LABORATORY 20

OVARY & FEMALE REPRODUCTIVE TRACT

OBJECTIVES:

At the end of this lab you should be able to:

1. identify the parts of a normal ovary: hilus, medulla, cortex, germinal epithelium, and tunica albuginea
2. identify ovarian follicles of all stages: primordial follicle, unilaminar primary follicle, multilaminar primary follicle, secondary follicle, mature (Graafian) follicle, and atretic follicle
3. identify the following structures within ovarian follicles of the appropriate stage: membrana granulosa cells, cumulus oophorus, corona radiata, theca interna, theca externa, zona pellucida, antrum, oocyte (and its cytoplasm vs. nucleus vs. nucleolus)
4. identify a corpus luteum and its component parts: the theca lutein and granulosa lutein
5. identify a corpus albicans
6. list the hormones produced by a follicle and a corpus luteum; know what cell type produces each, the target organ and action of each, and how the secretion of each hormone is regulated
7. describe the general histology of the oviduct and compare and contrast its different parts: infundibulum, ampulla, isthmus & interstitial (intramural) segment
8. identify the myometrium, endometrium, stratum basale, stratum functionale, endometrial glands and spiral arteries of the uterus
9. identify the following stages of the uterus based on changes in the endometrial glands and the stroma: proliferative stage, early secretory stage (~ day 17), late secretory stage, & menstrual stage
10. correlate the morphology of the uterus with that of the ovary at different stages of the menstrual cycle
11. describe the general histology of the cervix and vagina. Know which cells are studied in the Papanicolaou method (Pap smear).

LABORATORY:

Please study the following slides in your set:

I. OVARY

   Slide 85 (HU Box): Ovary, or
   Slide 86: Ovary

   In these sections of the ovary, the follicles are in various stages of development. Identify examples of primordial follicles, unilaminar primary follicles and multilaminar primary follicles. Primordial follicles lie just deep to the tunica albuginea and consist of the oocyte surrounded by a single layer of flat follicular cells. When the follicle is
stimulated, the follicle cells become cuboidal to form unilaminar primary follicles. Once they become cuboidal, the follicle cells are known as granulosa cells or membrana granulosa cells. They divide to form multiple layers, at which point the follicle is a multilaminar primary follicle. Next find either a secondary follicle or a mature (Graafian) follicle. Both have a central, fluid-filled cavity called the antrum, but a Graafian follicle is much larger than a secondary follicle. It should fill the entire width of the cortex and even cause a bulge on the surface of the ovary. Identify the following features in a secondary or Graafian follicle:

- oocyte
- zona pellucida
- membrana granulosa
- theca interna
- theca externa
- antrum
- cumulus oophorus
- cells that will become the corona radiata after ovulation

What hormones do the membrana granulosa cells secrete? (Answer: Mainly estrogens, especially estradiol) What hormones do the theca interna cells secrete? (Answer: They produce androstenedione, which serves as a precursor for estrogen production in the membrana granulosa cells.)

Slide 27 (HU Box): Ovary, Corpus Luteum of Ovulation, Human, or Slide 87 and 88: Ovary

Slides 27HU & 87 contain a large corpus luteum that formed from a ruptured follicle following ovulation. The corpus luteum includes a folded mass of granulosa lutein cells derived from the membrana granulosa cells of the follicle, and theca lutein cells derived from the theca interna. The theca lutein cells are small and dark-staining. They form thin strands of cells located between the folds of the granulosa lutein cells. The granulosa lutein cells make up the bulk of the corpus luteum. They are large cells whose cytoplasm is rich in lipid droplets. What hormone does each cell type secrete? (Answer: Theca lutein cells produce androgens such as androstenedione. Granulosa lutein cells convert the androgen to estradiol, and also produce progesterone from cholesterol supplied mainly by LDLs. The LDLs are carried to the granulosa lutein in blood vessels that grow into that layer following ovulation. Recall that the membrana granulosa of the follicle was avascular, and hence the cells had limited access to LDLs at that stage.) Find some of the small blood vessels that are present within the granulosa lutein.

In most of these slides the lumen of the corpus luteum still contains clotted blood, so the structure should more properly be called a corpus hemorrhagicum rather than a corpus luteum. Slide 88 shows several older corpora lutea where the folds of granulosa lutein cells have become so extensive that a central lumen is no longer visible.

