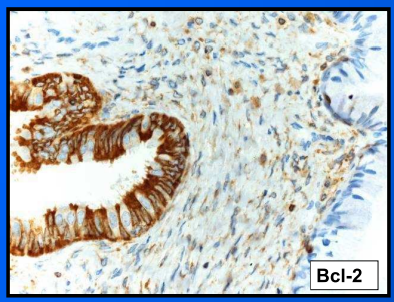


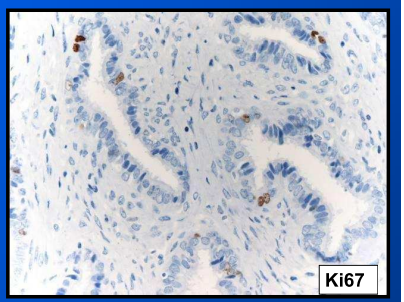


Florid TEM in deep localization within the cervical wall. Differential diagnosis with adenocarcinoma and cystadenofibroma









Discussion and conclusions:
TEM is localized preferentially in the squamo-columnar junction and it can mimic in situ adenocarcinoma since it has similar topography as endocervical adenocarcinoma. Confusion may occur, especially in biopsy material. Diagnosis should be performed by demonstration of ciliated and intercalary “peg” cells. When TEM occurs in a florid fashion with multiple intramuscular foci in the cervical wall it may be confused with minimal deviation adenocarcinoma, cystadenofibroma or adenosarcoma.
In these cases p16 positivity does not mean necessarily that this is a HPV-related lesion, as it is a constant marker of TEM in the endometrium and elsewhere. In this cases CEA and Ki67 should be performed. CEA has only apical positivity in TEM but diffuse in endocervical adenocarcinoma. Ki67 index is low in TEM but high in adenocarcinoma.

Horree N, et al. Cell Oncol. 2007;29:37-45.Cameron RI, et al. Histopathology. 2002;41:313-21. Vang R, et al. Int J Gynecol Pathol. 2005;24:391-398