The cells of the corpus luteum eventually degenerate, and are phagocytized by macrophages. All that remains of the former corpus luteum is a scar of poorly vascularized dense connective tissue called a corpus albicans. This structure persists through several cycles before it becomes indistinguishable from the surrounding stroma. Next to the corpus luteum on some versions of slides 27HU and 87 is a corpus albicans.
Slide 86 (HU Box): Ovary, Corpus Luteum of Ovulation, C.T. Stain

Identify the corpus luteum and confirm this one does not have a theca lutea. This is because it is from an animal species where the theca lutein cells intermingle with the granulosa lutein so that there is no separate theca lutein layer. Human corpora lutea have a distinct theca lutein. Note the lipid-rich cytoplasm of the granulosa lutein cells and the vascularity of the corpus luteum. Locate the dense connective tissue layer called the tunica albuginea near the surface of the ovary, just deep to the germinal epithelium. In places, the germinal epithelium may have been lost during tissue preparation.

Look at any of the ovary slides to try to identify an atretic follicle. Atretic follicles are those that are in the process of degenerating, and will never be ovulated. Atresia can occur at any stage of follicular development, and therefore atretic follicles differ greatly in size and morphology. During the atresia of any follicle, the oocyte dies and the granulosa cells detach from their basal lamina and degenerate. Often all that remains of a primary follicle that became atretic is a collapsed zona pellucida. In an atretic secondary follicle you may see individual granulosa cells or clumps of cells that have been sloughed off into the antrum. Sometimes the basal lamina of the membrana granulosa becomes thickened to form a glassy membrane.

II. FEMALE REPRODUCTIVE TRACT

Slide 23 (HU Box): Fallopian (Uterine) Tube, Fimbriated End, Human, c.s.

The lumen of the entire oviduct is lined by a simple columnar epithelium containing ciliated cells and non-ciliated (peg) cells. Peg cells are secretory in nature. The apical end of the peg cells often extends further into the lumen of the oviduct than do the ciliated cells, making them stand out like little pegs.

The oviduct can be divided histologically into four segments: the funnel-shaped free end (the infundibulum), the ampulla, the isthmus and the interstitial (intramural) segment, which passes through the wall of the uterus. As you progress from infundibulum to interstitial segment, the folding of the mucosa becomes less pronounced, the thickness of the smooth muscle layer gradually increases, and the diameter of the lumen changes.

Slide 23 is a transverse section through the uterine tube at the level of the infundibulum. Notice that the mucosal folds extending into the lumen are large and complex. Identify the peg cells and ciliated cells, noting the excellent preservation of cilia in these slides. The lumen of the infundibulum is in direct continuity with the peritoneal cavity, and the tube ends in numerous fringe-like projections called fimbriae. The same epithelium that lines the lumen extends over the fimbriae and then becomes continuous with the simple squamous epithelium that covers the outer surface of the rest of the uterine tube. Look around the outer edge of the section for areas where the section passed through fimbriae that were curving backward from the mouth of the uterine tube. The presence of fimbriae conclusively identifies this as the infundibulum. Another less obvious clue is the numerous large, thin-walled veins in the fimbriae and in the wall of the tube. Engorgement of these vessels helps extend the fimbriae toward the ovary just prior to ovulation, so that they almost surround the ovary, helping to ensure that the ovulated oocyte will enter the uterine tube rather than becoming lost in the peritoneal cavity. Note the presence of numerous nucleated cells in the lumen of the oviduct. What are these cells and what does their presence suggest? (Answer: They are neutrophils, and their presence suggests a mild case of acute inflammation, i.e., salpingitis).
Identify the three layers that make up the wall of the uterine tube: the mucosa, the muscularis and the serosa. The characteristic features of the ampulla that distinguish it from the other regions of the uterine tube are: a mucosa that is thrown into elaborate highly branched folds, fewer large veins in the serosa as compared to the infundibulum, the absence of fimbriae, and a wide lumen.

Compare the isthmus of the oviduct to the infundibulum and ampulla. In the isthmus the lumen is narrower, the mucosal folds are greatly reduced in number and height and the extent to which they branch, and the muscularis becomes thicker.

Identify the endometrium and the myometrium. The outermost layer of the uterus is not present on most of these slides. It is primarily a connective tissue layer containing large blood vessels, and is called the perimetrium. Most of the outer surface of the uterus is covered by a mesothelium called the peritoneum. The presence of a mesothelium makes that portion of the perimetrium a serosa. Over the more inferior portions of the uterus, near the cervix, there is no mesothelium, so the outermost layer of the perimetrium is an adventitia in that location.

See if it is possible to distinguish the two layers of the endometrium, stratum basale and stratum functionale, on your slide. Generally the stroma of the stratum basale is more cellular, and the presence of many nuclei makes this area stain somewhat darker than the stratum functionale. The stratum functionale is shed during menstruation while the stratum basale is not. During the proliferative phase of each uterine cycle, under the influence of estrogen, the stratum functionale is restored.

The lumen of the uterus is lined by a simple columnar epithelium containing ciliated cells and secretory cells, although it is not always easy to distinguish between them. This surface epithelium invaginates to form glands that contain mainly secretory cells. The glands are present in both the stratum functionale and the stratum basale. The portion of the glands in the stratum basale is coiled and does not undergo major changes in morphology during the uterine cycle. In contrast, the morphology of the glands in the stratum functionale undergoes marked changes, so that their appearance depends on the stage of the menstrual cycle.

In Slide 28HU & Slide 92 the uterus is in the proliferative phase of the cycle, and the glands in the functionale are long, narrow, relatively straight tubules. There is considerable mitotic activity in the epithelial cells of the glands and in stromal cells. The spiral arteries are short (having lost their terminal portions during menstruation) and only moderately coiled. They are therefore fairly inconspicuous.

Slide 92B could be considered to be in either the late proliferative or early secretory stage, in that the glands are becoming slightly wavy rather than straight.

Observe the myometrium and note that the smooth muscle cells are organized into bundles, and that neighboring bundles are oriented in many different directions rather than forming distinct layers.
In the secretory phase, the myometrium is under the influence of progesterone. The epithelium of the glands begins to secrete a mucoid material and there are major changes in glandular morphology, especially in the stratum functionale. These include the following:

- The glands first take on a coiled or corkscrew appearance where the entire gland curves back and forth like a sine wave. The walls of the gland are parallel to one another. Later in the secretory phase the glands become sacculated and the epithelial cells form tooth-like projections that give a serrated appearance to the edge of the lumen (the sawtooth appearance).

- In the early secretory phase (~ day 17) the nuclei of the gland cells are displaced toward the apical end of the cell by the accumulation of glycogen in the basal cytoplasm. A few days later the glycogen moves toward the apical cytoplasm to be secreted, and the nuclei return to their basal position.

- The spiral arteries elongate toward the surface of the endometrium, become progressively more coiled, and develop thicker walls, making them more apparent in the mucosal stroma. (These vessels, although called arteries, usually have the morphology of arterioles in most of the stratum functionale.) To find these vessels, look for multiple cross sections through an arteriole clustered together within a small area of the stroma. The multiple sections are probably all part of a single coiled vessel; in favorable specimens the sections through the vessel will be arranged in a column oriented from the lower portion of the stratum functionalis toward the surface epithelium.

- Edema of the stroma becomes even more pronounced, and then near the end of the secretory phase decreases precipitously. The excess extracellular fluid is removed by the venous system, causing irregularly shaped, thin-walled venous lacunae to become evident. The entire thickness of the functionalis decreases, which may contribute to the sacculcation of the glands.

- The stromal cells near the lumen of the uterus take on a "predecidual" appearance, meaning that they appear more like epithelial cells than fibroblasts, i.e., the cells and nuclei are rounder and surrounded by more cytoplasm. Later these cells will also cluster around spiral arteries, and finally become widespread throughout the stroma. (One version of Slide 93 labeled “Uterus Secretory H&E” is at a later stage than the other, and the predecidual changes are more pronounced.)

**Slide 91: Uterus Menstrual**

The menstrual phase of the uterine cycle is characterized by:

- Reduced edema of the stroma.
- Infiltration of the stroma by leukocytes, including neutrophils & eosinophils (leukocyte infiltration actually begins in late secretory phase).
- A surface epithelium that is disrupted or missing.
- Erythrocytes free in the stroma due to bleeding from ruptured spiral arteries.
Menstruation occurs as a result of ischemia caused by prolonged contractions of the spiral arteries. This leads to necrosis of the terminal portions of the arteries themselves as well as the surrounding tissue. When the spiral arteries then re-open, clumps of necrotic stratum functionale are washed away by blood flowing out of the disrupted arteries. This combination of necrotic tissue and blood constitutes the menstrual flow. Notice that the stratum basale is not affected because it is supplied by straight arteries that do not undergo prolonged contractions. Be aware that both spiral and straight arteries arise from the radial arteries of the uterus. The blood supply of the uterus is arranged as follows: the uterine artery gives off 6-10 arcuate arteries in the myometrium, radial arteries branch from these and enter the endometrium, straight arteries branch from the radial arteries in the stratum basale & supply it, and the main trunk of the radial artery continues on into the stratum functionale as a spiral artery.

The leukocytic infiltration typical of the menstrual phase serves a protective function. Normally the epithelial lining of an organ is its first line of defense, helping to keep antigens (including micro-organisms) from penetrating into the wall of the organ. Since the epithelium is lost during menstruation, an alternate defense mechanism, namely leukocytes, must be employed.

As the proliferative phase of the next cycle begins, epithelial cells from the bases of the glands in the stratum basale will begin to divide and migrate up to restore the glands in the stratum functionale as well as the surface epithelium. Stromal cells will divide and begin to produce the extracellular matrix of the growing stratum functionale, and the cells in the walls of the spiral arteries will divide as the vessels grow toward the uterine surface.

Slide 31 (HU Box): Cervix Uteri, Human, l.s., or
Slide 93A (Several Versions): Uterus L.S. Cervix, or Cervical Canal

The cervix is the inferior portion of the uterus, but it has a distinctly different histology and physiology from the rest of the uterus. The cervix is continuous with the uterine cavity superiorly through a constriction called the internal os and with the vagina inferiorly through the external os. The cervical canal or endocervix is the lumen of the cervix; it runs from internal os to external os. The mucosa of the cervical canal is lined mainly with tall mucus-secreting columnar cells that also line the highly branched, tubular cervical glands. The glands are oriented obliquely with reference to the cervical canal. The necks of the glands may become occluded, resulting in the accumulation of mucus within their lumens and forming large cysts called Nabothian cysts (Nabothian follicles). Is the surface layer of the cervix shed during menstruation? [Answer: No, the cervix is considered part of the uterus by gross anatomists, but it differs significantly from the rest of the uterus in terms of its histology and physiology. One example is that the rest of the uterus sheds its surface layer (stratum functionalis) whereas the cervix does not.]

Compare the connective tissue underlying the cervical epithelium with that of the rest of the uterus. You should notice that the cervix contains relatively dense collagenous connective tissue.

The lower part of the cervix extends downward into the lumen of the vagina. Clinicians refer to this part as the portio vaginalis. Histologists sometimes reserve this term to refer to the point of transition between the simple columnar epithelium of the cervix and the stratified squamous epithelium of the vagina. The exact location of this squamo-columnar junction changes with age.

Examine the epithelium of the vagina on these and the following slides.
The epithelium lining the vagina is minimally keratinized stratified squamous.
(Note that some versions of Slide 90 HU are somewhat atypical in that the epithelium looks like stratified cuboidal in many places.) Glycogen is a major component of the vaginal epithelial cells, and the pale-staining regions in the cytoplasm of the surface cells are areas where extensive glycogen deposits were extracted during fixation. The glycogen accumulates during the proliferative phase under the influence of estrogen, and is shed into the lumen in increasing amounts during the secretory phase under the influence of progesterone.

In addition to the high glycogen content, other characteristics of vagina are:

- The connective tissue layer beneath the epithelium usually has long papillae that project into the overlying epithelium.
- A rich complex of veins is present in the connective tissue layer (especially on Slide 90 HU), and these become engorged with blood during sexual excitement.
- The vagina is generally devoid of glands. Vaginal epithelium is lubricated in part by mucus from cervical glands, and in part by fluid transudate from vessels in the vaginal wall.

External to the connective tissue layer is a muscularis composed of smooth muscle and finally an adventitia.

In Slide 94, be sure to distinguish the vagina (at the edge of the section) from the cross section of the urethra near the center. Both structures are lined by minimally keratinized stratified squamous epithelium, although other types of epithelia (including transitional) line the more proximal portions of the urethra. Both the vagina and the urethra open into the vestibule (not shown on this slide), which is the space between the two labia minora.

III. ELECTRON MICROSCOPY (RHODIN)

A. OVARY

1. In Figure 34-3, identify the germinal epithelium on the surface of the ovary. It was at first thought that oocytes developed from these cells, but in fact they do not. Identify tunica albuginea, primordial follicles, the large follicle labeled secondary follicle, zona pellucida, membrana granulosa cells (#7), basal lamina, theca interna (note the lipid droplets beginning to accumulate in the cytoplasm of the cells as they differentiate into steroid-producing cells) and theca externa. The identification of the large follicle as a secondary follicle could be argued, since there is no obvious antrum visible in this micrograph. However the figure legend says this section came from a follicle such as that seen in Fig. 34-2, where an antrum is clearly visible.

2. Compare the relatively squamous follicular cells of a primordial follicle to the cuboidal follicular cells of a unilaminar primary follicle (Fig. 34-4 vs. 34-5). (NOTE THAT DESPITE WHAT THE LEGEND TO FIG. 34-4 CLAIMS, PRIMORDIAL FOLLICLES CONTAIN PRIMARY OOCYTES, NOT PRIMORDIAL GERM CELLS. PRIMORDIAL GERM CELLS ARE MITOTIC CELLS THAT EXIST ONLY DURING
FETAL DEVELOPMENT. PRIMARY OOCYTES ARE ARRESTED IN THE FIRST MEIOTIC PROPHASE.) Primary oocytes persist until a few hours before ovulation, at which time they complete the first meiotic division (giving off the first polar body), enter the second meiotic division, and become arrested as secondary oocytes. The second meiotic division is not completed until the time of fertilization.

3. Observe the structure of a secondary follicle (Figs. 34-6 & 34-7). (NOTE THAT SECONDARY FOLLICLES ALSO CONTAIN PRIMARY OOCYTES, NOT SECONDARY OOCYTES AS THE LEGEND TO FIG. 34-6 CLAIMS). Note the beginning of antrum formation as fluid has accumulated in the extracellular spaces between granulosa cells. These spaces will enlarge and fuse to become the antrum. Identify the nucleolus, nucleus and cytoplasm of the oocyte, the zona pellucida, and the cells that will form the corona radiata upon ovulation. Identify cortical granules in the superficial cytoplasm of the oocyte (Fig. 34-7) are modified lysosomes. Their contents will be released at fertilization (as part of the cortical reaction) and will then alter the properties of the zona pellucida (zona reaction). Notice that cytoplasmic processes from both the oocyte and the corona radiata cells penetrate into the zona pellucida (Fig. 34-10), where they contact one another and form gap junctions.

4. Identify the granulosa cells, theca interna (identified by the lipid droplets in some cells) and theca externa of a secondary follicle at high magnification (Fig. 34-12). Note that a basal lamina separates the granulosa cells (membrana granulosa) from the theca interna. This basal lamina can thicken greatly during atresia to form the glassy membrane of atretic follicles. Be sure you can distinguish the basal lamina from the zona pellucida (Fig. 34-6).

5. Observe how the granulosa lutein cells of the corpus luteum have acquired the ultrastructural appearance of steroid-secreting cells (Fig. 34-21). Compare this with the relatively undifferentiated appearance these cells had when they were membrana granulosa cells in a follicle (Fig. 34-6).

6. Study the light microscopic appearance of a corpus albicans (Fig. 34-19). A corpus albicans is the remnant of an old corpus luteum; it is composed of connective tissue. At a higher magnification than shown here, you would see that it contains relatively few cells (mostly fibroblasts) that are scattered among abundant collagen fibers. Compare this with the corpus luteum, which is highly cellular and contains little connective tissue of any kind.

7. Fig. 34-20 illustrates an atretic follicle with a thickened, folded glassy membrane (#2), and a remnant of the collapsed zona pellucida (#4). These features are not easy to see in this micrograph. Both would stain eosinophilic with H&E. Consult the videodisk for better examples of atretic follicles.

B. FEMALE REPRODUCTIVE TRACT

1. In the oviduct note the simple columnar epithelium with its ciliated cells and secretory (peg) cells (Fig. 34-27). Peg cells may have microvilli, but no cilia (note the absence of basal bodies in their apical cytoplasm).

2. Compare the LM appearance of uterine glands in the proliferative stage (Figs. 34-29) with the secretory stage (Figs. 34-32). Fig. 34-32 actually shows glands with
coiled or corkscrew glands rather than sacculated, so it might better be labeled early or mid-secretory, instead of late. Observe that in the secretory stage glycogen appears to be released from the cell in an apocrine fashion (Figs. 34-34 & 34-35).

3. Study the cervical glands (Figs. 34-40 to 34-43). Notice that, like the surface epithelium of the cervix, the epithelium of the glands consists mostly of mucous cells. There is also an occasional ciliated cell.

4. Be aware that the vagina is lined by a nonkeratinized (minimally keratinized) stratified squamous epithelium (Fig. 34-46), since the surface cells still contain recognizable nuclei. Note the presence of glycogen in the vaginal epithelium (Figs. 34-48 & 34-49).
**LABORATORY 20 CHECKLIST**  
**OVARY & FEMALE REPRODUCTIVE TRACT**

### LIGHT MICROSCOPY

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<td>corpus albicans</td>
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### ELECTRON MICROGRAPHS

| primordial vs. primary vs. secondary follicle | membrane granulosa cells |
| oocyte                                      | theca interna vs. theca externa               |
| zona pellucida                              | antrum                                       |
| follicular cells                            | ciliated vs. peg cells of oviduct             |

**NOTE:** These checklists include MOST of the structures that you should be able to identify. Exams may include structures not on these lists.
FOCUS QUESTIONS
LAB 20: OVARY & FEMALE REPRODUCTIVE TRACT

See whether you can answer the following questions. The correct answers are posted on the course website (http://neurobio.drexelmed.edu/education/ifm/microanatomy) under “Lab Focus Questions”.

1. Are the oocytes in primordial follicles interphase cells or are they arrested at some stage in mitosis or meiosis?

2. How does the location of a follicle in the ovary change as it develops?

3. Distinguish between the structure of primordial follicles, unilaminar primary follicles, multilaminar primary follicles and secondary follicles. When does the zona pellucida appear? The antrum?

4. How would you distinguish between a secondary ovarian follicle and a Graafian follicle? How can you use the oocyte to help you make this distinction?

5. Theca interna cells of the developing follicle produce androgens, but when the same cells become theca lutein cells in the corpus luteum they produce estrogen. What prevents them from producing estrogen during the follicular phase?

6. What is the only type of ovarian follicle that ever contains a secondary oocyte? Does it always contain a secondary oocyte?

7. The oocyte that is ovulated after the first meiotic division is a secondary oocyte. What is its DNA content and chromosome number? Why is the first meiotic division sometimes called a reduction division?

8. Why does the infundibulum of the oviduct have many large, thin-walled veins in its wall?

9. Why is the lumen of the ampulla of the oviduct almost completely filled by mucosal folds?

10. Compare the muscularis externa of the oviduct with that of the ureter and small intestine in terms of the number of muscle layers and their orientation.

11. How would you distinguish between parts of these different tubes (oviduct, ureter and small intestine) that have the same muscle arrangement?

12. Name the three phases of the uterine cycle. With which phases of ovarian function do these phases coincide?

13. What causes the stratum functionalis of the uterus to be shed during menstruation, whereas the stratum basalis is retained?

14. Why is it important that the stratum basalis is not lost during menstruation?

15. Distinguish between the uterus, the cervix and the vagina in terms of their epithelium and glands.

16. In what ways do the properties of cervical mucus change during the menstrual cycle? Why are these changes beneficial?
17. What is meant by the “fundus” of an organ? Name some organs that have a fundus.

18. The vagina does not undergo the dramatic cyclic changes that the uterus does, but there are some variations in the structure and function of the vaginal epithelium during the menstrual cycle. Describe these.

19. In a Pap (Papanicolaou) smear, also called a cervical smear, epithelial cells are swabbed from the region of the squamo-columnar junction, spread on a glass slide, fixed, stained and examined for evidence of a variety of conditions including cervical carcinoma. What cell type is the pathologist most interested in, and what morphological changes correlate with possible malignancy?

Congratulations!
You have completed Microscopic Anatomy! We hope you have enjoyed the course, and we wish you continued success and satisfaction in all your remaining studies.